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1 **Human skin commensals augment *Staphylococcus aureus* pathogenesis**

2

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25

26 **All bacterial infections occur within a polymicrobial environment, from which a pathogen**

27 **population emerges to establish disease within a host. Emphasis has been placed on**

28 **prevention of pathogen dominance by competing microflora acting as probiotics[1]. Here we**

29 **show that virulence of the human pathogen, *Staphylococcus aureus* is augmented by native,**

polymicrobial, commensal skin flora and individual species acting as "proinfectious agents". The outcome is pathogen proliferation but not commensal. Pathogenesis augmentation can be mediated by particulate cell wall peptidoglycan (PGN), reducing the *S. aureus* infectious dose by over 1000-fold. This phenomenon occurs using a range of *S. aureus* strains, infection models and is not mediated by established receptor-mediated pathways including Nod1, Nod2, Myd88 and the NLPR3 inflammasome. During mouse sepsis, augmentation depends on liver resident macrophages (Kupffer cells, KC), that capture and internalise both pathogen and 'proinfectious agent', leading to reduced production of reactive oxygen species, pathogen survival and subsequent multiple liver abscess formation. The augmented infection model more closely resembles the natural situation and establishes the role of resident environmental microflora in initiation of disease by an invading pathogen. As human microflora is ubiquitous[2] its role in increasing susceptibility to infection *S. aureus* highlights potential strategies for disease prevention.

Whilst *S. aureus* exists as part of a heterogeneous resident microflora[3], it often emerges as an invasive human pathogen, capable of *in vivo* persistence and dissemination[4]. The surrounding commensal community is protective in some contexts[5,6], while in others coinfection can be mutually beneficial for pathogen and commensal[7]. During pathogenesis, the population of *S. aureus* expands clonally, as individual organisms within the original infecting cohort found the characteristic abscesses[8,9]. As animal host mortality is dose dependent[10], most of the inoculum does not directly contribute to disease. We hypothesised that virulence might be enhanced by co-inoculation with non-infectious organisms. To test this, we first used the established zebrafish embryo infection model[10] and demonstrated that a virulence attenuated mutant (*pheP saeR*; deficient in an amino acid permease and a global regulator of virulence factors[10,11]) of *S. aureus* SH1000 is able to augment infection caused by low dose of a virulent strain (Fig. 1a). Only the low dose virulent organism substantially benefits (Supplementary Data Fig. 1a). To test if unrelated non-pathogenic organisms can similarly augment infection, the skin commensal *M. luteus* was co-injected with *S. aureus* (Fig. 1b). *M. luteus* alone does not cause disease and is swiftly eliminated from the host when injected alone or in combination with *S. aureus* (Fig. 1c,

59 Supplementary Data Fig. 1b). However, its presence significantly enhances *S. aureus* virulence
60 leading to host mortality and pathogen proliferation. Both pathogen and commensal can be
61 found co-localised in phagocytes *in vivo* (Fig. 1d), characteristic of *S. aureus* infection dynamics
62 in this model.
63
64 *S. aureus* infection of humans is often iatrogenic, resulting in co-inoculation of skin (or other)
65 microflora. The ability of human skin commensal organisms to augment *S. aureus* mammalian
66 infection was next tested. 1-2 x 10^8 CFU *S. epidermidis* or *M. luteus* led to augmentation (Fig. 1e-
67 h). Survival of *S. epidermidis* was not enhanced by *S. aureus*, and *M. luteus* was completely
68 cleared (Fig. 1f). 1 x 10^8 CFU *M. luteus* could augment as low as 1 x 10^5 CFU *S. aureus*
69 (Supplementary data Fig. 1c). Combining 1 x 10^6 CFU of both *S. aureus* and *M. luteus* gave a
70 significant increase in *S. aureus* liver CFU (Supplementary data Fig. 1d). Live commensal flora,
71 whilst able to augment infection are cleared by the host, likely because they do not have the
72 multiple mechanisms that enable *S. aureus* to avoid killing by the innate immune system[12,13]. The
73 number of commensal bacteria necessary to augment *S. aureus* infection is comparable to that
74 found on the skin, where punch biopsies have demonstrated at least 10^6 CFU/cm^2 [14]. Also in a
75 study of vascular catheters, a range of bacteria were found with numbers up to 10^7 CFU[15].
76 However, it was important to demonstrate the ability of the natural mix of mammalian skin
77 microflora to augment pathogenesis. Thus, skin-associated, microbiota containing material from
78 either naturally colonised or GF mice was harvested and used directly to augment *S. aureus*
79 infection. Pathogenesis of *S. aureus* could be augmented by material from mice colonised with
80 native microflora, whereas material from GF mice could not (Fig. 1i, $P<0.05$). This demonstrates
81 that native flora has the capacity to augment *S. aureus* infection. Given the varied molecular
82 moieties that can augment infection, we have named them “proinfectious agents”.
83
84 To determine the molecular basis of “proinfectious agents” we first established that heat killed
85 *M. luteus* can augment zebrafish infection, strongly suggesting a bacterial cellular component
86 may be responsible (Fig. 1b). Peptidoglycan (PGN) is a bacterial cell wall polymer, known to have
87 many host immune system interactions[16,17]. Particulate, but not soluble, *M. luteus* PGN can

88 augment infection and is co-localised within phagocytes with *S. aureus* (Fig. 2a, b, c,
89 Supplementary Data Fig. 1e). Latex beads are also co-phagocytosed but do not alter infection
90 dynamics or outcome (Supplementary Data Fig. 1f-i, Supplementary video 5), demonstrating this
91 is not a simple niche-filling phenomenon. PGN as a proinfectious agent was then tested in the
92 murine sepsis model of infection, where a mixed inoculum consisting of *S. aureus* NewHG (low
93 dose, $1x10^6$ CFU) and *M. luteus* PGN (500 µg) were injected intravenously, compared to each
94 component alone. PGN alone had no effect on animal weight or health status (Supplementary
95 Fig. 2a). Mice receiving the mixed inoculum lost significantly more weight than low dose controls
96 (P<0.001), with exceedingly high *S. aureus* numbers (around 10^8 CFU) recovered from livers (Fig.
97 2d, e). Only mixed inocula caused severe structural deterioration of liver parenchyma including
98 multiple, small abscesses (Fig. 2f). At 72 hours post infection (hpi), in the presence of PGN,
99 classical abscesses were formed where a central extracellular nidus of *S. aureus* is surrounded by
100 a dense neutrophilic infiltrate. Solubilised PGN and latex beads did not augment infection (Fig.
101 2d, e, Supplementary Data Fig. 2b-d). PGN augmented infection with the community acquired
102 MRSA strain JE2 (Supplementary Data Fig. 2e-g), leading to increased kidney CFU and weight loss.
103 As the augmentation phenomenon has been demonstrated with three distinct strains, ranging
104 from laboratory to emergent clinical epidemic strains[18], and including both methicillin sensitive
105 (MSSA) and methicillin resistant strains (MRSA), it is inferred that strain specificity does not play
106 a significant role.
107
108 Large inocula are required to reliably establish infection in *S. aureus* murine models, with 10^{7-8}
109 CFU injected as standard[19–21]. It is improbable that such large doses are mirrored in human
110 infection and early work notes that 'a nasal droplet of 100 µm diameter could not accommodate
111 this number, even if it consisted entirely of staphylococci[22]. However, the *S. aureus* infectious
112 dose can be drastically reduced when augmented with PGN. Significant weight loss occurred with
113 a dose of *S. aureus* as low as $1x10^5$ CFU in the co-inoculum (Supplementary Data Fig. 2h) and
114 strikingly, high liver bacterial numbers were recovered from all mice receiving $1x10^4$ CFU (Fig.
115 2g). Astonishingly, one mouse exhibited a liver burden of 10^6 CFU with an inoculum of only 700
116 CFU. A lower PGN dose of 250 µg also augmented (Fig 2h, Supplementary Data Fig. 2a).

117 To determine how augmentation enhances disease outcome, *S. aureus* population dynamics
118 during infection were evaluated. We have previously identified a phagocyte-dependent
119 immunological bottleneck, from which clonal expansion of a small number of bacteria results in
120 characteristic kidney abscesses[8]. Mice were injected with three marked but otherwise isogenic,
121 *S. aureus* strains in a 1:1:1 ratio totaling 1 x 10^6 CFU. 30 minutes post-infection, regardless of
122 PGN addition, the majority of the CFU were in the liver and without PGN, bacterial numbers
123 subsequently declined (Fig. 2i). As infection progressed there were significantly more *S. aureus*
124 in the liver, kidneys and spleens of mice receiving mixed inocula. To understand clonal expansion
125 in this context, we assessed contribution to the final bacterial load of the three marked strains,
126 in each organ. By 70 hpi, dominance by individual or pairs of strains indicated clonal expansion
127 in kidneys, but less so in the liver (Fig. 2j, Supplementary Data Fig. 2k,l). However, careful
128 dissection and bacterial enumeration of individual liver abscesses showed these were clonal (Fig.
129 2j, $P<0.001$).
130
131 To determine the molecular basis for infection augmentation, PGNs from a range of species
132 including *Staphylococcus epidermidis*, *Curtobacterium flaccumfaciens*, *Bacillus subtilis* and *S.*
133 *aureus* strains were used. Infection could be augmented in the murine sepsis model by PGN from
134 all species tested (Supplementary Data Fig. 3a-l), having a diversity of amino acids in the peptide
135 side chain, suggesting the conserved glycan moiety is important. *C. flaccumfaciens* has unusual
136 PGN for a Gram-positive organism as it contains glycine as the first peptide in the side chain as
137 opposed to L-alanine. *Bacillus subtilis* PGN contains meso-diaminopimelic acid (m-DAP) at stem
138 peptide position 3, an amino acid commonly found in the PGN of Gram-negative bacteria[23]. PGN
139 from *S. epidermidis* however is similar to that of *S. aureus*, the only difference being altered
140 composition of the crosslinking side chains. The presence of wall teichoic acids on PGN (i.e. the
141 PGN was not HF treated) did not alter augmentation (Supplementary Data Fig. 3m-o). *S. aureus*
142 lipoproteins are immunostimulatory via TLR2[24], however PGN from a lipoprotein deficient
143 mutant (lipoprotein diacylglyceryl transferase, *lgt*) could still augment pathogenesis
144 (Supplementary Data Fig. 3p–r). Furthermore, solubilisation of PGN abrogates augmentation
145 eliminating a contaminating moiety within the preparations as the mechanism of augmentation.

146 PGN can also augment infection in other murine models. Using *S. aureus* LS-1 and NMRI mice, *M.*
147 *luteus* PGN caused increased severity in both septic arthritis (Fig. 2k, l) and subcutaneous abscess
148 models (Supplementary Data Fig. 4a,b) in which PGN alone had no effect.
149
150 Augmentation circumvents the immune bottleneck during pathogenesis which we have
151 hypothesised occurs inside phagocytes[8,9] (Fig. 2c). Therefore, we depleted either neutrophils or
152 macrophages (and macrophage-like cells) prior to challenge in the murine sepsis model.
153 Depletions led to an expected increased susceptibility to *S. aureus*, thus requiring a reduced
154 inoculum of $1x10^5$ CFU. Macrophage depletion using clodronate liposomes resulted in multiple,
155 small liver abscesses reminiscent of PGN augmentation. However, addition of PGN to the
156 inoculum did not lead to augmentation suggesting a critical role for macrophages (or related
157 cells) in augmentation of pathogenesis (Fig. 3a). Conversely, infection of neutropenic mice was
158 still PGN augmented (Fig. 3b) and this is in agreement with *in vitro* data where co-incubation of
159 *S. aureus* and PGN with human derived neutrophils did not promote survival of *S. aureus*
160 compared to bacteria only controls (Supplementary Data Fig. 4c). However, survival of
161 intracellular *S. aureus* in human monocyte derived macrophages (MDMs) was increased in the
162 presence of PGN (Supplementary Data Fig. 4d; $P<0.01$), demonstrating a potential human
163 relevance for our findings.
164
165 Augmentation of infection by PGN leads to liver abscesses, so to decipher organ and cellular level
166 mechanisms, we employed spinning-disk intravital microscopy (SD-IVM) to visualise Kupffer cells
167 (KC; liver-resident macrophages), and other innate immune cells. Polymorphonuclear
168 neutrophils (PMNs) are crucial for *S. aureus* control by the host in both animal models[25,26] and
169 humans[27]. Additionally, an important role for KC in capturing and eliminating *S. aureus* during
170 bacteremia has been recently described[19,28]. Fluorescently labelled PGN and *S. aureus*-GFP were
171 co-injected into C57BL/6J mice and both were rapidly engulfed by KC (purple), but not
172 neutrophils (red) (Fig. 3c, Video 1 and 2). The rate of *S. aureus* capture by KC was not perturbed
173 by PGN augmentation (Fig. 3d). However, at 8 hpi mice receiving mixed inocula had significantly
174 more *S. aureus*-GFP within the liver than controls and by 24 hpi they contained small, multi-lobar

175 focal abscesses (Fig. 3e), concomitant with an elevated *S. aureus* burden (Fig. 3f). Augmentation
176 with PGN did not affect neutrophil recruitment at 8 hpi (Fig. 3g), but by 24 hpi there was a
177 significant increase (Fig. 3g) consistent with abscess formation (Figs. 2f and 3e). These data
178 suggest that a mixed inoculum leads to inadequate control of *S. aureus* inside KC.
179
180 It has been previously shown that during natural colonisation of mice an immune tolerance to *S.*
181 *aureus* infection occurs, mediated by Nod1[29]. To test whether prior exposure to commensal
182 organisms affects augmentation, we tested the phenomenon in germ-free (GF) mice, which are
183 incidentally more susceptible to *S. aureus*[30]. Using SD-IVM it was observed that the rate of
184 staphylococcal capture by KC is comparable to that seen in wildtype mice and importantly, *S.*
185 *aureus* pathogenesis is augmented by particulate PGN (Supplementary Data Fig. 4e-g).
186
187 Host PGN recognition has been attributed to TLR2 receptors, however this is now known to be
188 due to lipoprotein contamination[31]. We demonstrated no role for MyD88 dependent signaling in
189 augmentation and also ruled out the cytosolic PGN receptor Nod2 and the NLRP3 inflammasome
190 (Fig. 4a,b). Furthermore, the range of PGN structures able to augment precluded a role for Nod1[32]
191 and the NLRP3 inflammasome[33] (Supplementary Data Fig. 3a-l). However, Cybb$^{-/-}$ mice, missing
192 the NADPH oxidase[34], required by phagocytes to produce reactive oxygen species (ROS), showed
193 lack of augmentation at 8 hpi (Fig. 4b). Cybb$^{-/-}$ mice are highly susceptible to *S. aureus* infection
194 (Fig. 4b), not surviving until 24 hpi. At a lower inoculum of 10^5 CFU, 8 hpi imaging was not possible
195 due to low fluorescence levels, but at 24 hpi, augmentation was still not observed (Fig. 4c)
196 confirming the likely mechanistic involvement of ROS. Augmentation with PGN led to greatly
197 diminished oxidation ($P<0.0001$) and to less acidification ($P<0.01$) of the phagolysosomes
198 containing *S. aureus* in KC (Fig. 4d-f, Video 3 and 4), highlighting the critical role of ROS.
199
200 A characteristic feature of many *S. aureus* infection models is a high inoculum. Here we have
201 established that the majority of the infecting material can be commensal bacteria or even cell
202 wall peptidoglycan. This has important implications for infection prevention where both the
203 pathogen and other organisms or material, previously thought innocuous, need to be considered.

204 Deciphering the cellular and molecular mechanisms involved will allow exploitation for
205 development of novel interventions[17,35,36]. Potentially *S. aureus* responds to augmenting material
206 resulting in an increased capability of the pathogen to initiate infection. *P. aeruginosa* is known
207 to respond to PGN to enhance its virulence[37]. As well as at the initiation of infection,
208 augmentation could occur during the action of antibiotics, where death of a proportion of the
209 bacterial population may give rise to cell wall fragments. Also, indwelling medical devices, such
210 as intravascular catheters, reside *in-situ* for several days where the prosthetic material can
211 become colonised by commensal flora[15]. As catheters are regularly accessed, both commensal
212 flora (e.g. *M. luteus* and *S. epidermidis*) and pathogen (*S. aureus*) could be flushed into the
213 bloodstream simultaneously. Our work establishes a precedent for how a human pathogen can
214 initiate disease using proinfectious agents, as microbial crowdsourcing to circumvent immune
215 system control. In clinical practice, infection by all bacterial pathogens occurs from within a
216 microflora and is therefore initially polymicrobial. This raises the likelihood of a more general role
217 for proinfectious agents, requiring revision of existing models of bacterial pathogenesis and
218 highlighting the involvement of commensal organisms as unwitting accomplices in infection
219 initiation.
220
221 **Author Information**
222 The authors declare no competing interests. Correspondence and requests for materials should
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238

239

240 **Author Contributions**

241 E.B., B.G.J.S., D.S., M.N., Y.F., A.A., A.W., E.J.G.P., P.S., P.M., and T.K.P. performed and analysed
242 the experiments. K.D.M., T.J., D.H.D., J.A.G.S., P.K., S.A.R and S.J.F. contributed to study design
243 and data analysis. E.B. and S.J.F. wrote the manuscript. All authors discussed the results and
244 commented on the manuscript.

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249

250 **Figure 1 *S. aureus* virulence is augmented by live commensal flora**

251 **a,** Survival curves of fish injected with low dose *S. aureus* SH1000 (150 CFU, SA low) and/or *S.*
252 *aureus* SH1000 *phePsaeR* (1350 CFU). *S. aureus* SH1000 high dose (1500 CFU, SA high) was
253 injected as a positive control. Data are representative of three independent experiments; $n \geq 28$,
254 log-rank (Mantel-Cox) test. **b,** Survival of fish injected with low dose *S. aureus* SH1000 (150 CFU,
255 SA low) with or without live or heat killed (HK) *M. luteus* (2000 CFU, ML). Data are representative
256 of three independent experiments; $n \geq 28$, log-rank (Mantel-Cox) test. **c,** Growth of bacteria
257 within embryos after co-injection with *M. luteus* (2000 CFU) and *S. aureus* SH1000 (150 CFU).
258 Open circles, live and filled circles, dead embryos, *M. luteus* (red), *S. aureus* (black) CFU in each
259 fish. $n \geq 60$. **d,** *In vivo* imaging of pHrodo (red) labelled *M. luteus* (2000 CFU, ML indicated by
260 arrows) and *S. aureus* SH1000-GFP (150 CFU, SA indicated by arrows) 2 hpi. Within the zebrafish
261 circulation valley, phagocytes were viewed at x 60 magnification). Images are representative of
262 5 embryos from two independent experiments. Scale bar 10 µm. **e,f,** Co-injection of live $1x10^8$
263 CFU *S. epidermidis* (SE) and low dose ($1x10^6$ CFU) *S. aureus* NEWHG into mice (SA) with weight
264 loss **(e)** and liver CFU **(f)** recorded (*S. aureus*, black; *S. epidermidis*, red). $n = 10$ per group; median
265 value shown, Mann-Whitney two-sided test. **g,h,** Co-injection of live *M. luteus* (ML, $2x10^8$ CFU)
266 and low dose *S. aureus* NEWHG (SA, $1x10^6$ CFU) into mice with weight loss **(g)** and liver CFU (*S.*
267 *aureus*) **(h)** recorded. n = 10-20 per group; median value shown, Mann-Whitney two-sided test.
268 **i,** Dermonecrotic lesion size for C57BL/6J mice injected (on the left flank) with *S. aureus* NewHG
269 (SA, 10^7 CFU, n= 13) or co-injected with *S. aureus* NewHG 10^7 CFU and either isolated skin
270 commensals from SPF mice (SA & SPF harvest, n= 8) or skin commensals from GF mice (SA & GF
271 harvest, n=5). Median value shown, one-way ANOVA with Tukey post-test.

272

273 **Figure 2 Gram-positive PGN augments *S. aureus* pathogenesis in animal models**

274 **a,** Survival curves of fish injected with low dose *S. aureus* SH1000 (150 CFU, SA low) and 5 ng
275 polymeric *M. luteus* PGN (ML pPGN). *S. aureus* SH1000 high dose (1500 CFU, SA high) was injected
276 as a positive control. Data are representative of three independent experiments; $n \geq 28$, log-rank
277 (Mantel-Cox) test. **b,** Growth of bacteria within embryos after co-injection with low dose *S.*
278 *aureus* SH1000 (150 CFU) and 5 ng *M. luteus* PGN. Open circles, live and filled circles, dead
279 embryos. $n \geq 60$. **c,** *In vivo* imaging of Alexafluor 647 (blue) labelled *M. luteus* PGN (5 ng, ML PGN
280 indicated by arrow) and *S. aureus* SH1000-GFP (150 CFU, SA indicated by arrow) 2 hpi. Within the
281 zebrafish circulation valley, phagocytes were viewed at x 60 magnification). Images are
282 representative of 5 embryos from two independent experiments. Scale bar 10 µm. **d, e,** BALB/c
283 mice were injected i.v. with low dose ($1x10^6$ CFU) *S. aureus* NEWHG^{kan} with or without 500 µg *M.*
284 *luteus* particulate PGN (pPGN) or soluble PGN (sPGN). Weight loss **(d)** and liver **(e)** CFU were
285 measured. *n* = 10 per group; median value shown, Mann-Whitney two-sided test. **f,**
286 Representative images of histopathological changes during infection. Arrows show 1, large
287 abscess within liver parenchyma; 2, accumulation of extracellular *S. aureus*; 3, dense infiltrate of
288 polymorphonuclear leukocytes (PMNs). Inset box at x10 magnification (scale bar 100 µm) is
289 displayed at x100 (scale bar 10 µm) in bottom panels. *n* = 5 per group. **g,** Liver CFU recovered
290 from BALB/c mice injected i.v. with a decreasing dose of *S. aureus* NEWHG^{kan} with or without 500
291 µg *M. luteus* pPGN. *n* = 5 per group; median value shown, Mann-Whitney two-sided test. **h,** Liver
292 CFU recovered from BALB/c mice injected i.v. with low dose ($1x10^6$ CFU) *S. aureus* NEWHG^{kan} with
293 or without a decreasing dose of *M. luteus* pPGN. *n* = 5 per group; median value shown, Mann-
294 Whitney two-sided test. **i,** Liver CFU at various time points after co-injection of low dose ($1x10^6$
295 CFU) *S. aureus* with or without 500 µg *M. luteus* pPGN. *n* = 8 per group; median value shown,
296 Mann-Whitney two-sided test. **j,** Livers from mice injected with low dose ($1x10^6$ CFU; 1:1:1
297 mixture of NewHG Ery^R, Tet^R or Kan^R, *n* = 5 per group *S. aureus* NEWHG^{kan} plus 500 µg *M. luteus*
298 PGN were harvested. Individual abscesses were dissected and bacterial CFU enumeration from
299 each abscess was determined (A1-A4). Bacterial CFUs from residual liver tissue post dissection (-
300) was also enumerated and added to the abscess CFUs to provide a total CFU count for each liver
301 (L). **k,** Micro-CT imaging of knee and front right paw of an NMRI mouse injected i.v. with low dose
302 ($1x10^6$ CFU) *S. aureus* LS-1 with or without 1 mg *M. luteus* pPGN. Images are representative of 10
303 animals. **l,** Clinical arthritis severity of NMRI mice injected i.v. with *S. aureus* LS-1 low dose ($1x10^6$
304 CFU) and 1 mg *M. luteus* pPGN. dpi, days post infection. *n* = 10 per group; error bars, mean and
305 s.e.m, Mann-Whitney two-sided test. * $P < 0.05$.

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310 **Figure 3 Kupffer cells are key mediators of augmentation**

311 **a,** Liver CFUs of BALB/c mice injected i.v. with low dose ($1x10^5$ CFU) *S. aureus* NEWHG with or
312 without 500 µg *M. luteus* PGN post treatment with empty liposomes or clodronate. *n* = 10 per
313 group; median value shown, Mann-Whitney two-sided test. **b**, Liver CFUs of BALB/c mice injected
314 i.v. with low dose ($5x10^5$ CFU) *S. aureus* NEWHG with or without 500 µg *M. luteus* PGN post
315 treatment with anti-Ly6G. *n* = 9-10 per group; median value shown, Mann-Whitney two-sided
316 test. **c,** Representative SD-IVM images of liver neutrophils (Ly6g; red) and Kupffer cells (KC)
317 (F4/80; purple) at baseline or after i.v. injection of *S. aureus* ($5x10^7$ CFU, BSG1; green) or *S. aureus*
318 plus *S. epidermidis* PGN (PGN-AF647; blue) at 15 min in female C57BL/6J mice; scale bars 50 µm.
319 Insert shows higher magnification image of KCs with internalized *S. epidermidis* PGN and S.
320 *aureus*. Scale bar 10 µm. *n* = 5 per group. **d,** Quantification of SD-IVM images of *S. aureus* ($5x10^7$
321 CFU, BSG1) catching by KC in the livers of female C57BL/6J mice with (red) and without (black)
322 co-injection of 500 µg *S. epidermidis* PGN (FOV – field of view); *n* = 4; thin lines, mean and s.e.m.
323 **e,** Representative stitched SD-IVM images of mouse livers at 8 h (infected with *S. aureus*, 10^7 CFU,
324 BSG1) or 24 h (infected with *S. aureus* 10^6 CFU, BSG1) with and without co-injection of 500 µg *S.*
325 *epidermidis* PGN in male C57BL/6J; scale bar 250 µm; n = 5. **f,** Quantification of 2 mm^2 stitched
326 SD-IVM images for GFP-fluorescence (*S. aureus,* BSG1) in murine livers, assessed at 24 h post i.v.
327 injection of *S. aureus* with or without *S. epidermidis* PGN. *n* = 5 per group; mean value shown,
328 Mann-Whitney two-sided test. **g**, Quantification of $2mm^2$ stitched SD-IVM images for TdTomato
329 fluorescence (Neutrophils) assessed at 8 h post i.v. injection with *S. aureus* (10^7 CFU, BSG1) or at
330 24 h post i.v. injection with *S. aureus* (10^6 CFU, BSG1) with and without co-injection of *S*
331 *epidermidis* PGN in Catchup mice. *n* = 4-5 per group. Error bars, mean with s.e.m. Tukey's multiple
332 comparisons test applied.

333

334

335 **Figure 4 Reduced oxidative burst in KCs permits augmentation of *S. aureus* virulence**

336 **a,b,** Representative SD-IVM image of mouse livers **(a)** or quantification of 2 mm^2 stitched SD-
337 IVM images for GFP-fluorescence, scale bar 50 µm **(b)** at 8 h after i.v. infection with *S. aureus*
338 BSG1 (SA, 10^7 CFU) with and without co-injection of 500 µg PGN in male C57BL/6J, Nod2$^{-/-}$,
339 MyD88$^{-/-}$, NLRP3$^{-/-}$ or Cybb$^{-/-}$ mice, n = 4 per group; mean shown, error bar s.d, unpaired t-test
340 two-tailed. **c,** Quantification of 2 mm^2 stitched SD-IVM images for GFP-fluorescence at 24 h after
341 i.v. infection with *S. aureus* BSG1 (SA, 10^5 CFU) with and without co-injection of 500 µg PGN in
342 male C57BL/6J or Cybb$^{-/-}$ mice, n = 4 per group; error bar s.d. **d,** SD-IVM image of mouse livers
343 injected with pH-rodo *S. aureus* bioparticles (red) additionally labelled with AF647 (blue) as a
344 reference fluorophore and OxyBURST (green) with and without (control) co-injection of 500 µg
345 PGN at 5 and 50 min post infection, scale bar 50 µm. Arrows point to oxidized bioparticles. n = 3
346 per group. **e,f,** Quantification of intracellular acidification of pH-rodo **(e)** or oxidation of
347 OxyBURST labelled *S. aureus* bioparticles **(f)** in KC over time in C57BL/6J mice with and without
348 (control) co-injection of 500 µg PGN. Data represent the mean fluorescence of *S. aureus*
349 bioparticles compiled from five separate FOV per time point, n = 3 per group, error bars, s.e.m,
350 two-way ANOVA.
351

352

Species	Strain	Description	Reference
Staphylococcus aureus	SH1000	*rsbU*$^{+}$ derivative of *S. aureus* 8325-4	[38]
Staphylococcus aureus	NewHG	Newman with *saeS*^{L} allele from strain RN1	[39]
Staphylococcus aureus	Newman	NCTC **8178**	[40]
Staphylococcus aureus	BSG 1	NewHG carrying pCM29-GFP	[41,42]
Staphylococcus aureus	BSG 2	MW2 carrying pCM29-GFP	[42]
Staphylococcus aureus	JE2	USA300 LAC cured of p01 and p03	[43]
Staphylococcus aureus	NewHG^{ery}	NewHG *lysA*::pGM068 (Ery^{R}) *lysA*+	[8]
Staphylococcus aureus	NewHG^{kan}	NewHG *lysA*::pGM072 (Kan^{R}) *lysA*+	[8]
Staphylococcus aureus	NewHG^{tet}	NewHG *lysA*::pGM070 (Tet^{R}) *lysA*+	[8]
Staphylococcus aureus	TJ1	LS1	[44]
Staphylococcus aureus	SH1000 GFP	SH1000 carrying pMV158-GFP	This study
Staphylococcus aureus	SH1000 mCherry	SH1000 carrying pMV158-mCherry	This study
Staphylococcus aureus	*phePsaeR*	SH1000 *sae*::Ery^{R} *pheP*::Tet^{R}	This study
Staphylococcus aureus	SA113	*lgt::ermB*	[31]
Staphylococcus aureus	SJF4591	SH1000*lgt::ermB*	This study
Micrococcus luteus	SJF 256	ATCC 4698	Sigma
Micrococcus luteus	SJF4393	ATCC 4698 (Rif^{R})	This study
Staphylococcus epidermidis	SJF229	138	[45]
Staphylococcus epidermidis	SJF4381	138 (Rif^{R})	This study
Bacillus subtilis	SJF 1	168	Lab stock
Bacillus cereus	SJF 1657	ATCC 14579	Lab stock
Curtobacterium flaccumfaciens	SJF 449	Wildtype	Lab stock

353 **Table 1: Bacterial species and strains and plasmids used in this study**

Strain	Description	Source
BALB/c	Wildtype	Charles River Laboratories
C57BL/6J	Wildtype	The Jackson Laboratory
Cybb$^{-/-}$	Cybb-deficient	The Jackson Laboratory
MyD88$^{-/-}$	MyD88 deficient	The Jackson Laboratory
Nod2$^{-/-}$	Nod2 deficient	The Jackson Laboratory
NLRP3$^{-/-}$	NLRP3 deficient	The Jackson Laboratory
Catchup	TdTomato driven from Ly6G-cre	University of Duisbury-Essen[46]
NMRI	Wildtype	Charles River Laboratories
SPF	Wildtype C57BL/6J	Taconic or the Jackson Laboratory
Germ-free C57BL/6J	Germ-free wildtype C57BL/6J	Taconic, or The Jackson Laboratory and rederived under Germ-free conditions[47]

354 **Table 2. Mouse strains used in this study**

355
356
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361

362 **Methods**

363 **Bacterial strains and culture conditions**

364 *S. aureus, M. luteus, C. flaccumfaciens and S. epidermidis* strains (Table 1) were grown using brain
365 heart infusion (BHI) liquid or solid medium (Oxoid) at 37°C with the exception of *M. luteus* which
366 was grown at 30°C. *Bacillus* sp. strains were grown using nutrient agar liquid or solid medium
367 (Oxoid) at 37°C. Supplementation with the following antibiotics was added where appropriate:
368 kanamycin 50 µg/ml, tetracycline 5 µg/ml or erythromycin 5 µg/ml plus lincomycin 25 µg/ml
369 (Sigma-Aldrich). To distinguish bacterial populations in mixed inocula experiments, *M. luteus* was
370 serially passaged on BHI media with or without rifampicin (0.03 µg ml^{-1}) and incubated at 30°C
371 until a rifampicin resistant derivative was identified. The same was conducted for *S. epidermidis*.
372 For all murine experiments, pre-grown batches of bacteria were thawed, washed and diluted to
373 the desired concentration in endotoxin-free PBS (Sigma). Staining of bacterial cells for microscopy
374 was carried out as described for PGN below. SH1000 mCherry and SH1000 GFP were constructed
375 as follows. The pMV158mCherry plasmid was constructed by introducing a gene encoding
376 mCherry (Uniprot: X5DSL3-1) into pMV158GFP[48] and replacing the existing GFP gene. The
377 plasmids, pMV158GFP and pMV158mCherry, were then introduced into *S. aureus* RN4220 by
378 electroporation, resulting in transformants expressing GFP and mCherry, respectively. The
379 plasmids were subsequently transferred into *S. aureus* SH1000 by Φ11 transduction and the
380 obtained transductants were verified by fluorescence microscopy. To create an avirulent *S.*
381 *aureus* mutant, SH1000*pheP*[11] (deficient in amino acid permease) was transduced into
382 SH1000*saeR*[49] (mutation affecting the two component system required for innate immune
383 evasion) using ϕ 11 phage. The same phage was used to transduce *lgt::ermB* from *S. aureus*
384 SA113 background into SH1000.
385

386 **Preparation of latex beads, peptidoglycan and bioparticles**

387 For *in vivo* latex bead experiments, polystyrene latex beads (1.1 µm, Sigma) were washed in
388 endotoxin free PBS, diluted to the desired concentrations and sonicated for 3 x 30 sec (Soniprep
389 150, MSE, UK) prior to injection into infection models. To prepare PGN, a mid-exponential phase
390 bacterial culture was prepared in appropriate medium and purification was carried out as

391 previously described[50]. Endotoxin assay was carried out using Pyrochrome (Associates of Cape
392 Cod Inc.) as per manufacturer's instructions. No significant difference in endotoxin concentration
393 was detected between enzyme only control and the various solubilised Gram-positive PGN
394 preparations. To solubilise PGN, 250 μg ml^{-1} mutanolysin (50 mM sodium phosphate buffer, pH
395 5.5) was added and incubated at 37°C overnight on a rotary shaker (100 rpm). Thereafter, the
396 mixture was heated to 95°C for 5 mins before being centrifuged (13 000 rpm, 8 min) to remove
397 any remaining insoluble material. Succinimidyl esters (Life Technologies) were used to stain PGN.
398 pHrodo (2.5 mM), Fluorescein-5- EX (16.95 mM) or Alexa-Fluor 647 were used as per
399 manufacturer's protocols. In brief, 5 mg PGN was recovered by centrifugation (13 000 rpm, 2
400 min) and resuspended in an appropriate volume of PBS pH 9 and 200 μl of the suspension mixed
401 in a microcentrifuge tube with 1 μl of S-ester. After 30 min incubation at 37°C on a rotary shaker
402 (100 rpm), excess dye was removed by sequential washing in PBS pH 8, Tris pH 8.5 and again in
403 PBS pH 8 each followed by mixing, centrifugation and gentle removal of the supernatant. Stained
404 PGN was finally re-suspended in 200 μl PBS pH 7.4. Staphylococcal pHrodo bioparticles (Life
405 Technologies) were additionally labelled with Alexa-Fluor 647 Succinimidyl ester (Life
406 Technologies) and OxyBURST green (H2DCFDA SE) according to manufacturer's protocols to
407 generate reporter bioparticles for acidification and oxidation. pHrodo red *S. aureus* BioParticles
408 were labeled at 2 mg ml^{-1} with 50 μg ml^{-1} AlexaFluor 647 NHS ester and 100 μg ml^{-1} OxyBURST
409 in 100 mM bicarbonate, pH 8.3, buffered saline for 30 min at room temperature under vigorous
410 agitation. Activation of OxyBURST was accomplished by adding 250 μl 1.5 M hydroxylamine, pH
411 8.5, and incubating for 30 min on ice. Labelled BioParticles were washed twice with PBS and
412 checked for labeling efficiency by flow cytometry or injected i.v. into mice.
413
414
415 **Phagocyte bacterial challenge and quantification of viable intracellular bacteria**
416 Human blood was obtained from healthy volunteers, with informed consent, in compliance with
417 the guidelines of the South Sheffield Research Ethics Committee (07/Q2305/7).
418
419 Neutrophils were purified from anti-coagulated human blood as previously described[51]. In
420 triplicate and with two biological repeats, approximately $2.5x10^6$ cells/ml were co-incubated at

421 37°C with *S. aureus* NewHG bacteria to produce a multiplicity of infection (MOI) of 5 in the
422 presence or absence of *M. luteus* PGN. At 30 and 90 min post co-culture, extracellular and
423 intracellular CFU was calculated. To measure intracellular CFU, the co-culture was transferred to
424 a microcentrifuge tube, centrifuged at 2000 rpm for 2 mins before the supernatant was removed.
425 The neutrophils were then lysed with 1% (w/v) saponin for 10 mins at RT. Bacterial enumeration
426 was calculated from serial dilutions. At 90 mins, lysostaphin (20 μg ml^{-1}) was added to the
427 remaining co-culture wells for 30 mins to lyse extracellular bacteria for the final 120 minute
428 timepoint.
429
430 Monocyte derived macrophages (MDMs) were isolated from peripheral blood mononuclear cells
431 (PBMCs) from healthy donors, as previously described[52]. PBMCs were isolated by Ficoll Plaque
432 (GE Healthcare) density centrifugation, seeded at $2x10^6$ cells/ml in RPMI 1640 medium with 2
433 mmol/l L-glutamine (Lonza) supplemented with 10% v/v newborn foetal calf serum (Gibco) in 24
434 well plates (Corning) with 1 ml/well to achieve approximately $2x10^5$ MDM/ml. After 24 hours,
435 non-adherent cells were removed and adherent cells were cultured in RPMI 1640 medium with
436 2 mmol/l L-glutamine supplemented with 10% v/v low endotoxin heat inactivated foetal calf
437 serum (Biosera) and used at 14 days. Differentiated MDMs were challenged with *S. aureus*
438 NewHG at a MOI of 0.5. The bacteria were thawed, washed in PBS and added to the MDMs in
439 fresh media. *M. luteus* PGN (sonicated for 3 x 30 s bursts) was added to the bacteria-containing
440 media at a concentration of 100 μg/ml. The MDMs were incubated on ice for 1 hour then at 37°C,
441 5% v/v CO_2. Following a 4-h total challenge, infected media was removed and the MDMs washed
442 with ice cold PBS. Residual extracellular bacteria were killed by addition of 100 μg/ml gentamicin
443 in fresh media and incubated for 30 mins, then maintained in media containing 20 μg/ml of
444 gentamicin (Sanofi-Aventis) until the desired time point. MDMs were washed with PBS and
445 incubated with 2% v/v saponin (Sigma) at 37°C for 12 min. PBS was added and cells lysed by
446 scraping and pipetting. Estimation of viable intracellular bacteria was determined by surface
447 viable count[6]. To confirm complete killing of extracellular bacteria, some wells were fixed with
448 2% v/v paraformaldehyde before bacterial challenge, then exposed to gentamicin and lysed as
449 described, demonstrating absence of bacteria in lysates. Intracellular killing after initial bacterial

450 challenge was estimated by lysing cells maintained in 20 μg/ml of gentamicin for 0.5h – 3.5h.
451
452 **Animal experiments**
453 Murine work was carried out according to UK law in the Animals (Scientific Procedures) Act 1986,
454 under Project License PPL 40/3699; approved by the Animal Research Ethical Committee of
455 Gothenburg or approved by the University of Calgary Animal Care Committee (AC12 0162) in
456 compliance with the Canadian Council for Animal Care Guidelines.
457
458 **Murine models**
459 All mice (Table 2) were housed in designated animal facilities in standard environmental
460 conditions of temperature and light and fed laboratory chow and water *ad libitum*. For the
461 haematogenous septic arthritis model, *S. aureus* LS-1[44] was inoculated i.v. into the tail vein
462 female of NMRI mice (n=10), 6-8 weeks old (Charles River Laboratories, Germany) with 0.2 ml of
463 low dose LS-1 ($1x10^6$ CFU), with or without 1 mg *M. luteus* PGN. Mice were regularly weighed
464 and examined for clinical arthritis by observers blinded to the groups as previously described[53].
465 In brief, a clinical scoring system ranging from 0–3 was used for each paw (0- no inflammation;
466 1- mild visible swelling and/or erythema; 2- moderate swelling and/or erythema; 3- marked
467 swelling and/or erythema). The clinical arthritis severity overall score was constructed by adding
468 the scores from all 4 limbs for each animal. On day 10, mice were sacrificed and limbs were
469 resected for microcomputed tomography (micro-CT) radiological examination of bone erosion.
470
471 For the staphylococcal skin infection model, NMRI mice (n=18) were anaesthetised with
472 ketamine/medetomidine and the dorsum was shaved before subcutaneous (s.c.) injection of one
473 flank with 0.05 ml of *S. aureus* SH1000 ($1x10^6$ CFU/spot) and a mixture of SH1000 ($1x10^6$
474 CFU/spot) with PGN (250 µg/spot) in the other flank. PGN alone did not cause any inflammation
475 or skin lesions. Two observers blinded to the treatment groups measured the lesion size of each
476 mouse with a caliper on day 4. The skin lesion was calculated using the mathematical formula for
477 the area of an ellipse. After sacrificing the mice on day 4, skin was disinfected with 70% v/v
478 ethanol and skin biopsies encompassing the entire infected area were taken with a sterile 8 mm

479 biopsy puncher (Kai Medical, Seki, Japan). Biopsy samples were homogenised (Ultra Turrax T25
480 homogeniser, Germany) and viable counts of bacteria were assessed[54].
481
482 For the mouse sepsis model, female BALB/c mice (Charles River Laboratories, UK) or male and
483 female C57BL/6 at 7-8 weeks old (The Jackson Laboratory) were inoculated in the tail vein with
484 0.1 ml *S. aureus* (NEWHG or JE2), *M. luteus* or *S. epidermidis* either alone (dose range 10^2 – 10^7
485 CFU as indicated) or with PGN (50 µg - 1 mg as indicated) or with PGN only. Mice were monitored
486 and sacrificed at 72 hpi unless otherwise stated or according to experimental design. Mouse
487 organs were individually homogenised in PBS and after serial dilution, plated onto BHI agar
488 supplemented with antibiotics as needed for bacterial number enumeration. Germ-free (GF) and
489 C57BL/6(J) mice were bred and maintained in flexible-film isolators or in individually ventilated
490 cages (IVC) at the Clean Mouse Facility, University of Bern, Switzerland. At the age of 6 weeks
491 these mice were shipped under germ-free conditions to the University of Calgary. Alternatively,
492 GF mice were obtained from Taconic. GF status was routinely monitored by culture-dependent
493 and -independent methods and all mice were independently confirmed to be pathogen-free.
494
495 For the dermonecrosis model, mice were left in their cage without changing the bedding material
496 for at least 7 days. Mice were euthanized and skin samples ($2cm^2$) were surgically removed from
497 the abdominal region of specific pathogen free (SPF) C57BL/6J or GF mice. Isolation of skin
498 microorganisms was performed by incubation of the skin samples in PBS containing 0.1% Triton
499 X-100 for 1hr at 37°C at high agitation. Skins were gently massaged against a 100 µm filter with
500 additional PBS-Triton. Isolated microorganisms were centrifuged and washed 3x with PBS and
501 stored on ice until the infection experiment. Skin microbes were enumerated by culturing on TSA
502 blood agar plates for 48 hours at 25°C. 24 hours prior to infection experiments hair from the
503 dorsal region of C57BL/6J mice was removed by shaving and hair removal cream (Nair). The left
504 dorsal flank of each mouse was subcutaneously injected with 10^7 CFU *S. aureus* (injection volume
505 50 µL) and the right dorsal flank received a co-injection of the isolated skin microbes and 10^7 CFU
506 *S.* aureus. At 48 hpi mice were euthanized and skin lesions were photographed. Dermonecrotic
507 lesion area was measured using ImageJ.

508 For SD-IVM experiments, a tail vein catheter was inserted into mice after anesthetization with
509 200 mg kg^{-1} ketamine (Bayer Animal Health) and 10 mg kg^{-1} xylazine (Bimeda-MTC). Surgical
510 preparation of the liver for intravital imaging was performed as previously described[55]. Mouse
511 body temperature was maintained at 37°C with a heated stage. Image acquisition was performed
512 using an Olympus IX81 inverted microscope, equipped with an Olympus focus drive and a
513 motorized stage (Applied Scientific Instrumentation) and fitted with a motorized objective turret
514 equipped with 4×/0.16 UPLANSAPO, 10×/0.40 UPLANSAPO, and 20×/0.70 UPLANSAPO objective
515 lenses and coupled to a confocal light path (WaveFx; Quorum Technologies) based on a modified
516 Yokogawa CSU-10 head (Yokogawa Electric Corporation). Target cells were visualized using
517 fluorescently stained antibodies or fluorescent reporter bacteria. Typically, KCs and neutrophils
518 were stained by i.v. injection of 2.5 μg anti–F4-80 or 3.5 μg anti-ly6G fluorescent conjugated
519 mAbs. Laser excitation wavelengths 491, 561, 642, and 730 nm (Cobolt) were used in rapid
520 succession, together with the appropriate band-pass filters (Semrock). A back-thinned EMCCD
521 512 × 512 pixel camera was used for fluorescence detection (Hamamatsu). Volocity software
522 (Perkin Elmer) was used to drive the confocal microscope and for 3D rendering, acquisition, and
523 analysis of images. For quantification of staphylococcal catching or measurements of Bioparticle
524 oxidation and acidification in the liver, five random fields of view (FOV) with 10× objective were
525 selected before injection of bacteria. Fluorescent reporter bacteria or reporter Bioparticles were
526 injected i.v. into mice 1 min after initiation of acquiring background images. Find objects function
527 in Volocity software was used to identify individual captured bacteria or Bioparticles by Kupffer
528 cells (F4/80^+ cells in liver) and when appropriate autofluorescent spots were subtracted from the
529 final quantification. For Bioparticles the particles were selected in the AF647 reference channel
530 and an increase in pHrodo (acidification) and OxyBURST (oxidation) fluorescence was quantified
531 in the first hour after infection. Quantification of SD-IVM images; 4 hpi, computer generated
532 stitched images of $2mm^2$ were generated using the stitched image function in Volocity. Volocity
533 software was also used to quantify relative GFP fluorescence as a measurement of the presence
534 of *S. aureus*-GFP or TD-tomato for the quantification of neutrophils in the liver after
535 staphylococcal infection. SD-IVM images of uninfected mice were used to determine the

536 background fluorescence and *S. aureus*-GFP was quantified with the same settings for all mutants
537 and treatments.
538
539 **Macrophage and Neutrophil depletion**
540 Macrophages were depleted using clodronate liposomes (NvR). The mice were injected i.v. with
541 1 ml of liposomes per 100 g on day 1 as per manufacturer's instructions. The mice were then
542 injected with $1x10^5$ CFU of *S. aureus* NewHG (1:1:1 mixture of NewHG Ery^R, Tet^R or Kan^R) on day
543 2. Blank liposomes were used as a control. Macrophage depletion was confirmed using histology
544 sections of the liver stained with anti-macrophage antibody (rat anti mouse F4/80, AbD Serotec,
545 catalog number MCA497R). For antibody based neutrophil depletion, *in vivo* anti-ly6G mouse
546 antibody (1A8, BioXcell, catalog number BE0075-1) was used as per the previously published
547 protocol[56]. The mice were injected with 1.5 mg/mouse of antibody (200 µl per mouse) on day 1
548 with the mice being injected with $5x10^5$ CFU *S. aureus* NewHG (1:1:1 mixture of NewHG Ery^R, Tet^R
549 or Kan^R) on day 2. 100 µl of blood was collected via tail bleeding at the time of *S. aureus* injection
550 and at the end of the experiment via terminal anaesthesia and heart puncture. The blood samples
551 were mixed with 20 µl of Heparin each and then stained with APC Rat Anti-Mouse Ly-6G antibody
552 (BD biosciences, catalog number 560599) according to the BD bioscience protocol. The samples
553 were then processed using the BD LSRII flow cytometer to confirm neutrophil depletion.
554
555 **Histological analysis of mouse organs**
556 Carried out within a liquid nitrogen dewar, individual organs were placed in an embedding cube,
557 partially pre-filled with optimal cutting temperature (OCT) medium. Ensuring correct positioning
558 for optimal sectioning, the remaining cube was filled with medium and stored at -80°C. Pre-
559 sectioning, organs were placed at -20°C and 200 µm slices were taken before staining with
560 hematoxylin and eosin (H&E) stain as previously established[49].
561
562 **Zebrafish model**
563 Zebrafish embryos less than 5 days post fertilisation (dpf) are not protected under the Animals
564 (Scientific Procedures) Act 1986 but all zebrafish work was carried out according to the details

565 set out in Project License PPL 40/3574.

566

567 For zebrafish experiments, London wild-type (LWT) embryos were incubated in E3 medium at
568 28°C according to standard protocols[57]. Embryos were microinjected at 30 hours post fertilization
569 (hpf) into the circulation valley as previously described[10]. Following injection, embryos were kept
570 individually in 100 µl E3 medium and survival recorded for up to 90 hpi. For bacterial growth
571 experiments *in vivo*, at various time points, embryos were collected and bacterial numbers
572 enumerated as described for mouse organs above.

573

574 For microscopy, live anaesthetised zebrafish were mounted onto 15 mm petri dishes in 1% (w/v)
575 low melting point agarose and E3 solution. Images were acquired using either the TE-2000U
576 microscope (Nikon) with a Hamamatsu Orca-AG camera (objectives used: 4× Nikon Plan Fluor
577 objective NA 0.13 and 60× Nikon Plan Apo oil objective NA 1.4; fluorophores excited with either
578 488 nm (GFP) or 543 nm (mCherry)) or the UltraVIEW VoX spinning disk confocal microscope
579 (Perkin Elmer) (GFP, mCherry and Alexa Fluor® 647 were excited by the 457-51 nm argon laser,
580 561 nm sapphire laser and 642 nm diode laser, respectively). Image acquisition and processing
581 were performed with Volocity™ software.

582

583 **Statistical analysis**

584 Sample sizes were predetermined for mouse and zebrafish experiments based on previous
585 experimental data[8,10]. The refined murine sepsis model, augmenting *S. aureus* sepsis with PG,
586 increased reproducibility and decreased the spread of results. Revised power calculations (80%
587 power; 95% confidence) permitted comparisons with 5 animals per group (n=5) for both weight
588 loss (10% difference) and liver CFU (2 log difference). Animal experiments were not blinded but
589 to reduce bias, selected experiments were performed by different team researchers. Mice were
590 randomly selected for experimental or control groups and kept in separate cages throughout the
591 experiment. In instances of unexpected death in animals which had otherwise been showing
592 normal health, animals were excluded from analysis as per pre-established criteria. All statistical
593 tests were appropriate for the type of data obtained. For zebrafish embryo survival experiments,

594 the Kaplan-Meier method was employed. Comparison between survival curves was made using
595 the log-rank (Mantel Cox) test. For bacterial count comparison, skin lesion size or clinical arthritis
596 severity in murine experiments, the Mann-Whitney U and Wilcoxson signed rank tests were used
597 for comparison of unpaired and paired data, respectively. For comparison of two or more
598 independent samples (parametric) a one-way ANOVA was used with Tukey's multiple
599 comparison test. A two-way repeated measurements ANOVA compared pHrodo and OxyBURST
600 data as shown and the CFU quantification in neutrophils. For macrophage assays, a 2-way ANOVA
601 with Tukey's multiple comparison post-test was used between the first two time-points. To
602 compare the number of intracellular bacteria without and with PGN at all other time-points in
603 the macrophage assays, a paired t-test was used. Statistical analysis was performed using Prism
604 version 6.0 (GraphPad) and $P < 0.05$ was considered significant. Individual *P* values are reported.
605 Multinomial probability was calculated to compare the expected frequency of outcomes with
606 observed outcomes for strain ratios within individual abscesses in murine liver.
607
608 **Data availability**
609 The data supporting the findings of this study are available within the paper and its
610 Supplementary Information.
611

612
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