Manipulation of autophagy in phagocytes facilitates *Staphylococcus aureus* bloodstream infection.

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ABSTRACT

The capacity for intracellular survival within phagocytes is likely a critical factor facilitating *S. aureus* dissemination in the host. To date, the majority of work on *S. aureus*-phagocyte interactions has focused on neutrophils and to a lesser extent macrophages, yet we understand little about the role played by dendritic cells (DCs) in the direct killing of this bacterium. Using bone-marrow-derived DCs (BMDCs) we demonstrate for the first time that DCs can effectively kill *S. aureus,* however certain strains of *S. aureus* have the capacity to evade DC (and macrophage) killing by manipulation of autophagic pathways. Strains with high levels of Agr activity were capable of causing autophagosome accumulation, were not killed by BMDCs and subsequently escaped from the phagocyte, exerting significant cytotoxic effects. Conversely, strains that exhibited low levels of Agr activity failed to accumulate autophagosomes and were killed by BMDCs. Inhibition of the autophagic pathway by treatment with 3-Methyladenine restored the bactericidal effects of BMDCs. Using an *in vivo* model of systemic infection we demonstrated that the ability of *S. aureus* strains to evade phagocytic cell killing and to survive temporarily within phagocytes correlated with persistence in the periphery and that this effect is critically Agr dependent. Taken together our data suggests that strains of *S. aureus* exhibiting high levels of Agr activity are capable of blocking autophagic flux, leading to the accumulation of autophagosomes. Within these autophagosomes the bacteria are protected from phagocytic killing, thus providing an intracellular survival niche within professional phagocytes, which ultimately facilitates dissemination.

INTRODUCTION

Staphylococcus aureus causes a wide range of pathologies from superficial skin infections to more serious invasive infections associated with significant morbidity and mortality. In severe cases, localized infections can lead to bacterial invasion of the vascular system causing life-threatening conditions such as bacteremia and sepsis. A key factor facilitating this dissemination is the impressive arsenal of immune evasion strategies available to *S. aureus* that enables it to evade recognition and killing by the host immune system (1). Identifying and disarming the mechanisms by which this organism circumvents the host's immune system is an important strategy for identifying novel therapies.

Although classically considered an extracellular bacterium, *S. aureus* is capable of invading and persisting within a variety of non-professional phagocytic host cells (2) facilitating tissue persistence and relapsing disease. Strikingly, this organism is also capable of manipulating professional phagocytes and there is evidence that *S. aureus* can survive within monocytes, macrophages and even neutrophils (3-7). Unlike resident tissue cells, professional phagocytes are mobile and represent an opportunity for the bacterium to disseminate from the primary focus of infection to systemic sites. In a similar mechanism to that employed by traditional intracellular bacteria such as *Mycobacterium tuberculosis* and *Listeria monocytogenes* that utilize monocytes to disseminate via the bloodstream (8, 9), it has been proposed that *S. aureus* may be capable of subverting neutrophils to facilitate its dissemination (10). *S. aureus* has also been shown to persist within human

monocyte-derived macrophages (7) suggesting that these cells may also provide a potential intracellular niche to facilitate *S. aureus* dissemination *in vivo*. The bulk of the research conducted into the survival within or killing of *S. aureus* by phagocytes has focused on neutrophils and, to a lesser extent macrophages. To date, the contribution of dendritic cells to direct killing of *S. aureus*, and the capacity of *S. aureus* to manipulate these particular phagocytes has not been explored.

Despite the fact that the environment inside phagocytes is less than hospitable, gaining an intracellular niche, even briefly, within these cells affords a window of opportunity for extended survival and potential dissemination. Critical to survival is the ability to avoid destruction within phagolysosomes and *S. aureus* is equipped with a number of strategies to resist phagolysosomal killing (11-13). Having circumvented these killing mechanisms the bacterium can then escape into the cytoplasm, which in most cases, eventually leads to host cell death, releasing the bacteria into the extracellular space where it has the opportunity to replicate and infect other host cells. Phagosomal escape by *S. aureus* has been shown to depend upon the regulatory system encoded by the *agr* locus (7, 14, 15) which controls expression of a number of virulence factors including the secreted toxin α-haemolysin (Hla), a critical effector molecule essential for *S. aureus* survival within macrophages (7). Phenol-soluble modulins (PSMs) are small cytotoxic alpha-helical peptides. They are categorized into two classes, PSMα and PSMβ peptides. PSMα peptides are regulated by the Agr system and enable phagosomal escape by *S. aureus* from both non-professional (16) and

professional phagocytes (17, 18). Survival within neutrophils appears to be dependent upon the accessory regulator SarA, which facilitates the survival of *S. aureus* within large vacuoles that are not competent for fusion with lysosomes (5). While it is clear that phagocytes are critically important for effective clearance of *S. aureus* during an infection, it may be that the intracellular locale of the bacterium post-phagocytosis will dictate whether or not the phagocytes contribute to host protection or inadvertently play a deleterious role.

Autophagy is an important homeostatic process in eukaryotic cells critical for cell survival. Damaged cytosolic components are removed and recycled in double-membrane vacuoles called autophagosomes that are characterized by the recruitment of microtubule-associated protein 1 light chain 3 (LC3) conjugated to phosphatidylethanolamine (LC3-II) to its membrane (19). These autophagosomes then fuse with lysosomes and are digested. This process of autophagosome formation and eventual degradation is termed autophagic flux (20). Autophagy also plays an important role in host defense against bacteria that can invade host cells such as *Streptococcus pyogenes* (21) or facultative intracellular pathogens such as *Mycobacterium tuberculosis* (22). These organisms are sequestered in autophagosomes, which then deliver the bacteria to the lysosomes for destruction. Some microorganisms (e.g. *Coxiella burnetti* and *Porphyromonas gingivalis*) have evolved mechanisms to subvert the autophagic machinery of the cell, delaying autophagosomal maturation and lysosomal fusion thus creating a survival niche within autophagosomes (23). *S. aureus* can localize to autophagosomes and inhibit lysosomal fusion

within HeLa cells while proliferation of *S. aureus* was impaired within fibroblasts deficient in the autophagy protein Atg5 (24), indicating an essential role for the autophagy pathway in facilitating intracellular survival of *S. aureus* within non-professional phagocytic cells. In this study, a strain that expresses low levels of *agr* failed to colocalise with autophagosomal markers identifying the requirement for Agr-regulated genes to engage autophagosomes.

Whether or not *S. aureus* can manipulate the autophagic process in professional phagocytes as a means to evade killing remains to be established. We hypothesized that subversion of autophagy in professional phagocytes could provide *S. aureus* with a means to preserve a temporary intracellular survival niche, in order to facilitate dissemination. We demonstrate the strain-dependent ability of *S. aureus* to induce accumulation of autophagosomes in phagocytes, which appears to correlate with inter-strain differences in Agr expression. Strains with high levels of Agr activity became associated with autophagosomes, were not killed by phagocytic cells *in vitro* and demonstrated extended intracellular survival within phagocytes *in vivo.*

MATERIALS AND METHODS

Bacterial strains

S. aureus strains SH1000 (Clonal complex (CC) 8) and PS80 (CC30) have been previously described (25, 26). *S. aureus* clinical isolates were from blood culture bottles of patients diagnosed with *S. aureus* bacteremia at St George's Healthcare NHS Trust, London. Two isolates were used repeatedly throughout this study; Sa68 and Sa279. Both of these strains are methicillin sensitive and belong to the lineage CC1.

The expression of enhanced green fluorescent protein (GFP) (27) in the PS80 background was achieved through the integration of a non-replicative integrase vector (pIMC11-GFP) into the phage 11 attachment site. Expression of eGFP is under the control of the $P_{xyl/tetO}$ promoter, without repression from TetR. Chromosomal integration of PS80::pIMC11-GFP was validated with oligonucleotides IM293/IM294, which amplify across the site of integration yielding a 0.7kb product in PS80 and a 3.4kb product in PS80::pIMC11-GFP.

Deletion of the *agr* locus (*agrBDCA* genes) within PS80 was achieved by allelic exchange using pIMAY (28). Primers agr1 and agr2 amplified 532 bp of DNA upstream of *agrB* and primers agr3 and agr4 amplified 535 bp of DNA located downstream of the *agrA* gene (Table 1). The PCR products were denatured and allowed to reanneal via the complementary sequences in primers agr2 and agr3. This was used as template for PCR using primers agr1 and agr4. The amplimer was cloned into pIMAY (28) between SalI and EcoRI restriction sites using sequence and ligase independent cloning (29)

and the resulting plasmid (pIMAY::Δ*agr*) was transformed into DC10B and verified by DNA sequencing. The plasmid was transformed into electrocompetent PS80 and deletion of the *agr* genes was achieved by allelic exchange as previously described (28). The deletion was confirmed by DNA sequencing of a PCR amplimer generated using PS80Δ*agr* genomic DNA as template and the primers agr OUT F and agr OUT R. The mutant did not produce delta haemolysin on sheep blood agar.

All bacteria were cultivated from frozen stocks for 24 hours at 37°C on agar plates. Bacterial suspensions were then prepared in PBS and the concentrations estimated by measuring the absorbance of the suspension read at 600nm. CFUs were determined by plating serial dilutions of each inoculum.

In the case of PS80-GFP, log phase growth was required for optimal GFP expression. A single colony was inoculated into TSB overnight and a subculture to fresh TSB taken the following morning. The concentration of bacteria in the broth was determined by measuring absorbance at 600nm and confirmed by streaking on agar plates.

For immunoflourescent analysis, bacteria were stained with Cell Trace Violet (CTV, Life Technologies). Stationary phase bacteria in PBS at the appropriate OD were incubated with CTV for 20min at 37°C under rotation. They were then washed and resuspended in PBS prior to infection of cells.

Animals

Groups of wild type C57BL/6 mice (6-8 weeks) were housed under specific pathogen free (SPF) conditions in the Trinity College Dublin Comparative Medicines facility. All animal experiments were conducted in accordance with the recommendations and guidelines of the Health Products Regulatory Authority (HPRA), the competent authority in Ireland, and in accordance with protocols approved by Trinity College Animal Research Ethics Committee.

Cell Culture

Bone marrow derived dendritic cells (BMDCs) were prepared by culturing bone marrow cells isolated from C57Bl/6 mice with granulocyte-macrophage colony stimulating factor (GM-CSF) as described previously (30). On day 10, loosely adherent cells were collected, washed and reseeded at a concentration of $2x10^5$ cells/well in media without antibiotic, and rested overnight.

Peritoneal macrophages were isolated as previously described (31) and seeded at $2x10^5$ cells/well in media containing no antibiotics.

Immortalized Bone Marrow derived Macrophages (iBMM) stably expressing EGFP-LC3 (GFP-LC3) (32) were cultured in cRPMI (complete Roswell Park Memorial Institute) media under constant selection with 10µg/ml puromycin. Cells were seeded at 1 x 10^6 cells/well on poly-L-lysine coated 19mm coverslips in 12 well plates.

Infection of phagocytes

Cells were infected with live *S. aureus* at multiplicities of infection (MOI) of 10 or 100 for the indicated times. In some cases, prior to infection cells were incubated with 10mM 3-Methyladenine (3-MA, Sigma) for 30min. At 2 hours post infection media was replaced with fresh media containing gentamicin (200 μg/ml) for 1 hour to kill extracellular bacteria. This media was replaced with fresh media containing no antibiotics and this was considered time 0.

For assessment of total killing, cells were infected with live *S. aureus* at MOI 10 or 100 for the indicated time points and were not gentamicin treated.

Assessment of bacterial killing

At the indicated time point, infected cells were spun down, the supernatant removed and cells lysed by the addition of 20 μl 0.1% Triton-X 100. The supernatant was then re-introduced into the well and mixed with the cell lysate. Serial dilutions of the suspension were prepared in PBS and plated on TSA to determine the CFU/ml. Bacterial killing was determined as the % reduction of CFU in wells containing bacteria and phagocytes as compared to wells containing bacteria only.

Assessment of bacterial escape

S. aureus infected BMDCs underwent gentamicin treatment as described above. At specific time points the cell free supernatants were collected, serially diluted in PBS and plated on TSA to determine the number of bacteria

241 that had escaped into the media, measured as the fold increase in Log
242 CFU/well from time 0.
243
244 **Cell viability assays**
245 To assess *S. aureus* induced cytotoxicity, BMDCs were infected and treated
246 with gentamicin as described above. LDH release was measured using the
247 Pierce LDH Cytotoxicity Assay kit (Thermo Scientific) according to the
248 manufacturer's instructions. In some cases cell viability was assessed by the
249 addition of Propidium Iodine (PI, 1 µg/ml (eBioscience)) and analysis by flow
250 cytometry.
251
252 **Vesicle Lysis Test**
253 Phospholipid vesicles were prepared as described previously (33). Vesicle
254 lysis test (VLT) was performed using a 1:1 ratio of bacterial supernatant
255 (cultures grown for 18 h) and pure vesicles and fluorescence intensity
256 measured at excitation and emission wavelengths of 485-520 nm respectively
257 on a FLUOstar fluorometer (BMG labtech). Positive and negative controls
258 were pure vesicles with 0.01% Triton X-100 and HEPES buffer, respectively.
259
260 **Measurement of RNA III expression by qRT-PCR**
261 *S. aureus* RNA was isolated using the RNeasy Mini Kit (Qiagen) according to
262 the manufacturer's instructions with the addition of turbo DNase (Ambion)
263 following the purification step. RNA was quantified using RNA BR kit (Qubit)
264 and reverse transcription was performed using the ProtoScript Taq RT-PCR
265 kit (New England Biolabs) according to manufacturer's instructions using

266 random primers. Standard curves were generated for both gyrase B [*gyr*FW:
267 5'-CCAGGTAAATTAGCCGATTGC-3'; *gyr*RV: 5'AAATCGCCTGCGTTCTAGA
268 G] and RNAIII primers [*rna*IIIFW: 5'- GAAGGAGTGATTTCAATGGCACAAG-
269 3'; *rna*IIIRV: 5' GAAAGTAATTAATTATTCATCTTATTTTTTAGTG AATTTG-3']
270 using genomic DNA to determine primer efficiency. Real-time PCR was
271 performed using the SYBR green PCR master mix (Applied Biosystems) as
272 previously described (33).
273
274 **Western immunoblotting**
275 To detect LC3, BMDCs were infected and treated with gentamicin as
276 described above. At specified time points BMDCs were lysed in NP-40 lysis
277 buffer. The protein concentration of the lysates was measured using a
278 Bradford assay (Thermo Scientific) and equal concentrations of protein were
279 loaded to each lane of the gel. Samples were separated on a 15% SDS-
280 polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF)
281 membrane. The membrane was blocked with 5% (w/v) milk before being
282 probed with antibody (rabbit anti-LC3 - 1/1000 (Cell Signaling), HRP-
283 conjugated goat anti-rabbit immunoglobulin G (IgG) - 1/10000 (Jackson
284 Immune)). The membrane was developed with ECL (Mybio) on Bio-Rad
285 GelDoc.
286 To detect Hla expression, proteins from filtered bacterial supernatant were
287 concentrated by trichloracetic acid precipitation, separated on a 12.5% SDS-
288 polyacrylamide gel and transferred to PVDF. The membrane was blocked
289 in 10% (w/v) milk and probed with polyclonal rabbit anti-Hla IgG (1:1000, (34))
290 followed by HRP-conjugated protein A (Sigma). Reactive bands were

visualized using the LumiGLO reagent and peroxide detection system (Cell Signaling Technology).

Confocal imaging

BMDCs were infected, gentamicin treated as described above and Monodansylcadaverine (MDC, 50μM) added 15 minutes prior to cell fixation. Cells were then fixed in 2% paraformaldehyde (PFA (Thermo Scientific)). Alternatively, GFP+LC3-BMMs were infected and treated with gentamicin as described above. At specific time points post-infection, cells were fixed in 2% PFA and permeabilised in Triton-X100 (0.1% in PBS). Non-specific binding was blocked by incubation in 5% BSA before cells were incubated with Alexa Fluor 555 - conjugated phalloidin (1/100 (Life Technologies)) for 1 hour to stain actin.

The coverslips were mounted onto glass slides with fluorescent Mounting medium (DakoCytomation) and analyzed on an Olympus FV1000 laser scanning confocal microscope.

***In vivo* intraperitoneal infection model**

Mice were infected with *S. aureus* (5 x 10^8 CFU) via intraperitoneal (i.p.) injection. At specific time points post-infection peritoneal exudate cells (PEC) were isolated by lavage of the peritoneal cavity with sterile PBS. Lavage fluid was serially diluted in PBS and plated on TSA to determine the bacterial burden at the site of infection. Spleens were isolated and homogenized in 2ml of sterile PBS. Tissue homogenates were then serially diluted in PBS and plated on TSA to determine the tissue bacterial burden. Blood was collected

by cardiac puncture with a 27-gauge needle and a heparinized 1ml syringe. The CFU/ml of blood was determined by serial dilution and plating on TSA plates.

To isolate leukocytes, blood was layered onto Histopaque-1083 (Sigma) for density gradient centrifugation. Leukocytes were collected between the plasma layer and the pellet containing red blood cells (RBCs) and extracellular bacteria (35). Isolated leukocytes were then washed well and resuspended in Fcγ block for flow cytometric analysis or lysed in sterile water to quantify cell-associated CFU.

Flow cytometry

PEC or blood leukocytes were blocked in Fcγ block (1µg/ml, eBioscience) then surface-stained with fluorochrome-conjugated antibodies against Ly6G (clone 1A8, BDBioscience), F4/80 (clone BM8, eBioscience), CD11c (clone N418, eBioscience) and CD11b (clone M1/70, eBioscience). Flow cytometric data were acquired with a BD FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

To assess the rate of *S. aureus* phagocytosis by BMDCs, cells that had been infected with CTV-labeled *S. aureus* for 30 minutes or 2 hours, were incubated with gentamicin (200µg/ml) for 1 hour, washed and fixed in 2% PFA. They were then analyzed on BD FACSCanto II by gating on Forward-Scatter and Side-Scatter and % CTV+ cells assessed.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism statistical analysis software. Differences between groups were analyzed by the unpaired Students t test or analysis of variance (ANOVA) with appropriate post-test and using repeated measurers where required. $P<0.05$ was considered statistically significant.

RESULTS

Killing of *S. aureus* by dendritic cells and macrophages is strain dependent.

Despite the fact that dendritic cells have been shown to be involved in coordinating the immune response to *S. aureus* infection, their contribution to direct bacterial killing remains to be fully established (36, 37). We compared the bactericidal capacities of these phagocytic cells to that of macrophages which have a more clearly defined role in direct killing of *S. aureus* (38). Primary BMDCs were infected with two laboratory strains of *S. aureus* at an MOI 10 (Figure 1A) and MOI 100 (Figure 1B) and bacterial killing was monitored over time. Within 6 hours of infection, approximately 70% of SH1000 was killed and by 16 hours almost 100% of SH1000 had been killed by the BMDCs at either MOI. In contrast, the BMDCs were unable to kill *S. aureus* strain PS80.

Interestingly, the ability of BMDCs to kill SH1000 appeared to be MOI-dependent. It was reported previously that BMDCs were unable to kill SH1000 at an MOI 0.1 (36). We also failed to detect any killing of SH1000 by BMDCs at this low MOI but the ability of BMDCs to kill SH1000 by 16 hours became apparent at as low an MOI as 2 (97.7±1.7% killing).

To establish if the inability to kill *S. aureus* strain PS80 was specific to dendritic cells, we infected primary peritoneal macrophages with both strains of *S. aureus* at MOI 100. Similar to that observed with the BMDCs, peritoneal macrophages efficiently killed SH1000 but were unable to kill PS80 (Figure

1C). Interestingly, in our hands BMDCs and macrophages demonstrated a similar capacity to kill *S. aureus* strain SH1000 (the % killing at 16 hours was 90±6.8% in BMDCs compared to 78.3±6.6% in macrophages). Taken together these results suggest that BMDCs are capable of killing *S. aureus* but that strain-dependent differences may impact upon the ability of both macrophages and BMDCs to kill the bacterium.

S. aureus strain PS80 but not SH1000 can escape from dendritic cells causing associated cytotoxicity.

Given that BMDCs had a different capacity to kill *S. aureus* strains PS80 and SH1000, we wanted to confirm that both strains were phagocytosed by BMDCs at the same rate. BMDCs were infected with CTV-labeled *S. aureus* at MOI 100, and the uptake of bacteria into the BMDCs assessed after 30 minutes and 2 hours, following gentamicin treatment to kill any bacteria that had not been phagocytosed. At 30 min post infection PS80 and SH1000 were phagocytosed by BMDCs to the same extent, with ~30% of BMDCs staining positively for CTV-labeled PS80 or SH1000 (Figure 2A). At 2 hours post infection the % of cells that were CTV-PS80+ increased, alluding to the survival of this strain inside the cells.

S. aureus strains SH1000 and PS80 were both phagocytosed by BMDCs to the same extent but following phagocytosis PS80 was not killed. To establish whether PS80 escaped from the BMDCs, cells were allowed to phagocytose the bacteria and any extracellular bacteria were killed by the addition of the bactericidal antibiotic gentamicin. Cells were washed and incubated in fresh

medium and the escape of viable bacteria into the supernatant was measured after 6 and 12 hours incubation. By 6 hours there was evidence of PS80 but not SH1000 escaping from the BMDCs. By 12 hours the level of PS80 in the cell culture supernatant significantly higher than SH1000 (Figure 2B). Similar results were obtained following infection with MOI 10 (data not shown).

To establish if the escape of *S. aureus* from BMDCs was associated with cytotoxicity, LDH release from the infected BMDCs was measured. LDH activity was similar in uninfected BMDCs and BMDCs infected with SH1000 at both 6 h and 12 h post-infection, indicating that SH1000 had no effect on the viability of the infected cells. In contrast, BMDCs infected with PS80 had significantly higher levels of LDH in the supernatant compared to cells infected with SH1000 or uninfected BMDCs at both time points (Figure 2C), indicating significant cytotoxicity.

Identification of *S. aureus* bloodstream infection isolates with the ability to escape phagocytic killing.

S. aureus PS80 and SH1000 are both well-characterized laboratory strains. However, their relevance to clinical isolates may be limited. Accordingly, isolates that were recovered from *S. aureus* bacteremia patients were collected and screened for cytotoxic effects. BMDCs were infected with each isolate at MOI 100 and the viability of the infected DCs was assessed after 24 hours by staining with PI. The clinical isolates clustered together into one group that was cytotoxic to BMDCs in a similar manner to PS80, a second

group that did not exert any cytotoxic effects, akin to SH1000 and a third, intermediate group (Figure 3A).

A representative isolate from both the "PS80-like" group and the "SH1000-like" group were selected for analysis, *S. aureus* 68 (Sa68) and *S. aureus* 279 (Sa279). BMDCs were infected with Sa68 or Sa279 at MOI 100. The BMDCs were capable of killing strain Sa279 but were unable to kill strain Sa68 (Figure 3B). This data suggests that Sa68 is similar to PS80 and may be capable of escaping from phagocytes. We confirmed that both strains were phagocytosed by BMDCs at a similar rate by CTV-labeling the bacteria and infecting BMDCs as described above. Similar to the uptake of PS80 and SH1000, approximately 30% of BMDCs were associated with CTV+ Sa279 or Sa68 by 30 min post infection (Figure 3C). We then assessed the ability of Sa68 to escape from the BMDCs. After 12 hours the level of Sa68 in the cell culture supernatant was significantly higher than Sa279 (Figure 3D).

To establish if the ability of Sa68 to escape from the BMDCs correlated with cytotoxicity, cells were infected with Sa68, Sa279 or left uninfected. Following gentamicin killing of extracellular non-phagocytosed bacteria, the LDH release was monitored at 6 h and 12 h. The level of cytotoxicity (LDH release) associated with Sa68-infected cells was significantly higher than that of Sa279-infected cells or the uninfected control cells (Figure 3E).

Infection with PS80, but not SH1000, was associated with increased accumulation of LC3-II+ autophagosomes.

S. aureus has previously been shown to associate with autophagosomes in non-professional phagocytic cells. This provides a niche for the intracellular survival of *S. aureus* where it could replicate and eventually escape into the cytoplasm, ultimately leading to host cell death (24, 39). We postulated that *S. aureus* strain PS80 might employ a similar mechanism in BMDCs to evade killing. To assess autophagy in BMDCs, cells were infected and lysates prepared at intervals up to 6 hours post-infection and gentamicin killing of extracellular bacteria. Processing of the autophagic marker LC3 was then assessed by Western immunoblotting (40). Infection of BMDCs with *S. aureus* strain PS80 resulted in the persistence of substantial levels of LC3-II for at least 6 hours. In comparison, uninfected BMDCs or BMDCs infected with SH1000 showed no accumulation of LC3+ autophagosomes, although there was persistently a low level of LC3-II processing which was presumably due to homeostatic autophagy followed by autosome-lysosome fusion and degradation of LC3 (Figure 4A).

To confirm that PS80 was associating with autophagosomes, BMDCs were infected with CTV-labeled *S. aureus* strains PS80 or SH1000 and then treated with gentamicin to kill any extracellular bacteria. Staining with MDC (a fluorescent compound which accumulates specifically in autophagic vacuoles (41)) revealed colocalisation between PS80 and the autophagosome. SH1000 was not seen to colocalise to the same extent (Figure 4B). Additionally, RAW 264 macrophages that had been stably transfected with GFP-LC3 (42) were infected and gentamicin treated as above. Again, CTV-labeled PS80 was seen to colocalise with GFP-LC3 punctae at 3 hours post infection. In

comparison, SH1000 did not show the same level of association with GFP-LC3 punctae (Figure 4C).

To confirm that clinical isolates could also manipulate the autophagic process BMDCs were infected with Sa68 or Sa279 and lysates prepared after 6 hours. Processing of LC3 was assessed by Western immunoblotting. Similar to PS80, Sa68 infected cells had considerable levels of LC3-II present indicating a delay in the degradation of the autophagosomes. In addition, the level of LC3-II in Sa279 infected cells was similar to SH1000 infected cells or uninfected BMDCs, suggesting that these cells had normal autophagic flux (Figure 5A).

Engagement of autophagosomes facilitates escape of *S. aureus* from phagocytes.

To ascertain if the delay in turnover of autophagosomes was associated with the ability of *S. aureus* strains PS80 and Sa68 to escape phagocyte killing, BMDCs were pre-treated with 3-methyladenine (3-MA), a well-established PI3K inhibitor that inhibits the induction of autophagy (43), prior to infection with these two strains. The escape of *S. aureus* into the supernatant was then assessed at 6 and 12 hours. In the presence of 3-MA, PS80 and Sa68 escape into the cell culture supernatant was completely inhibited (Figure 5B). Associated with this, 3-MA treatment restored the viability of the infected BMDCs, with the level of LDH activity in the culture supernatant being significantly reduced following infection with both PS80 and Sa68 (Figure 5C). Importantly, 3-MA had no direct effect on bacterial viability after 18 hours

incubation (7.90±0.13 vs. 7.55±0.39 Log CFU/ml, for *S. aureus* alone vs. *S. aureus* + 3-MA).

Differential expression of Agr by *S. aureus* strains correlates with their ability to engage autophagosomes.

It has previously been shown that the ability of *S. aureus* to divert from the endosomal pathway to autophagosomes is driven by factors that are under the control of the Agr regulatory system (24). We hypothesized that the different abilities of strains to delay autophagic flux may be associated with the level of expression of Agr. Consequently, Agr activity was measured using a vesicle lysis test (VLT). This assay measures the interaction of PSM toxins with lipid vesicles (33). The PSMα peptide δ-toxin is translated from a short open reading frame located within the regulatory RNAIII molecule while transcription of the other *psm* genes is activated directly by the AgrA response regulator of the Agr two component signal transduction system that responds to high cell density. Expression of these membrane-damaging toxins is a direct manifestation of the level of expression of Agr in the stationary phase of growth (44). *S. aureus* strains PS80 and Sa68 induced significantly more vesicle lysis than SH1000 and Sa279 (Figure 6A) suggesting a greater level of Agr activity in these strains. To further assess the expression of Agr, RNAIII was measured. Consistent with the VLT, RNAIII was expressed at higher levels by *S. aureus* strains PS80 and Sa68 as compared to SH1000 and Sa279 (Figure 6B). Taken together we can conclude that the *S. aureus* strains PS80 and Sa68 that induce autophagosome accumulation, exhibit a greater

level of Agr activity than SH1000 and Sa279 which have no effect on autophagosomes.

Deletion of the *agr* locus prevents LC3-II accumulation and facilitates bacterial killing.

In order to investigate if strain dependent differences in bacterial killing and the delay of normal autophagic flux were under the control of Agr regulated genes we generated an *agr* mutant strain of PS80 by allelic exchange. BMDCs were infected with PS80 and PS80Δ*agr* and bacterial killing was monitored over time. By 6 h post infection almost 100% of PS80Δ*agr* were killed (Figure 7A) as compared to the parental strain, which failed to be killed. Furthermore, the escape of PS80 from the BMDCs was significantly inhibited in the absence of *agr* (1.29±0.28 fold increase in Log CFU/well from T0 PS80 versus 0.52±0.12 fold reduction in Log CFU/well compared to T0 PS80Δ*agr*) 12 hours post infection.

In addition, the accumulation of LC3-II in infected BMDCs was also measured after 6 h of infection with PS80 or PS80Δ*agr*. LC3-II expression was reduced in cells infected with PS80Δ*agr* compared to the wild type, further proving that the *agr* locus plays a role in PS80's ability to block autophagic flux. However, the LC3-II processing was not reduced to baseline levels (Figure 7B), suggesting that PS80 may be expressing alternative, non-Agr regulated genes, which have some capacity to delay autophagic flux.

Agr influences *S. aureus* persistence *in vivo*.

546 Having established that both laboratory and clinical strains of *S. aureus* can
547 subvert autophagy to evade phagocytic killing, it was important to determine
548 whether this phenomenon affected infection outcome *in vivo*. Groups of wild-
549 type mice were infected with *S. aureus* strains PS80, SH1000, Sa68, Sa279
550 or PS80Δ*agr* by i.p. injection. At 3 hours post challenge blood was collected
551 and total bacterial burden in the blood was quantified (Table 2). As expected,
552 there were significant differences in the bacterial burdens in the blood
553 following infection with different strains. It has previously been documented
554 that strains of *S. aureus* expressing Capsular Polysaccharide (CP) seed the
555 blood stream from the peritoneal cavity in greater numbers than acapsular
556 strains (45, 46). PS80 is known to express CP 8 (25), SH1000 and PS80Δ*agr*
557 are a-capsular (26, 47) and the CP-expression of the clinical strains are
558 unknown. In order to prove that the differential abilities of these strains to
559 seed the blood were not simply due to differences in CP expression levels,
560 mice were infected with PS80 or an isogenic mutant of PS80, RMS-1 that is a-
561 capsular (46). 3 hours post infection blood was isolated and total bacterial
562 burden quantified. There was no significant difference in the levels of bacteria
563 recoverable from the blood between the two groups (PS80 v RMS-1; 4±0.2 v
564 3.7±0.1 Log CFU/ml), confirming that the observed differences in bacteremia
565 levels were not as a result of differential CP expression.
566
567 To prove that differences in bacterial burden in the blood were due to the
568 differential abilities of individual strains to survive intracellularly, mice were
569 infected with *S. aureus* strains PS80, SH1000, PS80Δ*agr*, Sa68 or Sa279. At
570 3 hours post infection the total leukocytes were separated from the RBC and

extracellular bacteria by centrifugation through Histopaque 1083. Leukocytes were then washed thoroughly and lysed to quantify viable intracellular *S. aureus*. The number of intracellular bacteria recovered was significantly higher in PS80 infected animals compared to PS80Δ*agr* (Figure 8A) or SH1000 (Figure 8B) infected animals. The same trend was seen in the clinical strains, with significantly higher levels of Sa68 recovered from the blood leukocytes in comparison to Sa279 (Figure 8C). This suggests that PS80 and Sa68 are capable of surviving within phagocytes *in vivo*, potentially facilitating systemic dissemination and persistence. Consistent with this, animals infected with PS80 demonstrated a significantly increased bacterial burden in the spleen at 12 hours post challenge as compared to the animals infected with the PS80Δ*agr* (Figure 8D) or SH1000 (Figure 8E). Unfortunately, due to limitations in cell numbers we were unable to analyze autophagic flux in individual blood leukocyte populations *ex vivo*.

Finally, to establish which specific leukocyte populations in the blood were harboring intracellular *S. aureus,* GFP-expressing PS80 was injected into the peritoneum. At 3 hours post infection total leukocytes were isolated from the blood. These leukocytes were stained with a panel of antibodies against various surface markers in order to identify the phagocyte populations containing intracellular bacteria. As expected, the predominant cell type associated with GFP-expressing PS80 was found to be PMN. Surprisingly, DCs accounted for the cell type that contained the second largest population of PS80-GFP+ cells. In contrast, only a low number of monocytes were associated with PS80-GFP+ (Figure 8F). This supports the contention of this

study that DCs play an important direct role in phagocytosis and clearance of *S. aureus*.

Discussion

Undoubtedly, the success of *S. aureus* as a pathogen can be attributed to its inherent ability to disarm the host's protective immune responses. In particular, *S. aureus* possesses a unique arsenal of virulence factors that can circumvent the bactericidal effects of phagocytes and can manipulate these cells, even parasitizing them to facilitate an intracellular lifestyle. Here we provide significant new insights into the molecular mechanisms involved. Analysis of several *S. aureus* strains revealed that, despite being phagocytosed to similar extents, some strains could elude phagocytic killing, subsequently lysing phagocytes and escaping. The ability to evade killing was directly associated with the capacity of these strains to inhibit normal autophagic flux within the cells. We showed that the ability of *S. aureus* to subvert autophagic pathways and survive within phagocytes is associated with Agr activity as strains with lower levels of Agr exhibited normal, homeostatic turnover of autophagosomes. Moreover, we established that the level of Agr expression is directly linked with the ability of *S. aureus* to survive intracellularly within phagocytes *in vivo*, suggesting that this phenomenon is related to the ability of *S. aureus* to subvert autophagy.

Previous studies have documented a protective role for dendritic cells during *S. aureus* infection. Depletion of dendritic cells was associated with increased mortality during *S. aureus* blood stream infection (36), and impaired bacterial clearance in a *S. aureus* pneumonia model (37). In both cases the beneficial effects afforded by dendritic cells were dependent upon their ability to control the inflammatory response. In this study, we demonstrated for the first time

624 that dendritic cells also have the potential to contribute to host protection by
625 directly killing *S. aureus*. The bactericidal effects of dendritic cells were found
626 to be comparable to those of macrophages, with both cell types being
627 effective in reducing growth of *S. aureus* strain SH1000. Consequently
628 we chose to use DCs as a representative phagocyte to investigate the
629 mechanisms by which *S. aureus* can parasitize these cells. Of note, our
630 finding contrasted with a previously published study, which concluded that
631 BMDCs do not play a major role in direct killing of *S. aureus* (36). In that
632 study, dendritic cells were infected with *S. aureus* at a very low ratio (MOI
633 0.1). Given that the uptake of bacteria by macrophages has been directly
634 linked to MOI (48), we hypothesized that bacteria must reach a critical
635 threshold to ensure appropriate activation of the phagocyte before phagocytic
636 killing can occur. To test this, dendritic cell killing assays were repeated using
637 SH1000 at an MOI 0.1 and no killing was observed. However the ability of
638 BMDCs to kill SH1000 by 16 hours became apparent at as low an MOI as 2
639 (97.7±1.7% killing).
640
641 Our previous work demonstrated that *S. aureus* strains SH1000 and PS80
642 possess distinct capacities to activate innate signaling pathways in dendritic
643 cells resulting in different levels of IL-1β production (49). Accordingly, we
644 wanted to dissect the interaction of these particular strains with dendritic cells.
645 Interestingly, while both primary BMDCs and peritoneal macrophages were
646 able to kill *S. aureus* strain SH1000 they lacked the ability to kill PS80. PS80
647 avoided the bactericidal effects of phagocytes and instead escaped from the
648 cells by inducing cell death. In contrast, once phagocytosed, SH1000 did not

escape from the phagocyte, and cells that ingested this strain remained viable for up to 24 hours post infection. Importantly both strains of *S. aureus* were efficiently phagocytosed by the dendritic cells, implying that manipulation of the phagocyte response by PS80 was exerted once it became intracellular.

S. aureus strain PS80 has previously been shown to survive intracellularly within neutrophils isolated from *S. aureus* surgical site infections (50). We have now demonstrated that PS80 establishes its intracellular survival niche within phagocytes through subversion of the autophagic pathway. Following infection of BMDCs, PS80 prevented the constitutive degradation of autophagosomes by lysosomes, leading to the accumulation of LC3-II. In contrast, *S. aureus* strain SH1000 did not interfere with the homeostatic turnover of the autophagic machinery. Furthermore, BMDCs that had been treated with MDC (which accumulates in the autophagosome) post-infection, showed colocalisation between the autophagosome and PS80 but not SH1000. In addition, macrophages that were stably transfected with GFP-LC3 also demonstrated colocalisation of PS80 with LC3-II punctae, indicating the interaction of the bacterium with autophagosomes. Upon invasion of non-professional phagocytes *S. aureus* has been shown to subvert autophagy enabling replication within the autophagosome, and subsequent lysis of the host cell (24). Consistent with this we have demonstrated that the cytotoxic effects exerted by *S. aureus* strain PS80 on BMDCs are associated with the subversion of autophagy. Treatment of BMDCs with the autophagy inhibitor 3-MA protected cells from PS80-induced cytotoxicity and simultaneously prevented escape of the bacterium from the phagocyte.

Importantly, bloodstream infection isolates with comparable phenotypes to PS80 and SH1000 were identified, highlighting the clinical relevance of this phagocyte evasion strategy for facilitating systemic infection. Similar to PS80, Sa68 was not killed by BMDCs and could escape from the cells causing associated cytotoxicity. In contrast, Sa279 behaved more like SH1000 and was killed by the BMDCs. This was consistent with the observation that Sa68 induced significant LC3-II accumulation in BMDCs, while inhibition of autophagy using 3-MA reduced escape of Sa68 from BMDCs.

The ability of *S. aureus* to subvert autophagy in non-phagocytic cells is controlled by the Agr system and has been shown to specifically depend upon Agr regulated expression of α-toxin (Hla) (24, 39). *In vitro*, *agr* and *hla* mutants of *S. aureus* fail to trigger autophagy, are delivered efficiently to the lysosome where they are degraded and thus cannot survive intracellularly for extended periods. However, a recently published *in vivo* study has shown that while autophagy plays an important role in conferring protection against *S. aureus* lethality by mediating tolerance towards the cytotoxic effects of Hla, infection with a Hla mutant strain actually caused increased bacterial burden in wild-type mice in comparison to Atg16L1^{HM} mice (that display reduced autophagy). This indicates that Hla may actually be dispensable in the exploitation of autophagy in the context of intracellular bacterial survival (51). Interestingly, when we profiled Hla expression among our strains, it did not correlate with the abilities of these stains to inhibit autophagic flux in phagocytes. *S. aureus* strains PS80 and Sa68 were comparable in their abilities to manipulate autophagy in order to evade phagocytic killing however

PS80 was a high Hla producer whereas Sa68 was Hla negative. Furthermore, SH1000 and Sa279 are both killed by DCs and fail to accumulate autophagosomes but SH1000 does express low levels of Hla and there is no expression detectable in Sa279 (Supplemental Fig 1). *S. aureus* strains PS80 and Sa68 that evade phagocytic killing through the subversion of autophagy did express higher levels of Agr RNAIII and membrane-damaging cytolytic peptide toxins compared to SH1000 and Sa279 which did not have any appreciable effect on autophagy and were killed by the phagocytes. Crucially, we have also shown that Agr activity dictated the ability of *S. aureus* to survive within phagocytes *in vivo*. Using an *agr* mutant of PS80, we demonstrated a reduced ability of PS80Δ*agr* to survive within leukocytes isolated from the peripheral blood following systemic challenge compared to wild-type PS80. In addition SH1000 (which exhibited reduced Agr activity) also had a significantly reduced capacity to survive within peripheral blood leukocytes *in vivo* confirming that the inability of PS80Δ*agr* to survive in the phagocytes is not an artifact of the mutation to *agr*. Similarly, the clinical strain Sa279 (which also exhibits reduced Agr activity) shows significantly reduced survival in the circulating leukocytes in comparison to Sa68. It appears that the Agr-dependent predilection of PS80 and Sa68 for associating with autophagosomes enables them to survive within circulating leukocytes thus potentially increasing their capacity for systemic dissemination. Consistent with this, bacterial burdens in the spleen were significantly elevated in PS80 infected mice as compared to animals infected with PS80Δ*agr* or SH1000 suggesting that intracellular survival in the autophagosome facilitates increased persistence in the periphery of the host.

Until this study Hla was the only known *S. aureus* virulence factor implicated in the induction of autophagy (39). However, the pattern of Hla expression between the strains used in this study was not sufficient to explain the phenotypes observed and it raises the question whether other Agr-regulated factors might also be capable of manipulating autophagy. Intriguingly, the VLT used to assess Agr activity measures PSM activity in culture supernatants of *S. aureus* and the pattern of vesicle lysis corresponds exactly with the observed phenotypes (33). Thus it is tempting to speculate that these toxins may also have an as yet undocumented role in the induction of autophagy in phagocytic cells. Interestingly, melittin, a component of bee sting venom that is an α-helical, amphipathic antimicrobial peptide, similar to δ-toxin (52) has previously been shown to induce autophagic cell death in trypanosomes (53). In addition αPSMs trigger phagosomal escape by *S. aureus* in the monocytic cell line THP-1s (17), allowing the bacteria to replicate in the cytoplasm, leading to cell lysis (18). Autophagy has been shown to respond to both bacteria in the cytosol and within damaged phagosomes (54) supporting the notion that certain strains of *S. aureus* deliberately induce autophagy by causing damage to the phagosome. Then, by inhibiting the digestion of the autophagosomes by the lysosomes they survive within the autophagosome. A comprehensive analysis of the role played by PSMs in the induction of and engagement with autophagic pathways is warranted but is beyond the scope of this current study.

The precise mechanism by which *S. aureus* subverts autophagosomes has yet to be defined. It has previously been shown that autophagosomes may

form around a phagosome that has been damaged by internalized bacteria such as *Salmonella enterica* (55), suggesting that both strains of *S. aureus* may be phagocytosed normally but that PS80 may then damage the phagosome deliberately in order to secrete itself within an autophagosome. Alternatively, Gresham et al. have suggested that *S. aureus* can be taken up unconventionally by neutrophils via macropinocytosis into "large spacious vacuoles" (5). Other studies have shown that autophagy proteins can be recruited to single-membrane vacuoles such as macropinosomes (56). This may suggest an alternative internalization route for certain strains of *S. aureus.* While some strains are phagocytosed and killed by phagolysosomal fusion others may become internalized via macropinocytosis, which facilitates subversion of autophagic pathways in order to promote their survival.

Interestingly, PS80 can survive within several different phagocytic cell types *in vivo*. Consistent with previous studies (5, 50) we showed that neutrophils are the main intracellular reservoir for *S. aureus*. However DCs showed higher levels of viable intracellular bacteria than monocytes, further supporting our belief that these cells are critical in regulating the outcome of *S. aureus* infection. The primary role of DCs is to migrate to the lymph node following antigen uptake in order to activate the adaptive immune response. Therefore the ability to survive within these cells may be an attractive route of dissemination for *S. aureus*.

This study contributes to the growing literature that links subversion of autophagosomes by *S. aureus* with intracellular survival (24, 39). Our data

demonstrates that *S. aureus* strain PS80 and a comparable clinical isolate that express high levels of Agr prevent constitutive degradation of LC3-II+ autophagosomes in order to survive and escape killing by professional phagocytes. Strains that had a lower level of Agr expression did not affect the degradation of autophagosomes in BMDCs and were efficiently killed. This study implicates autophagy as a mechanism to facilitate temporary intracellular survival of certain *S. aureus* strains within different phagocytes, maximizing their potential for dissemination and persistence *in vivo*.

The notion that *S. aureus* could parasitize neutrophils to facilitate dissemination has already been proposed (57) and our studies support the hypothesis that other phagocytes may also act as “Trojan horses” for the metastasis of *S. aureus* provided that the infecting organism possesses the appropriate tools to subvert autophagosomes. Given that our findings were replicated in clinically relevant strains, it is tempting to speculate that identifying *S. aureus* isolates which can inhibit autophagic flux by measuring Agr activity may predict invasive disease potential.

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Table 1: Primers used in deletion of *agr* from PS80.

IM293	TATACCTCGATGATGTGCATAC
IM294	GCTGATCTAACAATCCAATCCA
agr1	CCTCACTAAAGGGAACAAAAGCTGGGTACCACTCTACTAG CAAATGTTACTC
agr2	CAAACTGGTCAATTTTGTTATC
agr3	CACATCGGTTGCTAAAATCCTTAATAAGATAATAAAGTCAG TTAAC
agr4	CGACTCACTATAGGGCGAATTGGAGCTCAGGATTTTAGCA ACCGATGTG
agr OUT F	AATACATAGCACTGAGTCCAAG
agr OUT R	GGGATGCCTTTATTGGTGCAG

Table 2: Bacterial burden in the blood

	PS80	SH1000	Sa68	Sa279	PS80Δ*agr*
Log CFU/ml (Mean±SEM)	3.63±0.12	2.93±0.26	3.92±0.39	2.37±0.49	2.28±0.29
Significance compared to PS80	n.s.	p<0.01 **	n.s.	p<0.001 ***	p<0.0001 ***

Figure Legends

Figure 1: Killing of *S. aureus* by dendritic cells and macrophages is strain dependent.

BMDCs were infected with PS80 or SH1000 at an MOI of 10 (A) or 100 (B). Alternatively, peritoneal macrophages were infected with either strain at MOI 100 (C). % killing of bacteria was determined by comparing the total CFU in the presence of phagocytes to bacteria in media only. Results expressed as mean ± SEM at each time point, n=3/4. *p<0.05, ***p<0.001 compared to other strain by repeated measures two-way ANOVA with Bonferroni post-test.

Figure 2: *S. aureus* strain PS80 but not SH1000 can escape from dendritic cells causing associated cytotoxicity.

BMDCs were infected with either CTV-labeled PS80 or SH1000 at MOI 100. % uptake of bacteria was measured at 30 min or 2 hours (A). Following infection of BMDCs with PS80 or SH1000 at MOI 100, escape of each strain

into the cell culture media was assessed at 6 hours and 12 hours (B). LDH levels were assessed in the supernatant of both infected and uninfected BMDCs (C). Results expressed as mean±SEM (A&B) or mean±SD (C). n=3/4 (A&B), representative of 3 independent experiments (C), *p<0.05, ***p<0.001 by repeated measures one or two-way ANOVA with appropriate post-test.

Figure 3: Identification of clinical bloodstream isolates with the ability to escape phagocytic killing.

BMDC viability was screened by PI staining 24 hours post infection with a panel of clinical strains, identifying “PS80-like” strains (black), “SH1000-like” strains (white) and “intermediate” strains (checked) (A). BMDCs were infected with Sa68 or Sa279 and % killing of bacteria was determined by comparing total CFU in the presence of phagocytes to bacteria in media only (B). BMDCs were infected with either CTV-labeled Sa68 or Sa279 at MOI 100 and % uptake of each strain was determined by flow cytometry at 30 min or 2 hours post infection (C). Following infection of BMDCs with Sa68 or Sa279 at MOI 100, escape of each strain into the cell culture media was assessed at 6 hours and 12 hours (D). LDH levels were assessed in the supernatant of both infected and uninfected BMDCs (E). Results expressed as mean±SEM (A-D) or mean±SD (E). n=2/6 (A-D), representative of 3 independent experiments (E), *p<0.05, ***p<0.001 by repeated measures one or two-way ANOVA with appropriate post-test.

Figure 4: *S. aureus* strain PS80 inhibits normal autophagic flux in phagocytes.

BMDCs were infected with *S. aureus* strains PS80 or SH1000. At indicated time points cells were lysed and expression of LC3 analysed by Western immunoblotting. Bands show conversion of LC3-I to LC3-II. β-actin was measured as a loading control. Representative blots from n=3 independent experiments are shown (A). At 6 hours post infection with CTV-labeled bacteria, BMDCs were stained with MDC and fixed to be viewed under a fluorescent microscope. Blue, bacteria; yellow, MDC; white arrows indicate colocalisation of bacteria and LC3-II (B). At 3 hours post infection with CTV-labeled bacteria, GFP-LC3 iBMM were fixed, permeabalised and stained for phalloidin to be viewed under a fluorescent microscope. Blue, bacteria; green, LC3; red, phalloidin; white arrows indicate colocalisation of bacteria and LC3-II (C). See also enlarged images showing the extent of co-localization.

Figure 5: Inhibition of autophagic flux facilitates escape of *S. aureus* from phagocytes.

BMDCs were infected with *S. aureus* strains PS80, SH1000, Sa68 and Sa279. At 6 hours cells were lysed and expression of LC3 analysed by Western immunolotting. Bands show conversion of LC3-I to LC3-II. β-actin was measured as a loading control. A representative blot is shown (A). BMDCs were pretreated with 3-MA for 30min and infected with either PS80 or Sa68 (MOI 100). Escape of each strain into the cell culture media was

assessed at 6 hours and 12 hours (B). LDH levels were assessed in the supernatant of 3-MA pretreated and untreated BMDCs that were infected with either PS80 or Sa68 (C). Results expressed as mean±SEM (A&B) or mean±SD (C), n=4/6 (A&B), representative of 3 independent experiments (C). ***p<0.001 by repeated measures two-way ANOVA with Bonferroni post-test.

Figure 6. *S. aureus* strains exhibit distinct levels of Agr activity as assessed by VLT and RNA III gene expression.

Bacterial supernatant was incubated at 1:1 ratio with lipid vesicles and fluorescence intensity recorded as a measure of vesicle lysis (A). RNAIII activity was measured using quantitative RT-PCR, as a ratio of RNA III and gyrB transcript number (B). Results expressed as mean±SEM. n=3/4, *p<0.05, **p<0.01 ***p<0.001 by one-way ANOVA with Tukey post-test.

Figure 7: PS80Δ*agr* is killed by BMDCs and leads to reduced accumulation of LC3-II.

BMDCs were infected with *S. aureus* strains PS80 or PS80Δ*agr* at MOI 100. % killing of bacteria was determined by comparing total CFU in the presence of BMDCs to bacteria in media only (A). At 6 hours cells were lysed and expression of LC3 analysed by Western immunoblotting. β-actin was measured as a loading control. (B). Results expressed as mean ± SEM. n=3

(A), representative of 3 individual experiments (B), *p<0.05, **p<0.01, ***p<0.001 by repeated measures two-way ANOVA with Bonferroni post-test.

Figure 8: Intracellular persistence of *S.* facilitates infection *in vivo*.

Groups of mice were challenged with *S. aureus* strains PS80 (A,B,D,E), PS80Δ*agr* (A&D), SH1000 (B&E), Sa68, Sa279 (C) or GFP-PS80 (F) (5×10^8 CFU) via the intraperitoneal route. At 3 hours post challenge blood was collected, total leukocytes isolated, washed and lysed. Cell-associated bacteria were expressed per 10^5 cells (A-C). At 12 hours post challenge, spleens were isolated, homogenized and the bacterial burden assessed (D&E). Leukocytes isolated 3 hours post challenge were also analysed by flow cytometry and CD11b+F480-Ly6G+ (neutrophil (PMN)), CD11c+ (dendritic cells (DC)) and CD11b+F480+Ly6G- (monocyte (MΦ)) populations that were GFP+ determined (F). Results expressed as mean±SEM, line indicates mean, n=5/12, *p<0.05, **p<0.01 by unpaired students t-test or one-way ANOVA with Tukey post-test.