Intracellular replication of *Staphylococcus aureus* in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination

Ronald S. Flannagan,1 Bryan Heit1,2* and David E. Heinrichs1,2*

1Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario, Canada, N6A 5C1.
2Centre for Human Immunology, The University of Western Ontario, London, Ontario, Canada, N6A 5C1.

Summary

The success of *Staphylococcus aureus* as a pathogen is partly attributable to its ability to thwart host innate immune responses, which includes resisting the antimicrobial functions of phagocytes. Here, we have studied the interaction of methicillin-resistant *S. aureus* (MRSA) strain USA300 with murine RAW 264.7 and primary human macrophages using molecular imaging and single cell analysis to obtain an unprecedented understanding of the interaction between the macrophage and MRSA. Herein we demonstrate that macrophages fail to control intracellular infection by MRSA USA300 despite trafficking the bacteria into mature phagolysosomes. Using fluorescence-based proliferation assays we also show that intracellular staphylococci proliferate and that replication commences while the bacteria are residing in mature phagolysosomes hours after initial phagocytosis. Finally, live-cell fluorescence video microscopy allowed for unprecedented visual insight into the escape of MRSA from macrophages, demonstrating that the macrophages die through a pathway characterized by membrane blebbing and activation of caspase-3 followed by acquisition of the vital dye propidium iodide. Moreover, cell death precedes the emergence of MRSA from infected macrophages, and these events can be ablated by prolonged exposure of infected phagocytes to gentamicin.

Introduction

*Staphylococcus aureus*, a bacterium for which there is no approved vaccine, is a leading cause of infections involving the skin, soft tissue, vasculature, bone and respiratory tract, in many developed countries (Lowy, 1998). Antibiotic resistance complicates treatment, and the spread of drug resistant strains of *S. aureus* has reached pandemic status. Strikingly, mortality associated with invasive methicillin-resistant *S. aureus* (MRSA) infections is approximately 20%, and, in the U.S., these infections are the leading cause of death by any infectious agent (Boucher et al., 2010; van Hal et al., 2012; Kochanek et al., 2012). Community-acquired MRSA, which emerged in the 1990s, evolved in the community setting, and one strain in particular, USA300, has achieved pandemic status across North America (Mediavilla et al., 2012; Diekema et al., 2014).

The success of *S. aureus* as a leading pathogen is undoubtedly attributed to its extensive repertoire of virulence factors that promote colonization, proliferation and dissemination, destroy leukocytes, alter leukocyte recruitment or function and inhibit complement and antimicrobial peptides (Bartlett and Hulten, 2010; Otto, 2010; Yoong and Torres, 2013). Professional phagocytes, such as macrophages, represent an essential facet of the innate immune system that must be overcome in order for *S. aureus* to succeed in establishing infection. This notion is supported by the fact that *S. aureus* produces toxins, such as LukAB, that specifically target and intoxicate phagocytes including macrophages (DuMont et al., 2013; Reyes-Robles et al., 2013; Spaan et al., 2014; Melehani et al., 2015).

The importance of macrophages to the innate immune response emerges from their unique ability to ingest invading microbes through phagocytosis, a receptor-driven process that culminates with the formation of a membrane-bound vacuole or phagosome that harbours ingested bacteria (Flannagan et al., 2012a). While the nascent phagosome is innocuous, a complex series of interactions with the endolysosomal network morphs the phagosome into a highly degradative organelle with potent microbicidal properties (Fairn and Grinstein, 2012). To counter the phagocytic function of macrophages, *S. aureus* employs anti-phagocytic proteins, such as protein...
A and Efb that neutralize host opsonins such as immunoglobulin G (IgG) and complement protein C3b, thus limiting Fc gamma- and complement receptor-mediated engulfment of cocci (Dossett et al., 1969; Ko et al., 2013). In contrast, staphylococcal anti-phagocytic factors affecting non-opsonic phagocytic receptors such as scavenger receptors (e.g. CD36 and SR-A) have not been described. The role of CD36 and SR-A in mediating phagocytosis of S. aureus by macrophages is well established, and in vivo infection models reveal that their function profoundly impacts upon resistance to S. aureus infection (Thomas et al., 2000; Stuart et al., 2005). This underscores the importance of macrophage function in combating bacterial infection.

Once considered an extracellular pathogen it is now firmly established that S. aureus can survive within many mammalian cell types, including macrophages (Schnait et al., 2007; Kubica et al., 2008; Tuchscherr et al., 2011; Hamza and Li, 2014). Despite this, descriptions of the interaction between host cells and S. aureus are fraught with discrepancies, which is likely a consequence of strain variation, inherent differences between cellular models and inconsistencies in experimental procedures (Kubica et al., 2008; Grosz et al., 2014; Hamza and Li, 2014; Müller et al., 2014). Given the importance of phagocyte function in shaping infection and the clinical relevance of MRSA, we sought to characterize the interaction of MRSA with macrophages and determine the intracellular fate of phagocytosed cocci. Prior evidence indicates that S. aureus survives for days within primary human macrophages until bacterial proliferation and lysis of infected macrophages occur (Kubica et al., 2008; Hamza and Li, 2014). Surprisingly, during this persistent intracellular phase, S. aureus reportedly remain confined to the lumen of membrane-bound vacuoles where survival requires expression of the lytic toxin α-hemolysin (Kubica et al., 2008). Conversely, other work employing human macrophage cell lines suggests that phagocytosed S. aureus express alpha phenol soluble modulins to escape the phagolysosome to gain access to the cytosol where bacterial proliferation proceeds within hours of engulfment (Grosz et al., 2014). Further confusion surrounding the macrophage/S. aureus interaction emerges from significant discrepancies between the reported outcomes of the cellular infection, as S. aureus has been reported to induce an anti-apoptotic programme, be killed within the phagolysosome or kill infected macrophages (Koziel et al., 2009; Grosz et al., 2014; Müller et al., 2014; Melehan et al., 2015).

In an attempt to add clarity to the aforementioned confusion, we sought to elucidate the fate of phagocytosed MRSA within macrophages using fluorescent fusion proteins and lipid biosensors to perform single cell molecular imaging analysis of infected cells. In this study, we show that murine and primary human macrophages fail to eradicate phagocytosed MRSA, and, using molecular imaging, the maturation pathway of MRSA-containing phagosomes was elucidated. This analysis revealed that staphylococcal-containing phagosome maturation is unperturbed and that engulfed bacteria remain confined to the membrane-bound vacuoles in which they reside, long after infection. Importantly, we show that phagocytosed bacteria undergo replication while remaining confined to LAMP-1-positive S. aureus-containing phagosomes (SaCPs) in intact macrophages. Finally, employing live cell fluorescence imaging we demonstrate that viable MRSA only emerge from within infected macrophages that undergo cell death. Dying macrophages demonstrate characteristics of both apoptosis and necrosis as revealed by the formation of dynamic membrane blebs and activation of caspase-3 followed by loss of membrane integrity. Our data support the contention that phagocytosed MRSA resist killing in the macrophage phagolysosome where they initiate intracellular replication and that failure to restrict intracellular growth leads to macrophage death and release of viable staphylococci. This represents an important strategy by which MRSA circumvent innate immune function to promote bacterial dissemination.

**Results**

**Macrophages fail to control intracellular S. aureus USA300 infection**

When extracellular, S. aureus USA300 bacteria deploy a multitude of toxins that kill macrophages with great potency; however, it is unclear whether phagocytosed bacteria similarly intoxicate infected macrophages (Kitur et al., 2015). To begin to elucidate the intracellular fate of S. aureus USA300 within macrophages classical gentamicin protection assays were performed with RAW 264.7 (RAW) macrophages using a range of multiplicity of infection (MOI) from 0.5 to 30. For each MOI employed, viable bacteria were recovered from within macrophages at 1.5 h post-infection (Fig. 1A). Moreover, macrophages at this early time remained adhered to the substratum and demonstrated their characteristic ability to form membrane ruffles, indicating that the intracellular bacteria were not acutely intoxicating infected cells (data not shown). Conversely, by 24 h post-infection, and irrespective of the MOI employed, the tissue culture medium bathing macrophages was heavily contaminated with staphylococci indicating that at least some bacteria had evaded the microbicidal functions of the macrophages and gained access to the extracellular environment where they proliferated to high numbers (Fig. 1A). To establish whether these observations were merely an artifact of the RAW line, primary human macrophage colony-stimulating factor (M-CSF)-derived macrophages were
also infected with *S. aureus* USA300 and gentamicin protection assays performed (Fig. 1B). Importantly, regardless of the MOI employed, viable bacteria were recovered at 1.5 h post-infection, and bacterial outgrowth was observed at 24 h post-infection (Fig. 1B). To visualize the infection, primary human macrophages were analysed by phase contrast microscopy which revealed that at ~2 h post-infection individual phase dark cocci resided within macrophages (Fig. 1C). In contrast, by ~10 h post-infection large aggregates of bacteria that were in close association with host cells were observed indicating the infection was not contained (Fig. 1C). Taken together these observations are consistent with the notion that *S. aureus* USA300 can resist killing within macrophages (RAW and primary human) and that outgrowth of the bacteria in the gentamicin protection assays is because of bacteria having gained access to the extracellular milieu, subsequent to escape from the macrophage.

Given that *S. aureus* USA300-infected macrophages cannot eradicate the bacteria, it was verified that the macrophages could, in principle, restrict the growth of Gram-positive bacteria. To this end the human skin commensal and opportunistic pathogen *Micrococcus luteus* was used to infect both primary human M-CSF-derived macrophages and RAW cells at an MOI of 30, and their survival was evaluated. Importantly, over a 24 h period, *M. luteus* was nearly eradicated from RAW macrophages and was undetectable in human primary macrophages at 24 h under our experimental conditions (Fig. 1A and 1B); in both cases, the macrophages remained intact and viable. These data indicate that the outgrowth of *S. aureus* USA300 from macrophages, under our experimental conditions, is not because of an inherent inability of the macrophages to restrict the growth of phagocytosed bacteria.

**Prolonged gentamicin treatment can modulate intracellular infection and can prevent bacterial egress from infected macrophages**

Given the implications of the observation that MRSA bacteria emerge from infected macrophages we felt it prudent to ensure that our protocol intended to eradicate extracellular bacteria was not altering the course of the infection or was insufficiently killing the extracellular bacteria. In the preceding experiments gentamicin treatment was restricted to 1 h in an attempt to avoid untoward effects that prolonged drug exposure could presumably have on the host–pathogen interaction. Macrophages are highly pinocytic and constitutively consume their fluid phase environment, and, as such, it stands to reason that antibiotics outside the cell can be internalized and delivered to phagocytosed cocci thereby modulating the intracellular infection (Flannagan *et al.*, 2010; Bohdanowicz *et al.*, 2013). Indeed, this has been shown to affect intracellular *Listeria monocytogenes*, and therefore we sought to...
determine if phagocytosed Staphylococci would be similarly affected. To determine if pinocytosed material from the fluid-phase was indeed delivered to engulfed staphylococci, RAW macrophages were infected with S. aureus USA300 and then, at 30 min post-infection, the culture medium was removed and replaced with medium containing fluorescein isothiocyanate (FITC)-dextran and gentamicin. Thirty minutes later multiple fluorescent dextran puncta were visible throughout infected macrophages, demonstrating sustained macrophage pinocytosis despite the presence of intracellular S. aureus USA300 (Fig. S1A). Moreover, internalized dextran could be seen completely surrounding numerous intracellular cocci, indicating that fluid phase components (i.e. dextran and, presumably, gentamicin) are indeed delivered to compartments containing the phagocytosed bacteria (Fig. S1A).

Next we determined if sustained drug treatment could modulate the outcome of our macrophage infections. To this end we performed experiments where after an initial 1 h gentamicin treatment the antibiotic was either removed or was maintained in the culture medium through the duration of a 24 h infection. As opposed to the bacterial outgrowth observed in experiments where gentamicin was removed after 1 h, sustained drug treatment completely prevented outgrowth of S. aureus USA300 at an MOI of 1 or 30 (Fig. S1B and S1C). Even with an MOI of 30 sustained gentamicin exposure resulted in an approximately 4 log reduction in the number of viable bacteria recovered 24 h after infection when compared to conditions where gentamicin was washed out (Fig. S1C). Moreover, comparison of the number of viable bacteria recovered after 24 h with sustained gentamicin treatment, to that of the input determined after the initial gentamicin treatment (1.5 h post-infection), reveals that there is an approximately 1.5 log reduction in the number of bacteria recovered at the end of the experiment (Fig. S1C). Not surprisingly, when a lower MOI of 1 is employed and infected macrophages are maintained in gentamicin we fail to detect any viable staphylococci after 24 h (Fig. S1B).

Given that our gentamicin protection assays (1 h treatment) indicate that some fraction of phagocytosed S. aureus USA300 escape from infected macrophages, we sought to confirm this using live cell time-lapse differential interference contrast (DIC) imaging to visualize infected macrophages. To this end, S. aureus USA300-infected macrophages were imaged at 5 h post-infection, 4 h after the extracellular cocci were killed by a 1 h treatment with gentamicin. In this video, where the first frame represents ~5 h post-infection, a few extracellular cocci can be seen on the cover glass in between macrophages; these bacteria never divide, consistent with gentamicin-mediated killing. Moreover, from this analysis it is evident that viable staphylococci emerge from within infected RAW macrophages and in the time lapse video presented, this occurs at ~12 h post-infection (Video S1). To support the contention that this outcome can be modulated by prolonged drug treatment live-cell video microscopy of infected primary human M-CSF-derived and RAW macrophages in the presence of gentamicin was also performed. This analysis revealed that with gentamicin treatment bacterial outgrowth did not occur and that macrophages remain adhered to the substratum while maintaining their dynamic ruffling behaviour (data not shown). Taken together, these observations support the contention that sustained drug treatment can indeed modulate the course of intracellular staphylococcal infections in macrophages. Thus, to limit this artifact, our antibiotic regime of 100 μg/ml gentamicin for 1 h, which was sufficient to kill all USA300 bacteria in in vitro viability assays (Fig. S1D), was employed for all experiments henceforth. Interestingly, in the absence of sustained drug treatment and at the time that extracellular cocci appear, there are large numbers of bacteria that become immediately present (Video S1). This, in and of itself, would suggest that the phagocytosed bacteria have indeed proliferated within infected macrophages prior to escape to the extracellular milieu. Considering this possibility, we next sought to characterize the intracellular niche in which MRSA bacteria reside over time in order to define the intracellular compartment in which the bacteria replicate.

The S. aureus-containing phagosome acquires early markers of phagosome maturation

Having established that our infection model shows that not only do MRSA USA300 withstand macrophage killing but, in fact, emerge from infected macrophages, we next sought to understand the intracellular dynamics underlying this phenomenon. To this end, we assessed the localization of established phagosome maturation markers to the SaCP. The small GTPase Rab5 is a master regulator of membrane dynamics on early phagosomes and endosomes and, to determine if it was present on the SaCP, a plasmid encoding Rab5-green fluorescence protein (GFP) was transfected into RAW macrophages (Duclos et al., 2000; Vieira et al., 2003). Macrophages expressing Rab5-GFP were infected with S. aureus USA300, and, by 9 min post-infection, the SaCP was demarcated by GFP, indicating Rab5 accumulation. Indeed, at this early time, 76% of the bacteria-containing phagosomes were GFP-positive while the remaining GFP-negative intracellular cocci presumably resided in phagosomes that had already progressed into a more mature Rab5-negative phagosome (Fig. S2A and S2C).

To ascertain if S. aureus perturbs Rab5 activation, we assessed the Rab5-dependent synthesis of phosphatidylinositol 3-phosphate (PI(3)P) on phagosomes containing bacteria (Christoforidis et al., 1999; Shin et al., 2005). RAW macrophages expressing tandem

© 2015 John Wiley & Sons Ltd, Cellular Microbiology, 18, 514–535
FYVE domains fused to GFP (2xFYVE-GFP), a biosensor for PI(3)P, were infected with S. aureus USA300. RAW macrophages expressing the PI(3)P probe had GFP positive puncta throughout the cytoplasm, consistent with its localization to early PI(3)P-positive endosomes (Fig. S2B). Moreover, GFP was accumulated on the 84% of the SaCP at 9 min post-infection (Fig. S2C), indicating local activation of the Rab5-dependent PI3 kinase Vps34 and production of PI(3)P. Taken together these data revealed that the classical pathway for early remodelling of the phagosome is not perturbed in macrophages harbouring phagocytosed S. aureus USA300.

S. aureus USA300-containing phagosomes mature into phagolysosomes without evoking the autophagy pathway

Many successful pathogens disrupt phagosome maturation to avoid the antimicrobial function of the phagolysosome (Flannagan et al., 2009). We therefore directly assessed for this, as it was conceivable that S. aureus exploits such a strategy. Concomitant with phagosome maturation is the progressive acidification of the phagosome lumen. To test whether, like some bacteria, S. aureus can interfere with phagosome acidification, macrophages infected with S. aureus USA300 were incubated at 30 min post-infection with the acidotropic fluorescent dye Lysotracker Green DND-26, which accumulates in acidic organelles such as late phagosomes and phagolysosomes. Live cell confocal fluorescence imaging revealed that 71% of the S. aureus USA300-containing phagosomes were lysotracker positive (Fig. 2A and 2B). Importantly, this was not significantly different from the 74% of phagosomes containing IgG-opsonized beads that were lysotracker positive at the same time point (Fig. 2A and 2B). These data indicate that normal phagosome acidification was unaffected by phagocytosed S. aureus USA300.

Next we investigated whether lysosome-associated membrane protein-1 (LAMP-1), a membrane spanning glycoprotein that is enriched on mature acidic phagosomes, also resided within the limiting SaCP membrane (Rabinowitz et al., 1992). RAW macrophages having phagocytosed S. aureus USA300 were immuno-stained to detect endogenous LAMP-1 and, at 30 min post-infection, pronounced LAMP-1 accumulation surrounding intracellular cocci was observed; 84% of the SaCP stained LAMP-1 positive at this time (Fig. 2C and 2D). Importantly, fluorescence corresponding to LAMP-1 was only observed on membranes surrounding phagocytosed cocci, indicating that the LAMP-1 immunodetection methodology was not confounded by non-specific binding of the antibody to the surface of protein A-expressing staphylococci (Fig. 2C). Using IgG-latex beads as a control, it was evident that the kinetics of LAMP-1 acquisition by the SaCP closely parallels that of IgG-bead containing phagosomes; 100% of these phagosomes are LAMP-1 positive at 30 min (Fig. 2C and 2D). Although a 16% difference in the fraction of LAMP-1 positive S. aureus – as compared to IgG-bead – containing phagosomes is observed, this discrepancy can readily be explained by the fact that engulfment of unopsonized S. aureus is likely less synchronous than FcγR-driven uptake of beads.

While the SaCPs harbouring MRSA USA300 transition to the late LAMP-1-positive stage, this does not directly demonstrate phagolysosomal fusion. To determine if engulfed S. aureus USA300 eventually reside in mature phagolysosomes, dextran pulse-chase experiments were performed. Tetramethylrhodamine (TRITC)-labelled dextran, an indigestible fluorescent fluid phase tracer, was loaded into RAW macrophages through pinocytosis and allowed to accumulate in lysosomes. Dextran-loaded macrophages were subsequently allowed to phagocytose S. aureus USA300, or IgG beads, and then imaged live to determine whether dextran was delivered to the phagocytosed targets, indicating lysosomal fusion with the maturing phagosome. Lysosomal loading of RAW cells with TRITC-dextran revealed the presence of an intricate tubular lysosomal network that emanated throughout the macrophages (Fig. 3A). Importantly, in macrophages containing phagocytosed S. aureus, approximately 72% of the phagosomes harbouring bacteria were dextran positive at 30 min post-infection (Fig. 3A and 3B). This frequency of co-localization closely paralleled the 74% of phagocytosed IgG-beads that also co-localized with dextran at the same time (Fig. 3A and 3B). To ensure that the observed phagolysosomal fusion in RAW macrophages is not a cell line artifact, M-CSF-derived primary human macrophages were also infected after performing dextran pulse-chase. Importantly, these experiments revealed that, as in RAW cells, a significant fraction (~75%) of the S. aureus USA300-containing phagosomes fuse with lysosomes by 30 min post-infection, as indicated by their co-localization with fluorescent dextran (Fig. 3C and 3D). Furthermore, the fraction of SaCP co-localizing with dextran is remarkably similar to the ~72% of IgG-bead containing phagosomes showing dextran co-localization at the same time point (Fig. 3D). Taken together, the lysotracker, LAMP-1 and dextran co-localization experiments revealed that, in macrophages, phagosome maturation remains unperturbed by S. aureus USA300 and that the phagocytosed cocci ultimately reside in mature, acidic phagolysosomes.

Previously, it was suggested that S. aureus, in an Agr quorum sensing dependent manner, activates autophagy pathways and that this is required for intracellular survival in epithelia and dendritic cells (Schnaith et al., 2007; O’Keeffe et al., 2015). To determine whether, in macrophages, the machinery needed for autophagosome biogenesis is involved in the maturation of the SaCP we expressed microtubule-associated protein 1A/1B-light
chain 3 (LC3) as an RFP fusion protein in RAW macrophages and assessed its localization to the SaCP. In LC3-RFP-expressing macrophages, fluorescent puncta were present throughout the cytoplasm, consistent with the presence of some LC3-positive endomembranes. Despite this, however, fewer than 4% of the phagosomes containing *S. aureus* USA300 (measured at 30 min, 3 h, 6 h and 12 post-infection) showed LC3-RFP accumulation (Fig. S3A and S3B, and data not shown). These data are an indication that, in macrophages, the vast majority of phagosomes harbouring MRSA are not comprised of autophagy-related proteins. Moreover, the absence of LC3 from the SaCP indicates that the limiting phagosomal/phagolysosomal membrane is not significantly damaged by *S. aureus* USA300.

**Intracellular *S. aureus* USA300 do not promote disruption of the phagolysosomal membrane**

Having established that phagocytosed *S. aureus* USA300 reside within mature phagolysosomes, we next sought to determine if the bacteria promote either remodelling or disruption of the limiting phagolysosomal membrane. The

© 2015 John Wiley & Sons Ltd, *Cellular Microbiology*, 18, 514–535
proteins CD63 (LAMP-3) and LAMP-1 are integral to phagosomal and lysosomal membranes and can therefore function as membrane markers (Metzelaar et al., 1991; Huynh et al., 2007; Huynh et al., 2010). To determine if these proteins demarcate the SaCP long after initial phagolysosome formation (i.e. 6 to 6.5 h post-infection), we performed microscopy on RAW macrophages expressing either a CD63-GFP fusion protein or cells immunostained for LAMP-1. Analysis of CD63-GFP revealed that there is prominent GFP accumulation around intracellular cocci even at these later time points (Fig. S4A).

Fig. 3. Phagocytosed S. aureus reside in mature phagolysosomes in RAW and primary M-CSF-derived human macrophages. Fluorescent dextran, pulse chased into the lysosomes of RAW macrophages, or primary human M-CSF-derived macropahges was analysed for co-localization with phagocytosed S. aureus USA300 and IgG opsonized beads. In (A) representative confocal fluorescence micrographs acquired at 30 min post exposure of RAW cells to S. aureus USA300 (top panels) and IgG beads (bottom panel) demonstrate the accumulation of TRITC-dextran around intracellular cocci and beads. Bacteria and beads remaining fluid-phase accessible are marked with green fluorescence, and white arrows point to instances of dextran co-localization. Bar is equal to 10 μm. In (B) the bar graph depicts the percentage of dextran-positive phagosomes at 30 min after exposure to S. aureus USA300 and IgG opsonized beads for RAW macrophages. The data are the mean ± standard error of the mean obtained from three independent experiments. In (C) representative micrographs depicting the localization of TRITC-dextran (in red) and S. aureus USA300 (in blue) in M-CSF-derived primary human macrophages are shown. Extracellular bacteria (in green) were marked with fluorescent IgG. The hashed box demarcates the area of the cell presented in the inset, and the white arrows point to cocci that are co-localizing with dextran. In (D) the graph depicts the fraction of S. aureus and IgG bead containing phagosomes that are dextran positive at 30 min exposure to bacteria or beads. Shown is the mean ± standard error of the mean derived from the analysis of three independent experiments.

© 2015 John Wiley & Sons Ltd, Cellular Microbiology, 18, 514–535
membranes are intact. To directly assess whether \textit{S. aureus} disrupts the phagosomal membrane, the accumulation of lysotracker in the phagosomes of infected RAW macrophages was analysed at the same time point. Because lysotracker accumulation is dependent upon the retention of protons in the lumen of the phagosome, staining would be readily lost from phagosomes following even minute perturbations of the phagosomal membrane, and thus this assay represents a sensitive measure of phagosomal membrane integrity (Birmingham et al., 2008). At 6.5 h post-infection, RAW macrophages were stained with lysotracker green, and fluorescent puncta were visible throughout infected cells, indicating an absence of gross defects in vesicular acidification (Fig. S4C). Furthermore, close inspection of infected cells revealed that the vast majority of intracellular cocci co-localized with lysotracker fluorescence (Fig. S4). Taken together, these observations reveal that \textit{S. aureus} USA300 bacteria are retained within membrane-bound vacuoles that remain intact for many hours (> 6.5) after bacterial engulfment.

**Phagocytosed \textit{S. aureus} USA300 initiates intracellular replication within the phagosome**

The images from video S1 indicate a large number of bacteria are escaping the macrophages, suggesting that intracellular replication had occurred. However, our previous observations also indicate that this likely occurs at some point beyond the 6 h post infection time point because we observe detectable but low intracellular bacterial numbers up to this time point (see Fig. S4A–C). To firmly establish that \textit{S. aureus} USA300 commences replication while remaining inside macrophages we utilized a fluorescence-based cell proliferation assay where bacterial replication would be apparent by loss of fluorescence. To this end \textit{S. aureus} USA300 bacteria expressing GFP were labelled with eFluor-670 cell proliferation dye marking all GFP-positive bacteria with far-red fluorescence at the outset of the experiment. These labelled bacteria were phagocytosed by RAW macrophages, and at 30 min post-infection all GFP-positive bacteria were eFluor-670 positive (Fig. 4A, top panels). To demarcate the plasmalemma of macrophages and to mark uninternalized bacteria tetramethyl rhodamine-labelled wheat germ agglutinin (TMR-WGA) was used and clearly revealed the presence of GFP and eFluor670-positive bacteria that were indeed phagocytosed (Fig. 4A, top panels). At 12 h post-infection and approximately the time point when we observed in video S1 \textit{S. aureus} USA300 emerging from RAW macrophages, intracellular bacterial populations (i.e. TMR-WGA negative bacteria) that were GFP-positive but devoid of the far-red fluorescence are readily observed (Fig. 4A, middle panels). While these observations would indicate that \textit{S. aureus} USA300 undergoes intracellular replication it could not be ruled that the intracellular environment is without effect on the eFluor670 dye and compromises its fluorescence. To control for this scenario, macrophages were also incubated with GFP-eFluor670 positive \textit{S. aureus} USA300 that were inactivated and unable to replicate. At 30 min post-infection inactivated bacteria that were positive for GFP and eFluor but TMR-WGA negative were observed indicating that inactivated bacteria were indeed phagocytosed (data not shown). Importantly, at 12 h post-infection internalized inactivated bacteria were still few in number and maintained their intense proliferation dye staining indicating the environment of the phagosome does not in of itself compromise the eFluor-670 signal (Fig. 4A, bottom panels). Taken together these data indicate that loss of the far-red signal from viable USA300 is a consequence of dilution of the dye amongst intracellularly replicating bacteria. To complement these imaging experiments we also performed gentamicin protection assays where the number of bacteria recovered from infected RAW macrophages at 1.5 h, 5 h and 10 h post-infection were enumerated (Fig. S5). Importantly, these data support our earlier assertion that bacterial replication occurs only hours after infection and are in agreement with our ability to detect bacterial replication using the eFluor-670 fluorescence-based proliferation assay.

Given that \textit{S. aureus} USA300 is proliferating inside RAW macrophages we next analysed whether the bacteria can similarly proliferate within primary human macrophages. To this end M-CSF-derived macrophages were infected with live GFP-expressing bacteria that were also labelled with proliferation dye and imaged at 1.5 h and 8 h post-infection. Early after infection and just after gentamicin treatment all MRSA bacteria were marked with fluorescent proliferation dye showing the bacteria have not replicated (Fig. 4B, top panels). Importantly, all phagocytosed bacteria that are indicated by a lack of WGA staining were also marked with GFP and eFluor-670 (Fig. 4B, top panels). In contrast, at 8 h post-infection intracellular foci of GFP-positive \textit{S. aureus} bacteria that are devoid of eFluor-670 fluorescence can be observed inside intact macrophages (Fig. 4B, bottom panels). As a control, inactivated bacteria were also employed, and, as expected, at 8 h post-infection all inactivated bacteria, including those that had been phagocytosed, retained intense proliferation dye staining (data not shown). Taken together these data demonstrate that viable \textit{S. aureus} USA300 commence replicating intracellularly in RAW and primary human macrophages confirming our earlier assertion from video S1 that MRSA bacteria emerge from macrophages in large numbers as a result of intracellular bacterial growth.

**Replicating intracellular MRSA reside within LAMP-1-positive vacuoles**

Interestingly, in the preceding experiments many of the bacteria that are GFP-positive, yet eFluor-670-negative (i.e. replicating) appear in spherically shaped foci within

**© 2015 John Wiley & Sons Ltd, Cellular Microbiology, 18, 514–535**
the macrophages, suggesting that the bacteria may be constrained to the lumen of a vacuole. To test the notion that *S. aureus* USA300 is proliferating while contained inside phagosomes we performed similar proliferation assays but expressed in RAW macrophages the probe LactC2-RFP, a lipid biosensor of the glycerophospholipid phosphatidylserine (PS) (Yeung *et al.*, 2008). PS is enriched in the inner leaflet of the plasmalemma and is found on the cytosolic face of membranes throughout the endocytic network including phagosomes (Yeung *et al.*, 2008; Fairn *et al.*, 2011). As expected, LactC2-RFP accumulated at the RAW cell plasmalemma and was on structures throughout the cytoplasm (Fig. 5A and data not shown). Importantly, when LactC2 expressing RAW cells

**Fig. 4.** *S. aureus* USA300 replicate inside macrophages hours after being phagocytosed. Bacterial proliferation was established through dilution of a fluorescent dye conjugated directly to GFP expressing bacteria. In (A) and (B) the confocal micrographs depict macrophages infected with GFP expressing *S. aureus* USA300 (in green) that are labelled with proliferation dye (in blue) at the indicated times post-infection. The macrophage plasmalemma and extracellular bacteria are demarcated by WGA staining (in red). In (A) the top row of micrographs represent RAW macrophages 30 min post-infection where the white arrows point to GFP-positive and eFluo-670 proliferation dye-positive bacteria that are intracellular. The middle row depicts RAW macrophages at 12 h post-infection, and the white arrows highlight foci of intracellular GFP-positive bacteria that are eFluo-670 proliferation dye negative. The bottom panels depict RAW macrophages harbouring at 12 h post-infection bacteria that were inactivated prior to phagocytosis. The white arrows point to engulfed inactive bacteria that even at 12 h post-infection are positive for both GFP and eFluo-670 proliferation dye fluorescence. These images are representative of three independent experiments. Bars equal 10 μm. In (B) primary M-CSF-derived macrophages were analysed for intracellular replication of *S. aureus* USA300 as described above. Micrographs presented in the top row represent 1.5 h post-infection whereas the bottom row depicts macrophages at 8 h post-infection. In the bottom panels the white arrows point to foci of GFP-positive but eFluo-670 proliferation dye negative *S. aureus* USA300. Shown are representative fluorescent micrographs of two independent experiments from two independent macrophage donors. Bars equal 10 μm.
were infected with *S. aureus* USA300 expressing GFP that were also proliferation dye-positive, the ingested bacteria at 30 min post-infection were completely encircled by RFP indicating that the bacteria resided within PS-positive membranous compartments (Fig. 5A, top panels). Again at 12 h post-infection GFP-positive bacteria that were devoid of far-red fluorescence were observed, and in the presence of the LactC2-RFP probe it was clear that those bacteria, often in circular foci, were contained within a PS-positive vacuole demarcated by LactC2-RFP (Fig. 5A, bottom panel). To ascertain whether the membranes delineated by LactC2-RFP were indeed phagosomal, LAMP-1 immunostaining of infected cells at this 12 h time point was performed. In uninfected cells expressing LactC2-RFP intracellular PS positive puncta can be observed and all of the features that are LAMP-1 positive co-localize with LactC2-RFP illustrating the normal association between these cellular components (Fig. 5B, top panels). Importantly, immunodetection of LAMP-1 in RAW cells at 12 h post-infection revealed that populations of GFP-positive bacteria are enveloped by LactC2-RFP and are proliferation dye negative. The yellow arrows indicate the presence of some bacteria in the same macrophage that have not replicated as indicated by their retention of eFluor-670 (in blue). Bar equals ~10 μm.
whether replicating *Staphylococcus aureus* USA300 can also reside within LAMP-1 positive vacuoles within primary human macrophages. To this end the cell proliferation assays using the eFluor-670 dye were performed as described, but at 8 h post-infection infected cells were immunostained to detect the intracellular distribution of LAMP-1 (Fig. 5C). At this time foci of GFP positive USA300 bacteria were readily observed that were devoid of proliferation dye indicating the presence of replicating bacteria as expected. Importantly, many replicating bacteria (i.e. GFP-positive but proliferation dye negative) within macrophages were encircled by LAMP-1 protein (Fig. 5C). Interestingly, at this time point there were clearly bacteria that had also replicated but were not encircled by LAMP-1 which is in contrast to what was observed in RAW cells where all intracellular replicating cocci that we observed were membrane bound (Fig. 5A–C). Presumably some of the bacteria in the primary cells had commenced replicating inside phagosomes but that, at the time of imaging, the phagosomes that kept them constrained had become compromised. Nevertheless, it is clear that primary macrophages, like the RAW cells, can harbour replicating *S. aureus* USA300 inside LAMP-1 positive vacuoles.

Taken in their entirety the experiments presented in Figs. 4 and 5 clearly reveal that MRSA bacteria replicate intracellularly before gaining access to the extracellular milieu and that these bacteria can indeed commence replication while confined to the lumen of LAMP-1 positive phagosomes.

**RAW macrophages infected with *S. aureus* USA300 are not overtly intoxicated**

*Staphylococcus aureus* is armed with a profusion of genes encoding toxic effectors that can cause damage to host cell membranes; however it is unclear if these toxins contribute to the egress of USA300 bacteria from the macrophage. Conceptually, if membrane-damaging toxins contribute to bacterial escape then perturbation of the macrophage plasmalemma should be evident. To monitor non-invasively at the single cell level the integrity of the plasma membrane of infected cells the fluorescent lipid biosensor for phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), the pleckstrin homology (PH) domain of PLCδ fused to RFP (PH-Plcδ-RFP), was expressed in RAW macrophages that were either uninfected or were infected for up to 12 h. Monitoring of PI(4,5)P₂ was chosen because PI(4,5)P₂ levels and membrane localization of the PH-Plcδ probe are sensitive to minute changes in cytosolic calcium where sudden increases in cytosolic calcium, which would occur in instances of pore formation, cause PI(4,5)P₂ depletion and displacement of the lipid biosensor from the plasma membrane (Várnai and Balla, 1998; Fairn et al., 2009). In uninfected macrophages the PH-Plcδ-RFP probe showed a characteristic cellular distribution where it accumulated prominently at the plasma membrane and in membrane ruffles (Fig. 6A). In infected cells at 12 h post-infection this characteristic distribution remained unaffected by the presence of intracellular cocci (Fig. 6A). Moreover, macrophages harbouring large numbers of cocci that have obviously replicated show maintenance of PH-Plcδ-RFP probe at the plasma membrane indicating that PI(4,5)P₂ hydrolysis is not occurring despite the presence of a significant intracellular bacterial burden (Fig. 6A). Moreover, even in the cells with large numbers of GFP-positive bacteria, the bacteria still appear to be confined to cytoplasmic vacuoles as evidenced by their circular pattern of confinement and the noticeable void in soluble RFP signal.

While the preceding experiments demonstrated to us that the plasma membrane of USA300 infected macrophages appear uncompromised even in the presence of many intracellular bacteria, other cellular factors could be targeted by intracellular staphylococci to cause macrophage intoxication and eventual emergence of the bacteria. Phagocytosis requires an array of host processes that act in concert to bring about engulfment and, as such, phagocytosis represents a powerful test that can be employed to uncover defects in important cellular processes such as signal transduction and cytoskeletal remodelling (Flannagan et al., 2012a). In an attempt to uncover whether internalized *S. aureus* USA300 perturbed macrophage function, RAW macrophages that remained uninfected, or were infected for 12 h with *S. aureus* USA300, were exposed to IgG-laden beads. Uninfected RAW macrophages readily ingested IgG beads, which were distinguished from external beads by fluorescence microscopy (Fig. 6B and 6C). Interestingly, the majority of RAW macrophages infected with *S. aureus* USA300 also voraciously ingested IgG beads, with a phagocytic index that was comparable to uninfected cells (Fig. 6B and 6C). These data provide convincing evidence that, even after prolonged infection with *S. aureus* USA300, RAW macrophages are not overtly intoxicated, retain their membrane integrity and are able to sustain their phagocytic function.

**Macrophages harbouring phagocytosed *S. aureus* USA300 demonstrate hallmarks of apoptotic and necroptotic/necrotic death prior to bacterial escape**

In an attempt to better understand the events surrounding release of intracellularly replicating *S. aureus* USA300 we again employed live cell microscopy so that the emergence of staphylococci from individual macrophages could be captured in real time. This analysis clearly reaffirms our contention that MRSA bacteria emerge from within macrophages and also revealed that in several instances large populations of intracellular cocci can be
seen constrained within the dimensions of the infected phagocyte just prior to release (Video S2, left panel). Interestingly, before bacterial egress infected macrophages also undergo significant morphological changes characterized by lamellodium retraction, the appearance of dynamic membranous blebs followed by osmotic swelling of the infected cell (Video S2, left panel). Analysis of cells from several experiments performed on different batches of RAW macrophages demonstrated that the onset of these morphological changes is extremely asynchronous and can occur anywhere between ~7 and 14 h post-infection, but is commonly detected at ~12 to 13 h post-infection as is observed in video S1. Importantly, despite the asynchronous escape of \textit{S. aureus} USA300 from infected RAW macrophages, live-cell imaging clearly reveals that these morphological features consistently precede egress of the bacteria. Conceivably deformation of the macrophage facilitates release of intracellular bacteria, and this notion was tested by pharmacological induction of membrane blebs using the phosphatase inhibitor calyculin A (CalA) to prevent dephosphorylation of non-muscle myosin IIA and its activation (Wilson et al., 2010). Indeed, CalA treatment induced the formation of numerous membrane blebs that are noticeably absent from control cells (Fig. S6A and S5B). To determine if bleb formation facilitates bacterial egress, infected RAW cells that were treated with CalA after gentamicin exposure were imaged over time. Incredibly, despite the induction of profound blebbing that persisted for more than 7 hours, bacterial escape was not accelerated and occurred within the expected time frame (Fig. S6C and Video S3).

Cellular deformation often accompanies apoptotic and necrotic modes of cell death and as such we next determined if hallmarks of these modes of death could be detected prior to MRSA escape (Mills et al., 1998; Vanden Berghe et al., 2010). Cell retraction and bleb formation are a commonly associated with apoptosis and therefore we first investigated whether we could detect the activation of caspase 3, an apoptosis hallmark. To determine if caspase-3 is indeed activated in response to \textit{S. aureus} USA300 infection, RAW macrophages at 12 h post-infection were stained with a fluorescent caspase-3/7 reporter that marks the nucleus of cells entering the irreversible stage of apoptosis (Nicholson, 1999). As a positive control, RAW macrophages were treated with staurosporine to induce apoptosis, resulting in virtually every cell in the imaging field becoming marked with nuclear fluorescence (Fig. S7A and S6B). Importantly, RAW cells that were uninfected and without staurosporine failed to label, indicating a very low level of background caspase activation. In (C) quantitation of the phagocytic index for uninfected and infected RAW cells (12 h post-infection) is shown. The graph represents the mean ± the standard error of the mean for each condition derived from three independent experiments.

© 2015 John Wiley & Sons Ltd, \textit{Cellular Microbiology}, 18, 514–535
with the presence of a significant bacterial burden, as macrophages that were heavily populated by bacteria were never observed to accumulate the caspase-3 reporter (Fig. S7A). To demonstrate that caspase 3 can, in principle, be activated in S. aureus USA300 infected macrophages, RAW cells already having phagocytosed bacteria were treated with staurosporine at 6 h post-infection and were then analysed for the presence of active caspase-3 activation 6 h later (i.e. 12 h post-infection). In contrast to staurosporine alone, infection with S. aureus USA300 made RAW cells refractory to staining with the caspase-3/7 fluorescent reporter however there was an increase in cells with green nuclear fluorescence above S. aureus USA300 infection alone. These data reveal that in principle caspase 3 can be activated in infected RAW cells but also that the bacteria could perturb staurosporine induced caspase activation (Fig. S7B), as has been previously demonstrated (Koziel et al., 2009).

To validate the interpretation of the preceding experiments we also performed live cell fluorescence imaging experiments to evaluate in real-time whether infected RAW macrophages truly fail to activate caspase-3. Moreover, we also included the vital dye propidium iodide (PI), which will only stain cells where the integrity of the plasma membrane has also become compromised as is seen in necrotic cells (Vanden Berghe et al., 2010). From these videos it was evident that cells that are undergoing the aforementioned morphological changes also demonstrate green fluorescence that persists transiently for 12 to 18 min (3 frames) and then is lost (Video S2, middle panel). These data indicate that indeed caspase-3 activation does occur in dying cells from which USA300 bacteria emerge and the transient nature with which we detect caspase-3/7 activation explains, in part, why our single time point analysis presented in Figure S6 failed to detect a significant fraction of cells with active caspase-3. Interestingly, cells demonstrating caspase-3 activation also become marked with PI, which always follows, typically within ~12 to 18 min, acquisition of the green caspase-3/7 reporter fluorescence (compare Video S2, right panel and middle panel). This would indicate that caspase-3 activation precedes lost membrane integrity. In video S2 initiation of morphological changes commence at ~5 to 5.5 h post-infection after which the caspase reporter is detected followed by PI accumulation. Interestingly, in the cell shown in the video, replicating bacteria can be seen as fine phase dark spheres in the DIC channel, however escape does not occur until ~8h post-infection when there is a sudden release of bacteria (Video S2).

To ascertain whether the observed cell death events preceding escape of S. aureus USA300 from infected RAW macrophages is a RAW cell artifact, primary human M-CSF-derived macrophages were similarly analysed. As a control, uninfected primary human macrophages were imaged in the presence of caspase-3/7 reporter and the phosphatidyl serine-binding reporter annexin V. In this latter case, we did not observe even a single cell (>100 macrophages analysed) that became fluorescent over 18h time-lapse experiments (data not shown). Moreover, uninfected cells did not demonstrate morphological changes that would indicate apoptosis or necrosis. In contrast, primary human macrophages infected at an MOI of 1 or 30 with live S. aureus USA300 underwent morphological changes, accumulated the green caspase 3/7 indicator and stained with far-red annexin V which binds PS (Fig. 7A and Video S4). Moreover, acquisition of green fluorescence (i.e. caspase-3 activation) is also followed by staining with PI indicating that, as observed in the RAW cells, primary M-CSF-derived macrophages also lose integrity of the plasmalemma (data not shown). Interestingly, the induction of cell death in primary macrophages in response to the infection appears to be more rapid than that observed in RAW cells as the onset of caspase activation is seen between 5 and 10h post-infection however the time to the onset of death is still extremely heterogeneous (Fig. 7A and Video S4). Importantly, replicating S. aureus USA300 emerge from these apoptotic macrophages, form large colonies that later dislodge and diffuse into the extracellular milieu where they replicate to high cell density (Video S4). Taken together, our live cell imaging analyses reveal that macrophages infected with S. aureus USA300 die prior to the release of intracellular bacteria and that the mode of death displays hallmarks of both apoptosis and necrosis.

In agreement with our earlier results demonstrating that macrophages are proficient at killing M. luteus (Fig. 1), M. luteus-infected macrophages, when incubated in the presence of the caspase-3/7 reporter, annexin V and PI, did not become marked with green (i.e. activated caspase-3-dependent) fluorescence nor did they demonstrate any significant morphological changes or stain with annexin V or PI, indicating macrophage cell death was not induced (data not shown). Next, we determined if the observed response of primary human macrophages to intracellular S. aureus was induced only by metabolically active bacteria. Heat-killed S. aureus USA300 were incubated with primary human M-CSF-derived macrophages and, after phagocytosis and gentamicin treatment, these macrophages were imaged for caspase-3/7 activation. Similar to what we observed with live M. luteus, killed S. aureus USA300 failed to induce death, indicating that only metabolically active staphylococci elicit this response (Fig. 7B and Video S5). Considering our previous data presented in Figure S1 demonstrating that prolonged use of gentamicin completely abrogated the outgrowth of S. aureus USA300 from macrophages, we sought to determine if the inclusion of gentamicin also perturbed...
the onset of caspase-3 activation. To this end, when gentamicin was present throughout the live cell imaging experiment and, consistent with our previous observations, using even an MOI of 30, gentamicin completely prevented the outgrowth of USA300 and prevented cell death indicated by the fact that the cells maintain their dynamic ruffling behaviour. Moreover, in the presence of gentamicin macrophages fail to acquire green fluorescence associated with processing of the caspase-3/7 reporter.

Together, these results demonstrate that sustained gentamicin treatment significantly impacts the host-pathogen interaction.

We reasoned that since cell death, presumably initiated by the activation of caspase-3, precedes the egress of S. aureus from macrophages, it was conceivable that perturbation of this process by blocking caspase activation could prevent bacterial escape. To test this notion, M-CSF-derived human macrophages were pre-treated with
the pan-caspase inhibitor z-VAD-FMK, which was maintained in the culture medium throughout the duration of the infection. Despite the presence of z-VAD-FMK, however, an absolute block in caspase-3/7 reporter labelling was not achieved consistent with previous reports using this compound (Hanley et al., 2012). Nonetheless, the fraction of human macrophages that became labelled with the caspase reporter was significantly reduced, and the time required for the cells to become apoptotic lengthened significantly. In the absence of z-VAD-FMK, ~ 40% of the primary human macrophage population is apoptotic at the time that extracellular bacteria are first observed, which occurs between 5 and 9 h post-infection. In the presence of the caspase inhibitor z-VAD-FMK the fraction of cells becoming apoptotic is reduced to 7% at the time when extracellular bacteria can be observed, which occurs between 10 and 12 h post-infection (Fig. 7C and 7D). These data imply that inhibiting caspase activation facilitates containment of S. aureus, however, in the absence of an absolute block it is unclear if viable staphylococci would remain contained indefinitely or would eventually emerge as a result of sustained bacterial replication or alternative cell death pathways.

Discussion

Staphylococcus aureus is a highly successful pathogen and underpinning its infectious proficiency is its ability to thwart immune attack. The capacity of S. aureus to kill immune cells like macrophages through the extracellular secretion of toxins has been well characterized (Alonzo et al., 2012; DuMont et al., 2013; Reyes-Robles et al., 2014), however, intoxication of macrophages from an intracellular niche has remained ill-defined. This investigation was initiated in an attempt to add clarity to our understanding of how intracellular S. aureus interacts with macrophages by performing single cell analyses in an attempt provide an unprecedented view of the S. aureus/macrophage interaction. In this study we show that phagocytosis of MRSA by macrophages results in delivery of ingested bacteria to mature, acidic phagolysosomes where the bacteria persist for extended periods of time. Remarkably, despite phagolysosome biogenesis that should be microbicidal, we find that MRSA bacteria survive and proliferate in this inhospitable niche. This is in stark contrast to other successful intracellular pathogens such as L. monocytogenes or Mycobacterium tuberculosis that evade phagolysosomal fusion through the production of effectors that perturb phagosome maturation (reviewed in (Flannagan et al., 2009)). Indeed, M. tuberculosis arrests phagosomes at an early Rab5-positive but PI(3)P-negative stage and the selective depletion of PI(3)P is catalysed by a secreted bacterial phosphatase SapM (Vergne et al., 2005). Clearly such an effector is not operational in macrophages harbouring S. aureus USA300 as we find that bacteria-containing phagosomes show localized production of PI(3)P and do fuse with lysosomes with kinetics that are similar to inert IgG-laden beads. In contrast to arresting phagosome maturation L. monocytogenes catalyses the dissolution of the limiting phagosomal membrane and vacuolar escape through the concerted activity of the pore-forming toxin listeriolysin O and two distinct phospholipases (Portnoy et al., 1988; Camilli et al., 1993; Smith et al., 1995). Previous work has suggested that in some cell types S. aureus can catalyse escape from the phagosome however this has been contentious as discrepancies in the toxin(s) that catalyse these events have been reported (Jarry and Cheung, 2006; Jarry et al., 2008; Lâm et al., 2010; Giese et al., 2011; Grosz et al., 2014). Moreover, the ability to escape phagosomes is a property of very few select strains and it has been reported that most S. aureus strains actually do not exhibit this behaviour (Groz et al., 2014). Consistent with the latter notion we fail to identify phagosome escape, at least until a point just preceding cell death, as a strategy employed by S. aureus USA300 as the bacteria are found in PS-positive membrane bound vacuoles that contain LAMP-1 and this is where they eventually commence replication hours after engulfment. Interestingly, the data in Fig. 5C demonstrate that some S. aureus USA300 having replicated intracellularly may exit the phagosome while still inside the macrophage, however this appears unique to primary human macrophages as it was never observed in RAW cells.

Clearly the data presented in this study support the notion that phagocytosed S. aureus USA300 employ a strategy whereby they resist the antimicrobial functions of the phagolysosome in lieu of redirecting phagosome maturation, however, how the bacteria resist the onslaught of antimicrobial effectors in the phagolysosome is unclear. Presumably enzymes (e.g. KatA or SodM) that detoxify vacuolar reactive oxygen species or modify bacterial membranes and peptidoglycan (e.g. MprF and OatA) help counteract macrophage effectors and contribute to intracellular survival (Peschel et al., 2001; Kehl-Fie et al., 2011; Damo et al., 2013). Nevertheless other bacterial proteins presumably also participate in intracellular survival and future analyses of S. aureus USA300 gene deletion mutants will undoubtedly uncover these bacterial genes and their products.

Using fluorescence-based proliferation assays our data convincingly show that intracellular MRSA USA300 proliferate and that proliferation precedes macrophage death and bacterial escape. Moreover, our data indicate that intracellular growth in fact precipitates the demise of infected macrophages, a contention supported by the observation that prolonged gentamicin treatment blocks
growth and macrophage death. These data are compatible with previous work that indicated that phagocytosed *S. aureus* persist within macrophages for at least 5 days prior to intracellular bacterial proliferation and host cell escape (Kubica et al., 2008). In that study, gentamicin (50 μg/ml) intended to kill the extracellular bacteria is reportedly employed for 24 h prior to its removal (Kubica et al., 2008). Presumably in this instance the number of viable intracellular bacteria was greatly reduced by the concerted action of macrophage effectors and antibiotic treatment and the few surviving bacteria are what eventually recover, replicate and escape. Nevertheless, these data in conjunction with ours indicate that once antibiotics are removed from the tissue culture medium phagocytosed *S. aureus* replicate within macrophages leading to phagocyte death. Conceivably modulation of the intracellular infection by more efficient systems of intracellular antibiotic delivery and/or by perturbing intracellular nutrient acquisition needed for growth may represent plausible means of improving immunity to infection in vivo.

Using time-lapse live-cell fluorescence imaging, death of MRSA USA300 infected macrophages was visualized and revealed that dying cells display hallmarks of apoptosis (i.e. blebbing and caspase-3 activation) and necrosis (i.e. lost membrane integrity). Interestingly, these observations parallel some aspects of the described interaction between *S. aureus* and neutrophils in terms of host cell death despite there being significant functional differences between these phagocytes in terms of phagosome maturation and their microbialic properties (reviewed in (Nordenfelt and Tapper, 2011)). The death that is observed in the presence of USA300 is not conventional apoptosis as the transition to a necrotic state, approximately 12 to 18 min after caspase-3 activation is detected, is extremely fast and this could be because of the presence of intracellular bacterial products. *S. aureus* is recognized for its profound capacity to produce and secrete a variety of cytolytic toxins that are known to contribute to pathogenesis in vivo and to kill phagocytes in vitro when produced in the extracellular milieu (Voyich et al., 2006; Nygaard et al., 2012; DuMont et al., 2013; Kitur, et al., 2015; Melehanli et al., 2015). Whether these toxins function in the lumen of the macrophage phagosome remains enigmatic and our data support the notion that phagocytosed *S. aureus* USA300 need not deploy its arsenal of toxins in order to escape the phagocyte or promote membrane disruption. Indeed, localization of the PS lipid biosensor LactC2-RFP, in RAW macrophages, reveals that virtually all phagocytosed USA300 bacteria, including those that are proliferating, remain confined to the lumen of PS-containing, LAMP-1-positive vacuoles. This is consistent with the finding of Kobayashi et al., who reported that, in neutrophils, phagocytosed *S. aureus* USA300 remain confined to phagosomes without replicating at least until neutrophil lysis occurs (Kobayashi et al., 2010). It is clear from our molecular imaging that phagosomal *S. aureus* USA300 replicate to a high cell density, which at some point must cause disruption of the limiting vacuolar membrane. The timing between rupture of the phagosome, the initiation of cell death and release of the bacteria because of macrophage rupture is not yet resolved however, host cell death appears to precede escape. Support of this assertion comes from our observation that membrane deformation, caspase activation and plasmalemmal permeabilization precedes bacterial egress from infected macrophages (see videos S2 and S4). Given the large bacterial cell density that is achieved prior to disruption of the cell membrane it is unlikely that an efficient cytolytic toxin is being deployed or simply that it must reach some critical concentration to effect macrophage rupture from within. Alternatively, membrane rupture may be because of the physical forces imposed by the profound number of intracellular bacteria occupying the interior of the dying macrophage, however phenotypic screening of defined gene deletion mutants will be required to properly test these scenarios.

Previous studies have proposed that *S. aureus* evoke an anti-apoptotic signaling cascade in macrophages to prolong host cell survival (Koziel et al., 2009; Koziel et al., 2013; Koziel et al., 2015). As such it is interesting that as a result of our live cell imaging experiments we are able to demonstrate caspase-3 activation in fact precedes release viable *S. aureus* USA300. In these previous studies where anti-apoptotic programmes are up-regulated prolonged use of gentamicin was employed and presumably explains why bacterial outgrowth and cell death was not observed (Kubica et al., 2008; Koziel, et al., 2009). Interestingly, our data do support the conclusion that *S. aureus* bacteria perturb the induction of caspase-3 in response to staurosporine treatment, however in the absence of gentamicin, sustained bacterial replication eventually leads to cell death where caspase is activated (see Figure S6 and videos S2 and S4). In these instances the host cell may "win" and successfully evoke caspase-3 activation in an attempt to apoptose, as it is perceived that the intracellular infection is not contained. Indeed, macrophages that ingest apoptotic debris containing *M. tuberculosis* are better able to contain and eradicate the intracellular infection (Martin et al., 2012). This scenario may represent a futile response as *S. aureus* is capable of withstanding phagolysosomal killing in the macrophage altogether.

In summary, phagocytosed *S. aureus* USA300 withstand killing in the macrophage phagolysosome where the bacteria initiate intracellular replication. Intracellular replication precedes the onset of host cell death, which is characterized by membrane blebbing, caspase-3 activation and a rapid loss of membrane integrity. Importantly,
failure to curtail intracellular growth and to contain bacteria enables their escape to the extracellular milieu where they can disseminate. A better understanding, at the molecular level, of how MRSA accomplish this feat will facilitate the development of intervention strategies to augment host immunity and the eradication of infection.

**Experimental procedures**

**Reagents**

Cell proliferation dye eFluor-670 was from eBiosciences and the DNA dye Hoescht 33342 and human IgG suspension were form Sigma-Aldrich. Molecular probes fluorescent WGA was from ThermoFisher Scientific. The pharmacological agent z-VAD-FMK was from Enzo Life Sciences and CalyculinA was from Santa Cruz Biotechnology, Inc. All cytokines were purchased from PeproTech®. Monoclonal rat anti-mouse LAMP-1 antibody clone 1D4B developed by A.J. Thomas, Johns Hopkins School of Medicine, was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. The monoclonal mouse anti-human LAMP-1 antibody clone H4A3 was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and deposited by J.T. August and J.E.K. Hildreth, of the Johns Hopkins School of Medicine. The following fluorophore conjugated antibodies were from Jackson Immunoresearch: donkey anti-rat Alexa Fluor® 488 Fab, goat anti-human Alexa Fluor® 647, rabbit anti-sheep TRITC, FITC, and DyLight-647, donkey anti-rabbit antibodies were from Jackson Immunoresearch; donkey anti-rat Alexa Fluor® 488 Fab, goat anti-human Alexa Fluor® 647, rabbit anti-sheep TRITC, FITC, and DyLight-647, donkey anti-rabbit Alexa Fluor® 488 and goat-anti-rabbit Cy3.

**Bacterial strains and culture conditions**

The bacterial strains used in this study are summarized in Table 1. *S. aureus* and *M. luteus* were routinely cultured in Tryptic Soy Broth (Difco) at 37°C with shaking. *E. coli* DH5α was cultured in LB (Difco) medium also at 37°C with shaking. Sheep blood agar plates were purchased from BD. For the cultivation of strains carrying antibiotic resistance genes, antibiotics were used as follows: tetracycline (4 μg/mL), erythromycin (3 μg/mL), chloramphenicol (10 μg/mL) for selection of *S. aureus* and ampicillin (100 μg/mL) and kanamycin (50 μg/mL) for *E. coli*.

**Mammalian cell culture**

RAW 264.7 macrophages from the American Type Culture Collection were maintained in RPMI 1640 buffered with sodium bicarbonate and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 10% FBS. This RPMI medium was used for all mammalian cell culture, macrophage infection assays and for all live-cell imaging experiments. Macrophages were cultured at 37°C in the presence of 5% CO₂, and RAW macrophages were passaged for no more than 6 weeks at which time new cells were revived from liquid nitrogen frozen stocks.

Primary human macrophages were derived from peripheral blood monocytes isolated from whole blood taken from healthy volunteers in compliance with the Office of Research Ethics at The University of Western Ontario (protocol number 104010). Mononuclear cell fractions were isolated with lympholyte®-poly (Cedarlane Laboratories) according to the manufacturer’s instructions. Monocytes were purified from mononuclear fractions as previously described and were cultured in RPMI containing 10% (v/v) FBS supplemented with antibiotic/antimycotic cell culture solution (Wisent Bioproducts) and recombinant human M-CSF at 10 ng/mL (Flannagan et al., 2012b). After 5 days of differentiation, adhered cells were washed with sterile PBS, and the culture medium was replaced with fresh medium containing M-CSF and excluding antibiotic/mycotic solution. Macrophages were differentiated through to day 7 and used experimentally until day 10.

To generate primary human macrophages polarized to the M1 state, adhered monocytes were cultured as above but with recombinant human G-M-CSF at 20 ng/ml instead of M-CSF. At

---

**Table 1. Bacterial strains and plasmids used in this study.**

| Bacterial strain | Description*                                           | Source/reference |
|-----------------|--------------------------------------------------------|------------------|
| *Staphylococcus aureus* | USA300 USA300 LAC; hypervirulent community-associated MRSA; cured of antibiotic resistance plasmid | Laboratory stock (75) |
|                 | RN4220 rKΦ80 ϕλ, capable of accepting foreign DNA |                  |
| *Micrococcus luteus* | H3129 Skin commensal                              | Laboratory stock (79) |
| *Escherichia coli* | DH5α F 80d lacZΔ M15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK mcr) supE44 relA1 deo R (lacZYA-argF) U169 phoA λ | Laboratory stock |
| pAH9            | Constitutive staphylococcal mCherry expression vector; Ery | (76) |
| Ppsa:gfp        | GFP expression vector where GFP expression is driven by constitutive psa promoter; Ery | (77) |
| GFP-Rab5       | Mammalian expression vector encoding human Rab5 fused to GFP; Kan | (78) |
| 2xFYVE-GFP     | Mammalian expression vector encoding tandem FYVE domains fused to GFP; Kan | (79) |
| CD63-GFP       | Mammalian expression vector encoding murine CD63 fused to GFP; Kan | S. Grinstein |
| PH-Ptc-i-RFP   | Mammalian expression vector encoding the pleckstrin homology domain of phospholipase-Cα fused to RFP; Kan | (43) |
| LactC2-RFP     | The C2 domain of lactadherin fused to RFP           | (41) |

Abbreviations: Ery, Tet and Kan indicate resistance to erythromycin, tetracycline and kanamycin respectively.

© 2015 John Wiley & Sons Ltd, Cellular Microbiology, 18, 514–535
day 5, fresh RPMI containing serum, G-M-CSF, recombinant human IFN-γ 10 ng/mL and lipopolysaccharide (Sigma-Aldrich) at 250 ng/mL were added to the cells that were differentiated through to day 7 and used experimentally until day 10.

**RAW cell transfection and mammalian expression plasmids**

Mammalian expression vectors used in this study are summarized in Table 1. To transfect DNA into RAW macrophages, cells were plated onto glass coverslips and cultured overnight. RAW macrophages were transfected with mammalian expression vectors using lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocol. Briefly, macrophages were transfected using 1 μg of DNA and 2 μL of lipofectamine 3000 per well of a 12-well tissue culture plate. After 7 h incubation with the lipofectamine/DNA mixture, RAW cells were rinsed with serum free RPMI and incubated overnight in RPMI with 10% (v/v) FBS.

**Macrophage infections and enumeration of gentamicin protected bacteria**

Primary human M-CSF-derived and RAW macrophages were infected with an MOI of 30 to 1, unless otherwise indicated. Bacteria cultured overnight in TSB were pelleted, re-suspended in warm serum free RPMI and then diluted in serum free RPMI to the appropriate cell density for infections. To synchronize phagocytosis, bacteria added to individual wells containing macrophages were centrifuged at 277 × g for 2 min followed by incubation at 37°C in the presence of 5% CO₂. In instances where infections were for 30 min or less, gentamicin treatment was omitted. For infections that were 30 min or greater, gentamicin was applied as described below.

For the enumeration of gentamicin protected bacteria, macrophages were washed at 30 min post-infection with sterile PBS and incubated for 60 min with serum free RPMI containing gentamicin (100 μg/mL). After gentamicin treatment, macrophages were rinsed with PBS and incubated further in RPMI containing 10% (v/v) FBS without antibiotic, unless otherwise indicated. Enumeration of antibiotic-protected bacteria (i.e. intracellular bacteria) was performed by lysing infected macrophages with 0.1% (v/v) Triton X-100 in PBS. Macrophage lysates were serially diluted and plated on tryptic soy agar plates. To enumerate bacteria at 24 h post-infection the tissue culture medium containing extracellular bacteria was collected and centrifuged to obtain a bacterial pellet. The resulting bacterial pellet was combined with the corresponding macrophage lysate, which was then serially diluted and plated to obtain a value that represents the number of intra and extracellular bacteria.

For the inactivation of *S. aureus*, bacteria were incubated in 8% (v/v) PFA for 45 min at room temp followed by incubation at 95°C for 15 min. PFA was quenched with TSB, and the inactivated bacteria were re-suspended in RPMI, prior to adding to macrophage cultures.

**Detection of extracellular staphylococci**

By virtue of protein A expression, extracellular staphylococci were detected by incubation with FITC, TRITC or DyLight-647 conjugated rabbit anti-sheep IgG at 3 μg/mL for 2 to 4 min prior to macrophage fixation. To enhance the fluorescence intensity of extracellular bacteria donkey anti-rabbit Alexa Fluor® 488 or goat anti-rabbit Cy3 IgG Fab fragments were also added during primary labelling. Alternatively, extracellular *Staphylococci* were labelled with WGA conjugated with tetramethyl rhodamine or Alexa Fluor® 647.

**Immunofluorescence staining of lysosome-associated membrane protein 1**

Murine LAMP-1 was detected using hybridoma supernatants containing the rat anti-mouse LAMP-1 and human LAMP-1 was detected using mouse anti-human LAMP-1. Macrophages were fixed with 4% (v/v) PFA for 20 min at room temperature and then permeabilized with ice-cold methanol for 3 min. Macrophages were then blocked in 1 mL of undiluted human serum for at least 4 h and then incubated with the anti-LAMP-1 antibody diluted 1/100 in human serum for 1.5 h. After washing, the appropriate secondary antibody was added at a concentration of 0.75 μg/mL, incubated for 30 min and then rinsed. Stained 18 mm coverslips were mounted on glass microscope slides using permafluor-mounting media (Thermo Scientific) according to the manufacturer's recommendations and imaged by laser scanning confocal microscopy.

**Lysotracker and dextran loading of macrophages**

For the detection of acidic compartments, macrophages grown on glass coverslips were incubated with Lysotracker green DND-26 (Molecular Probes®, Thermo Fisher Scientific) at a final concentration of 250 nM for 7 to 10 min. Cells were washed 3x with serum free RPMI and then immediately imaged by confocal microscopy. To load macrophages with dextran, macrophages previously adhered to coverslips were incubated overnight with 10,000 MW dextran conjugated with FITC or TRITC at 100 μg/mL (Molecular Probes®, Thermo Fisher Scientific). Prior to phagocytosis dextran-loaded macrophages were washed 3x with PBS and then incubated 1 to 1.5 h in serum-free RPMI at 37°C without dextran to label lysosomal compartments. After phagocytosis of *S. aureus* or IgG-beads, macrophages were imaged live by confocal fluorescence microscopy. In instances where FITC-dextran was used and prior to imaging, 20 mM ammonium chloride was added to cells to un-quench FITC fluorescence in acidic lysosomes and phagolysosomes.

**Fluorescence cell proliferation assay**

*S. aureus* USA300 expressing GFP were labelled for 5 min in 1x PBS with eFluor-670 cell proliferation dye (0.5 μg/mL). Labelled cells were centrifuged and re-suspended in 1 mL LB broth for 3 min to quench unreacted eFluor-670 dye. To inactive labelled cells bacteria were fixed in 8% (v/v) PFA for 30 min at
IgG-bead opsonization and phagocytosis

Silica beads (3.14 μm, Bangs Laboratories) were opsonized with human IgG (0.8 mg/mL) for at least 1 h prior to washing. IgG-opsonized beads were added to individual wells containing macrophages and then centrifuged at 277 × g for 1 min to synchronize binding of targets to the macrophages. After phagocytosis, cells were washed vigorously to remove unbound silica beads. Beads remaining extracellular were detected by staining for 3 min with anti-human fluorophore-conjugated secondary antibodies (0.75 μg/mL) prior to fixation with 4% (v/v) PFA. Phagocytic index represents the average number of beads internalized by macrophages and was determined by calculating the total number of internalized beads divided by the total number of macrophages. For each experiment the uninfected phagocytic index was set to 1 for normalization.

Induction of membrane blebs

RAW macrophages were treated with the phosphatase inhibitor calyculin A at a concentration of 20 nM for 20 min to induce blebbing, then stained with WGA AlexaFluor®-647 (1 mg/mL for 1 min) before fixing with 4% (v/v) PFA.
References

Alonzo, F. 3rd, Kozhaya, L., Rawlings, S.A., Reyes-Robles, T., DuMont, A.L., Myszka, D.G., et al. (2012) CORS is a receptor for Staphylococcus aureus leukotoxin ED. Nature: 493: 51–55.

Bartlett, A.H., and Hulten, K.G. (2010) Staphylococcus aureus pathogenesis: secretion systems, adhesins, and invasins. Pediatr Infect Dis J 29: 860–861.

Birmingham, C.L., Canadien, V., Kaniuk, N.A., Steinberg, B.E., Higgins, D.E., and Brumell, J.H. (2008) Listeriolysin O allows Listeria monocytogenes replication in macrophage vacuoles. Nature 451: 350–354.

Bohdanowicz, M., Schlam, D., Hermansson, M., Rizzuti, D., Fairn, G.D., and Ogata, K., et al. (2013) Phosphatidic acid is required for the constitutive ruffling and macropinocytosis of phagocytes. Mol Biol Cell 24: 1700–1712.

Boucher, H., Miller, L.G., and Razonable, R.R. (2010) Serious infections caused by methicillin-resistant Staphylococcus aureus. Clin Infect Dis 51: S183–S197.

Camilli, A., Tilney, L.G., and Portnoy, D.A. (1993) Dual roles in Listeria monocytogenes pathogenesis. Mol Microbiol 8: 143–157.

Christoforidis, S., Miaczynska, M., Ashkan, K., Wilm, M., Zhao, L., Yip, S.C., et al. (1999) Phosphatidylinositol-3-0H kinases are Rab5 effectors. Nat Cell Biol 1: 249–252.

Damo, S.M., Kehl-Fie, T.E., Sugitani, N., Holt, M.E., Rathi, S., Murphy, W.J., et al. (2013) Molecular basis for manganese sequestration by calprotectin and roles in the innate immune response to invading bacterial pathogens. Proc Natl Acad Sci U S A 110: 3841–3846.

Diekema, D.J., Richter, S.S., Heilmann, K.P., Dohrn, C.L., Riahi, F., Tendolkar, S., et al. (2014) Continued emergence of USA300 methicillin-resistant Staphylococcus aureus in the United States: results from a nationwide surveillance study. Infect Control Hosp Epidemiol 35: 285–292.

Dossett, J.H., Kronvall, G., Williams, R.C., and Quie, P.G. (1969) Antiphagocytic effects of staphylococcal protein A. J Immunol 103: 1405–1410.

Duclos, S., Diez, R., Garin, J., Papadopoulou, B., Descoteaux, A., Stenmark, H., and Desjardins, M. (2000) Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome leishmanicidial properties in RAW 264.7 macrophages. J Cell Sci 113: 3531–3541.

DuMont, A.L., Yoong, P., Day, C.J., Alonzo, F., McDonald, W.H., Jennings, M.P., and Torres, V.J. (2013) Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. Proc Natl Acad Sci U S A 110: 10794–10799.

Fairn, G.D., and Grinstein, S. (2012) How nascent phagosomes mature to become phagolysosomes. Trends Immunol 33: 397–405.

Fairn, G.D., Ogata, K., Botelho, R.J., Stahl, P.D., Anderson, R.A., De Camilli, P., et al. (2009) An electrostatic switch displaces phosphatidylinositol phosphate kinases from the membrane during phagocytosis. J Cell Biol 187: 701–714.

Flannagan, R.S., Cosio, G., and Grinstein, S. (2009) Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol 7: 355–366.

Flannagan, R.S., Harrison, R.E., Yip, C.M., Jaqaman, K., and Grinstein, S. (2010) Dynamic macrophage “probing” is required for the efficient capture of phagocytic targets. J Cell Biol 191: 1205–1218.

Flannagan, R.S., Jaumouillé, V., and Grinstein, S. (2012a) The cell biology of phagocytosis. Annu Rev Pathol 7: 61–98.

Flannagan, R.S., Jaumouillé, V., Huynh, K.K., Plumb, J.D., Downey, G.P., Valvano, M.A., and Grinstein, S. (2012b) Burkholderia cenocepacia disrupts host cell actin cytoskeleton by inactivating Rac and Cdc42. Cell Microbiol 14: 239–254.

Giese, B., Glowinski, F., Paprotka, K., Dittmann, S., Steiner, T., Sinha, B., and Fraunholz, M.J. (2011) Expression of θ-toxin by Staphylococcus aureus mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of θ-toxin. Cell Microbiol 13: 316–329.

Grosz, M., Kolter, J., Paprotka, K., Winkler, A.-C., Schäfer, D., Chatterjee, S.S., et al. (2014) Cytoplasmic replication of Staphylococcus aureus upon phagosomal escape triggered by phenol-soluble modulin α. Cell Microbiol 16: 451–465.

van Hal, S.J., Jensen, S.O., Vaska, V.L., Espedido, B.A., Paterson, D.L., and Gosbell, I.B. (2012) Predictors of mortality in Staphylococcus aureus bacteremia. Clin Microbiol Rev 25: 362–386.

Hamza, T., and Li, B. (2014) Differential responses of osteoblasts and macrophages upon Staphylococcus aureus infection. BMC Microbiol 14: 207.

Hanley, P.J., Kronlage, M., Kirschning, C., del Rey, A., Di Virgilio, F., Leipziger, J., et al. (2012) Transient P2X7 receptor activation triggers macrophage death independent of Toll-like receptors 2 and 4, caspase-1, and pannexin-1 proteins. J Biol Chem 287: 10650–10663.

Huynh, K.K., Eskelinen, E.-L., Scott, C.C., Malevanets, A., Saftig, P., and Grinstein, S. (2007) LAMP proteins are required for fusion of lysosomes with phagosomes. EMBO J 26: 313–324.

Huynh, K.K., Plumb, J.D., Downey, G.P., Valvano, M.A., and Grinstein, S. (2010) Inactivation of macrophage Rab7 by Burkholderia cenocepacia. J Innate Immun 2: 522–533.

Jarry, T.M., and Cheung, A.L. (2006) Listeriolysin O and α-toxin. Cell Microbiol 8: 329–333.

Jarry, T.M., Memmi, G., and Cheung, A.L. (2008) The expression of alpha-haemolysin is required for Staphylococcus aureus phagosomal escape after internalization in CFT-1 cells. Cell Microbiol 10: 1801–1814.

Kehl-Fie, T.E., Chitayat, S., Hood, M.I., Damo, S., Restrepo, N., Garcia, C., et al. (2011) Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of Staphylococcus aureus. Cell Host Microbe 10: 158–164.

Klit, K., Parker, D., Nieto, P., Ahn, D.S., Cohen, T.S., Chung, S., et al. (2015) Toxin-induced necroptosis is a major mechanism of Staphylococcus aureus lung damage. PLoS Pathog 11: e1004820.

Ko, Y.-P., Kuipers, A., Freitag, C.M., Jongerius, I., Medina, E., van Rooijen, W.J., et al. (2013) Phagocytosis escape by a
Staphylococcus aureus protein that connects complement and coagulation proteins at the bacterial surface. *PLoS Pathog* 9: e1003816.

Kobayashi, S.D., Braughton, K.R., Palazzolo-Ballance, A.M., Kennedy, A.D., Sampaio, E., Kristosturyan, E., *et al.* (2010) Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun* 2: 560–576.

Kochanek, K.D., Xu, J., Murphy, S.L., Miniño, A.M., and Kung, H.-C. (2012) Deaths: final data for 2009. *Nati Vital Stat Reports* 60: 1–116.

Koziel, J., Chmiest, D., Blyzek, D., Kmieciak, K., Miggelska, D., Maciag-Gudowska, A., *et al.* (2015) The Janus face of α-toxin: a potent mediator of cytoprotection in staphylococci-infected macrophages. *J Innate Immun* 7: 187–198.

Koziel, J., Kmieciak, K., Chmiest, D., Maresz, K., Miggelska, D., Maciag-Gudowska, A., *et al.* (2013) The role of McI-1 in *S. aureus*-induced cytoprotection of infected macrophages. *Mediators Inflamm* 2013: 427021.

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., *et al.* (2008) A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS One* 3: e1409.

Läm, T.-T., Giese, B., Chikhkabali, D., Kühn, A., Wolber, W., Pané-Farré, J., *et al.* (2010) Phagolysosomal integrity is generally maintained after *Staphylococcus aureus* invasion of nonprofessional phagocytes but is modulated by strain 6850. *Infect Immun* 78: 3392–3403.

Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N Engl J Med* 339: 520–532.

Martin, C.J., Booty, M.G., Rosebrock, T.R., Nunes-Alves, C., Desjardins, D.M., Keren, I., *et al.* (2012) Efferocytosis is an innate antibacterial mechanism. *Cell Host Microbe* 12: 289–300.

Mediavilla, J.R., Chen, L., Mathema, B., and Kreiswirth, B.N. (2012) Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). *Curr Opin Microbiol* 15: 588–595.

Melehani, J.H., James, D.B.A., DuMont, A.L., Torres, V.J., and Duncan, J.A. (2015) *Staphylococcus aureus* leukocidin A/B (LukAB) kills human monocytes via host NLRP3 and ASC when extracellular, but not intracellular. *PLoS Pathog* 11: e1004970.

Metzelaar, M.J., Wijngaard, P.L., Peters, P.J., Sixma, J.J., Nieuwenhuis, H.K., and Clevers, H.C. (1991) CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *J Biol Chem* 266: 3239–3245.

Mills, J.C., Stone, N.L., Erhardt, J., and Pittman, R.N. (1998) Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. *J Cell Biol* 140: 627–636.

Müller, S., Faulhaber, A., Sieber, C., Pfeifer, D., Hochberg, T., Ganz, M., *et al.* (2014) The endolysosomal cysteine cathepsins L and K are involved in macrophage-mediated clearance of *Staphylococcus aureus* and the concomitant cytokine induction. *FASEB J* 28: 162–175.

Nicholson, D.W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 6: 1028–1042.

Nordenfelt, P., and Tapper, H. (2011) Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* 90: 271–284.

Nygaard, T.K., Pallister, K.B., DuMont, A.L., DeWald, M., Watkins, R.L., Pallister, E.Q., *et al.* (2012) Alpha-toxin induces programmed cell death of human T cells, B cells, and monocytes during USA00i infection. *PLoS One* 7: e36532.

O’Keeffe, K.M., Wilk, M.M., Leech, J.M., Murphy, A.G., Laabei, M., Monk, I.R., *et al.* (2015) Manipulation of autophagy in phagocytes facilitates *Staphylococcus aureus* bloodstream infection. *Infect Immun*. Doi: 10.1128/IAI.00358–15

Otto, M. (2010) Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu Rev Microbiol* 64: 143–162.

Peschel, A., Jack, R.W., Otto, M., Collins, L.V., Staubitz, P., Nicholson, G., *et al.* (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J Exp Med* 193: 1067–1076.

Portnoy, D.A., Jacks, P.S., and Hinrichs, D.J. (1988) Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J Exp Med* 167: 1459–1471.

Rabinowitz, S., Horstmann, H., Gordon, S., and Griffiths, G. (1992) Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. *J Cell Biol* 116: 95–112.

Reyes-Robles, T., Alonzo, F., Kozhaya, L., Lacy, D.B., Unutmaz, D., and Torres, V.J. (2013) *Staphylococcus aureus* leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. *Cell Host Microbe* 14: 453–459.

Schnaufer, A., Kashkar, H., Leggio, S.A., Addicks, K., Krönke, M., and Krut, O. (2007) *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death. *J Biol Chem* 282: 2695–2706.

Shin, H.-W., Hayashi, M., Christoforidis, S., Lacas-Gervais, S., Hoepfner, S., Wenk, M.R., *et al.* (2005) An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. *J Cell Biol* 170: 607–618.

Smith, G.A., Marquis, H., Jones, S., Johnston, N.C., Portnoy, D.A., and Goldfine, H. (1995) The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. *Infect Immun* 63: 4231–4237.

Spaan, A.N., Vrielin, M., Wallet, P., Badiou, C., Reyes-Robles, T., Ohneck, E.A., *et al.* (2014) The staphylococcal toxins γ-haemolysin AB and CB differentially target phagocytes by employing specific chemokine receptors. *FASEB J* 485.

Stuart, L.M., Deng, J., Silver, J.M., Takahashi, K., Tseng, A.A., Hennessy, E.J., *et al.* (2005) Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. *J Cell Biol* 170: 477–485.

Thomas, C.A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S.C., and Khoury, J.E. (2000) Protection from lethal gram-positive Staphylococcus aureus infection. *J Immunol* 164: 4116–4125.
infection by macrophage scavenger receptor-dependent phagocytosis. J Exp Med 191: 147–156.
Tuchscher, L., Medina, E., Hussain, M., Vöker, W., Heitmann, V., Niemann, S., et al. (2011) Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med 3: 129–141.
Vanden Berghe, T., Vanlangenakker, N., Parthoens, E., Deckers, W., Devos, M., Festjens, N., et al. (2010) Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell Death Differ 17: 922–930.
Várnai, P., and Balla, T. (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. J Cell Biol 143: 501–510.
Vergne, I., Chua, J., Lee, H.-H., Lucas, M., Belisle, J., and Deretic, V. (2005) Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 102: 4033–4038.
Vieira, O.V., Bucc, C., Harrison, R.E., Trimble, W.S., Lanzetti, L., Gruenberg, J., et al. (2006) Is Panton–Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? J Infect Dis 194: 1761–1770.
Wilson, C.A., Tsushida, M.A., Allen, G.M., Barnhart, E.L., Applegate, K.T., Yam, P.T., et al. (2008) Myosin II contributes to cell-scale actin network treadmill through network disassembly. Nature 465: 373–377.
Yeung, T., Gilbert, G.E., Shi, J., Silvius, J., Kapus, A., and Grinstein, S. (2008) Membrane phosphatidyserine regulates surface charge and protein localization. Science 319: 210–213.
Yoong, P., and Torres, V.J. (2013) The effects of Staphylococcus aureus leukotoxins on the host: cell lysis and beyond. Curr Opin Microbiol 16: 63–69.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Pinocytic function of macrophages delivers fluid-phase material to intracellular cocci, and sustained antimicrobial treatment modulates the outcome of the S. aureus USA300/macrophage interaction.
Fig S2. The S. aureus-containing phagosome acquires early markers of phagosome maturation.
Fig S3. The S. aureus-containing phagosome does not acquire the marker of autophagy microtubule-associated protein 1A/1B-light chain 3 (LC3).
Fig. S4. Phagocytosed S. aureus UAS300 remain confined to membrane-bound phagolysosomes long after phagocytosis.
Fig. S5. Detection of S. aureus replication after macrophage infection.
Fig. S6. Induction of bleb formation in RAW macrophages does not accelerate escape of S. aureus USA300 from infected macrophages.
Fig. S7. Single time point analysis of caspase-3/7 activation fails to detect apoptotic RAW cells.
Video S1. Viable S. aureus USA300 emerge from within infected RAW macrophages.
Video S2. S. aureus USA300 emerge from infected macrophages that demonstrate properties apoptotic and necrotic modes of death.
Video S3. The induction of macrophage blebbing does not accelerate escape of S. aureus USA300 from within RAW macrophages.
Video S4. S. aureus USA300 emerges from primary human M-CSF-derived macrophages that display membrane blebbing and caspase activation.
Video S5. Inactivated S. aureus USA300 do not evoke death of primary human M-CSF-derived macrophages.