Neutrophils use selective autophagy receptor p62 to target *Staphylococcus aureus* for degradation in the zebrafish model

Josie F Gibson[1,2,3], Tomasz K Prajsnar[1,2,4], Justyna J Serba[1,2], Rebecca D Tonge[5], Andrew J Grierson[2,5], Philip W Ingham[3,6#], Stephen A Renshaw[1,2#], Simon A Johnston[1,2#*].

1. Department of Infection, Immunity and Cardiovascular disease, Medical School, University of Sheffield, S10 2TN, UK.
2. The Bateson Centre, University of Sheffield, Sheffield, S10 2TN, UK
3. Institute of Molecular and Cell Biology, Agency of Science, Technology and Research (A-Star), Singapore
4. Institute Biology Leiden, Leiden University, Leiden, The Netherlands
5. Sheffield institute for Translational Neuroscience, Department of Neuroscience, University of Sheffield, S10 2HQ, UK
6. Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

*Correspondence: s.a.johnston@sheffield.ac.uk
#Joint senior author

Abstract

Autophagy leads to degradation of cellular components, and has an important role in destroying intracellular pathogens. Autophagy receptors, including p62, target invading intracellular pathogens to the autophagy pathway for degradation. p62 is able to co-localise with intracellular *Staphylococcus aureus,* previously shown *in vitro*, however, whether p62 is beneficial or detrimental in host defence against *S. aureus* had not been determined.

The intracellular fate of *S. aureus* over time following phagocytosis by neutrophils had not previously been analysed *in vivo*, and is not readily possible in other *in vivo* models. Here we use the excellent imaging and genetic capabilities of the zebrafish to analyse the fate and location *S. aureus* within neutrophils throughout infection. This information adds context to the dynamic temporal recruitment of autophagy proteins Lc3 and p62, accomplished using transgenic reporter lines.

We demonstrate, using a new *p62* zebrafish mutant to block host p62 function, that p62 is required for a favourable host outcome in the zebrafish *S. aureus* infection model. We examine the fate of *S. aureus* throughout the time-course of infection, including bacterial degradation and cellular location, and establish that Lc3 and p62 recruitment to *S. aureus* within the neutrophil is altered depending on bacterial location within the neutrophil. We suggest that *in vivo* Lc3 marking of bacterial phagosomes within neutrophils may lead to bacterial degradation and that p62 is important for controlling cytoplasmic bacteria.

Introduction

Autophagy (macroautophagy) is a process of cellular self-degradation, in which damaged or redundant cellular components are taken into an autophagosome and subsequently trafficked to the lysosome for degradation; these degraded components can then be recycled for alternative uses by the cell (Mizushima *et al.*, 2008; Tanida, 2011). During infection, autophagy is used by host cells to degrade invading pathogens, termed xenophagy (Gatica, Lahiri and Klionsky, 2018; Sharma *et al.*, 2018).

Autophagy is considered largely non-selective of the cargo to be degraded, classically being induced by starvation conditions. However, selective autophagy is a process which enables specific cargo to be targeted to the autophagy pathway, which can be used to target invading pathogens. Selective autophagy uses autophagy receptors (ARs), proteins that interact with both autophagy machinery and the cargo to be degraded (Popovic and Dikic, 2012; Rogov *et al.*, 2014). Many ARs are involved in targeting invading pathogens, including p62 (also named sequestosome 1 (SQSTM1)), neighbour of Brca1 gene (NBR1), optineurin (OPTN) and nuclear dot protein 52 (NDP52) (Farré and Subramani, 2016). Within an infected cell, pathogens may be cleared by autophagy, however in some cases it may be beneficial to the pathogen to be targeted to the autophagy pathway where they are able to exploit the pathway for their own end.

Loss of autophagy function, for example through mutations in key autophagy genes, can increase the risk of infection from intracellular pathogens (Levine, Mizushima and Virgin, 2011). It is well established that pathogen presence can induce host cell autophagy and that pathogens can be degraded by this pathway. Intracellular pathogens such as *Mycobacterium marinum*, *Shigella flexneri* and *Listeria monocytogenes* (Mostowy *et al.*, 2011; Zhang *et al.*, 2019) can be targeted by ARs for degradation. Conversely, pathogens have evolved to be able to block or subvert immune defences, and autophagy is no exception. Indeed, many bacterial pathogens are able to inhibit autophagy induction, reside within the autophagy pathway by preventing lysosomal fusion, or avoid making contact with autophagic machinery at all (Deretic and Levine, 2009). In some cases it is beneficial to the pathogen to up-regulate the autophagy pathway, for example *Legionella pneumophila, Coxiella burnetii* and *Salmonella enterica* serovar typhimurium (Hernandez *et al.*, 2003; Amer and Swanson, 2005; Gutierrez *et al.*, 2005). The outcome of host cell autophagy is therefore varied between different invading pathogens.

Staphylococcus aureus is a bacterial pathogen that is able to reside within neutrophils as an intracellular niche (Thwaites and Gant, 2011; Prajsnar *et al.*, 2012). Although autophagy has been implicated in *S. aureus* infection, there are conflicting reports suggesting autophagy might be beneficial or detrimental for *S. aureus* (Schnaith *et al.*, 2007; Neumann *et al.*, 2016). Intracellular pathogens, including *S. aureus*, are able to escape the phagosome into the cytoplasm (Bayles *et al.*, 1998), likely through toxins excreted by the bacteria or membrane rupture due to bacterial growth. Once in the cytoplasm, bacteria can be ubiquitinated and targeted by ARs (Farré and Subramani, 2016). Indeed, p62 in fibroblasts and epithelial cells has been shown to localise to cytoplasmic *S. aureus* leading to autophagsome formation *in vitro* (Neumann *et al.*, 2016; Singh *et al.*, 2017). Therefore, we investigated whether p62 recruitment is employed by neutrophils in *S. aureus* infection and what influence selective autophagy has on infection outcome *in vivo*.

In order to examine the role of neutrophil autophagy in *S. aureus* infection we compared the fate of bacterial cells following Lc3 and p62 recruitment. We tested the role of p62 in pathogen handling *in vivo*, using the genetic tractability of the zebrafish to create a neutrophil specific p62 transgenic reporter, and a *p62* mutant. We show that p62 is recruited to intracellular *S. aureus* and loss of *p62* function adversely affects infection outcome for zebrafish following *S. aureus* infection.

Results

Staphylococcus aureus location within neutrophils changes throughout infection

Autophagy responses have been demonstrated to vary through the progression of infection and targeting by autophagy receptors is likely to occur later in infection. Therefore, to determine the fate and location of *S. aureus* in neutrophils over infection, *S. aureus* was and imaged at early (2 to 5 hours post infection (hpi)) and late (24 to 28hpi) time points. Initially, the established *Tg(mpx:eGFP)i114* line was used to analyse the fate of intracellular *S. aureus* throughout infection. We found a significant reduction in the number of bacterial cells within individual neutrophils occurs between early and late infection time points (Figure 1A), and that the number of neutrophils containing *S. aureus* is also significantly reduced throughout infection (Figure 1B). This suggested to us that neutrophils are able to effectively degrade intracellular *S. aureus* throughout infection.

We next sought to determine the location of bacteria, and relationship to autophagic machinery, within neutrophils. To do this we used the newly generated *Tg(lyz:RFP-GFP-Lc3)sh383* (Prajsnar *et al.*, 2019). We first confirmed that the infection dynamics were similar

to the *Tg(mpx:eGFP)i114* line, with a significant reduction in intracellular bacteria by 26hpi, and a significant reduction in infected neutrophils observed (Figure 1C, D). The labelling of *S. aureus* containing vesicles enabled the identification of intracellular bacteria that were within a vesicle (Figure 1E, Supplementary Figure 1 A) or free in the cytoplasm (Figure 1F). We found that the proportion of bacteria within vesicles was significantly reduced throughout infection, whereas the number of bacteria within the cytoplasm remains relatively constant at a low level, despite becoming proportionally higher (Figure 1G). Thus, *S. aureus* phagocytosed by a neutrophil are initially located in a phagocytic vesicle and are subsequently degraded. However, a smaller proportion of *S. aureus* is able to survive to later infection time points, and these predominantly reside in the cytoplasm.

Generation and characterisation of an *in vivo* neutrophil GFP-p62 reporter line

A previous study identified colocalisation of p62 with *S. aureus* in non-immune cells (Neumann *et al.*, 2016). Our findings demonstrated a small but significant population of bacteria that were cytoplasmic, and therefore a possible target for p62 binding. Therefore, we generated a transgenic neutrophil-specific p62 reporter zebrafish line to examine whether p62 and intracellular pathogens are co-localised *in vivo*. We used GFP attached with a small linker region to the N-terminus of *p62* in order to produce a fluorescently marked fusion protein expressed within neutrophils via the lysozyme C (*lyz*) promoter (Hall *et al.*, 2007). We were able to identify GFP expressing cells co-expressing with 98% of separately marked neutrophils (*Tg(lyz:nfsB-mCherry)sh260*) (Buchan *et al.*, 2019), and within the *Tg(lyz:GFP-p62)i330* reporter line (hereafter called GFP-p62 reporter; Supplementary figure 2A-C)

We next examined whether the GFP-p62 protein is able to function as expected. Interestingly, in the double labelled larvae, GFP puncta but not mCherry puncta were seen (Supplementary figure 2D). Similar p62 puncta have been observed *in vitro* for endogenous p62, that required UBD to function (Bjørkøy *et al.*, 2005). To test whether the GFP-p62 puncta observed in the GFP-p62 reporter line respond as expected, GFP-p62 reporter larvae were treated with autophagy inhibitor Bay-K8644 which is can block autophagy in the zebrafish model (Williams *et al.*, 2008). When autophagy is blocked, p62 degradation is not expected, as p62 proteins are degraded when targeting cargo for autophagic degradation. There was a significant increase in the number of neutrophils which contained GFP-p62 puncta following Bay-K8644 treatment in comparison to non-treated controls (Supplementary figure 2E), as well as a significant increase in the number of GFP-p62 puncta within individual neutrophils as expected for endogenous p62 (Supplementary figure 2F). This suggests that the GFP-p62 puncta are not being degraded through autophagy and build up within the cell, as reported for endogenous p62 (Bjørkøy *et al.*, 2009).

As we had for neutrophils and Lc3 positive vesicles we examined the location of *S. aureus* throughout infection with our GFP-p62 reporter (Figure 1) and found that the overall fate and location of *S. aureus* within neutrophils was consistent. There was a reduction in the number of bacteria within neutrophils at 26hpi in comparison to 2hpi (Supplementary figure 3A) in addition to a significant reduction in the number of infected neutrophils from 2hpi to 26hpi (Supplementary figure 3B), suggesting that neutrophils are efficiently degrading these bacteria. We observed bacteria in neutrophils with low GFP fluorescence (relative to the cytoplasm; p62GFP^{low} Supplementary figure 3C) that represented *S. aureus* vacuoles as well as GFP labelled bacteria either in a damaged phagosome, or located in the cytoplasm (Supplementary figure 3D). We counted *S. aureus* in a vesicle or labelled with GFP-p62 and compared bacterial locations at 2hpi and 26hpi. We determined that the proportion of *S. aureus* within vesicles was significantly reduced by 26hpi (Supplementary figure 3E), and that the number of bacteria within the cytoplasm is similar at both time points (Supplementary figure 3E), in agreement with our *Tg(lyz:RFP-GFP-Lc3)sh383* data (Figure 1) and further evidencing that p62GFP^{low} regions observed in the GFP-p62 reporter line were *S. aureus* containing phagosomes.

Lc3 and p62 are recruited to *Staphylococcus aureus* within neutrophils

We determined that GFP-p62 puncta co-localise with *S. aureus* either marking a vesicle containing *S. aureus* (Figure 2A), or directly in contact with *S. aureus* located in the cytoplasm (Figure 2B). For puncta marking *S. aureus* in vesicles, no difference in the proportion of vesicles marked was observed at 2 or 26hpi, although the actual number of puncta marking was dramatically reduced by 26hpi (Figure 2C) as most bacteria were degraded. GFP-puncta marking bacteria in the cytoplasm was significantly decreased in marking of bacteria at 26hpi (Figure 2D), as expected given that p62 is degraded along with cargo targeted for degradation (Bjørkøy *et al.*, 2009). Cytoplasmic GFP-p62 puncta were modulated by *S. aureus* infection and autophagy machinery targeting drugs. In agreement further with this, comparison between infected and uninfected neutrophils showed there was no difference in the number of cytoplasmic GFP-p62 at 2hpi but a significant reduction by 26hpi (Figure 2E, F). We next examined whether Lc3 is able to localise to vesicular and cytoplasmic *S. aureus*. At 2hpi and 26hpi there is no difference in the proportion of Lc3 marking vesicles, but most vesicular bacteria are degraded by 26hpi (Figure 2G), showing that a rapid Lc3 response to *S. aureus* infection occurs. In contrast, vesicles containing *S. aureus* are significantly more likely to have Lc3 puncta associated at 2hpi, (Figure 2H, Supplementary figure 1B), most bacteria are still cleared by 26hpi and there is no significant change in the association of Lc3 puncta to *S. aureus* in the cytoplasm over time (Figure 2I).

Loss of p62 reduces zebrafish survival following *Staphylococcus aureus* infection

Thus, we were able to comprehensively demonstrate the steps of Lc3 and autophagy receptor recruitment *in vivo* in the degradation of *S. aureus* by neutrophils. However, the question remained what was the requirement of p62 in the outcome of *S. aureus* infection. Our molecular studies suggested p62 was associated with the degradation of bacteria that escaped the phagosome. To test this prediction we examined the role of p62 in *S. aureus* zebrafish infection using a morpholino-modified antisense oligonucleotide (morpholino) targeting *p62* (van der Vaart *et al.*, 2014) to knock-down *p62* expression in the zebrafish larvae. Knock down of *p62* resulted in a significant reduction in survival compared to control larvae suggesting that *p62* was required for control of cytoplasmic *S. aureus* (Figure 3A). To confirm this result we further generated a *p62* mutant zebrafish (sh558) that lacked a functional UBD, inhibiting the ability of p62 to bind to ubiquitinated cargo. In agreement with our knock down study the *p62* mutant zebrafish (sh558) larvae were significantly more susceptible to *S. aureus* infection than wild-type siblings (Figure 3B). Thus, in addition to demonstrating how Lc3 and p62 were localised during intracellular handling of *S. aureus* by neutrophils we could independently show the requirement of p62 in the outcome of infection.

Discussion

Using the unique attributes of long-term high-resolution imaging and genetic manipulation of zebrafish larvae we have shown the dynamics of Lc3 on the *S. aureus* containing vacuole, its relation to bacterial degradation, and how p62 recognises cytoplasmic bacteria meaning that loss of p62 activity is sufficient to decrease zebrafish survival following *S. aureus* infection.

Loss of zebrafish *p62*, through morpholino-mediated knockdown, significantly increases susceptibility to infection to *S. aureus*. This is to our knowledge the first *in vivo* evidence that p62 is important in the outcome of intracellular handling of *S. aureus*. To confirm the *p62* knockdown data, we generated a zebrafish *p62* mutant lacking the UBD domain, which confirmed a significant increase in susceptibility to zebrafish in *S. aureus* infection. This suggests that for *S. aureus* infection control, the *p62* UBD, which is able to bind to ubiquitinated *S. aureus* (Neumann *et al.*, 2016; Singh *et al.*, 2017), is important for host control of infection. In addition to its role as an autophagy receptor, p62 can aid killing of pathogens through delivery of antimicrobial peptides (Ponpuak *et al.*, 2010), it may be possible anti-microbial peptides delivered by p62 are important in neutrophil control of *S. aureus* infection. The *p62* zebrafish mutant represents a valuable tool in the analysis of selective autophagy in infection, which may also be useful for study of other intracellular pathogens or in other autophagy diseases, for example in neurodegenerative disorders.

The GFP-p62 reporter line was generated to determine whether selective autophagy protein p62 co-localises to intracellular pathogens *in vivo.* Indeed, we show for the first time that GFP-p62 puncta are recruited to *S. aureus* inside neutrophils during zebrafish infection. Furthermore, we analysed the intracellular pathogen location as infection progresses with the GFP-p62 reporter in combination with the Lc3 line to provide context for the changes in Lc3 and p62 marking of these pathogens over time. These capabilities are not readily possible in other *in vivo* models such as the mouse.

The zebrafish *S. aureus* infection model was used to analyse the infection dynamic in terms of pathogen location and survival, in addition to autophagy proteins Lc3 and p62 co-localisation. Although *in vitro* studies have described co-localisation of p62 and autophagy in pathogen handling, until now, no evidence of direct p62 interactions with these pathogens has been shown in neutrophils or *in vivo*. Furthermore, the intracellular fate of *S. aureus* over time within neutrophils had not been examined *in vivo*, meaning the context of any temporal changes of autophagy protein co-localisation is unknown. We show that most *S. aureus* is contained within a vesicle soon after infection and that by 26hpi most *S. aureus* is absent from neutrophils, likely being degraded. The large reduction of neutrophils containing bacteria from 2hpi to 26hpi, leaving a small population at 26hpi may be representative of the immunological "bottleneck" previously described to be neutrophil dependent (Prajsnar *et al.*, 2012). Interestingly, it appears that Lc3 marks the majority of bacterial vesicles. Lc3 localisation to *S. aureus* may represent Lc3 recruitment to autophagosomes, however since recruitment is observed at early infection time points, it may represent Lc3-associated phagocytosis (LAP), which is also observed in *Listeria monocytogenes* infection of macrophages (Gluschko *et al.*, 2018). Since most bacteria is degraded it appears that Lc3 marking of vesicles could lead to bacterial degradation in the zebrafish (Figure 4). Here, we demonstrate study of autophagic Lc3 interactions *in vivo* using the zebrafish model and suggest that Lc3 marking of *S. aureus* vesicles within neutrophils precedes bacterial degradation.

Interaction of p62 with *S. aureus* has been demonstrated in *in vitro* studies using fibroblasts and epithelial cells (Neumann *et al.*, 2016; Singh *et al.*, 2017). We demonstrate for the first time *in vivo* that loss of *p62* in *S. aureus* infection is detrimental towards infection outcome in the zebrafish model. *S. aureus* is known to be capable of escaping the phagosome to survive in the cytoplasm. *In vitro* data shows *S. aureus* can be targeted for autophagic degradation by p62 (Neumann *et al.*, 2016; Singh *et al.*, 2017), where puncta appear to be co-localised with *S. aureus*. Our new zebrafish GFP-p62 reporter shows cytoplasmic puncta formation which has also been observed in other cell culture studies, using similar GFP-p62 reporter systems (Bjørkøy *et al.*, 2005; Pankiv *et al.*, 2007; Larsen *et al.*, 2010). We observe both cytoplasmic and vesicular *S. aureus* co-localised with GFP-p62 puncta, allowing us to visualise puncta

marking both cytoplasmic and vesicular *S. aureus*. By comparing GFP-p62 puncta marking of intracellular *S. aureus* with the location of bacteria over time, it is interesting to note that p62 marking is reduced overtime for cytoplasmic bacteria (which appear to be a small population which persist throughout infection), this may indicate that cytoplasmic bacteria marked with p62 are degraded (Figure 4). Furthermore, at later time points in *S. aureus* infection the number of GFP-p62 puncta is reduced within infected cells, suggesting when bacteria escape the phagosome, p62 becomes important in controlling cytoplasmic bacteria.

In conclusion, we have demonstrated that the zebrafish infection model provides an excellent model to study host-pathogen interactions, specifically the role of neutrophil autophagy in *S. aureus* infection. We utilise the imaging potential of zebrafish to demonstrate for the first time *in vivo* that p62 co-localises to *S. aureus* within neutrophils. Furthermore, we examine how autophagy proteins p62 and Lc3 interactions with intracellular pathogens change during the course of infection and suggest that Lc3 marking of *S. aureus* vesicles leads to degradation of the majority of bacteria, whereas p62 is important in the degradation of cytoplasmic bacteria at later stages of infection. Finally, we demonstrate for the first time, that host p62 is beneficial for the host outcome following *S. aureus* infection.

Materials and methods

Ethics statement

Animal work was carried out according to guidelines and legislation set out in UK law in the Animals (Scientific Procedures) Act 1986, under Project License PPL 40/3574 or P1A4A7A5E). Ethical approval was granted by the University of Sheffield Local Ethical Review Panel. Animal work completed in Singapore was completed under the Institutional Animal Care and Use Committee (IACUC) guidelines, under the A*STAR Biological Resource Centre (BRC) approved IACUC Protocol #140977.

Zebrafish husbandry

Zebrafish strains were maintained according to standard protocols (Nüsslein-Volhard and Dahm, 2002). For animals housed in the Bateson Centre aquaria at the University of Sheffield, adult fish were maintained on a 14:10-hour light/dark cycle at 28 °C in UK Home Office approved facilities. For animals housed in IMCB, Singapore, adult fish were maintained on a 14:10-hour light/dark cycle at 28°C in the IMCB zebrafish facility. LWT and AB wild-type larvae were used in addition to transgenic lines, *Tg(lyz:GFP-p62)i330* created in this study, *Tg(lyz:RFP-GFP-Lc3)sh383* (Prajsnar *et al.*, 2019), *Tg(lyz:nfsB-mCherry)sh260* (Buchan *et al.*, 2019) and *Tg(mpx:eGFP)i114* (Renshaw *et al.*, 2006). Generation of p62 sh558 mutant zebrafish is described below. Larvae were maintained in E3 plus methylene blue at 28°C until 5dpf.

S. aureus culture

The *Staphylococcus aureus* strain SH1000 was used in this study. A single bacterial colony was placed in 10ml brain heart infusion (BHI) medium (Oxoid number 1) overnight at 37°C, 250rpm. 500µl of this overnight culture was then added to 50ml of BHI medium and incubated at 37°C, 250rpm until OD_{600} 1. The bacteria were then pelleted at 4500rpm, 4°C for 15 minutes. The bacteria were then re-suspended in PBS (Oxoid, BR0014G), using a volume to dilute to the required dose, with 1500cfu/nL being standard. Bacteria were incubated on ice for a short period, until use. Strains used: SH1000 wild-type strain (Horsburgh *et al.*, 2002), SH1000-pMV158-mCherry (Boldock *et al.*, 2018), SH1000-pMV158-GFP (Boldock *et al.*, 2018).

Zebrafish micro-injection

For p62 morpholino micro-injections: Larvae were injected immediately after fertilisation using a *p62* morpholino (van der Vaart *et al.*, 2014). A standard control morpholino (Genetools) was used as a negative control. For injection of *S. aureus,* zebrafish larvae were injected at 2 dpf and monitored until a maximum of 5dpf. Larvae were anesthetised by

immersion in 0.168 mg/mL tricaine in E3 and transferred onto 3% methyl cellulose in E3 for injection. For *S. aureus* 1nl of bacteria, containing 1500cfu, was injected into the yolk sac circulation valley. Larvae were transferred to fresh E3 to recover from anaesthetic. Any zebrafish injured by the needle/micro-injection were removed from the procedure. Zebrafish were maintained at 28°C.

Generation of *Tg(lyz:GFP-p62)i330* transgenic line

The generation of the *Tg(lyz:GFP-p62)i330* line was completed through use of the Gateway™ system in combination with Tol2 transgenesis. To make the required expression clone, *pDest(lyz:GFP-p62)*, the *p5E-lyz* entry clone (Elks *et al.*, 2011) and the *pME-GFP-nonstop* middle entry vectors were used. The destination vector *pDestTol2CG*, was chosen which included Tol2 sites for integration into the genome, in addition to a GFP heart marker. The required p62 3' entry vector, and expression clone *pDest(lyz:GFP-p62)* were constructed following the Multisite Gateway™ three-fragment vector construction kit. To generate Tol2 mRNA, a *pT3Tol2* plasmid was used. The DNA *pT3Tol2* plasmid was linearised through a restriction site digest. Tol2 mRNA was generated by a transcription reaction (Ambion T3 mMessage Machine) from the linear *pT3Tol2* plasmid. Tol2 mRNA and *pDest(lyz:GFP-p62)* were co-injected into a single cell (at the single cell stage) of wild-type AB larvae. A 1nl injection contained 30pg of Tol2 mRNA and 60pg of *pDest(lyz:GFP-p62)*.

Microscopy of infected zebrafish

Larvae were anaesthetized 0.168 mg/mL tricaine in E3 and mounted in 0.8% low melting agarose onto glass bottom microwell dishes (MatTek P35G-1.5-14C). An UltraVIEW VoX spinning disk confocal microscope (Perkin Elmer, Cambridge, UK) was used for imaging neutrophils within larvae. 405nm, 445nm, 488nm, 514nm, 561nm and 640nm lasers were available for excitation. A 40x oil objective (UplanSApo 40x oil (NA 1.3)) was used for cellular level imaging. GFP, TxRed emission filters were used and bright field images were acquired using a Hamamatsu C9100-50 EM-CCD camera. Volocity software was used. Between early and late time points zebrafish larvae were placed back into E3 and maintained at 28°C.

Image analysis

Image analysis was performed using ImageJ software, to quantify the number of *S. aureus* cells within neutrophils, and to quantify GFP-p62 puncta and Lc3 co-localisation to these pathogens.

Drug treatment of zebrafish

Larvae were treated with autophagy inducers and inhibitors through immersion in E3 medium. All drugs were sourced from Sigma-Aldrich, UK. The Bay K8644 was added to the E3 to the required concentration, Bay-K 6844 1µM. Larvae were incubated at 28°C for 24 hours before microscopy. Zebrafish were not anaesthetised for immersion drug treatments.

Generation of *p62* mutant

A zebrafish *p62* mutant was generated using CRISPR/Cas9 mutagenesis. A guide RNA targeting exon 8 of zebrafish *p62* (ACAGAGACTCCACCAGCCTA) was inserted into a published oligonucleotide scaffold (Talbot and Amacher, 2014) and injected together with recombinant Cas9 protein (New England Biolabs) into 1-2 cell stage zebrafish (AB strain). Efficiency of mutagenesis was confirmed using high resolution melt curve analysis and several founders were identified. $P62^{sh558}$ carries a 10 base pair deletion resulting in a frameshift and premature truncation of p62 in the ubiquitin-associated (UBA) domain.

Statistical analysis

Statistical analysis was performed as described in the results and figure legends. We used Graph Pad Prism 7 (v7.04) for statistical tests and plots.

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Author contributions

JFG, TKP, SAJ, AJG, PWI and SAR conceived this study and designed experiments. JFG prepared manuscript with input from TKP, SAJ, AJG and SAR. JFG and JJS conducted bacterial fate and location analysis. JFG performed GFP-p62 reporter line generation and characterisation, zebrafish infection, intracellular imaging and subsequent analysis. TKP performed all p62 morpholino experiments with some assistance from JFG. AJG and RDT generated the p62 stable mutant line.

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Figure Legends

Figure 1: *Staphylococcus aureus* location within neutrophils changes from vesicular to cytoplasmic throughout infection

A-B *Tg(mpx:eGFP)*i114 larvae were injected at 1dpf with 1500cfu SH1000-mCherry *S. aureus*, and imaged at early (1-5hpi) and late (24-28hpi) time points **A** Number of bacteria contained in neutrophils, with maximum 100 bacterial cells counted (n=11-13, unpaired t-test, ***p<0.001, +/- SEM) **B** Proportion of neutrophils containing bacteria (n=11-12, unpaired t-test, ****p<0.0001, +/- SEM) **C-G** *Tg(lyz:RFP-GFP-Lc3)*sh383 larvae were injected at 2dpf with GFP *S. aureus*, and imaged at 2hpi, and ~26hpi. **C** The number of infected or non-infected neutrophils at 2hpi and 26hpi (n=3, 17 2hpi larvae, 11 26hpi larvae unpaired t-test, **p<0.01, +/- SEM) **D** The proportion of infected or non-infected neutrophils at 2hpi and 26hpi (****p<0.0001 Chi Square test, n=3, 17 2hpi larvae, 11 26hpi larvae) **E** *S. aureus* with Lc3 marking the entire vesicle, scale 9um, demonstrating a vesicle **F** *S. aureus* in the cytoplasm, scale 9um **G** Proportion *S. aureus* events observed within vesicles or cytoplasm at 2hpi and 26hpi (***p<0.001, Fisher's exact test, n=3, 17 larvae at 2hpi, and 11 larvae at 26hpi)

Figure 2: *In vivo* recruitment of GFP-p62 puncta during *S. aureus* infection

A Representative image of *S. aureus* observed within a likely "vesicle" with GFP-p62 puncta localisation, scale 7um **B** representative image of *S. aureus* observed within the cytoplasm with GFP-p62 puncta localisation, scale 9um **C** *S. aureus* within vesicles, co-localised with GFP-p62 at 2hpi and 26pi (ns, Fisher's exact test, n=3, 14 larvae at 2hpi, and 12 larvae at 26hpi) **D** *S. aureus* in the cytoplasm, co-localised with GFP-p62 at 2hpi and 26hpi (*p<0.05, Fisher's exact test, n=3, 14 larvae at 2hpi, and 12 larvae at 26hpi) **E** GFP-p62 puncta in the cytoplasm of infected and non-infected at 2hpi (ns, Mann-Whitney test, n=3, error bars +/- SD, 14 larvae) **F** GFP-p62 puncta in the cytoplasm of infected and non-infected at 26hpi (**p<0.01, Mann-Whitney test, n=3, error bars +/- SD, 12 larvae) **G-I** 2500cfu of GFP *S. aureus* injected into *Tg(lyzC:RFP-GFP-Lc3)*, larvae imaged in the CHT at 2hpi (2hpi) and 26hpi (26hpi). **G** Lc3 association to the entire *S. aureus* vesicle at 2hpi and 26hpi (ns, Fisher's test, n =3, 17 2hpi larvae, 11 26hpi larvae) **H** The number of *S. aureus* vesicles with Lc3 puncta (*p<0.05, Fisher's test, n =3, 17 2hpi larvae, 11 26hpi larvae) **I** The number of *S. aureus* events in the cytoplasm with Lc3 puncta at 2hpi and 26hpi (ns, Fisher's test, n =3, 17 2hpi larvae, 11 26hpi larvae).

Figure 3: Zebrafish survival is reduced following infection with *Staphylococcus aureus* in the absence of p62

A-B Zebrafish survival following *S. aureus* infection, larvae were injected with 1500cfu of SH1000 at 30hpf. **A** *p62* morphants or control morphants survival (n=3, 74-80 larvae per group, p=0.004, Log-rank, Mantel-Cox test) **B** *p62* mutant or wild-type sibling survival (n=3, 57-60 larvae per group, p=0.0168, Log-rank, Mantel-Cox test)

Figure 4: Prospective mechanism of Lc3 and p62 in neutrophil *S. aureus* infection

A *S. aureus* infection: 1. The majority of bacteria are enclosed in vesicles early in infection 2. The large proportion of vesicular bacteria are degraded over infection, there may be a role of LC3-assocaiated phagocytosis 3. Most bacteria are degraded by neutrophils 4. Some *S. aureus* is able to escape the phagosome into the cytoplasm 5. A small population of bacteria survives in the cytoplasm 6. p62 mediated degradation of cytoplasmic bacteria 7. Some bacteria are able to survive in the cytoplasm potentially being responsible for uncontrolled infection

Supplementary figure legends

Supplementary figure 1: Lc3 marking of *Staphylococcus aureus in Tg(lyz:RFP-GFP-Lc3)sh383* larvae

A-B *Tg(lyz:RFP-GFP-Lc3)sh383* larvae were injected at 2dpf with GFP *S. aureus*, and imaged at 2hpi, and ~26hpi. **A** *S. aureus* without Lc3 marking the entire vesicle, scale 5.8um. Also demonstrating a clear vesicle structure **B** *S. aureus* with Lc3 puncta marking the edge of a vesicle, scale 9um

Supplementary figure 2: The GFP-p62 reporter line expresses a GFP-p62 fusion protein within neutrophils

A Illustration of the plasmid created to make the GFP-p62 zebrafish reporter line, pDest(*lyz*:*GFP-p62*). **B** Representative image of neutrophils within larva resulting from crossing *Tg(lyz:nfsB-mCherry)sh260* (which has mCherry fluorescent neutrophils), with *Tg(lyz:GFP-p62)i330* which shows GFP expression within neutrophils. Image taken of the caudal haematopoietic tissue shown in red box of diagram of larvae. **C** The percentage of neutrophils which express fluorescence of GFP alone, mCherry alone, or both GFP and mCherry using six representative larvae from a cross of *Tg(lyz:nfsB-mCherry)sh260* with *Tg(lyz:GFP-p62)i330* zebrafish lines (one-way ANOVA, Tukey's multiple comparisons ****=p<0.001). **D** High magnification imaging of an individual neutrophil showing GFP puncta, but not mCherry puncta, from a *Tg(lyz:nfsB-mCherry*)*sh260* cross with *Tg(lyz:GFP-p62)i330*. **E-F** Quantification of images taken of *Tg(lyz:GFP-p62)i330* larvae treated with autophagy blocking drug Bay K8644. **E** Percentage of neutrophils with puncta present (Chi-square test, **p<0.01, n=3, 15-20 larvae per group) **F** Number of puncta observed within individual neutrophils (Error bars +/- SD, Unpaired T-test **p<0.01, n=3, 15-20 larvae per group)

Supplementary figure 3: The intracellular location of *Staphylococcus aureus* within neutrophils changes throughout infection

A-E *Tg(lyz:GFP-p62)i330* larvae were injected with mCherry *S. aureus* at 2dpf, and imaged at 2hpi, and ~26hpi. **A** The number of *S. aureus* events observed within neutrophils at 2hpi and 26hpi (n=3, 14 larvae at 2hpi, and 12 larvae at 26hpi) **B** Proportion of neutrophils infected or non-infected with *S. aureus* at 2hpi and 26hpi (****p<0.001, Fisher's exact test, n=3, 14 larvae at 2hpi, and 12 larvae at 26hpi) **C** Representative image of *S. aureus* observed within a likely "vesicle" **D** representative image of *S. aureus* observed within the cytoplasm **E** Proportion *S. aureus* events observed within vesicles or cytoplasm at 2hpi and 26hpi (****p<0.001, Fisher's exact test, n=3, 14 larvae at 2hpi, and 12 larvae at 26hpi)

Figures

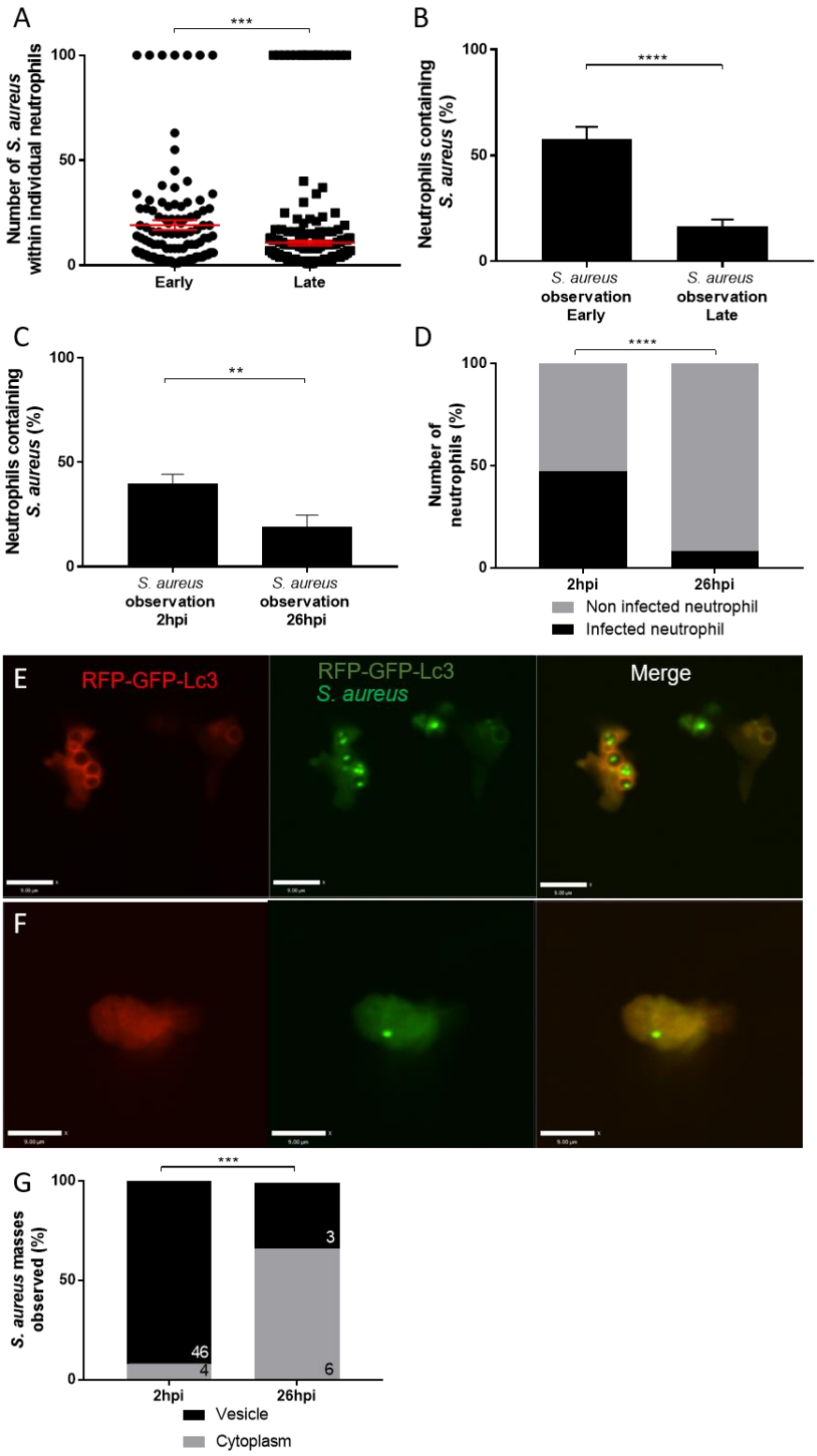


Figure 1: *Staphylococcus aureus* location within neutrophils changes from vesicular to cytoplasmic throughout infection

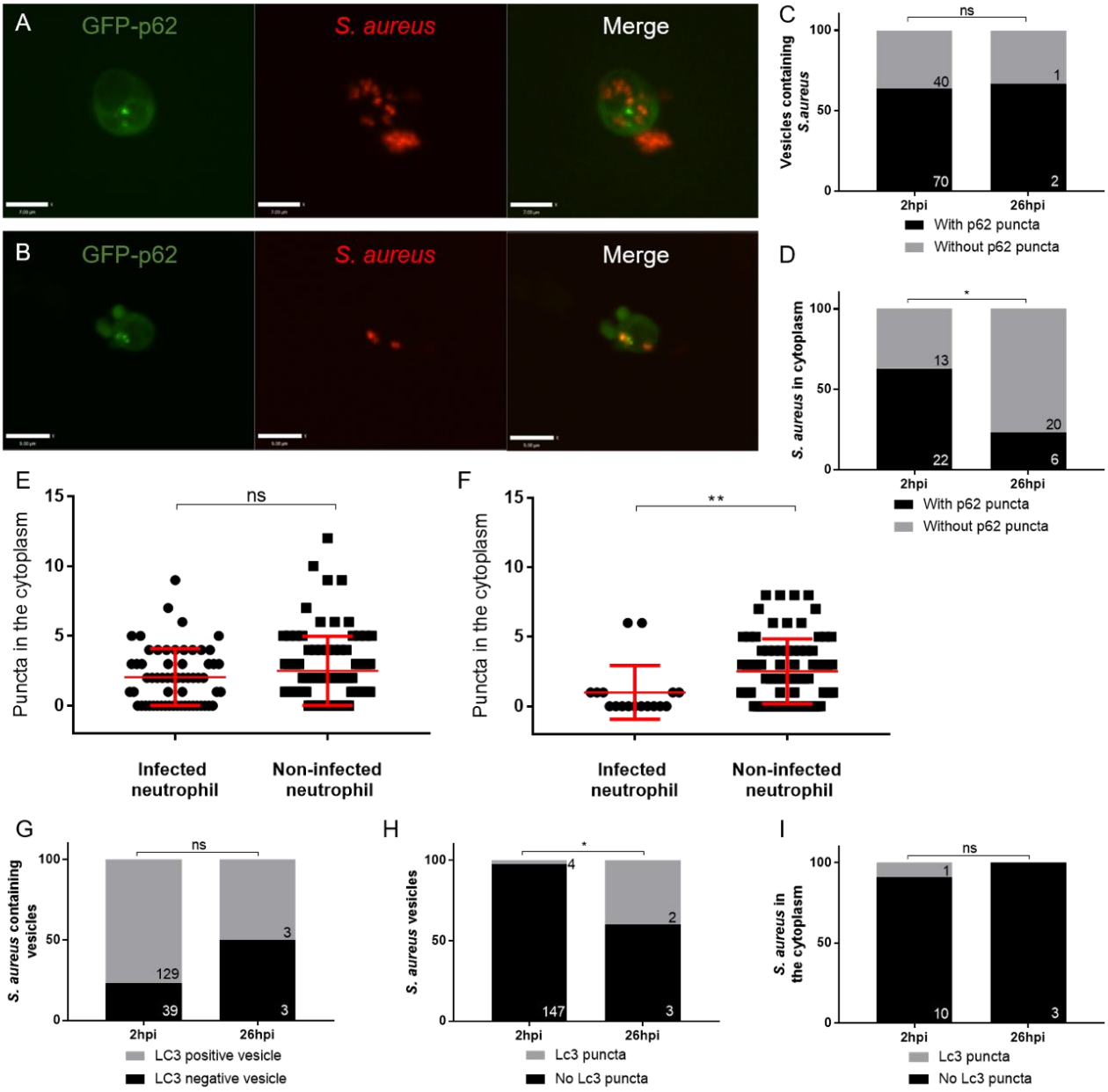
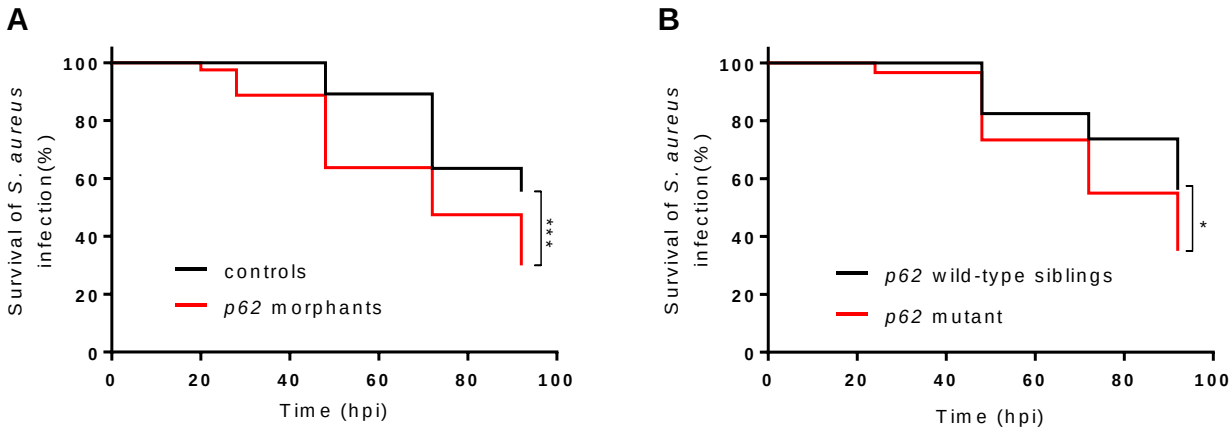


Figure 2: *In vivo* recruitment of GFP-p62 puncta during *S. aureus* infection



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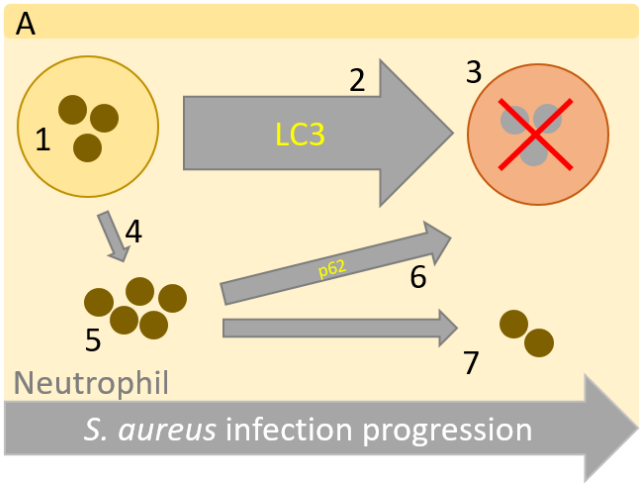

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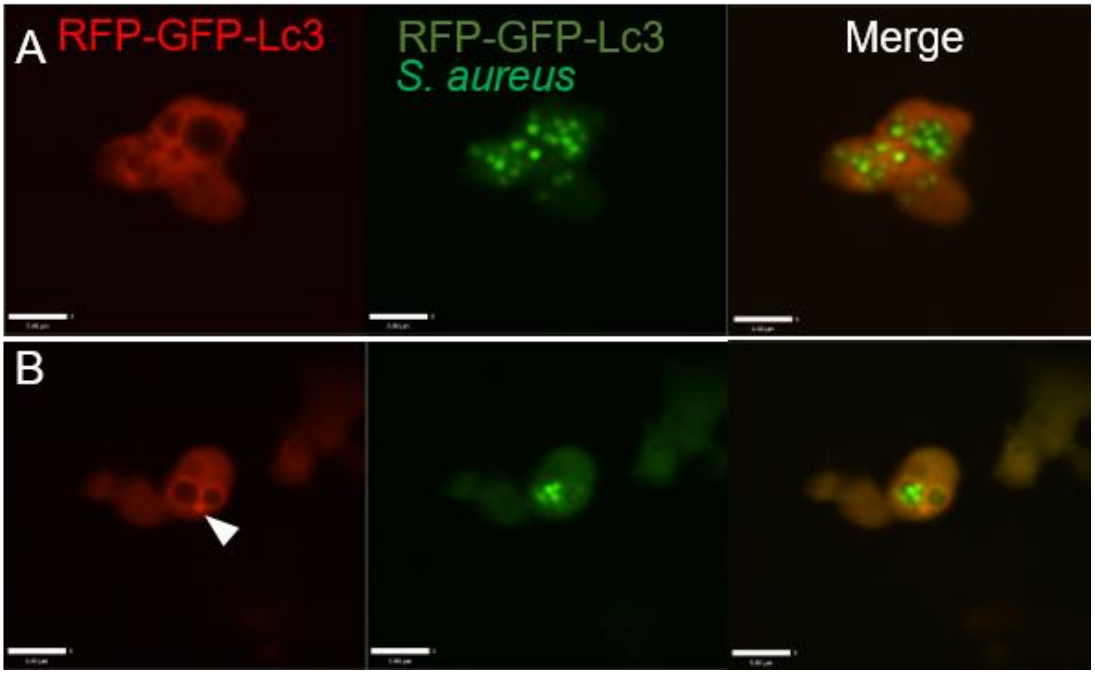
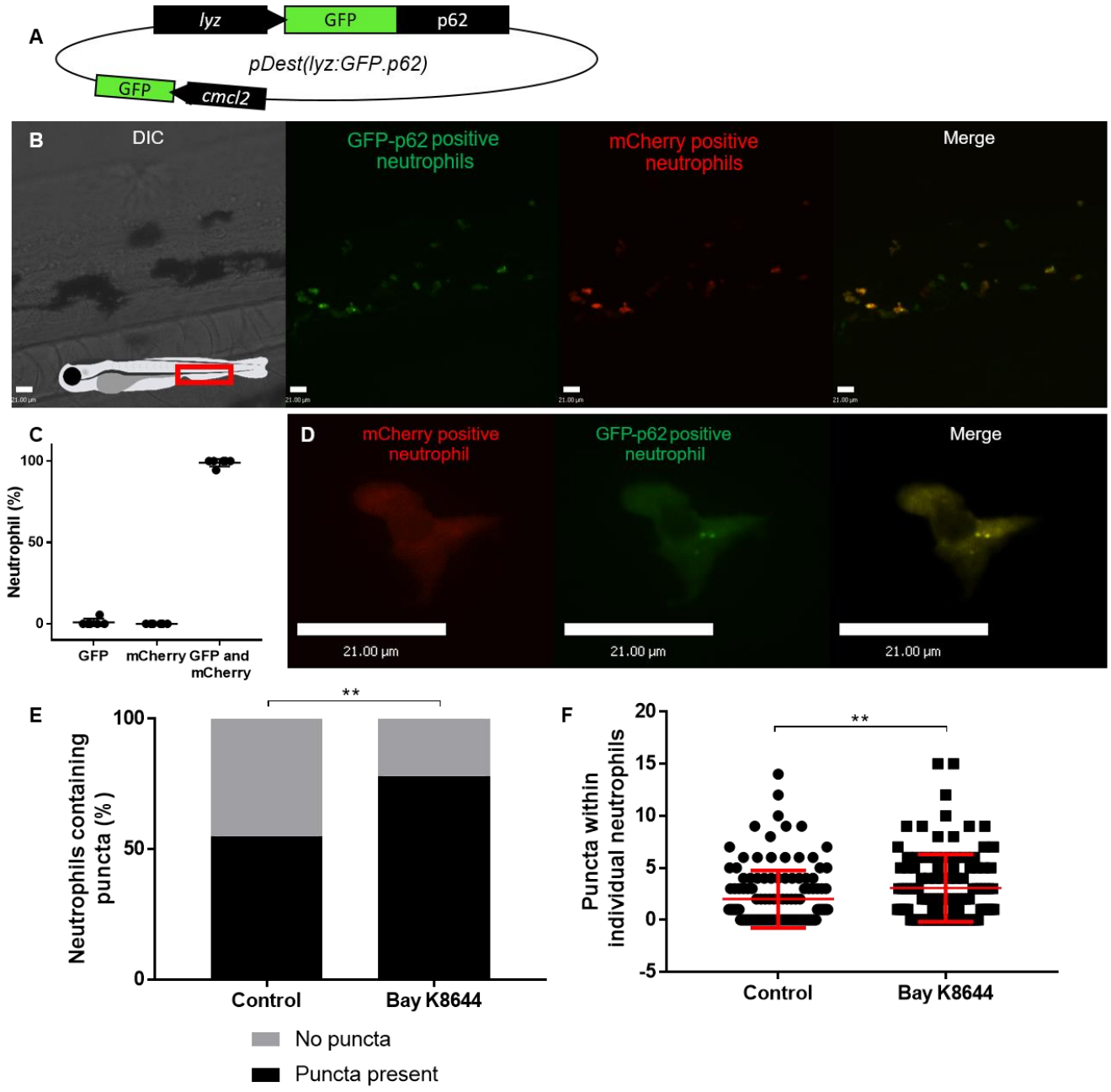
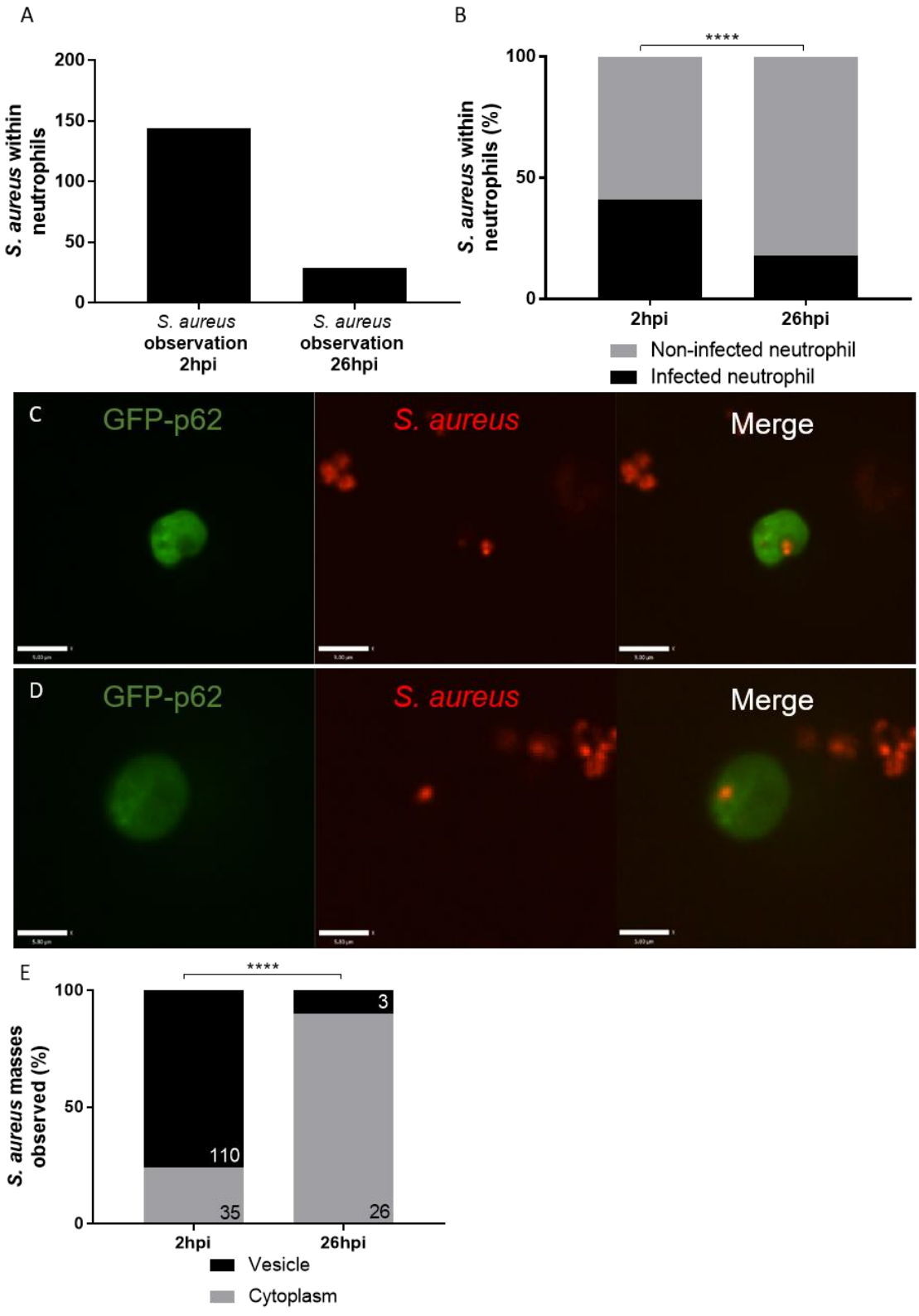
Supplementary figures



Supplementary Figure 1: Lc3 marking of of *Staphylococcus aureus in Tg(lyz:RFP-GFP-Lc3)sh383* larvae



Supplementary figure 2: The GFP-p62 reporter line expresses a GFP-p62 fusion protein within neutrophils



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