Autophagy mediates intracellular killing of *Burkholderia pseudomallei* in human neutrophils

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Abstract

Neutrophils play a key role in control of the melioidosis pathogen *Burkholderia pseudomallei* in murine models and defects in neutrophil function are associated with the risk of human infection. *B. pseudomallei* is rapidly eliminated from human neutrophils infected *ex vivo*, however the relative contribution of oxidative killing pathways and autophagy in this process is ill-defined. The net survival of intracellular *B. pseudomallei* significantly increased upon treatment with the autophagy inhibitor 3-methyladenine, or inhibition of cathepsin activity. Electron microscopy demonstrated that *B. pseudomallei* were sequestered into vacuoles with multiple membranes, with no evidence of bacterial escape to the cytosol. Co-localization of LC3, a marker of autophagosomes, with the bacteria increased along with co-localization with LAMP-1 and Cathepsin D over time. Here we report for the first time that autophagy is induced in human neutrophils infected with *B. pseudomallei*, and that this plays a key role in the killing of internalized bacteria.

Introduction

Neutrophils are highly specialized effector cells of the innate immune system involved in host inflammatory responses and immune surveillance. They play a key role in controlling bacterial infections including those caused by *Burkholderia pseudomallei*, a Gram-negative bacterium which causes melioidosis, a serious invasive disease of humans and animals. Melioidosis is endemic in Northern Australia, and Southeast Asia, especially in the Northeast part of Thailand. The mortality rate from melioidosis can be as high as 50%, and when associated with septic shock the mortality rate is close to 90%.[1, 2] Depletion of neutrophils renders mice exquisitely susceptible to experimental *B. pseudomallei* infection[3] and defects in neutrophil function are believed to underlie the elevated risk of melioidosis in humans with diabetes mellitus.[4]

B. pseudomallei is a facultative intracellular pathogen that can invade both phagocytic and non-phagocytic cells.[5] Following internalization in epithelial cells and macrophages the bacteria have been reported to escape from the phagosome into the host cytoplasm in a manner dependent on the Bsa type III protein secretion system (T3SS). In J774 murine macrophage-like cells such escape is complete within 3 h after infection and is presumed to allow the bacteria to escape oxidative killing mechanisms.[6] Once in the host cell cytoplasm, *B. pseudomallei* can replicate and induce actin polymerization at one pole of bacterial cell. Such actin-based motility is mediated by the BimA protein[7] and facilitates spread of *B. pseudomallei* into neighboring cells leading to the formation of multinucleated giant cells, which have been observed both in cultured cells and in tissues from infected patients.[8] It is unclear if endosome escape and actin-based motility detected in immortalized macrophages occurs in primary human neutrophils.

Recently, we reported that neutrophils could kill more than 90 % of intracellular *B. pseudomallei*, and neutrophils from patients with diabetes mellitus were impaired in their

phagocytosis, migration, apoptosis[4] and production of neutrophil extracellular traps (NETs) in response to *B. pseudomallei* infection.[9] Understanding of the intracellular lifestyle of *B. pseudomallei* in neutrophils and the response of such cells to infection is therefore essential to understand the basis of pathogenesis and protection during melioidosis.

Autophagy is a cellular pathway that acts as an autonomous defense against intracellular bacteria. *B. pseudomallei* induces autophagy in a mouse macrophage cell line[10] and partially evades killing in autophagosomes by producing BopA, a putative effector protein secreted by the Bsa T3SS.[11] The role of autophagy in clearance of bacterial pathogens by neutrophils has received relatively little study. In 1984, Rikihisa reported that rickettsiae induce the rapid formation of autophagosomes in guinea pig peritoneal PMNs.[12] In mice deficient for Atg5 in neutrophils, there is increased susceptibility to infection with *Listeria monocytogenes*, *Toxoplasma gondii*, and uropathogenic *E. coli* (UPEC).[13] Narni-Mancinelli *et al* reported that cytolytic memory T lymphocytes can enhance the functional pathogen-killing capacities of neutrophils by inducing autophagy.[14] Remijsen *et al* reported that autophagy plays an essential role in NET formation in neutrophils that can trap and degrade microbes.[15] Given the key role of neutrophils in resistance to *B. pseudomallei* infection, we investigated whether autophagy plays a role in intracellular killing of *B. pseudomallei* in human neutrophils *ex vivo*. We found that *B. pseudomallei* is exclusively confined to membrane-bound vacuoles in primary human neutrophils, co-localises with markers of autophagy and is killed in a manner sensitive to inhibitors of autophagy and cathepsins.

Materials and Methods

Bacteria

B. pseudomallei strain K96243[16], and mStrawberry red fluorescent protein (RFP)-expressing *B. pseudomallei* strain K96243[17] were grown in Luria-Bertani broth for 18 h at 37°C. After washing twice with phosphate-buffered saline (≤; pH 7.4), the number of bacteria was estimated from the absorbance of the bacterial suspension at 600 nm. In general, an absorbance at 600 nm of 0.33-0.35 was equivalent to approximately 10^8 CFU/ml of viable bacteria. The number of viable bacteria used in infection studies was determined by retrospective plating of serial ten-fold dilutions of the inoculum to LB agar. Live *B. pseudomallei* was handled under the US Centers for Disease Control regulations for biosafety containment level 3.

Neutrophil isolation

Human neutrophils were isolated from heparinized venous blood by 3.0% dextran T-500 (Pharmacosmos, Cat No. 551005004007) sedimentation and Ficoll-Paque PLUS centrifugation ((Sigma Aldrich, Cat No. 10771)) as previously described by Chanchamroen et al.[4] The purity of isolated cells was generally more than 95% as determined by FACSCalibur flow cytometry (Becton Dickinson).

Immuno-fluorescence microscopy

Purified neutrophils were placed into tissue culture Lab-Tek Chambers (Nunc International, Cat No. 154534) at a concentration of 2.5 x 10^6 cells/ml with medium control or RFP-*B. pseudomallei* K96243 at a multiplicity of infection (MOI) of 10 for 30 min. The extracellular bacteria were killed by 250 μg/ml of kanamycin (KM) in complete RPMI 1640 medium (RPMI 1640 containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS))

for 30 min and then cells were washed with PBS and maintained with 20 μg/ml of Km in complete RPMI1640 in the presence or absence of 3-methyladenine (3-MA) (Sigma Aldrich, Cat No. M9281) at 5 mM or a cathepsin-inhibitor cocktail (10 μg/ml of E64d, a membrane-permeable inhibitor of cathepsins B,H, L and 10 μg/ml of Pepstatin A, an inhibitor of cathepsins D and E)[18] for indicated periods. After infection, the cells were fixed in 4% (wt/vol) paraformaldehyde in PBS and incubated with 50 mM NH_4Cl for 10 min. Then, cells were permeabilized by 0.5% (vol/vol) Triton X-100 in PBS for 30 min and non-specific binding blocked by incubation with 3% (wt/vol) bovine serum albumin (BSA) in PBS for 30 min. Cells were stained with rabbit-anti-LC3B (Cell Signaling Technology (CST), Cat No. 3868s), mouse anti-LAMP-1 (Abcam, Cat No. ab25630), and rabbit-anti-cathepsin D antibodies (kindly provided by Dr. Talkashi Ueno) for 45 min at 37 ˚C. After washing with PBS, the cells were stained with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Cat No. A11034) and Alexa Fluor 647-conjugated goat anti-mouse IgG (Molecular Probes, Cat No. 4410) for 45 min at 37 ˚C. The stained cover slips were mounted using Pro-long anti-fade (Invitrogen, Cat No. P7481) and kept in the dark at 4 ˚C. Images were obtained by confocal microscopy LSM510 (Zeiss).

Transmission electron microscopy (TEM)

Uninfected neutrophils or neutrophils infected with live *B. pseudomallei* at an MOI of 10 for 3 hours were pre-fixed with 2.5% (wt/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at indicated time points for 2 h at room temperature. After pre-fixation, cell were collected and washed with 0.1 M cacodylate buffer containing 0.1 M sucrose twice and post-fixed for 1 h at room temperature with 1% (wt/vol) osmium tetroxide and subsequently incubated with 2% (wt/vol) uranyl acetate for 1 h. After dehydration and embedding in Epon resin, ultra-thin 70 nm sections were cut and stained with lead citrate and uranyl acetate.

TEM images were obtained at 80kV using a Hitachi H-7600 transmission electron microscope fitted with a Gatan Multiscan 791 CCD camera.

Western blotting

Neutrophils were infected with *B. pseudomallei* K96243 at an MOI of 10 in the presence or absence of 10 μg/ml of E64d and 10 μg/ml of Pepstatin A for 3 h. The cells were lysed with 1x SDS-PAGE sample buffer, and subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Pall Corporation, Cat No. 66543). Membranes were blocked with 5% (wt/vol) skimmed milk in Tris-buffered saline with 0.1% (vol/vol) Tween-20 (TBST) and were incubated with rabbit primary antibodies against LC3B (CST, Cat No. 7074, 1:1,000 dilution) for 1 h at room temperature. After three TBST washes, the membranes were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (CST, 1:2,000 dilution) for 1 h at room temperature. After three TBST washes, bound antibody was detected by enhanced chemiluminescence (Pierce, Cat No. 32106).

Assay of bacterial net intracellular survival

Purified neutrophils were co-cultured with *B. pseudomallei* at an MOI of 10 in the presence or absence of 3MA (5 mM) or a cathepsin-inhibitor cocktail (10 μg/ml of E64d and 10 μg/ml of Pepstatin A) at 37°C, 5% CO_2 for 30 min. Extracellular bacteria were then killed by addition of complete RPMI 1640 containing 250 μg/ml Km at 37°C, 5% CO_2 for 30 minutes, after which time the number of internalized bacteria were enumerated by lysis of the cells with 0.5% (vol/vol) Triton X-100 in PBS and plating of serial ten-fold dilutions of lysates to Luria-Bertani agar plates (1 h p.i.). Net intracellular survival over time was then determined by maintaining separate cultures with RPMI containing 20 μg/ml of Km with or

without inhibitors for 2 h (3 h p.i.) or 5 h (6 h p.i.) followed by lysis of the neutrophils and plating of serial ten-fold dilutions of lysates to Luria-Bertani agar.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 5 software (Graphpad). For quantitative analysis of micrographs, at least 100 bacteria were counted for each condition in each experiment. A p value of ≤ 0.05 was considered to be statistically significant).

Results

Inhibition of autophagy enhances survival of *B. pseudomallei* in human neutrophils

To investigate whether autophagy plays a role in bacterial killing in human neutrophils, we first analyzed the rate of survival of the prototype *B. pseudomallei* strain K96243 in primary human neutrophils *ex vivo* in the presence or absence of an inhibitor of autophagy (3-methyladenine). When neutrophils were infected with *B. pseudomallei* in the absence of 3MA the majority of intracellular *B. pseudomallei* were killed in a time-dependent manner, as previously reported.[4] When 3MA was added to the medium during the bacterial infection, the number of intracellular bacteria within neutrophils was about 1 log-fold (or ten-fold) higher than those in the absence of 3MA at 3 and 6 h (Fig. 1; $p \leq 0.05$). When the lysosomal cathepsin-inhibitors E64d and pepstatin A were added to the medium during infection, the number of intracellular bacteria was about 1.5 log-fold (or about 13-14 fold) higher than those without inhibitors (Fig. 1; $p \leq 0.05$). These results indicate that a 3MA-sensitive and lysosome-dependent pathway contributes to intracellular killing of *B. pseudomallei* in primary human neutrophils, consistent with a role for autophagy.

Autophagy is activated in response to *B. pseudomallei* infection of human neutrophils.

We next investigated whether intracellular *B. pseudomallei* co-localize with the autophagosome marker LC3 (microtubule-associated protein 1 light chain 3) in infected human neutrophils (Fig. 2A). Co-localization of LC3 with intracellular bacteria was observed at 3 and 6 h p.i., but not 1 h p.i. The lysosome marker LAMP-1 was also co-localized with *B. pseudomallei*, and the co-localization of bacteria with LC3 and LAMP-1 with *B. pseudomallei* increased to 67% of bacterial cells imaged by 6 h p.i (Fig. 2B).

Induction of autophagy during *B. pseudomallei* infection of human neutrophils was further examined by detection of a cleaved variant of LC3 (LC3B-II), a specific and sensitive

marker of activation of autophagy [19, 20] by Western blotting. As shown in Fig. 2C, when neutrophils were infected with *B. pseudomallei* K96243 LC3B-II levels were slightly increased, however the amount of LC3B-II was significantly increased in the presence of E64d and pepstatin A. Consistently, the population of LC3- and LAMP-1-positive *B. pseudomallei* in neutrophils dramatically increased in the presence of these lysosomal enzyme inhibitors (Fig. 2D and supplementary Fig. 1). These results indicate that autophagy is activated and lysosomal turnover of LC3B-II increased in *B. pseudomallei*-infected neutrophils. Furthermore, cathepsin D, a lysosomal protease, also co-localized with *B. pseudomallei* at 3 h p.i. and 6 h p.i. (supplementary Fig. 2).

B. pseudomallei-containing vacuoles in primary human neutrophils are bound by double- or multiple- membranes.

We performed electron microscopic analyses of *B. pseudomallei*-infected neutrophils to determine whether intracellular *B. pseudomallei* were sequestered within membranous compartments or free in the cytosol. When purified neutrophils were infected with *B. pseudomallei* at an MOI of 10, *B. pseudomallei*-containing vacuoles were surrounded by double- or multiple membranes at 3 h p.i. (Fig. 3). No bacteria were observed to be free in the cytosol. Consistent with this, co-staining for *B. pseudomallei* and polymerized F-actin with fluorophore-conjugated phalloidin failed to detect any intracellular bacteria with actin tails at 6 h p.i. (data not shown). Such tails only form once *B. pseudomallei* is in the cytosol and can be detected at this time in infected macrophage and epithelial cells.[6, 7] Thus, in contrast with events in immortalized macrophage and epithelial lines described to date, *B. pseudomallei* is exclusively confined to membrane-bound vacuoles in primary human neutrophils. Taken together with evidence of co-localisation with LC3, LAMP-1 and the effect of inhibitors of

autophagy and cathepsins, it is reasonable to conclude that *B. pseudomallei* is frequently sequestered in autophagosomes in such cells.

Discussion

Autophagy has been recognized as an important defense system to combat intracellular pathogens. However, the role of autophagy in neutrophils for the control of bacterial infection is poorly understood.[21, 22] To our knowledge, this is the first report showing that primary human neutrophils can kill intracellular *B. pseudomallei* by autophagy. We have demonstrated that survival of *B. pseudomallei* strain K96243 is significantly enhanced by inhibition of autophagy. We have also shown that the autophagy marker protein LC3, a late endosome marker LAMP-1, and cathepsin D are recruited to vacuoles containing *B. pseudomallei*. We have demonstrated that LC3 and bacteria in autophagosomes accumulate after treatment with cathepsin inhibitors, and autophagic flux was increased during *B. pseudomallei*-infection into neutrophils, as evidenced by elevated levels of LC3B-II. Whilst these data imply that autophagy is an important mechanism deployed by neutrophils to control intracellular bacterial infection, the number of intracellular *B. pseudomallei* still decreased when autophagy is inhibited, suggesting that other killing mechanisms are employed to control this pathogen. Neutrophils can kill engulfed bacteria in their phagosomes mostly by fusion of granule contents including proteolytic enzymes.[23] Activated neutrophils also produce reactive oxygen species (ROS) and reactive oxygen intermediate (RNI), which act as effector molecules to damage bacteria. It is likely that these mechanisms function as an alternative pathway to kill *B. pseudomallei* when autophagy is blocked.

Induction of autophagy was clearly diminished when neutrophils phagocytosed killed *B. pseudomallei* (supplementary figure 3). This suggests that pathogen-associated molecular patterns (PAMPs) of *B. pseudomallei* are insufficient to induce autophagy. The mechanism by which *B. pseudomallei* induces autophagy in neutrophils requires further study. It is possible that this involves damage to the integrity of the membrane by the Bsa type III

secretion system (T3SS) that mediates phagosome escape or the injection of bacterial factors directly into host cells. However, other data have suggested that *B. pseudomallei* may use the Bsa system to evade autophagy, as *bipD* and *bopA* mutants exhibit increased co-localisation with LC3 and LAMP-1 and impaired survival in immortalized RAW264.7 macrophage-like cells.[11] In contrast to the study by Gong et al, which detected *B. pseudomallei* free in the cytosol and in vacuoles with single membranes[11], we found no evidence of escape of *B. pseudomallei* to the cytosol of primary human neutrophils and intracellular bacteria were frequently confined to vacuoles with multiple membranes. Preliminary data using neutrophils from two donors have indicated that null *bsaZ* and *bsaQ* mutants exhibit net intracellular survival kinetics in human neutrophils *ex vivo* that are comparable to the wild-type strain (data not shown), indicating that strategies used to evade autophagy and escape the entry vesicle are sensitive to the cell type. Although we have established a role of autophagy in control of *B. pseudomallei* human neutrophils, the fate of intracellular *B. pseudomallei* in vacuoles with different properties remains difficult to discern.

In summary, our study highlights a role for autophagy in control of intracellular *B. pseudomallei* by a cell type that plays a vital protective role against melioidosis. It benefits from use of primary cells from the target human host, and indicates that the fate of internalized bacteria in neutrophils differs from studies with immortalized macrophage-like lines. With future research it may be feasible to potentiate autophagic control of *B. pseudomallei*, as current antibiotic therapy is often ineffective.

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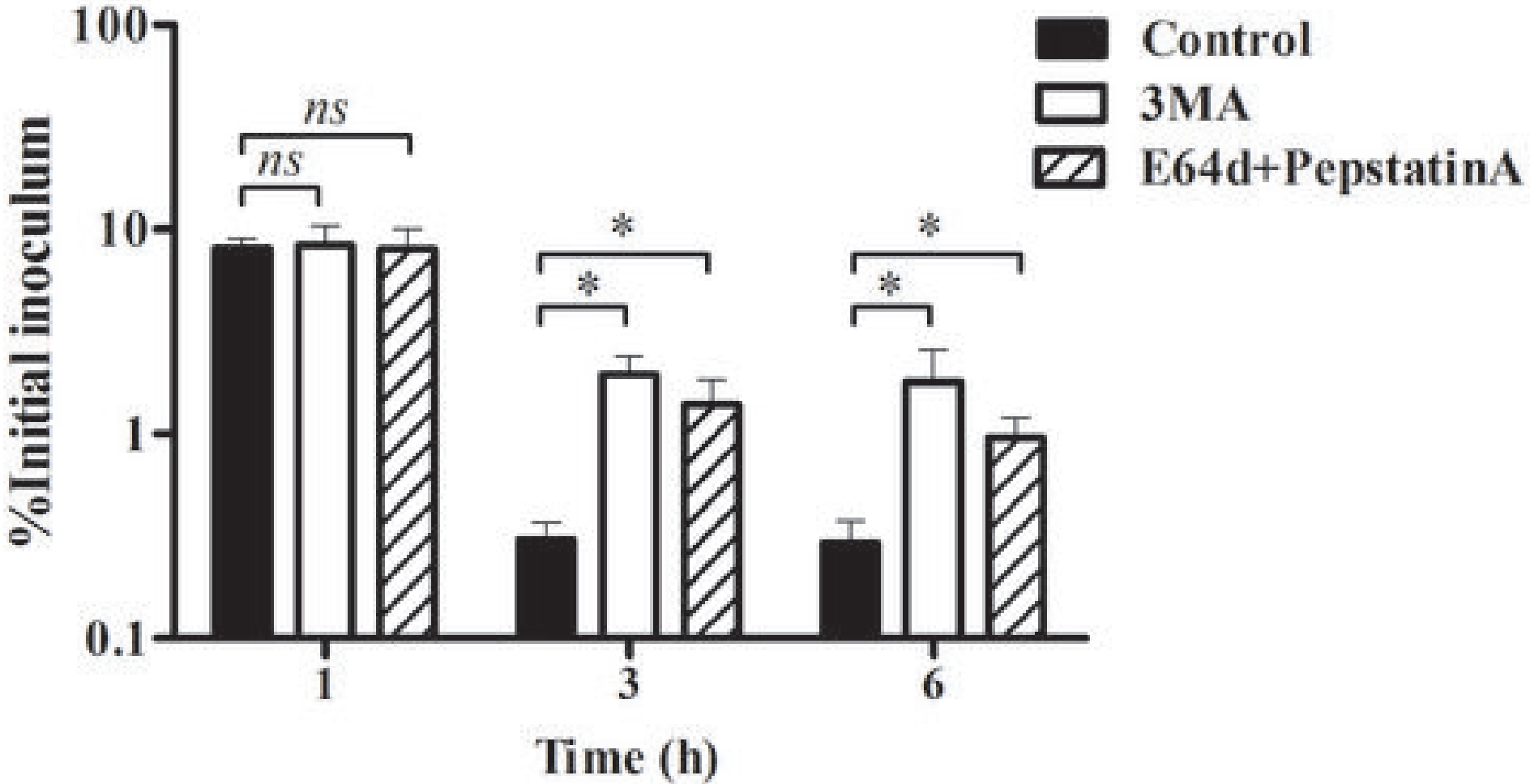
Figure legends

Figure 1: Blocking the induction of autophagy enhances survival of *B. pseudomallei* in human PMNs. Neutrophils purified from the blood of healthy subjects (*n*=3) were infected with live *B. pseudomallei* strain K96243 at an MOI of 10. After 30 min of incubation to allow uptake, the medium was removed and the cells were incubated with fresh medium in the presence or absence of 5mM 3MA or 10 μg/ml each of E64d & Pepstatin A. After incubation for indicated periods, the cells were treated with 250 μg/ml of kanamycin at 37°C for 30 min to kill extracellular bacteria. The intracellular survival of *B. pseudomallei* in neutrophils was determined by bacterial colony count after infection at 1, 3 and 6 hours as indicated in each graph. The results are expressed as percentages of the initial inoculum for individuals, which were calculated by dividing the number of recovered bacteria by the total number of *B. pseudomallei* cells added. All results are shown as the mean ± standard error of the mean (SEM) of duplicate measurements of three samples. Statistical significance was determined using unpaired t test. ns denotes not significant, asterisks denote $p \leq 0.05$.

Figure 2: *B. pseudomallei* co-localizes with the autophagy marker LC3 and the lysosome marker LAMP-1 in human neutrophils. Representative confocal micrographs images of neutrophils infected with *B. pseudomalleui* K96243 for indicated periods are shown (A). K96243 expressing red fluorescent protein (RFP) appear red, LC3B is stained green, LAMP-1 is stained blue and nuclei were stained purple. Scale bars = 5 μm. Data shown are from one donor representative of experiments performed with five subjects. Quantitative analysis of bacteria co-localized with LC3 and LAMP-1 (B). All results are shown as the mean ± SEM of duplicate measurements of all samples. Western blot analysis for LC3B-I and LC3B-II in lysates of infected

(Bp) or non-infected (M) neutrophils in the presence or absence of cathepsin inhibitor cocktail at 3 hours p.i. (C). Quantitative analysis of bacteria co-localized with LC3 and LAMP1 of neutrophil treated and non-treated with E64d+PepstatinA were infected with *B. pseudomallei* K96243, at 3 and 6 h p.i. by confocal microscopy assay (D). Statistical significance was determined using unpaired t test. Ns denotes not significant, asterisks denote $p \leq 0.05$.

Figure 3: Electron microscopic analysis of *B. pseudomallei*-containing vacuoles in primary human neutrophils. Transmission electron micrographs show the intracellular location of *B. pseudomallei* in human neutrophils at 3 h post infection (p.i.). Arrow indicates ultra membrane structure. Scale bars represent 500 nm (A, B) and 200 nm (C, D).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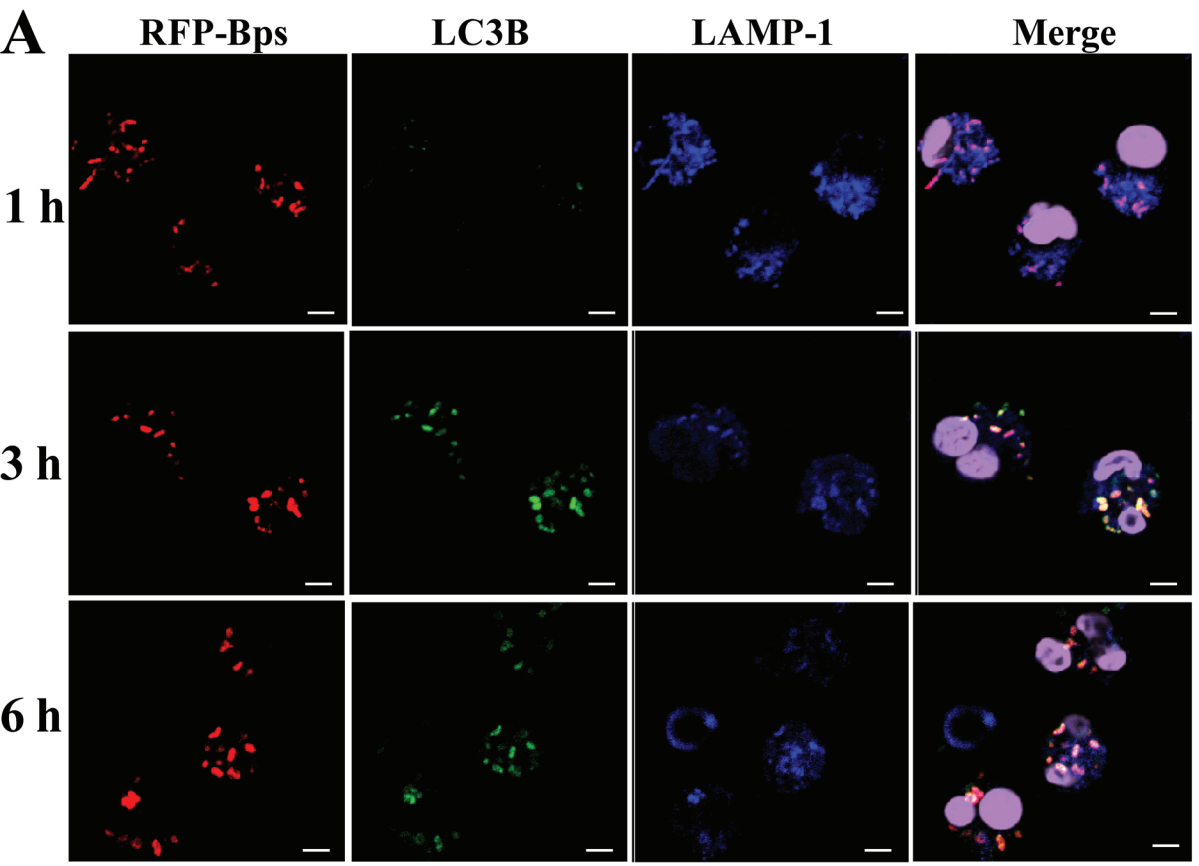
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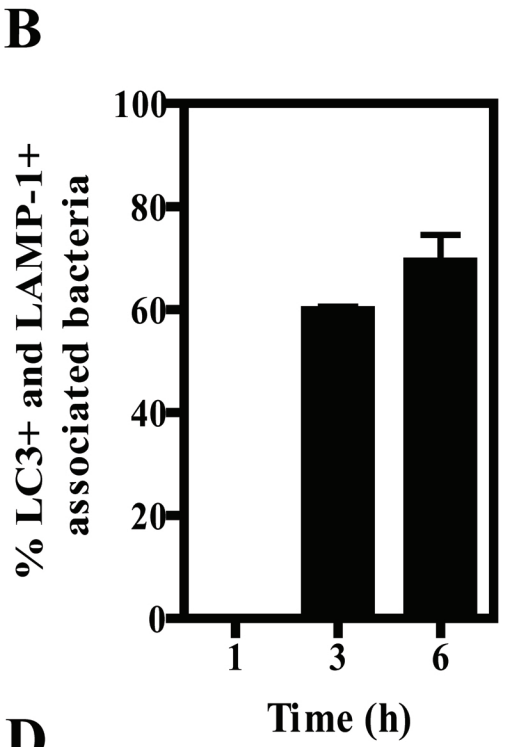
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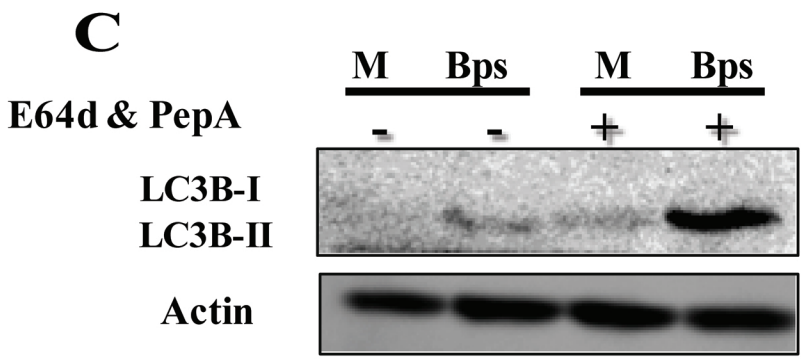
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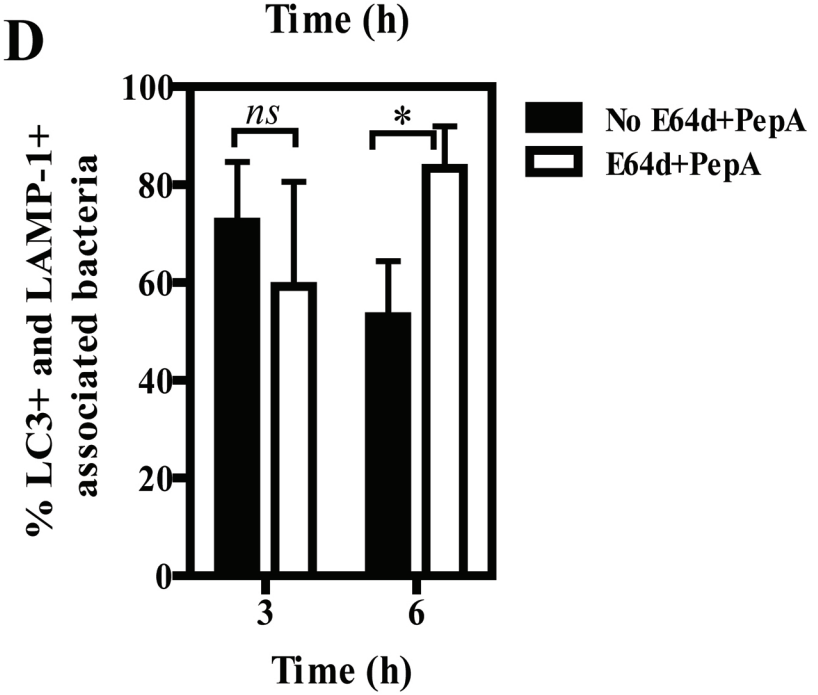
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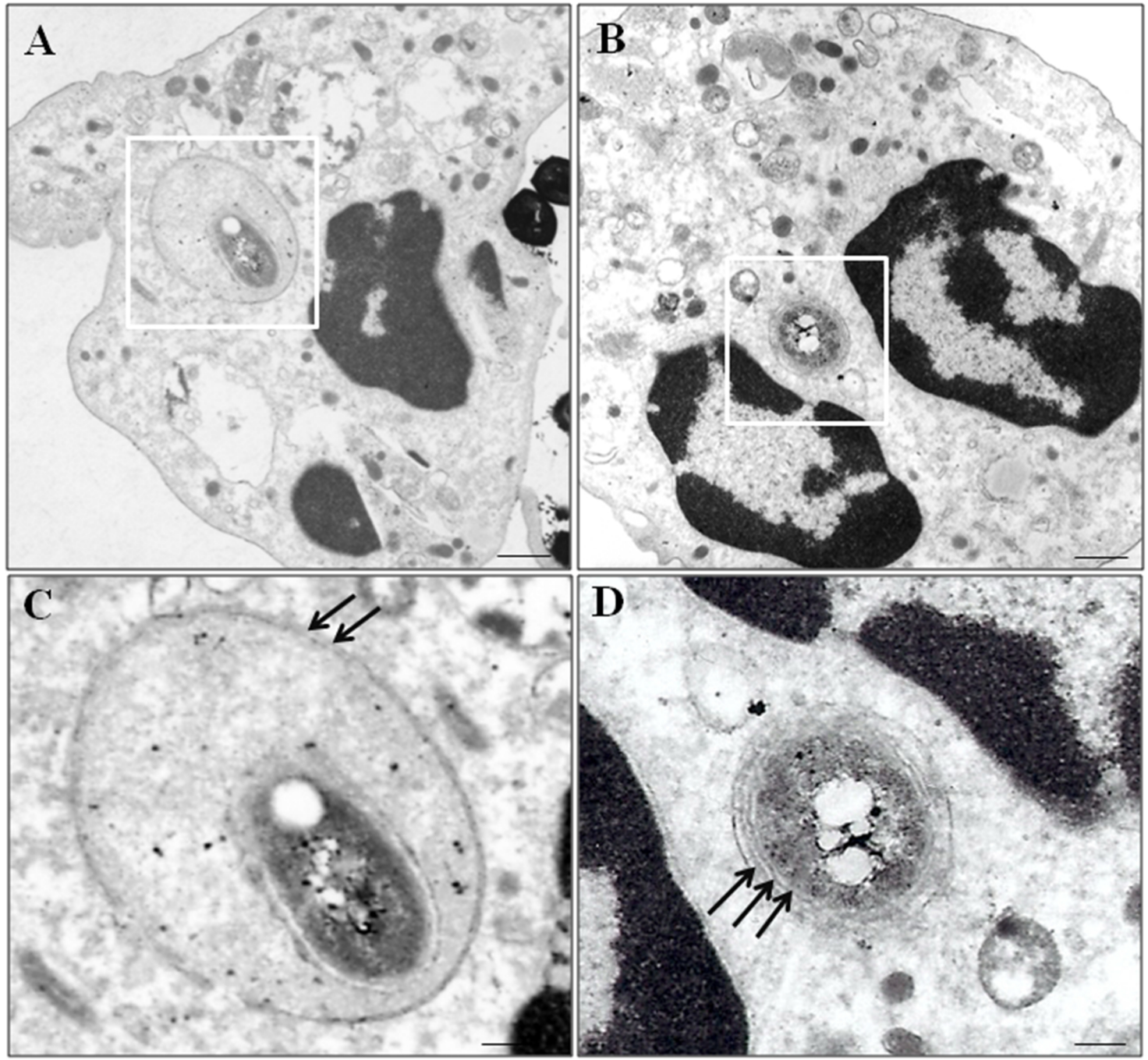
3
6
Time (h)
No E64d+PepA
E64d+PepA


A
B
C
D