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Macroautophagy is essential for killing of intracellular *Burkholderia pseudomallei* in human neutrophils

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Abbreviations: 3MA, methyladenine; Bp, *Burkholderia pseudomallei*; Bsa, *Burkholderia* secretion apparatus; KM, kanamycin; LAMP1, lysosomal-associated membrane protein 1; LC3-I, unlipidated form of LC3; LC3-II, LC3-phospholipid conjugated and phagophore or autophagosome-associated form of LC3; MAP1LC3/LC3, microtubule-associated protein 1 light chain 3; MOI, multiplicity of infection; NET, Neutrophil Extracellular Taps; p.i., postinfection; T3SS, Type III secretion system; WT, wild type

Neutrophils play a key role in the control of *Burkholderia pseudomallei*, the pathogen that causes melioidosis. Here, we show that survival of intracellular *B. pseudomallei* was significantly increased in the presence of 3-methyladenine or lysosomal cathepsin inhibitors. The LC3-flux was increased in *B. pseudomallei*-infected neutrophils. Concordant with this result, confocal microscopy analyses using anti-LC3 antibodies revealed that *B. pseudomallei*-containing phagosomes partially overlapped with LC3-positive signal at 3 and 6 h postinfection. Electron microscopic analyses of *B. pseudomallei*-infected neutrophils at 3 h revealed *B. pseudomallei*-containing phagosomes that occasionally fused with phagophores or autophagosomes. Following infection with a *B. pseudomallei* mutant lacking the *Burkholderia* secretion apparatus Bsa Type III secretion system, neither this characteristic structure nor bacterial escape into the cytosol were observed. These findings indicate that human neutrophils are able to recruit autophagic machinery adjacent to *B. pseudomallei*-containing phagosomes in a Type III secretion system-dependent manner.

Introduction

Neutrophils are highly specialized effector cells of the innate immune system, which are 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 that causes melioidosis, a serious invasive disease in 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%; when associated with septic shock, it 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 nonphagocytic cells.[5] Following internalization into epithelial cells and macrophages, *B. pseudomallei* can escape from the phagosomes into the cytosol in a Bsa Type III secretion system (T3SS)-dependent manner. In J774 murine macrophage-like cells, bacterial escape into the cytosolis completed within after 3 h of infection, which allows the bacteria to circumvent oxidative antimicrobial agent and gain replicative niche.[6] Once migrated into the cytosol, *B. pseudomallei* can replicate and form actin comet tail at one pole of the bacterium. Such F-actin polymerization-driven motility is mediated by the bacterial surface exposed BimA protein[7] and facilitates intercellular spreading of *B. pseudomallei* into neighboring cells, leading to formation of multinucleated giant cells, which have been observed in both cultured cells and tissues from infected patients.[8]

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Recently, we reported that neutrophils could kill more than 90% of intracellular *B. pseudomallei*, and that neutrophils from patients with diabetes mellitus are impaired in phagocytosis, migration, apoptosis,[4] and production of neutrophil extracellular traps (NETs) in response to *B. pseudomallei* infection.[9] Elucidation of the intracellular life cycle of *B. pseudomallei* in neutrophils and the response of host cells to *B. pseudomallei* infection is therefore essential to our better understanding of the basis of pathogenesis and protection during melioidosis. However, whether *B. pseudomallei* escapes into the cytoplasm following phagocytosis by primary human neutrophils, and how the bacteria are eliminated, remains unclear.

Autophagy is a cellular activity that acts as an autonomous defense against intracellular bacteria, such as Group A *Streptococcus*,[10] *Salmonella*,[10,11] *Shigella*[12] and *Listeria*.[13] It has been reported that *B. pseudomallei* can induce autophagy and be engulfed by LC3-associated phagosomes in a mouse macrophage cell line,[14] and partially evade killing in these phagosomes by producing BopA, a putative effector protein secreted by the Bsa T3SS.[15] The role of autophagy in the 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 neutrophils.[16] Mice with ATG5-deficient neutrophils show increased susceptibility to infection with *Listeria monocytogenes, Toxoplasma gondii*, and uropathogenic *Escherichia coli*.[17] Narni-Mancinelli et al. report that cytolytic memory T lymphocytes can enhance the functional pathogen-killing capacities of neutrophils by inducing autophagy.[18] Remijsen et al. report that autophagy plays an essential role in NET formation in neutrophils that can trap and degrade microbes.[19] 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 the autophagic pathway contributes to killing of *B. pseudomallei* in human neutrophils that possess a characteristic membranous structure associated with phagophore-like structures, in a Bsa T3SS-dependent manner.

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 survivability of the full-genome-sequenced prototype *B. pseudomallei* strain K96243 in primary human neutrophils ex vivo in the presence or absence of the autophagy inhibitor, 3-methyladenine (3MA). 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] Upon treatment with 3MA, the number of intracellular bacteria within neutrophils at 3 and 6 h p.i was about 10-fold higher than that in the absence of 3MA (**Fig. 1**; $P \leq 0.05$). Furthermore, upon treatment with E64d and pepstatin A, inhibitors of lysosomal hydrolases, the number of intracellular bacteria was also increased to the same level of 3MA-treated cells (**Fig. 1**), suggesting that a 3MA-sensitive(PtdIns3P-dependent) and lysosomal enzymes-dependent pathway contributes to intracellular killing of *B. pseudomallei* in primary human neutrophils. The viability of neutrophils was not affected by treatment with these inhibitors (**Figs. S1 and S2**).

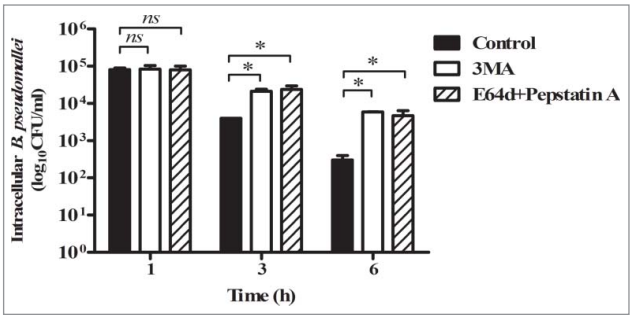

Figure 1. Blocking the induction of autophagy enhances survival of *Burkholderia pseudomallei* in human PMNs. Neutrophils purified from the blood of healthy subjects ($n = 3$) were infected with a live *B. pseudomallei* strain (K96243) at an MOI of 10. After 30 min of incubation to allow uptake, the medium was removed and the cells were incubated with fresh medium in the presence or absence of 5 mM 3MA or 10 μg/ml each of E64d and pepstatin A. After incubation for the 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 counts at 1, 3, and 6 h p.i., as indicated in each graph. All results are shown as the mean ± standard error of the mean (SEM) of duplicate measurements of 3 samples. Statistical significance was determined using an unpaired Student *t* test. ns denotes not significant, asterisks denote $P \leq 0.05$.

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

To clarify the contribution of the autophagic activity to *B. pseudomallei*-elimination in neutrophils, we first investigated the accumulation of LC3-II during infection (**Fig. 2A**).[20,21] LC3-II is an established autophagosome marker specifically localized to autophagosomes and autolysosomes. When uninfected neutrophils were treated with rapamycin, an inducer of autophagy via inactivation of MTORC1, accumulation of LC3-II-positive puncta was observed in the cells, indicating that autophagy can be induced in neutrophils (**Fig. 2A**). When neutrophils were infected with *B. pseudomallei* K96243, LC3-II was accumulated, as in rapamycin-treated neutrophils, indicating that an autophagic response was induced in neutrophils during *B. pseudomallei* infection (**Fig. 2A**). Treatment of *B. pseudomallei*-infected neutrophils with rapamycin produced little additional effect on the increase of LC3-II level (**Fig. 2A**).

We next focused on autophagic activity during infection, since temporal accumulation of LC3-II is not always observed when autophagy is activated and it is observed either when autophagy is activated or impaired.[22] To clarify whether accumulation of LC3-II during *B. pseudomallei* infection reflects activated autophagy or impaired autophagy, next we performed the autophagy flux assay during bacterial infection using E64d and pepstatin A,

inhibitors of lysosomal cathepsins. If autophagy is activated in *B. pseudomallei*-infected neutrophils, a further accumulation of LC3-II in the infected cells will be observed with inhibition of lysosomal hydrolases. Upon treatment with E64d and pepstatin A for 3 h during infection as previously reported,[22] the amount of LC3-II was increased 2-fold compared to untreated infected neutrophils (**Fig. 2A** and **Fig. S3**). Our data strongly suggested that *B. pseudomallei* infection activates autophagy flux in human neutrophils.

If autophagy contributes to the intracellular clearance of *B. pseudomallei* in neutrophils, LC3 will colocalize with intracellular *B. pseudomallei*. Next we investigated the colocalization of intracellular *B. pseudomallei* with LC3 in *B. pseudomallei*-infected human neutrophils (**Fig. 2B and C**). At 1 h postinfection (p.i.), LAMP1, but few LC3 signal, was associated with *B. pseudomallei*. LC3 was associated with intracellular bacteria at 3 h and 6 h p.i. The association of *B. pseudomallei* with LC3 was not observed when killed bacteria were used, suggesting that some biological activities of *B. pseudomallei* may be required for the recruitment of LC3 adjacent to the bacterium (**Fig. S4**).

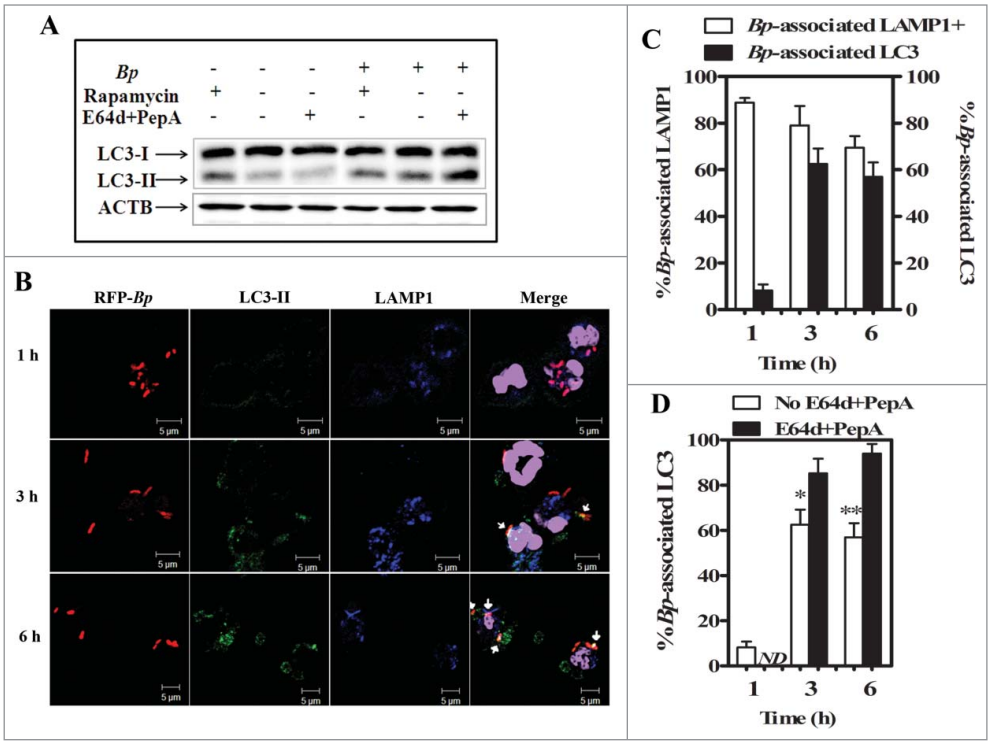

Figure 2. *Burkholderia pseudomallei* colocalizes with the autophagy marker LC3 and the lysosome marker LAMP1 in human neutrophils. Western blot analysis for LC3-I and LC3-II in lysates of infected or uninfected neutrophils in the presence or absence of rapamycin or cathepsin inhibitor cocktail at 3 h p.i. (**A**). Representative confocal micrograph images of neutrophils infected with *B. pseudomallei* K96243 for the indicated periods are shown (**B**). K96243 bacteria expressing red fluorescent protein (RFP) are shown in red; LC3 is shown in green, LAMP1 in blue, and nuclei in purple. Arrows represent LC3-positive bacteria. Scale bars: 5 μm. Data shown are from a single donor representative of experiments performed with 5 subjects. Quantitative analysis of bacteria colocalized with LC3 and LAMP1 (**C**). All results are shown as the mean ± SEM of duplicate measurements of all samples. Quantitative analysis of bacteria colocalized with LC3 and LAMP1 in neutrophils treated with and without E64d+pepstatin A at 3 and 6 h postinfection with *B. pseudomallei* K96243 (**D**). Statistical significance was determined using an unpaired Student *t* test. *ND* denotes not determined, * $P \leq 0.05$ and ** $P < 0.01$. *Bp, Burkholderia pseudomallei*.

Upon treatment with E64d and pepstatin A, the population of LC3-associated bacteria was increased in a time-dependent manner (**Fig. 2D** and **Fig. S5**), suggesting that LC3 in *B. pseudomallei*-containing vacuoles was degradated by lysosomal hydrolases. These results were consistent with the results of western blot analysis using anti-LC3 antibody, as shown in **Figure 2A**, and suggested that the recruitment of LC3 around bacterium and lysosomal hydrolases contributes to the elimination of *B. pseudomallei* in human neutrophils.

B. pseudomallei-containing phagosomes in human neutrophils are associated with phagophore-like structures containing cytosol and granules

Next, we performed electron microscopic analyses of *B. pseudomallei*-infected neutrophils at 3 h p.i. to investigate the *B. pseudomallei*-containing LC3-positive membranous structures. At 3 h p.i., *B. pseudomallei* was surrounded by characteristic membranous structures composed of partial phagosome-like single membranes and partial elongated membranes. These *B. pseudomallei*-containing membranous structures also contained granules and cytoplasm. In some cases, the phagophore-like or autophagosome-like structures were partially fused with these *B. pseudomallei*-containing membranous structures (**Fig. 3D to F**).

Few LAMP1-negative bacteria were observed in neutrophils during the course of infection, suggesting that few bacteria could escape from phagosomes into the cytoplasm. Consistent with this, no bacteria in the cytosol of neutrophils were detected in EM examination (**Fig. 3** and **Fig. S6**) and the actin comet tails formed at one pole of the bacterium could not be detected by fluorescence microscopy (data not shown).

Collectively, the results of confocal and electron microscopy analyses indicate that the *B. pseudomallei*-containing membranous structures partially associated with LC3 signal could be a novel elongated single-membrane structure, which is fused with autophagosomes or phagophore in part and with granules in part. Our EM results suggest that the *B. pseudomallei*-containing membranous structures in human neutrophils are likely distinct from those that perform LC3-associated phagocytosis, in which

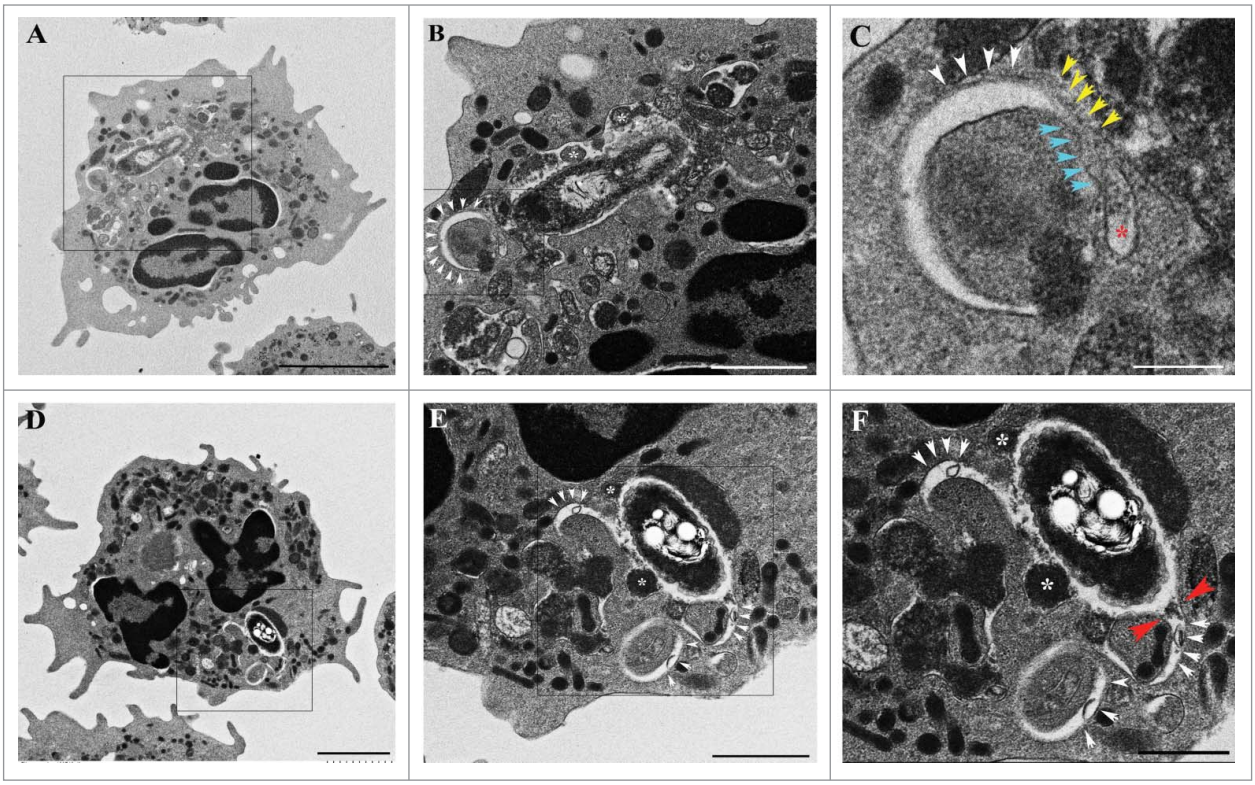

Figure 3. Electron microscopy analysis of *Burkholderia pseudomallei*-containing vacuoles in primary human neutrophils. Representative transmission electron micrographs showing the intracellular location of *B. pseudomallei* in human neutrophils at 3 h p.i. The image shows a *B. pseudomallei*-containing single-membrane-limited phagosome. The limiting membrane of this phagosome is continuous with a phagophore-like structure, containing a portion of the cytoplasm (white arrowheads) and/or granules (white asterisks) (**A to F**).Boxed areas are shown as magnified images, as in panels (**B and E**). The autophagosome-like membranous structure was occasionally fused with a bacteria-containing phagosome; white arrowheads in panels (**B, C, E, and F**) indicate the outer limiting membrane of the phagophore-like structure. Blue arrowheads in panel (**C**) indicate the region where the 2 limiting membranes of the phagophore are in close proximity. Yellow arrowheads in panel (**C**) indicate the single limiting membrane of the phagosome and red asterisks indicated the lumen of a small vesicle or tubule. The 2 red arrowheads in panel (**F**) indicate continuity of the phagophore and phagosome membranes (**F**). Scale bars: 2000 nm (**A, D**), 1000 nm (**B, E**), and 500 nm (**C, F**), respectively

LC3 can be recruited to normal-shaped single-membrane phagosomes in mouse macrophage cell line.

B. pseudomallei induces autophagosome-like structures in a T3SS-dependent manner

As shown in **Figure S4**, some biological activities of *B. pseudomallei* were suggested to be required for autophagy induction after neutrophil infection. Then we assessed the possibility of the autophagy induction via *B. pseudomallei* Bsa T3SS. It is reported that the Bsa T3SS of *B. pseudomallei* plays an important role to evade killing in infected macrophages.[6] Since the K96243-derived Bsa T3SS fully null mutant strain is unavailable for this study, we employed another *B. pseudomallei* clinically isolated strain 10276, which was isolated from a severe case of human melioidosis.[23] As previously reported,[6] 10276 exhibits the same infectious activities as K96243, including Bsa T3SS-dependent escape into the cytosol and intracellular replication, as well as actin comet tail-driven motility in macrophages. To investigate whether the Bsa T3SS influences the induction of the LC3-associated *B. pseudomallei*-containing membranous structures in human neutrophils, we employed a *B. pseudomallei* 10276 derived *bsaZ* mutant lacking a functional Bsa T3SS. Confocal microscopy analyses revealed that the population of LC3-positive bacteria is significantly decreased in the *bsaZ* mutant compared to wild-type (**Fig. 4A and B**). Electron microscopy analyses revealed that the *bsaZ* mutant resided in normal-shaped phagosomes (**Fig. 4E to G**), whereas wild-type bacteria were engulfed in the phagophore-like structure-associated *B. pseudomallei*-containing membranous structures, similarly observed in K96243 wild-type infected cells (**Fig. 4D and G**). Taken together, these data suggested that the T3SS machinery or effector proteins secreted via Bsa T3SS play a role in the induction of these membranous structures in *B. pseudomallei*-infected neutrophils.

Discussion

This is the first report showing that primary human neutrophils can kill intracellular *B. pseudomallei* through autophagy. We have demonstrated that intracellular survival of the *B. pseudomallei* K96243 strain is significantly enhanced by inhibition of the autophagic activities. Autophagic flux is increased during *B.*

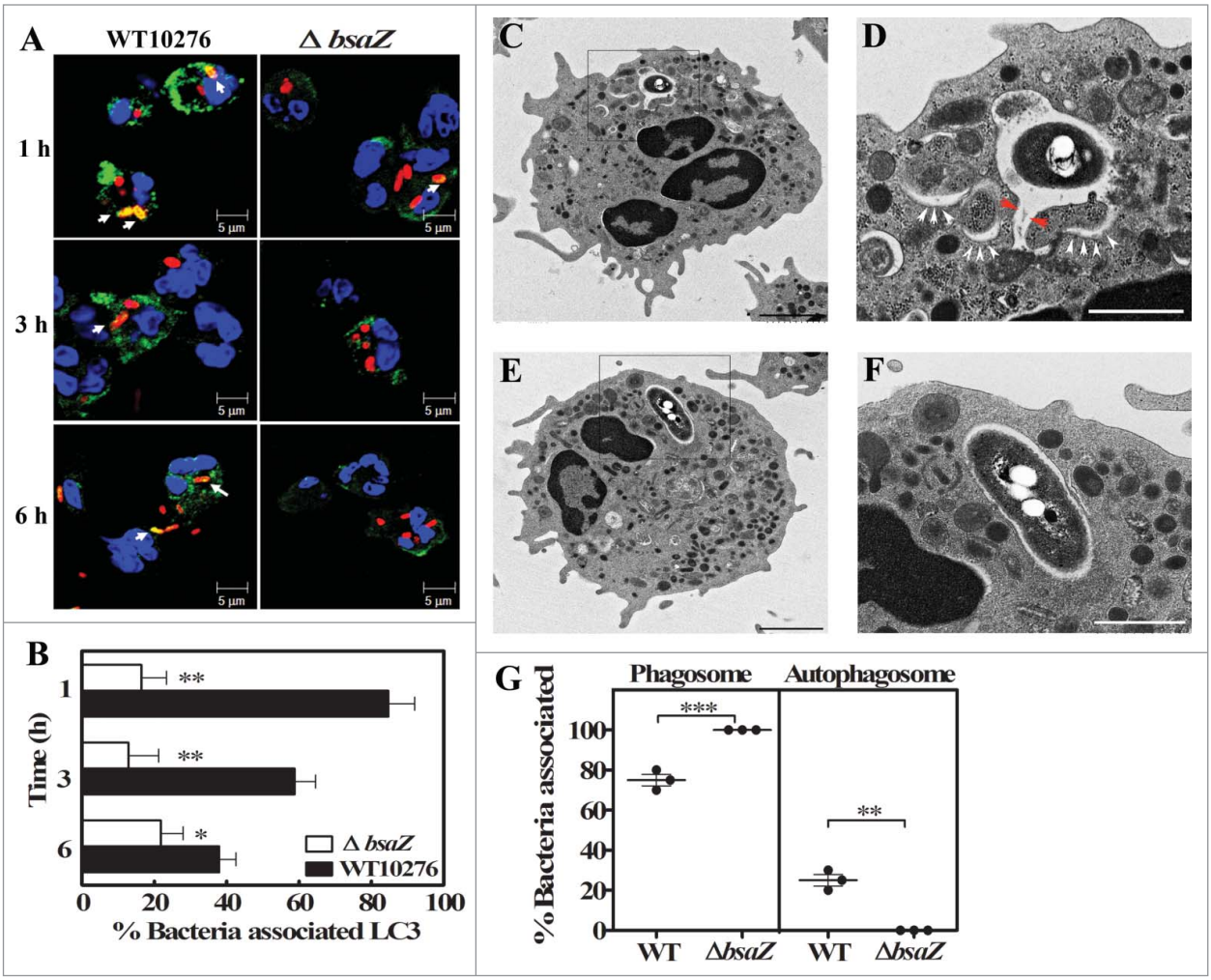

Figure 4. The *Burkholderia pseudomallei* Bsa machinery is necessary to induce autophagosome-like structures in human neutrophils. Representative confocal micrograph images of neutrophils infected with *B. pseudomallei* WT10276 or *bsaZ* mutant for the indicated periods are shown (**A**). Bacteria are shown in red, LC3 in green, and nuclei in blue. Arrows represent LC3-positive bacteria. Scale bars: 5 μm. Data shown are from a single donor representative of experiments performed with 3 subjects. Quantitative analysis of bacteria colocalized with LC3. All results are shown as the mean ± SEM of duplicate measurements of all samples (**B**). Transmission electron micrographs show the intracellular location of *B. pseudomallei* WT10276 (**C and D**) or *bsaZ* mutant (**E and F**) in human neutrophils at 3 h p.i. The image shows a *B. pseudomallei*-containing single-membrane-limited phagosome. The limiting membrane of this phagosome is continuous with a phagophore-like structure, containing a portion of the cytoplasm (white arrowheads). Putative continuity between the phagosome-limiting membrane and the outer limiting membrane of the autophagosome is indicated by red arrowheads (**D**). Boxed areas are shown as magnified images, as in panel (**D and F**). Scale bars: 2000 nm (**C and E**) and 500 nm (**D and F**), respectively. Quantitative analysis of bacteria associated with phagosomes or autophagosomes (**G**). Statistical significance was determined using an unpaired Student *t* test. ns denotes not significant, * $P \leq 0.05$. ** $P < 0.01$.

pseudomallei infection in neutrophils. LC3 could be recruited to the phagosomes via alternative routes: directly recruited to the phagosomal membranes from the cytoplasm, by fusion of phagosomes with LC3-positive autophagosomes or phagophores, or both. Strikingly, electron microscopic analyses revealed the presence of bacteria-containing membranous structures in *B. pseudomallei*-infected neutrophils. The structures containing *B. pseudomallei* were variable in shape, composed of a single membrane that was partially fused with phagophore- or autophagosome-like structures. The formation of these membranous structures were dependent on the Bsa T3SS, suggesting that membrane damage by the T3SS apparatus or effector proteins secreted via the T3SS can trigger the formation of these characteristic membranous structures.

It is of interest that the autophagic pathway contributes to the elimination of *B. pseudomallei* in human neutrophils, as neutrophils contain few acidic compartments, but possess numerous neutral neutrophilic granules in the cytoplasm. Once neutrophils phagocytose bacteria, early phagosomes containing bacteria promptly fuse with granules, followed by killing of bacteria;[24] this differs from other types of cells, showing that only late phagosomes that have fused with lysosomes are LAMP1positive. TEM data from neutrophils at 0.5-h p.i. clearly show that *B. pseudomallei*-containing endosomes and phagosomes are closely associated

with high-electron-density granules (shown as asterisks in figures), which may play a role in bacterial killing in advance of autophagy. Autophagy is likely the second line of defense of neutrophils against *B. pseudomallei* remaining within phagosomes. This kind of fusion between LC3-positive phagophores/autophagosomes and bacteria-containing phagosomes has not previously been described, and differs from the giant autophagosomes that observed at late stage of group A *Streptococcus* infection.[25] Formation of the membranous structure required the presence of live bacteria and the Bsa T3SS. It is noteworthy that a related T3SS from *Salmonella* is required to trigger autophagy in a manner associated with damage to the membrane of the *Salmonella*-containing vacuole.[26,27] We investigated the colocalization of ubiquitin and SQSTM1/p62 with intracellular bacteria; however, little colocalization was observed (data not shown). It has been reported that *B. pseudomallei* may use the Bsa system to evade LC3-associated phagocytosis in RAW264.7 macrophage-like cells.[15] In macrophage-like cells, *B. pseudomallei* escapes from phagosomes to the cytoplasm in a Bsa-dependent manner. However, in primary human neutrophils, no escape of *B. pseudomallei* to the cytoplasm and no actin comet tail could be detected by EM and confocal microscopy observation. Therefore, it is difficult to compare the novel membranous structure observed in *B. pseudomallei*-infected neutrophils with LC3-associated phagosomes in macrophage-like cells. Strains of *B. pseudomallei* are genetically diverse and vary widely in infectivity. The differing kinetics of LC3 accumulation between K96423 and 10276 are perhaps due to variation in the clinical isolates of *B. pseudomallei*. However, the similarity in the membranous structures observed in infected neutrophils suggests that this particular structure is common to *B. pseudomallei*-infected neutrophils. It is conceivable that *B. pseudomallei* injects Bsa-secreted effectors into the cytosol to induce the formation of phagophores and autophagosomes, resulting in the fusion of *B. pseudomallei*-containing phagosomes with phagophores and autophagosomes; however, it will be difficult to distinguish T3SS effector-mediated autophagy from autophagy caused by Bsa-mediated phagosomal membrane damage.

In summary, our study highlights a role for autophagy in the control of intracellular *B. pseudomallei* by neutrophils, a cell type that plays a vital protective role against melioidosis. We propose a novel type of autophagy, which is induced by live *B. pseudomallei* in phagosomes of primary neutrophils in a Bsa T3SS-dependent manner. Defects in autophagy may predispose individuals to melioidosis. Current antibiotic therapy is often ineffective against melioidosis; with further research, it may be feasible to potentiate autophagic control of *B. pseudomallei*.

Materials and Methods

Bacterial strains

The *B. pseudomallei* wild-type (WT) strain K96243,[28] the mStrawberry red fluorescent protein (RFP)-expressing *B. pseudomallei* strain K96243,[29] the WT strain 10276, and the 10276 *bsaZ* mutant lacking a functional Bsa T3SS apparatus have been described.[6] *B. pseudomallei* was grown in Luria-Bertani (LB) broth for 18 h at 37°C. After washing twice with phosphate-buffered saline (PBS, pH 7.4; Gibco, 10010023), the number of bacteria was estimated by measuring the absorbance of the bacterial suspension at 600 nm. In general, an absorbance of 0.33 to 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 10-fold dilutions of the inoculum to LB agar. Live *B. pseudomallei* was handled according to the regulations set forth by the US Centers for Disease Control for biosafety containment level 3.

Neutrophil isolation

Human neutrophils were isolated from heparinized venous blood by 3.0 % dextran T-500 sedimentation (Pharmacosmos, 551005004007) and Ficoll-Paque PLUS centrifugation (Sigma-Aldrich, 10771), as previously described by Chanchamroen et al.[4] The purity of isolated cells was generally greater than 95%, as determined with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and FlowJo software (Treestar).

Immunofluorescence microscopy

Purified neutrophils were placed into tissue culture Lab-Tek Chambers (Nunc International, 154534) at a concentration of 2.5×10^6 cells/ml with medium control or *B. pseudomallei* K96243 was added at a multiplicity of infection (MOI) of 10 and incubated for 30 min. The extracellular bacteria were killed by addition of 250 μg/ml of kanamycin (KM) in complete RPMI 1640 medium (RPMI 1640 containing 10% [vol/vol] heat-inactivated fetal bovine serum) 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, M9281) at 5 mM or a cathepsin inhibitor cocktail (10 μg/ml of E64d [Peptide Inst., 4321-v] and 10 μg/ml of pepstatin A [Peptide Inst., 4397-v]) for the indicated periods. After incubation, the cells were fixed with 4% (wt/vol) paraformaldehyde in PBS and incubated with 50 mM NH_4Cl for 10 min. Then, cells were permeabilized with 0.5% (vol/vol) Triton X-100 (Sigma-Aldrich, 9002931) in PBS for 30 min and nonspecific binding was blocked by incubation with 3% (wt/vol) bovine serum albumin (Sigma-Aldrich, 9048) in PBS for 30 min. Cells were stained with rabbit-anti-LC3B (Cell Signaling Technology [CST], 3868), mouse anti-LAMP1 (Abcam, ab25630), or rabbit-anti-CTSD/cathepsin D antibodies (kindly provided by Dr. Takashi Ueno, Juntendo University) for 45 min at 37°C. After washing with PBS, cells were stained with DAPI for staining of nuclei, Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, A11034), and Alexa Fluor 647-conjugated goat anti-mouse IgG (Molecular Probes, 4410) for 45 min at 37°C. The stained cover slips were mounted using ProLong Antifade (Invitrogen, P7481) and kept in the dark at 4°C. Images were obtained by confocal microscopy using a LSM 510 META microscope (Carl Zeiss, Göttingen, Germany).

Transmission electron microscopy (TEM)

Uninfected neutrophils or neutrophils infected with live *B. pseudomallei* strains at an MOI of 10 for 1 and 3 h were prefixed

with 2.5% (wt/vol) glutaraldehyde in PBS (pH 7.2) at the indicated time points for 2 h at room temperature. Cells were post-fixed with 2% OsO_4 and embedded in Epon 812 (TAAB Laboratories Equipment, T002) after dehydration, essentially as previously described.[30] Ultrathin sections were cut with an ultramicrotome (UC6, Leica, Vienna, Austria), stained with uranium acetate and lead citrate, and examined with a Hitachi HT7700 electron microscope (Hitachi High-Technologies, Tokyo, Japan).

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 1× SDS-PAGE sample buffer, subjected to 12.5% SDS-PAGE, and transferred to polyvinylidene fluoride membranes (Pall Corporation, 66543). Membranes were blocked with 5% (wt/vol) bovine serum albumin fraction V (Sigma-Aldrich, 9048) in Tris-buffered saline with 0.1% (vol/vol) Tween-20 (Sigma-Aldrich, P1379) (TBST; 1 ml of Tween-20 and 1000 ml of 1X PBS [Gibco, 10010023]) and incubated with rabbit primary antibodies against LC3B (CST, 7074, 1:1,000 dilution) overnight at 4°C. After washing with TBST 3 times, the membranes were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution; CST, 7074) for 1 h at room temperature. After washing with TBST 3 times, bound antibody was detected by enhanced chemiluminescence (Pierce, 32106), and the intensity of each band was analyzed by densitometry using the software ImageJ.

Assay of bacterial net intracellular survival

Purified neutrophils were cocultured with *B. pseudomallei* strains 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 the addition of complete RPMI 1640 containing 250 μg/ml KM at 37°C, 5% CO_2 for 30 min, and 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 10-fold dilutions of lysates to LB agar plates (1 h postinfection; 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 neutrophils and plating of serial 10-fold dilutions of lysates to LB agar.

Statistical analysis

Statistical analysis (unpaired Student *t* test) was performed using the software GraphPad Prism version 5 (GraphPad). For quantitative analysis of micrographs, at least 100 bacteria were counted for each condition in each experiment. A *P*-value of $\leq$ 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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