Burkholderia pseudomallei Toxins and Clinical Implications

3

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Abstract

Burkholderia pseudomallei is the causal agent of melioidosis. In spite of ongoing studies, the molecular mechanisms underlying toxin-induced pathogenesis of this bacterium are not clearly elucidated for this potential biological warfare pathogen. In this review, we highlight current information of *B. pseudomallei* toxins and their roles in pathophysiological effects in various experimental models. Several secretary proteins/lethal factors show lethal toxicity to cells in culture via filtrates of *B. pseudomallei* culture. These toxins are released in culture from

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P. Gopalakrishnakone et al. (eds.), *Microbial Toxins*, Toxinology,
https://doi.org/10.1007/978-94-007-6449-1_12

strains isolated from soil, animals and humans. Toxins are also found in infected patients, which strongly correlate with severity of melioidosis. Melioidosis progression begins with an environmental reservoir and bacterial attachment in the host, invasion of epithelial/macrophage cells and subsequent intercellular spread. The molecular and cellular basis of pathogenesis in melioidosis will provide a better, rational understanding toward design and development of new drugs with novel mechanisms of action.

Keywords
Melioidosis • Soil pathogen • Lethal factors • Exotoxins

Contents

Introduction

The gram-negative bacterium *Burkholderia pseudomallei* causes melioidosis and has become a serious public health issue with potential bioterrorism implications worldwide (Gilad 2007). This is an emerging disease in Vietnam (Leelarasamee 2000), Northern Thailand (Waiwarawooth et al. 2008), Singapore, and Malaysia. In

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Northern Australia, 46% of severe cases become bacteremic, and 19% of these patients died (Currie et al. 2000). The first cases of pulmonary melioidosis have also been reported in Southern Cambodia (Overtoom et al. 2008), the Western Province of Papua New Guinea with most cases being in children (Warner et al. 2008), New Caledonia (Le Hello et al. 2005), as well as various Indian states (Whitmore and Krishnaswami 1912; Saravu et al. 2008). Recently, there were reported cases of acute and travel-related fatal melioidosis (Morosini et al. 2013). Not only *B. pseudomallei* but also other species such as *Burkholderia cepacia* are an important threat to patients in North America, as well as Europe (Hauser et al. 2001).

These bacteria are opportunistic pathogens in patients with cystic fibrosis or chronic granulomatous disease (Whitby et al. 2006). This bacterium is resistant to many antibiotics, and there is cross infection involving patient-to-patient transmission, especially nosocomially (Hunt et al. 2004). Almost 20% of colonized patients have acute necrotizing pneumonia and septicemia (Kitt et al. 2016). Pneumonia is one of the most frequent clinical presentations of melioidosis, especially acute, subacute, as well as chronic forms of pneumonia due to *B. pseudomallei* infection (Currie 2003). A recent clinical study also clearly shows that consolidated with cavitary lesions, hepatosplenomegaly and sputum analysis confirm the presence of *B. pseudomallei*. These melioidosis patients were also completely treated for primary pneumonia (Afroze et al. 2016). Other complications, such as coinfection of pulmonary tuberculosis (TB) in a diabetic condition, have also been reported (Sulaiman et al. 2013). However, approximately 60–80% of melioidosis patients possess high risk factors for this infection along with diabetes mellitus, chronic pulmonary infection, and heavy alcohol use (Wiersinga et al. 2012). Melioidosis and TB can be associated diseases, which have been reported in a neck abscess (Shenoy et al. 2009) and lung mass (Truong et al. 2015). Such complicated cases of neck melioidosis, defined as a parapharyngeal abscess, are treated by incision, drainage, and intravenous combination of antibiotics for 6 weeks along with oral antibiotic administration (Zulkiflee et al. 2008). In addition, *B. pseudomallei* has also been isolated from the patient's blood and pus of a left gluteal abscess in an imported case (Pelerito et al. 2016). Nearly 34 confirmed human, and 3 animal, cases have been reported from the USA/Puerto Rico during 2008–2013 that had histories of travel into areas endemic for melioidosis (Benoit et al. 2015).

Naturally, these bacteria can survive in the environment for more than a year. During nitrogen/amino acid starvation, these bacteria can use sigma factors such as RNA polymerase nitrogen-sigma factor (RpoS)/RNA and polymerase nitrogen-limitation (RpoN) to modulate gene expression for their adaptation and survival (Diep et al. 2015). In addition, this bacterium can survive in host cells by escaping reactive oxygen species (ROS) through the regulation of stress-responsive sigma factors (RpoS) (Chutoam et al. 2013). However, sigma factors E (σE) play an important role in regulating extra-cytoplasmic stress responses in various gram-negative bacteria (Daimon et al. 2015). Many bacterial toxins are proteins, encoded by the bacterial chromosomal genes, plasmids, or even phages (Lubran 1988). In addition, a variety of bacterial secretion systems have been characterized to date, and they are known as types I–VII (Kimelman et al. 2012). These toxins, when delivered

by these secretion systems, can locally damage the host at the site of bacterial infection (Henkel et al. 2010).

Various microbial or bacterial toxins are important virulence factors that induce pathological changes to hosts, causing severe diseases (Martin 2012). Bacterial toxins from all types of bacteria can cause sepsis-related mortality globally, for instance, in the USA alone there was an incidence rate of 240 per 100,000 people in 2013 (Martin 2012). However, *B. pseudomallei* also causes sepsis and leads to an uncontrolled inflammatory response by the host cells, which results in multiple organ failure and death (Morgan et al. 2016). Endotoxins are derived from the outer cell membrane of gram-negative bacteria (Michael and Silverman 1998). In particular, lipopolysaccharide (LPS) or endotoxin contains lipid A (core molecule) that induces diverse clinical-pathological effects in humans. These cell wall components are also responsible for severe cellular toxicity (Brown et al. 2015), and higher doses (6–25 ng/kg) of these toxins elicit fatal shock to humans, as well as other mammals. Generally, LPS produces high fever in the host via release of interleukin-1 (IL-1) and other mediators that cause a systemic inflammatory response, leading to cell death and organ dysfunction (Silverman and Ostro 1999).

Some pathogens secrete exotoxins containing enzymatic activity that is implicated in the necrosis of tissues (proteases, lipases, lecithinases, catalases, and hemolysins). These exotoxins can also be polypeptides devoid of enzymatic activity, such as those produced by *Staphylococcus aureus* that include toxic shock syndrome toxin-1, staphylococcal enterotoxins, and leukocidin (Martin et al. 2004). These protein toxins bind to specific receptors on cells and are also fatal to hosts, even in very small doses (Brown et al. 2015). Commonly, the membrane-perforating bacterial toxins are known as pore-forming toxins (Gurnev and Nestorovich 2014). However, small peptides such as alpha-hemolysin of *S. aureus* form channels that cause lesions on cellular membranes that simply punch holes in membranes. Currently, there are no enzymatic subunits that enter a cell (Yan et al. 2013). *Burkholderia pseudomallei* lethal factor 1 (BPSL1549) was the first discovered toxin from this bacterium, using X-ray crystallography studies of hypothetical proteins (Cruz-Migoni et al. 2011). Later, BPSL1549 was renamed as *Burkholderia* lethal factor 1 (BLF1). A recombinant version of BLF1 is toxic and kills mice (challenged intraperitoneally) and cultured macrophages (Hautbergue 2012). Further characterization of BLF1 has revealed its deadly molecular mechanism of action in human cells, identifying molecules that may prevent the modification of eukaryotic initiation factor 4A (Eif4A) by BLF1. The elevated levels of BLF1 promote or inhibit pathogenesis. However, BLF1 promotes deamidation of glutamine-339 (Gln339) of the translation initiation factor Eif4A, completely abolishing its helicase action and preventing translation. In addition, inactivation of BLF1 by various means might lead to an interesting vaccine candidate (Guillaume et al. 2009). *B. pseudomallei* has been isolated from various sources, yet bacteria isolated from melioidosis patients release higher levels of endotoxin in vitro compared to those isolated from other sources such as soil/animals (Chen et al. 2015). Collectively, these *B. pseudomallei* toxins contribute to poor clinical outcomes. The current review will discuss *B. pseudomallei* toxins and their clinical implications at a cellular, and molecular level of melioidosis.

Severity of Potential Soil Pathogens

The soil pathogen, *B. pseudomallei*, causes severe diseases such as melioidosis, which is endemic to Southeast Asia and other parts of the world. This pathogen is isolated from soil of agricultural lands; and also, the prevalence of *B. pseudomallei*-specific antibodies in humans increases significantly among residents affected by a typhoon/flood incident (Chen et al. 2015). *B. pseudomallei* has been isolated from seroma using sheep blood agar (Zong et al. 2012). Recent serology studies support the presence of melioidosis in Myanmar (Wuthiekanun et al. 2006), from where the original case of melioidosis was first recognized in 1911 (Whitmore and Krishnaswami 1912). All empyema aspirates of melioidosis patients test positive for arginine dihydrolase, and serological testing reveals a minimal 1:2560 antibody titer against *B. pseudomallei* (Tsang and Lai 2001). Following diagnosis, the patient's symptoms improve after treatment with high doses of ceftazidime (Lee et al. 2005). The bacterium is typically sensitive to co-trimoxazole, Augmentin, ceftazidime, cefoperazone, ciprofloxacin, chloramphenicol, and imipenem, while being resistant to aminoglycosides (Goldberg and Bishara 2012).

The most important routes for melioidosis infection are via aerosol, ingestion, or cutaneous inoculation via muddy water or wet soil. As a result, infection severely affects the main organs such as the liver, spleen, kidney, prostate, and brain, leading to bacteremia and septicemia, which elicits larger numbers of pro-inflammatory cytokines that trigger oxidative stress as well as cell death (Fig. 1). Thus, the bacterium invades cells via endocytic vesicles, as the bacterium attaches onto the host cell wall, disrupts the membrane, escapes into the cytosol, and finally travels to adjacent cells via cell-to-cell membrane contact. Bacteria can release endotoxin from the cell wall components (O-antigen/LPS), which promote secretion of interferon gamma that is linked to cellular signaling molecules of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway.

Clinical Implications of Bacterial Exotoxin

Until now, there are no vaccines available for the treatment of clinically relevant, multidrug-resistant (MDR) *B. pseudomallei*. Recent structural studies of a cytolethal exotoxin (CLT) demonstrate the molecular mechanism underlying the function of this *B. pseudomallei* protein. The toxin induces a more severe form of melioidosis (Haase et al. 1997). In particular, the exotoxin is one of the most important agents responsible for septicemic melioidosis in humans. The molecular weight of CLT is 10 kDa, as purified from the culture filtrates of *B. pseudomallei* grown in vitro. Bacterial isolates from soil, animals, and humans possess differential cytotoxic effects in in vitro systems. The toxic effects of culture filtrates include lethality and hemorrhagic dermonecrosis in mice (Haase et al. 1997). Additional studies show that two other *B. pseudomallei* proteins (31 and 35 kDa) possess proteolytic, as well as toxic, activities (Mohamed et al. 1989). Furthermore, BLF-1 (23 kDa) is responsible for inhibiting host protein synthesis during melioidosis (Ahmad et al. 2015), which is similar to that of the catalytic domain of *Escherichia coli* cytotoxic necrotizing factor

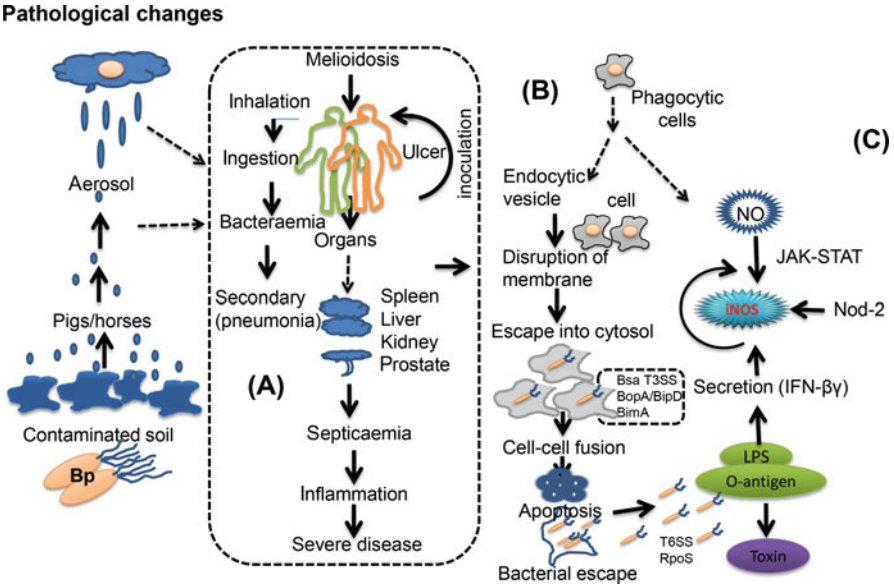

Fig. 1 (**a**–**c**) Muddy water and soil contaminated with *Burkholderia pseudomallei* in an endemic region transmit melioidosis by aerosol or inhalation, ingestion, inoculation, and human-to-human contact. This disease severely affects various organs such as the liver, spleen, kidney, prostate, and brain, leading to bacteremia/septicemia and increased pro-inflammatory cytokines that trigger oxidative stress as well as cell death. Bacteria enter phagocytes via endocytic vesicles, disrupt the membrane, and escape from the cytosol by cell-to-cell fusion. Bacteria also release toxin from their cell wall components (O-antigen/LPS), eliciting secretion of interferon gamma linked to cellular signaling molecules of JAK-STAT through Nod2

1 (CNF1-C). This further highlights that BLF-1 serves as a potent cytotoxin to not only eukaryotic cells but is also lethal to mice and promotes pathogenesis.

B. thailandensis (E264) also possesses contact-dependent growth inhibition (CDI) systems similar to the *B. pseudomallei* (strain 1026b), which modulates CDI-facilitated delivery of CdiA-CT toxins derived from other strains (Nikolakakis et al. 2012). This CDI system encodes CdiI immunity proteins that specifically bind to CdiA-CT, neutralize its toxin activity, and, as a result, protect CDI-positive cells from auto-inhibition. In addition, these variations in CDI toxin, as well as immunity proteins, reveal that these systems play an important role in bacterial self and nonself-recognition in microbial communities (Willett et al. 2015). The toxin-antitoxin (TA) system is commonly distributed in bacteria and linked to the formation of antibiotic-tolerant cells involved in chronic diseases (Daimon et al. 2015). A previous study shows that overexpression of *B. pseudomallei* HicA toxin (13 kDa) retards bacterial growth and generates cells tolerant to antibiotics such as ciprofloxacin/ceftazidime (Butt et al. 2014). These toxins are more pathogenic and responsible for severe disease.

Structure Activity Relationship of Bacterial Toxins

Structure analysis indicates that the HicA(a) toxin, HicA (H24A), is a mutant with histidine to alanine change at position 24. The protein consists of 95–135 amino acid (AA) residues. The N-/C-terminal AA residues and secondary structure reveal that the HicA (H24A) conserved side chains help form a hydrophobic core containing Val36, Val38, and Phe27, with charge distribution on the surface of HicA (H24A) (Fig. 2a, b). The active site consists of strongly conserved residues and includes cysteine, histidine, and multiple hydrogen bonds (Fig. 3a, b). However, the gene fragment containing amino acids 95–135 encodes a biologically active toxin that interacts with diverse cellular components like RNA, ribosomes, and DNA, which subsequently leads to killing of host cells (Yamaguchi et al. 2011). Earlier studies also show the toxin-antitoxin (TA) systems consisting of five different types (I–V), based on the gene products (Schuster and Bertram 2013). However, this antitoxin (RNA) or protein binds to the toxin and blocks its activity (Hayes 2003).

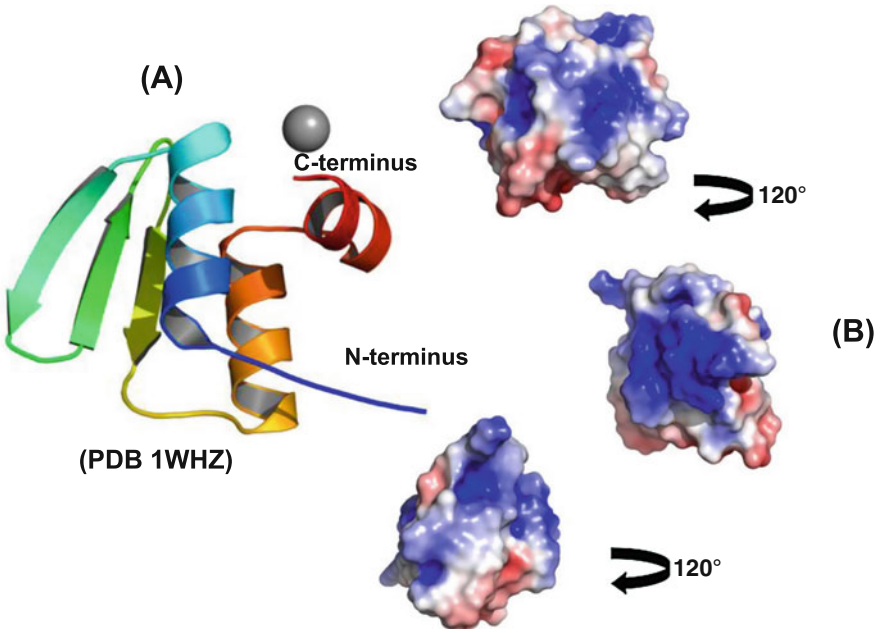

Fig. 2 Structure analysis of HicA(a) toxin. (**A**–**B**) The N-/C-terminal and secondary structure demonstrate that the HicA (H24A) conserved side chains contribute to the hydrophobic core containing Val36, Val38, and Phe27, with charge distribution on the surface of HicA (H24A) (PDB code 1 WHZ)

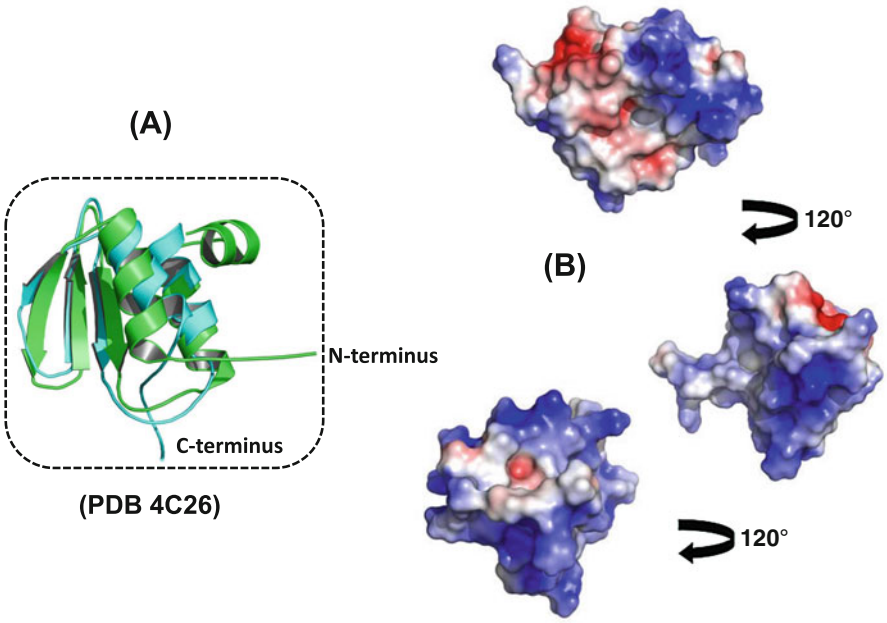

Fig. 3 (**A**) X-ray crystal studies clearly indicated that HicA bacterial toxin from *Burkholderia pseudomallei* plays an important role in persister cell formation and toxicity. (**B**) Crystal structure of the HicA toxin from *B. pseudomallei* responsible for persister cell formation (Butt et al. 2014)

Effect of Exotoxin (CLT) on Pathogenicity

Culture and Infection of Invasive Rodent Models

Toxic substances are produced by various bacteria that cause deleterious effects to targeted host cells as well as promote bacterial proliferation (Ashida et al. 2014). In the case of CLT, cell death is confirmed by formation of a pore in the endosomal membrane, which facilitates *B. pseudomallei* escape into the cytosol (Dubail et al. 2000). Murine melioidosis models of acute (BALB/c, Th2 phenotype) and chronic (C57BL/6, Th1 phenotype) infections mimic the disease stages in humans (Lazar Adler et al. 2009; Leakey et al. 1998). Infected C57BL/6 mice exhibit an early influx of neutrophils, followed by better activation and clearance of *B. pseudomallei* with moderate pro-inflammatory cytokine secretion contributing to chronic melioidosis (Ulett et al. 2000). Whereas, infected BALB/c mice lead excessive inflammation (Gan 2005) and exhibit increased levels of pro-inflammatory cytokines such as IL-6, IL-12, IL-15, IL-18, TNF-α, and IFN-γ at 24–48 h, with reduced macrophage/lymphocyte recruitment and activation, contributing to the development of acute disease (Leakey et al. 1998; Wiersinga et al. 2007). *B. pseudomallei* is an important biological warfare agent because it poses a threat to humans (Aquino and Wu 2011).

This species is the main causal agent of melioidosis, causing deadly sepsis in various areas (Williams et al. 2015). Currently, there is much mortality due to higher rates of relapse and severe inflammatory responses (Currie et al. 2000). Inflammation is an important biological response of the human body to harmful stimuli, such as bacterial pathogens or damaged cells. As a result of inflammation, a protective host response is initiated by various mediators such as immune cells, blood vessels, and specific cellular mediators. Inflammation also eliminates cell injury, necrotic cells, and damaged tissues from the injury site and initiates tissue repair mechanisms.

Several rodent models have been developed for the study of melioidosis. BALB/c mice are highly susceptible to *B. pseudomallei* by the aerosol route of infection in an acute model, with higher bacterial counts in the lungs/spleen (1×10^3–1×10^5 per gram of tissue versus other organs). Focal points of acute inflammation and severe necrosis are in the lungs, liver, and spleen (Lever et al. 2009). The murine aerosol model is well established and most suitable for not only *B. pseudomallei* infection but also other respiratory pathogen infections. Such murine models can also be useful for identifying biomarkers that may lead to effective treatments (Massey et al. 2014). Besides mice, marmosets can be used as a model for melioidosis. When the latter are challenged with different strains of *B. pseudomallei*, there ensues a severe acute disease with mild dissimilarity of the time of death and pathologic appearance versus mice. Fevers with bacteremia, bacterial dissemination, necrotizing hepatitis, splenitis, and pneumonia are common observations (Nelson et al. 2015). In our study, more foci were found in the lung of challenged animals versus the liver and spleen (unpublished work). Viable bacteria are found in the liver, spleen, and kidney that rapidly increase during 24 and 48 h postinfection, with the number of viable bacteria drastically increasing to a peak of 1×10^5 cfu/ml in 48 h (Fig. 4a–f).

Histologic analysis demonstrates acute necrotizing alveolitis and pneumonia in the lungs of mice, 24 h after exposure to *B. pseudomallei,* particularly in alveolar spaces and walls that are infiltrated by neutrophils/macrophages. Larger foci of consolidation exist, consisting of enlarged macrophages predominating in some bronchi lesions and thickening of alveoli walls, 48 h postinfection. Infected mouse lung sections show diffuse inflammatory infiltrates, including neutrophils and macrophages. Furthermore, lobes are filled with cellular debris, while proteinaceous fluid and inflammatory cells are found in airways/bronchial lumen on day 3 postinfection. In addition, there are small lesions containing infected macrophages and large lesions surrounded by noninfected neutrophils.

Several studies prove that human macrophages and neutrophils play a vital role in preventing infection by highly virulent strains of *B. pseudomallei* versus less virulent strains (Massey et al. 2014). Capsular polysaccharide influences the deposition of critical complement component C3, which essentially controls this bacterium through nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase stimulation by human neutrophils (Woodman et al. 2012). Polymorphonuclear neutrophils (PMNs) are also implicated in the pathogenesis of melioidosis, which includes formation of weblike structures called neutrophil extracellular traps (NETs); however, NETs killing of *B. pseudomallei* until now has been rather ambiguous (Riyapa

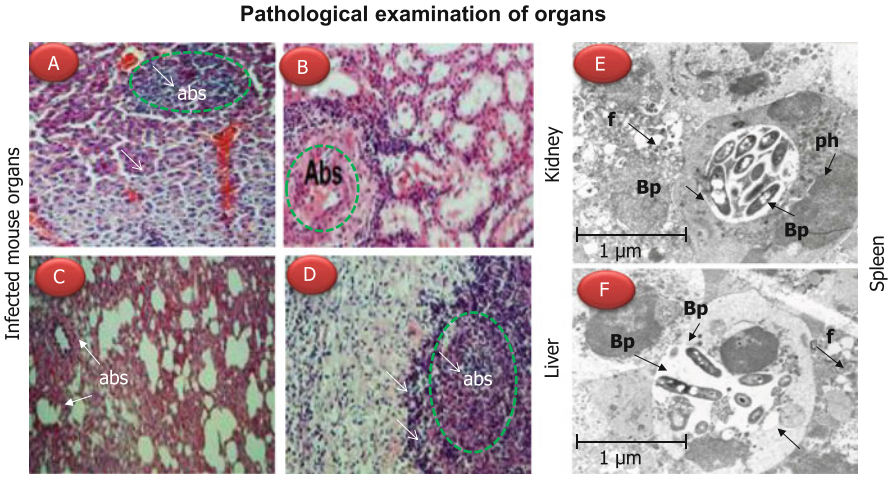

Fig. 4 Light micrograph showing *Burkholderia pseudomallei* infection in mouse spleen, liver, and kidney sections stained by hematoxylin and eosin, magnification 10 & ×20. (**A**–**D**). Large abscesses (*Abs*) with focal areas of necrosis, surrounded by a rim of meshed fibrous tissue, are evident after 2-week intraperitoneal (i.p.) challenge with 1.7×10^5 CFU/ml. The bacterial invasion is more pronounced in the spleen and liver than the kidney. (**E**–**F**) Transmission electron microscopic examination of BALB/c mouse spleen infected with *B. pseudomallei* 2 weeks following i.p. challenge with 1.7×10^5 CFU/ml. Large abscesses (*Bp*) with focal areas of cell, surrounded by a rim of meshed fibrous tissue, are evident. Abbreviations: *Bp B. pseudomallei, n* nucleus, *ph* phagocytosis, *abs* abscess, *f* fibrous tissue

et al. 2012). However, these NETs effectively trigger innate activation of plasmacytoid dendritic cells (pDCs) through Toll-like receptor-9 (TLR-9). The pDCs are potent producers of type I interferons (IFN), and a recent study has investigated whether the pDCs and type I IFN play a role during the early stages of *B. pseudomallei* infection (Williams et al. 2015). Virus-induced transient immunosuppressed C57BL/6 mice, infected with a low dose of biofilm-defective mutant (M10) of *B. pseudomallei*, relapse with severe inflammation (Panomket et al. 2016). Glycogen synthase kinase-3β (GSK3β) serves a vital role during an innate inflammatory response caused by bacterial pathogens. The effect of lithium chloride (LiCI), a GSK3β inhibitor, in an experimental murine model of acute melioidosis reveals improved survival of infected mice. These animals have elevated levels of anti-inflammatory cytokines such as IL-10/IL-1Ra in the sera as well as organs, while pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ are greatly reduced by LiCI (Tay et al. 2012). In this study, *B. pseudomallei*-infected peripheral blood mononuclear cells (PBMC), as well as infected diabetic/normal rats, generate raised levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-10, IL-12, and IL-18. This occurs via the NOD-like receptor nucleotide-binding domain, leucine-rich pyrin domain-containing-3 (NLRP3), or Nod-like receptor protein-3/pyroptosis through NLRC4 (Bast et al. 2014; Abderrazak et al. 2015) and phosphorylation of NF-kB in certain cells (Maniam et al. 2015). However, the blocking of dysregulated

GSK3β in PBMC from diabetic animals leads to inactivation of NF-kB and modulation of inflammatory cytokines.

Several gram-negative bacterial pathogens have secretion systems that inject structural proteins (flagellin) into the host cytosol, leading to caspase-1 activation/pyroptotic cell death (Abderrazak et al. 2015). A previous study shows that *B. pseudomallei* factors trigger caspase-1 activation, downstream signaling pathways, and effector mechanisms of caspase-1. Furthermore, the type 3 secretion system (T3SS3) modulated-NLRC4, caspase-1 activation, pyroptosis, and caspase-1-dependent/-independent cell death mechanisms contribute to the pathogenesis of *B. pseudomallei* infection of macrophages (Bast et al. 2014).

Effect of Exotoxin (CLT) on Virulence

The bacterial virulence factors playing an important role during an acute infection were determined using gene expression profiles in the spleen, lung, and liver of BALB/c (Th2 phenotype) and C57BL/6 (Th1 phenotype) mice via DNA microarrays. This analysis identified BPSS1521 (*bprD*), a predicted transcriptional regulator located in the type III secretion system (T3SS-3) operon, to be upregulated and specifically so in C57BL/6 mice. Whereas, BALB/c mice infected with a *bprD* mutant (a knockout) also resulted in death but in a shorter time and with more inflammation, as determined by histopathological analysis/enumeration of bacteria in the spleen. A large number of multinucleated giant cells (MNGCs), a hallmark of human melioidosis, were detected in animals infected with either wild-type or *bprD* mutants. One striking observation was the increased expression of BPSS1520 (*bprC*), located downstream of *bprD*, in the *bprD* mutant. BprC is a regulator of T6SS-1 that is required for the virulence of *B. pseudomallei* in murine infection models. Deletion of *bprD* led to the overexpression of *bprC* and a decreased time to death. The *bprD* expression was elevated in C57BL/6 mice, as compared to BALB/c, which suggests a role for BprD in the natural resistance of C57BL/6 mice to *B. pseudomallei*.

The negative regulation of *bprC* by BprD sheds further light on the complexity of regulation between T3SS-3 and T6SS-1, suggesting further investigation of suppression upon T6SS-1, as the latter is considered one of the most important *B. pseudomallei* virulence factors (Stevens et al. 2005). The actin-based motility of *B. pseudomallei* involves a distinctive mechanism of activation. For example, *bimA* homologs in *B. mallei* and *B. thailandensis* induce actin-based motility in J774.2 cells (Stevens et al. 2005). Another study also shows that T6SS-1 plays a vital role in actin-based motility in RAW 264.7 cells. Mutants (*tssE*) undergo vacuolar escape, and in the cytoplasm of host cells containing these bacteria, there are defects in actin polymerization, as evidenced by decreased intra-, as well as inter-, cellular spreading (Burtnick et al. 2010). Whereas, *B. pseudomallei*-induced actin polymerization is due to $bimA_{Bm}$ genes being highly expressed, as evidenced in 556 melioidosis cases (human) from Australia (Sarovich et al. 2014). In addition, a filamentous hemagglutinin gene, *fhaB3*, has been observed in positive blood cultures; however, it was

negatively correlated with localized skin lesions without sepsis (Sarovich et al. 2014).

Secretion of Bacterial Molecules Responsible for Virulence

Molecules that play key roles in *B. pseudomallei* virulence include capsular polysaccharide, lipopolysaccharide, adhesins, specialized secretion systems, actin-based motility, and various secreted factors (Stone et al. 2014). After internalization, bacteria escape from endocytic vacuoles into the cytoplasm of infected cells, subsequently forming membrane protrusions by inducing actin polymerization at one pole of the bacterium that helps to propel it out of the host cell. Survival within phagocytic cells, and cell-to-cell spread via actin protrusions, is required for full virulence. Previous studies reveal the role of a putative type III protein secretion apparatus (Bsa) during the interaction of *B. pseudomallei* with host cells. These murine-based findings indicate that the Bsa type III secretion system critically modulates the intracellular behavior of *B. pseudomallei* (Stevens et al. 2002). *B. pseudomallei* bipD mutants, lacking a component of the translocation apparatus, are significantly attenuated as determined by intraperitoneal or intranasal challenge studies in BALB/c mice. Furthermore, a bipD mutant is attenuated in C57BL/6 IL-12 p40 (−/−) mice, which are highly susceptible to *B. pseudomallei* infection. Mutation of bipD impairs bacterial replication in the liver and spleen of BALB/c mice during the early stages of infection (Stevens et al. 2004).

Deletion of Hcp from cluster 1 (Hcp1) strongly attenuates *B. pseudomallei*, suggesting a prominent role of the T6SS cluster 1 (T6SS1, BPSS1496 to BPSS1511) for virulence in mammalian hosts (Chirakul et al. 2014). The type VI secretion system (T6SSs) and their effectors play an important role in pathogenesis and inter-bacterial competition (Attar 2015). A previous study shows that the bacterial toxin-antitoxin system mediates this transition by controlling bacterial motility in response to extracellular stress (Hadjifrangiskou et al. 2011). However, the bacterial capsule and a type III protein secretion apparatus enable *B. pseudomallei* to survive intracellular killing and facilitate intercellular spread. Since one of the capsules produced by *B. pseudomallei* is important in virulence, the genes encoding the proteins responsible for its biosynthesis may be considered as potential targets to disable the bacterium and halt disease progression (Reckseidler-Zenteno et al. 2009).

Effect of Exotoxin (CLT) on Resistance and Drug-Efflux Mechanism

Currently, recommended antibiotics such as chloramphenicol, doxycycline, co-trimoxazole, and kanamycin are often ineffective in patients with severe melioidosis infections (Estes et al. 2010). The main resistance mechanisms affecting these antibiotics include enzymatic inactivation, target deletion, and efflux from the bacterium, which are all mediated by chromosomally encoded genes (Schweizer

2012). Overexpression of efflux pumps strongly correlates with clinically relevant drug resistance (Sun et al. 2014; Chan et al. 2004, 2007; Chan and Chua 2005). Excessive release of mutations in the class A PenA beta-lactamase causes ceftazidime and amoxicillin-clavulanic acid resistance. Removal of penicillin binding protein-3 (PBP-3) leads to ceftazidime resistance. Whereas, the BpeEF-OprC efflux pump expression causes trimethoprim and trimethoprim-sulfamethoxazole resistance (Schweizer 2012). Of clinical relevance, particularly in gram-negative bacteria, efflux pumps of the resistance nodulation cell division (RND) play a vital role. Diverse efflux pumps exist in *B. cenocepacia*, which confer resistance to many potent antibiotics such as chloramphenicol, tetracyclines, and aminoglycosides (Podnecky et al. 2015). BpeAB-OprB strains of KHW mediate the efflux of aminoglycosides, while macrolides play a major role in quorum sensing and virulence (Mima and Schweizer 2010).

Toxins Modulating the Mechanism of Action and Cellular Signaling

Mouse macrophages (RAW 264.7), treated with LPS from *B. pseudomallei* (BP-LPS), produce significantly less nitric oxide (NO) and TNF-α than those treated with *Escherichia coli* or *Salmonella typhi* LPS (Utaisincharoen et al. 2000). Interestingly, plasma levels of endotoxin are an important predictor of multiple organ failure and death in systemic infections (Brandtzaeg et al. 1989). Mammalian cells respond to LPS by activating protein kinase cascades, which leads to new gene expression. This includes mitogen-activated protein kinase (MAPK), targeted by endotoxin, and hyperosmolarity in mammalian cells (Han et al. 1994). However, the p38 MAPK signaling pathway is activated by various stress stimuli such as osmostress, which also regulates multiple biological processes that include the p38 signaling pathway (Ben Messaoud et al. 2015). In addition, MAPK-interacting protein kinases 1 and 2 (Mnk1/Mnk2) play a vital role in controlling signals essential for mRNA translation (Joshi and Platanias 2014). The extracellular regulated kinase (Erk)/p38 MAPK pathways play key roles in mediating various biological functions such as development, apoptosis, autophagy, and inflammation (Roux and Blenis 2004). Bacterial pathogens can release toxic proteins to outmaneuver the host's immune system. The NADPH oxidase enzyme family generates ROS that contributes to cell signaling, the development of immune responses, as well as promoting proliferation and transcription (Bokoch et al. 2009). On the other hand, these proteins target regulatory GTPases belonging to the RHO family that organize the host's actin cytoskeleton (Aktories 2011). Therefore, the molecular basis of actin-based motility of these bacterial pathogens will be useful to understand fully the novel insights of pathogenesis and host-cell pathways (Stevens et al. 2006).

Melioidosis is a severe form of infection endemic to Southeast Asia, Northern Australia, and other parts of the world. Present treatment of melioidosis is highly challenging and complicated because of resistance to many existing antibiotics. It is very difficult to treat this disease as it results in very high mortality and morbidity.

Currently, there is no vaccine, and the organism has become multidrug resistant, which often induces relapse. Bacterial cell wall-secreted proteins or toxins responsible for severe inflammation/tissue destruction, pathological changes, cell death, and organ failure lead to death of the host. These bacterial toxins play an important role in invasion, virulence, drug-efflux mechanisms, and cellular signaling. Melioidosis is manifested by an acute/chronic septicemia that can be asymptomatic, leading to septic shock. Pathology examination of human melioidosis, via solid organs, is usually performed in the lungs, liver, and spleen. Disease-attributed abnormalities can also be detected in the brain or kidney of mice infected with *B. pseudomallei.* Necrotizing lesions of melioidosis can be attributed to bacteria-released toxins that kill host cells. *B. pseudomallei* produce numerous exotoxins that lead to acute progression of the disease but are not found in aerosol-infected BALB/c mice (Lever et al. 2009). The bacterial burden is higher in the lung versus other organs in aerosol-infected mice. Experimental evidence clearly shows elevated levels of toxins secreted from in vitro cultured bacteria isolated from patients suffering from severe melioidosis. Several culture methods, as well as rodent models, are well established and useful for studying the pathophysiology of melioidosis. However, the lack of diagnostics presents a challenge not only in endemic regions but also globally.

Conclusion and Future Directions

In conclusion, further experimental studies are required to understand *B. pseudomallei* toxins, their clinical implications, and to subsequently design and develop new drug targets against melioidosis in the near future.

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