Innate immune response to *Burkholderia mallei*

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Purpose of review

Burkholderia mallei is a facultative intracellular pathogen that causes the highly contagious and often fatal disease, glanders. With its high rate of infectivity via aerosol and recalcitrance towards antibiotics, this pathogen is considered a potential biological threat agent. This review focuses on the most recent literature highlighting host innate immune response to B. mallei.

Recent findings

Recent studies focused on elucidating host innate immune responses to the novel mechanisms and virulence factors employed by B. mallei for survival. Studies suggest that pathogen proteins manipulate various cellular processes including host ubiquitination pathways, phagosomal escape, and actin-cytoskeleton rearrangement. Immune signaling molecules such as TLRs, NOD, MyD88, and pro-inflammatory cytokines such as IFN-γ and TNF-α, play key roles in the induction of innate immune responses. Modifications in B. mallei LPS, in particular, the lipid A acyl groups, stimulate immune responses via TLR4 activation that may contribute to persistent infection.

Summary

Mortality is high due to septicemia and immune-pathogenesis with B. mallei exposure. An effective innate immune response is critical to controlling the acute phase of the infection. Both vaccination and therapeutic approaches are necessary for complete protection against B. mallei.

Keywords: Innate Immune response, Burkholderia mallei, immune signaling, cellular immunity, vaccine.
INTRODUCTION

*Burkholderia mallei* is the etiological agent of a highly contagious, acute or chronic, usually fatal disease of solipeds, known as glanders. This obligate mammalian, facultative intracellular pathogen is a gram-negative, non-motile, non-spore forming bacilli which is widely regarded as a host-adapted deletion clone of *Burkholderia pseudomallei*, an environmental saprophytic pathogen that causes the disease melioidosis. Although horses, donkeys, and mules constitute the only known natural reservoirs for *B. mallei*, humans and other mammalian hosts (e.g., camels, non-human primates, goats, dogs, cats, rabbits, hamsters, guinea pigs, and mice) are susceptible to infection and display similar disease progression and pathology (1-7).

Glanders transmits amongst animals via respiratory secretions and exudates from skin lesions. In human infections, the primary modes of *B. mallei* transmission are via direct contact with damaged skin, invasion of mucous membranes, and deposition into the lung. Depending on the route of exposure, the disease course of glanders infection can range from acute to chronic and manifest in multiple forms, such as localized, pulmonary, disseminated and septicemic. The clinical and pathological presentation of *B. mallei* infections bare a striking resemblance to *B. pseudomallei* infections, including their ability to remain quiescent and persist in the host following apparent clinical resolution (8). Due to the reasons above, in addition to their highly infectious nature as an aerosol, both pathogens are classified as Tier 1 select agents by the federal select agent program. Currently, no licensed vaccines are available for either disease, and medical therapeutic options are limited.

Both *B. pseudomallei* and *B. mallei* thrive intracellularly via modulation of host immune responses, which attributes to their resilience against current medical countermeasures.
Despite the characterization of many *B. pseudomallei* virulence factors, its strategies for circumventing intracellular host defenses remain ill-defined. Comparatively, even less is known for *B. mallei*. Limited understanding of these survival tactics poses a major challenge in the development of effective therapeutics. Thus, delineating the specific molecular mechanisms utilized by these pathogens to dysregulate host immune responses, is paramount. The majority of research and review articles are focused on host immune responses to *B. pseudomallei*. This review will concentrate on recent advances in characterizing *B. mallei* specific host immune responses, specifically innate immune responses.

**HOST-PATHOGEN INTERACTIONS AND INNATE IMMUNE RECOGNITION OF *BURKHOLDERIA MALLEI***

Although mechanisms can vary amongst *Burkholderia* spp., adhesion and invasion of host epithelial cells are vital steps during infection and appear to contribute to the overall virulence (9). For successful infection of host cells, *B. mallei* depends on the strategic utilization of a multitude of virulence factors and mechanisms to manipulate many host processes and pathways. Recently, a combined computational and experimental approach was utilized to systemically assess nine *B. mallei* virulence factors and their interactions with host proteins to elucidate mechanisms of *B. mallei* pathogenicity (10). Topological analyses of *B. mallei*-host protein-protein interactions (PPIs) suggest that *B. mallei* targets multifunctional intracellular host proteins, host proteins that interact with each other, and proteins with a large number of interacting partners. Host processes broadly influenced by these PPIs include the ubiquitination degradation system and focal adhesion pathways (10). These results are consistent with the previous work that reported TssN protein interactions with the polyubiquitin-B protein (UBB)
and with the cullin-1a protein (CUL1). These host proteins interact with TNF receptor-associated factor 6 (TRAF-6) and I-kappa-B inhibitor alpha (IκB-α), components central to Toll-like receptor (TLR) signaling (11). These studies provide some insights into \textit{B. mallei} pathogenesis, and on the proposed hypothesis that \textit{B. mallei} modulates innate immune responses by interfering with host ubiquitination directly or in combination with other pathogen proteins.

A comprehensive assessment of murine macrophages infected with a diverse panel of \textit{Burkholderia} spp. resulted in the uniform production of cytokines interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and murine keratinocyte-derived protein chemokine, a murine homolog of human IL-8 (12). Compared to \textit{B. pseudomallei} infected macrophages, \textit{B. mallei} infected macrophages secreted significantly higher levels of IL-6 and IL-10, which suggest these two pathogens differentially modulated host signaling cascades. Additionally, macrophages expressed IL-1β, IL-10, tumor necrosis factor receptor superfamily member 1B (Tnfrsf1B), and IL-36α mRNA, at significantly higher levels when infected with \textit{B. mallei} compared to the other \textit{Burkholderia} spp. (12), suggesting the existence of gene-based differences in the host inflammatory response that is unique to \textit{B. mallei}.

Infected macrophages further assessed for changes in their host signaling dynamics showed increased phosphorylation of adenosine monophosphate-activated protein kinase (AMPK); regulators of NF-κB signaling pathway (e.g. IκBα, GSK3β, Src, and STAT1) and mitogen-activated protein kinases (e.g. p38, ERK1/2 and c-Myc) (13). The degrees in which target host proteins or processes are modulated correlated to the differences in pathogenicity observed amongst \textit{Burkholderia} species. In infected macrophages, \textit{B.mallei} was a stronger inducer of
iNOS expression and IFN-β production compared to \textit{B. pseudomallei}. Based on these data, in addition to current knowledge of signaling transduction, a representative network of signaling pathways and axes was constructed to illustrate the activation of signaling cascades in response to \textit{Burkholderia} spp infection (13). Based on canonical pathways downstream of TLR4, induction of phosphorylated forms of AMPK-α1, GSK3β, and Src play key roles in regulating the inflammatory response of \textit{Burkholderia} spp. infections.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria, and a potent stimulator of host innate immune responses. Structure-activity relationship studies of TLR4 agonist suggest the biological activity of LPS correlates with the composition of its lipid A moiety (14). Evaluation of \textit{B. mallei} LPS showed the acylation of lipid A had a greater effect on its biological activity than their length (15). Thus, overall differential macrophage activation may be related to \textit{B. mallei} LPS, which is similar to the \textit{B. pseudomallei} LPS and bares a penta-acylated lipid A with 4, amino-4-deoxyarabinose (Ara4N) in almost half of its molecules, and appears to be a weaker macrophage activator as compared to enterobacterial LPS. Consistent with this, a significant reduction in mRNA expression or secretion of IL-6, TNF-α, and IL-1β is exhibited when stimulated with purified \textit{B. mallei} LPS compared to \textit{E. coli}-LPS-treated macrophages. Compared to \textit{E.coli}-infected macrophages, \textit{B. mallei}-infected macrophages also produce reduced levels of both IFN-dependent genes and mediators (IFN-β and NO) and cytokines (TNF-α, IL-6, IL-10, GM-CSF, and RANTES).

\textit{B. mallei} must overcome a gamut of antibacterial mechanisms and products (e.g., AMPs, and reactive oxygen and nitrogen species) critical to innate immunity to establish persistent infection. \textit{B. mallei} FMH isolates collected from mice spleens 60 days post-infection showed
attenuated abilities to replicate and induce cytotoxicity in macrophage assays (16). One B. mallei isolate displayed a change in its LPS phenotype, from smooth to rough, resulting from the loss of its O-polysaccharide (OPS) during the course infection (16). These phenotypic changes were conceived to stem from the infection shifting from an acute to a chronic or subclinical form, which is less prone to stimulate host immune responses. Earlier studies highlighted that genetic and phenotypic characteristics potentially associated with persistence of both B. pseudomallei and B. mallei (17, 18). Further studies including sequencing the OPS biosynthetic gene cluster of this B. mallei FMH strain may provide insight into the genetic basis for the loss of OPS. Intriguingly, OPS modification and loss is a hallmark of chronic Pseudomonas aeruginosa infection (19).

**CYTOKINES AND CHEMOKINE REGULATING INNATE IMMUNITY TO B. MALLEI INFECTION**

Highlighting the susceptibility of B. mallei to cell-mediated immune responses, previous studies compared the survival rates of infected BALB/c and IFN-γ knockout mice. BALB/c mice survived 37+ days longer than IFN-γ knockout mice and showed significantly lower levels of bacterial colonization, which illustrates the importance of IFN-γ-mediated immunity for control of infection (20). Macrophages and human pulmonary alveolar type II cells contribute to innate immunity by secreting inflammatory cytokines during B. mallei infection (21). When exposed to heat-killed B. mallei, primary PBMCs from non-human primates (NHPs) and humans elicit the strong production of IFN-γ, TNF-α, IL-6 and IL-1β (22). Cytokine responses varied among the NHPs, in which the African Green Monkey appears to be most responsive, compared to Rhesus or Cynomologus species, suggesting the inflammatory responses vary within mammalian
species (22). Similar results were observed with aerosol exposure of *B. mallei* FMH strain to NHPs, where most of the African Green Monkeys died but all Rhesus or Cynomologous species survived (Personal communication). The immune signaling mechanism for the strong cellular response demonstrated that MyD88-mediated signaling contributes to pro-inflammatory cytokine responses (22). These results were consistent with earlier reports which showed that MyD88/- mice were highly susceptible to pulmonary challenges with *B. mallei* and had significantly short survival time, increased bacterial burdens, and severe organ pathology compared to wild-type mice (23). Recruitment of inflammatory monocytes and DCs to the lungs and local production of IL-12, followed by NK cell production of IFN-γ, are the key cellular responses required for early protection from *B. mallei* infection.

**LACK OF AUTOPHAGY AND PERSISTENCE OF *B. MALLEI***

*B. pseudomallei* demonstrates an ability to escape autophagosomes in host phagocyte *in vitro* as well as in murine models and human cases of melioidosis, thus avoiding immune responses (24). The recurring illness of melioidosis patients in endemic areas can potentially be due to relapse or reinfection. Bacteria can become quiescent and subclinical to avoid host immune mechanisms of clearance. An earlier report indicated that non-functional mutations in *BPSS0180*, a type VI cluster-associated gene capable of inducing autophagy in both phagocytic and non-phagocytic mammalian cells, resulted in significant colocalization of *B. pseudomallei* with autophagy marker LC3 and impaired intracellular survival (25). A recent report suggests that *B. pseudomallei* evade autophagy (26). Consistent with these reports, recent results from our laboratory also suggest that lack of autophagy correlate with intracellular persistence of bacteria with aerosol exposure not only of *B. pseudomallei* but also *B. mallei* in spleens of
BALB/c and C57/BL6 mice with chronic infection (Alam et al. 2016; manuscript submitted). Mimesevic et al. suggests that multiple B. mallei virulence factors such as BMAA1865, BMAA0728 (TssN) and BMAA0553 influence critical host processes related to modulation of host ubiquitination, phagosome escape, interference with host cycloskeleton rearrangement and focal adhesion and a means to modulate and adapt the host-cell environment to advance infection (10). Further studies may shed light on whether any of these B. mallei proteins are directly or indirectly linked in the evasion of host autophagy processes.

POTENTIAL THERAPEUTIC AND PREVENTIVE STRATEGY TO GLANDERS:

Antibiotic resistance associated with Burkholderia infection is on the rise (27). Even with optimal antibiotic treatment, the mortality from acute severe melioidosis is high (30%-50% in Thailaland, 19% Australia) and mortality rates can be as high as 40% for cases of glanders (28-30). Recently, Waag reported that mice experimentally exposed to B. mallei suggest that although antibiotics can be efficacious after prolonged interval between exposure and treatment, but only if the animals were previously vaccinated (31). Thus, it is likely that both vaccination against B. mallei and post-exposure therapeutic approaches would be required for complete protection against B. mallei exposure.

THERAPEUTIC STRATEGY: MyD88 targeted therapy in preventing perturbed inflammation and septicemia

Primary cellular responses by analyses of IL-1β and other inflammatory cytokine responses by comparison to E. coli LPS, African Green Monkeys appears to be most responsive to B. mallei or B pseudomallei than Cynomolgus or Rhesus (22). Characterization of the immune signaling mechanism for cellular inflammatory response revealed that MyD88 mediated
signaling contributed to the \textit{B. mallei} and \textit{B. pseudomallei} induced pro-inflammatory responses. Notably, \textit{B. mallei}, \textit{B. pseudomallei} or purified LPS from these pathogens induced reporter activity inhibited and inflammatory cytokine production was attenuated by a MyD88 inhibitor (22). In the scenario of dysregulating inflammatory responses with established \textit{B. mallei} infection that often leads to septicemia and immune-pathogenesis, thus MyD88 targeted therapeutic intervention may be a potential strategy for therapy.

**VACCINE STRATEGY: Vaccine modulation of innate immunity**

For complete protection against Burkholderia pathogens, previous vaccine efforts focused on inducing both cellular and humoral immune responses (32). Possible candidates include whole-cell killed, subunit-glycoconjugate, and live-attenuated vaccines, as recently reviewed by Aschenbroich, SA et al. (33). These vaccines showed limited efficacy that resulted in partial protection and bacterial dissemination in murine models of infection. Live-attenuated recombinant Salmonella expressing \textit{B. mallei} LPS O antigen showed protection in a murine infection model of \textit{B. thilandensis}, a surrogate for biothreat \textit{Burkholderia spp.}, and suggest a promising platform for vaccine development (34).

Recently, two live-attenuated \textit{B. mallei} strains consisting of mutations in ubiquitination and phagosomal escape (\textit{ΔtssN}) or iron transport (\textit{ΔtonB}) show protection against lethal challenges in models of murine glanders (35,36). Analysis of the immune responses observed in vaccination-challenge studies was performed to understand how these mutants modulate immune responses. BALB/c mice surviving exposure to aerosolized \textit{ΔtssN} showed elevated expression of pro-inflammatory cytokines and chemokines: IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-4, IL-10, IL-12, MIG, MIP-1\(\alpha\), and TNF-\(\alpha\), and VEGF (35). This modulation of host responses showed \textit{ΔtssN}
capable of inducing prolonged innate immunity despite its high degree of attenuation. Mice immunization with ΔtssN demonstrated 67% survival rates at 21 days post-wild-type challenge (35). Authors suggested the partial protection afforded by ΔtssN immunization was mainly driven by innate immunity as BALB/c mice failed to show increased expression of pro-inflammatory cytokines and chemokines after ΔtssN prime and boost regimens.

BALB/c mice immunized with ΔtonB provided up to 100% survival at 21 days post-wild-type challenge (36). Compared to controls, immunized mice expressed moderated inflammatory cytokine/chemokine profiles with significant reductions reported in IL-6, GM-CSF, MCP-1, and RANTES (36). Authors correlated these results with reduced immune-mediated tissue damage observed in immunized mice. In cross-protection studies, ΔtonB immunized mice challenged with B. pseudomallei K96243 demonstrated 75% survival 36 days post-infection (36). Although these studies displayed protection and resulted in wild-type clearance, ΔtonB immunization was noted to result in persistence infection of the live-attenuated mutant in the spleens of surviving mice. Despite persistence, the B. mallei tonB mutant shows potential as a candidate for further vaccine development and optimization.

CONCLUSIONS

B. mallei target intracellular host immune signaling pathways for intracellular survival. Recent studies provide some understanding of pathogen-host protein interactions, dysregulation of macrophage activation, and immune evasion by B. mallei. Still, considerable gaps exist regarding the understanding of specific B. mallei protein(s) and signaling pathways that likely contribute to intracellular survival and evasion of host immune effector mechanisms. More focused research in delineating the molecular basis for host inability or dysregulation of
the host immune effector mechanism manipulated by this pathogen is needed. This may limit persistent infection, and likely provide direction towards developing medical countermeasures.

KEY POINTS

- *B. mallei* proteins interact with multifunctional host proteins that have large numbers of interacting partners to broadly influence host cellular mechanisms such ubiquitin-mediated proteolysis and focal adhesion
- Compared to other *Burkholderia spp.*, *B. mallei* displays unique manipulation of host signaling architectures and mechanisms of evading host innate immune responses
- The biological activity of *B. mallei* LPS is directly correlated with the acylation status of its lipid A molecule.
- MyD88-targeted post exposure therapy may be a potential strategy against *B. mallei* infection.
- Live-attenuated *B. mallei* mutants may be promising candidates for vaccine development against acute glanders.

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Conflict of interest

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Disclaimers
Army: Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as
▪ of special interest
▪▪ of outstanding interest

   ▪ This review outlines the mechanisms of disease with a special focus to host-pathogen interactions between Burkholderia species and lung epithelial cells
   ▪▪ This study provides the mechanisms to modulate and adapt the host cell environment for the succesful establishment of host infections and intracellular spread.
   ▪ This study reports the phosphorylation of novel targets of host proteins during *Burkholderia* species infection. Based on their study and with what is known in the literature, authors constructed a representative network that contains signaling axes that are likely modulated by *Burkholderia* infection.
   ▪▪ This study demonstrate a correlation between the biological activity of LPS from *B. mallei* and other pathogenic bacteria and extent of their lipid A acylation.
   ▪ This in vitro study describes pro-inflammatory responses of PBMCs from NHPs and humans exposed to *B. mallei* and *B. pseudomallei* at the equivalent levels that are contributed by MyD88-mediated signaling and possible therapeutic strategy targeting MyD88 in preventing immuno-pathogenesis.
   ▪ This study reports the live-attenuated B. mallei tssN mutant as a vaccine candidate that provides partial protection against aerosolized B. mallei infection. Although the tssN mutant show limited potential as a vaccine candidate, these studies highlight the role of innate and cellular immunity in mitigating glanders infection.
   ▪ This study reports the live-attenuated B. mallei tonB mutant as a promising vaccine candidate that provides excellent protection against both B. mallei and B. pseudomallei infection. While studies show complete clearance of wild-type strains, the B. mallei tonB mutant remains, thus further work focused on preventing chronic infection is needed.