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Characterization of cellular immune response and innate immune signaling in human and nonhuman primate primary mononuclear cells exposed to *Burkholderia mallei*

Shahabuddin Alam [a], Kei Amemiya [b], Robert C. Bernhards [b], Robert G. Ulrich [a], David M. Waag [b], Kamal U. Saikh [a, *]

[a] Department of Immunology, Army Medical Research Institute of Infectious Diseases, Frederick, MD, USA
[b] Department of Bacteriology, Army Medical Research Institute of Infectious Diseases, Frederick, MD, USA

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ABSTRACT

Burkholderia pseudomallei infection causes melioidosis and is often characterized by severe sepsis. Although rare in humans, *Burkholderia mallei* has caused infections in laboratory workers, and the early innate cellular response to *B. mallei* in human and nonhuman primates has not been characterized. In this study, we examined the primary cellular immune response to *B. mallei* in PBMC cultures of non-human primates (NHPs), *Chlorocebus aethiops* (African Green Monkeys), *Macaca fascicularis* (Cynomolgus macaque), and *Macaca mulatta* (Rhesus macaque) and humans. Our results demonstrated that *B. mallei* elicited strong primary pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, and IL-6) equivalent to the levels of *B. pseudomallei* in primary PBMC cultures of NHPs and humans. When we examined IL-1β and other cytokine responses by comparison to *Escherichia coli* LPS, African Green Monkeys appears to be most responsive to *B. mallei* than Cynomolgus or Rhesus. Characterization of the immune signaling mechanism for cellular response was conducted by using a ligand induced cell-based reporter assay, and our results demonstrated that MyD88 mediated signaling contributed to the *B. mallei* and *B. pseudomallei* induced pro-inflammatory responses. Notably, the induced reporter activity with *B. mallei*, *B. pseudomallei*, or purified LPS from these pathogens was inhibited and cytokine production was attenuated by a MyD88 inhibitor. Together, these results show that in the scenario of severe hyper-inflammatory responses to *B. mallei* infection, MyD88 targeted therapeutic intervention may be a successful strategy for therapy.

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1. Introduction

Burkholderia mallei, an obligate mammalian pathogen is the causative agent of glanders found primarily in equidae. *Burkholderia pseudomallei*, is an opportunistic soil bacterium causes melioidosis in humans and can infect a wide variety of mammalian species. Both organisms have been classified as category B select agents by the Centers for Disease Control and Prevention and both pathogens are considered to be potential bioweapons. *B. mallei* has been used deliberately against animals and humans in the past [1]. Inhalational exposure with either of these bacteria elicits rapidly fatal pneumonia and sepsis in the host. *B. mallei* appears to be derived from *B. pseudomallei* and has lost genetic material in association with a host adapted parasitic existence [2]. *B. mallei* infection in humans can present as either an acute or chronic nasal-pulmonary (glanders) or cutaneous (farcy) infection [3]. Clinical manifestations of glanders in humans are similar to those of melioidosis. *B. pseudomallei* infections are an important cause of community acquired sepsis and mortality in endemic regions (around 80% in individuals with bacteremia or sepsis) and have been well documented [4]. Although rare in humans, *B. mallei* has caused infections in laboratory workers [5,6], and respiratory inoculation with either of these bacteria in humans is characterized by a rapid onset of fatal pneumonia, bacteremia and sepsis [7–10]. However, little is known of the early cellular immune responses with exposure to *B. mallei* in non-human primates (NHPs) and humans, and in addition, the pro-inflammatory signaling mechanisms required in generating protection from pneumonic *B. mallei*

* Corresponding author.
E-mail address: kamal.u.saikh.civ@mail.mil (K.U. Saikh).

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infection or immune-pathogenesis, as the host response to *B. mallei* may be both protective and damaging. Even with optimal treatment, the mortality from acute severe melioidosis is high (30%–50% in Thailand, 19% in Australia) [10,11]. Serum levels of TNF-α, IFN-γ, and soluble IL-2 receptors, as well as IL-6, IL-8, IL-10, IL-12 p40, IL-18, granzymes A and B, procalcitonin, and CXC chemokines are elevated in patients with severe melioidosis, and some of these correlate with disease severity and outcome [12–15]. Support for a role for hyper production of IFN-γ and other pro-inflammatory cytokines in pathogenesis of septic shock in melioidosis has also been obtained from animal models [16,17].

Since the mortality of severe melioidosis remains high during early stages of treatment, it is likely that a similar pro-inflammatory response may happen with early exposure of *B. mallei* infection. Gram negative intracellular pathogens including *Burkholderia* spp., *Brucella* spp. and *Coxiella burnetti* induce host directed response through TLR engagement, particularly TLR4 by lipopolysaccharide (LPS). LPS, a major component of the outer membrane of gram-negative bacteria, is a potent activator of innate immune cells, and induces fatal endotoxin shock syndrome with exaggerated production of inflammatory cytokines in experimental animals and humans that often leads to organ failure and death. Clinically, septicemic melioidosis has many of the features of endotoxemia, and it is likely that LPS contributes to immune pathogenesis. Upon engagement, TLR4 recruits universal adaptor molecule such as MyD88. Activation of TLR4 by LPS facilitates recruitment of IRAK1 and TRAF6 via MyD88. This leads to activation of JNK, and NF-Kβ, and thereby induces expression of the genes of inflammatory cytokines. The MyD88-mediated signaling pathway predominantly induces a pro-inflammatory response. Earlier reports from our laboratory and others have demonstrated that MyD88$^{-/-}$ mice do not show any inflammatory cytokine production in response to *Escherichia coli* LPS [18–20]. Since *B. mallei* and *B. pseudomallei* are closely related pathogens, their clinical manifestations are anticipated to be very similar. Septicemic melioidosis and hyper production of pro-inflammatory cytokines has been reported to be associated to immune-pathogenesis with *Burkholderia* spp. infection, thus, thereby indicating that a differential cellular immune response may be associated with disease outcome and pathogenesis. We characterized the early cellular immune responses in primary cells of NHPs and humans by analyzing the innate immune signaling pathway. Our results demonstrate that MyD88-mediated immune signaling contributes to cellular immune response to *B. mallei* and MyD88 inhibition has significant potential for therapeutic intervention of cellular response from immune-pathogenesis.

2. Materials and methods

2.1. Reagents

E. coli LPS (055:B5) was purchased from Sigma–Aldrich (Saint Louis, MO). Pooled human AB sera were obtained from Pel–Freez (Brown Deer, WI). The *B. mallei* strain used in this study was obtained from Dr. Dave Waag (USAMRIID) as described elsewhere [21]. The Meso Scale Discovery (MSD) multi spot array ultrasensitive cytokine assay kit was purchased from MSD (Gaithersburg, MD). Ficoll–Hypaque was purchased from GE Healthcare Biosciences (Piscataway, NJ). The HEK 293-TLR4-NF-kB-SEAP cell line was obtained from In Vivogen (San Diego, CA).

2.2. Bacterial strains

B. mallei GB 15-1-3 and *B. pseudomallei* MSHR305 strains (obtained from D. Waag, USAMRIID) were used for all experiments. Work involving these bacterial strains such as culture conditions and irradiation for using in BSL2 lab was carried out as described elsewhere [21]. Whole irradiated *B. mallei* and *B. pseudomallei* were prepared as described elsewhere [22]. Briefly, 10–20 μl of stock bacterial culture was inoculated into 50 ml of glycerol tryptone broth (GTB) growth medium in a 250 ml flask. The bacterial culture was incubated overnight in 37 °C in a shaker incubator at 200 rpm. The bacterial culture was centrifuged in a 50 ml tube and pellet suspended in 25 ml of Hepes buffered saline solutions (HBSS) and placed in a heat sealed bag to freeze at −70 °C overnight. The bacterial culture was sterilized by irradiation at 2.1 M rads. Killed bacteria were pelleted by centrifugation, washed, and pellet suspended in sterile deionized water and concentration of killed bacteria at OD 660 nm was estimated using a standard curve. Initially, the standard curve was prepared by making dilutions of irradiation-killed bacteria, measuring OD at 660 nm, and placing 1 ml in tared vials to determine the dry weight (in mg) corresponding to the respective OD.

2.3. LPS preparation

B. pseudomallei MSHR 305 and *B. mallei* GB15.1-3 were cultured in GTB at 37 °C with shaking at 200 rpm overnight. The *B. pseudomallei* MSHR 305 culture was heat-killed at 90 °C for 90 min, while *B. mallei* GB15.1-3 was irradiated at 2.1 M rad. Sterility was confirmed for each sample then LPS extractions were performed using approximately 10 mg of killed cells following the procedure from Yi and Hackett [23]. Sample purity was examined by western blots with polyclonal antibody ABE 335 (Critical Reagents Program, Frederick, MD). Each sample was estimated to be >99% pure. LPS quantitation was performed using the LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA).

2.4. Blood collection and separation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were obtained from consenting healthy donors in accordance with an Institutional Review Board-approved research donor protocol. PBMCs were isolated by standard density gradient centrifugation with Ficoll–Hypaque, harvested from the interface, washed, and re-suspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA). Isolated cell populations were >98% purity (24). Blood from *Chlorocebus aethiops* (African Green Monkeys, "AGM"), *Macaca fascicularis* (Cynomolgus macaque, "Cyno"), and *Macaca mulatta* (Rhesus macaque, "Rhesus") were drawn according to the Institutes approved animal protocol, and the NHP PBMCs were purified similar to human samples by Ficoll–Hypaque density gradient centrifugation.

2.5. Cytokine analysis

Cell cultures were incubated for 16 h at 37 °C with 5% CO_2. Cytokines in culture supernatants were measured by a Meso Scale Discovery (MSD) multi spot array ultrasensitive cytokine assay kit (according to the manufacturer's protocol) as described [24].

2.6. Secreted alkaline phosphatase (SEAP) assay

TLR4 ligand (LPS) - induced cell based SEAP reporter assay was utilized to characterize the MyD88-mediated downstream immune signaling using a previously reported MyD88 inhibitor [25]. HEK 293 cells TLR4/MD-2/NF-kB/SEAPorter (5×10^5 cells/ml/well) were cultured with LPS (1 μg/ml) with varying concentrations of EM-163 in a 96-well plate and incubated at 37 °C for 16 h. The culture