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Short communication

# Neutrophils are the predominant cell-type to associate with *Burkholderia pseudomallei* in a BALB/c mouse model of respiratory melioidosis☆

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ABSTRACT

A variety of studies have implicated neutrophils and the rapid induction of cytokine in the host response in melioidosis. Here a BALB/c mouse model of infection with aerosolised *Burkholderia pseudomallei* K96243 has been used to understand the immune response to infection in this model and verify other infection models that show rapid growth of bacteria, colonisation of tissues and periphery, induction of cytokines and influx of neutrophils. Uniquely, this study has also determined the association of *B. pseudomallei* to host cells *in vivo* using flow cytometric techniques. Neutrophils were found to be the predominant cell-type exhibiting *B. pseudomallei* antigens during infection and it is likely that bacteria have been internalised. This data confirms that neutrophils are likely to play an important and active role in fighting infection with *B. pseudomallei*.



## 1. Introduction

*Burkholderia pseudomallei* causes a variety of infections collectively termed as melioidosis, which is endemic to parts of South Asia and Northern Australasia [1,2].

The lack of suitable antibiotics for treatment of infection, it's ease of culture and it's virulence by the aerosol route make *B. pseudomallei* a potential bio-threat agent [3].

The experimental animal model that is predominantly used for understanding melioidosis is the mouse. Susceptibility to *B. pseudomallei* is strain-dependant for both bacteria and the host [4]; moreover, death occurs more rapidly when the mice are infected by the respiratory route [5,6]. Studies indicate that the BALB/c mouse is more susceptible to *B. pseudomallei* infection than the C57BL/6 mouse [5]. Using these models, previous work has begun to dissect the immunological factors that are important in host defence. Interferon-γ signalling is essential in limiting the effects of the disease and antibody ablation or gene deletion studies have rendered C57BL/6 mice substantially more susceptible to *B. pseudomallei* when compared to control antibody-treated or wild-type mice [7,8]. This interferon-γ is produced in a functionally redundant fashion by NK and $CD8^+$ T-cells (i.e. production in both cells needs to be ablated for biological effects) [9]. The importance of interferon-γ is further typified when macrophages were incubated with this cytokine and exhibited a capacity to kill *B. pseudomallei* [10]. Furthermore, activated neutrophils are recruited to infected sites [11,12]. Additionally, neutrophils are believed to play a key role in host defence against *B. pseudomallei* infection since antibody ablation causes mice to rapidly succumb to infection [11]. In these ablation experiments the GR-1 antibody was used. This antibody can also affect other cells important in infection [13]. It is therefore important to further verify the importance of neutrophils during infection.

Given the vital role that neutrophils play in melioidosis, further exploration of the function of professional phagocytes in *B. pseudomallei* infection is warranted. In this study, we use a BALB/c mouse model of acute infection with aerosolised *B. pseudomallei* to confirm which phagocytes associate with the invading bacteria.

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## 2. Materials and methods

### 2.1. Bacterial culture

*B. pseudomallei* strain K96243 was cultured in Luria broth at 37 °C, overnight on a rotary shaker. The suspension was then adjusted using phosphate buffered saline (PBS) until the optical density at 600 nm was 0.32–0.35, where the estimated bacterial density would be $10^8$ CFU/ml. Bacterial numbers were determined by enumeration on agar plates following serial dilution (1:10) of samples.

### 2.2. Animals

Six to eight week old female BALB/c mice (Charles River) were transferred to a high containment Class III rigid isolator, where they were given unlimited access to food and water. Mice were challenged with *B. pseudomallei* by aerosol as previously described, using a Henderson-type apparatus [14] and a Collison nebuliser [15]. Survival times were recorded for some mice and others were culled for analysis of tissues at different time points. Where mice were culled, they were exsanguinated using cardiac puncture following terminal anaesthesia. Blood was placed into 1.5 ml heparin tubes. Lungs, spleen and liver were removed and placed into bijoux tubes filled with 2 ml of PBS. Duplicate experiments were performed. Mice received a calculated retained challenge dose of 99 CFU in the first experiment and 460 CFU in the second experiment. Culls were performed at 0 (pre-challenge), 2, 10 and 24 h in the first experiment and at 0 (pre-challenge), 3, 10, 24 and 36 h in the second experiment. All procedures and housing were in accordance with the Animal (Scientific Procedures) Act (1986).

### 2.3. Sample preparation

Organs were processed at less than 1 h post-mortem. Blood was diluted 1:10 in PBS. All organs collected (lungs, liver and spleen) were placed into 6-well trays containing 40 μm cell sieves with 1800 μl of PBS. Organs were disrupted through the cell sieve using the plunger of a 2 ml syringe and cell suspensions were collected. Subsequently, 100 μl aliquots of the cell suspension or blood were used for enumeration of bacteria on agar plates following serial dilution in PBS. For flow cytometry, 200 μl aliquots of cell suspension or blood were spun down in a micro-centrifuge for 5 min at 700 rpm. Supernatants were removed for cytokine analysis and stored in flat bottom 96-well trays at −80 °C. The supernatants were subsequently investigated using Cytometric Bead Array flex sets (Becton Dickinson™), performed according to manufacturer's instructions, with the additional step of fixing the samples in 4% paraformaldehyde in PBS for at least 48 h at 4 °C. Cells were resuspended in 100 μl red cell lysis buffer (Sigma™) and incubated at room temperature for 5 min. Red cell lysis buffer was quenched using 800 μl of PBS and micro-centrifuged at 700 rpm for 5 min. Supernatants were aspirated and resuspended in 100 μl of blocking, permeablising solution (5% foetal calf serum, 10 mg/ml bovine serum albumin, 10 mM EDTA, 0.2% Saponin; filter sterilised, then 20 μl/ml Fc block [Becton Dickinson™, anti CD16/32] added) and incubated for 20–30 min at room temperature. Flow cytometry antibodies were added and incubated for 15–20 min at room temperature. Cells were washed with 800 μl PBS and micro-centrifuged at 700 rpm for 5 min, then supernatants were removed and resuspended in 4% paraformaldehyde in PBS. Samples were subsequently stored for at least 48 h at 4 °C, for the paraformaldehyde to render the *B. pseudomallei* inactive.

### 2.4. Flow cytometry

All flow cytometry was performed using a 6 colour FACSCanto II (Becton Dickinson™) and the analysis/acquisition program FACSDiva (Becton Dickinson™). The standard sample stain for professional phagocytes comprised of 2 μl of the following antibodies, in mixture: FITC-labelled α-*B. pseudomallei* (4VIH12 (×20 FITC coupling ratio) at 0.01 mg/ml, produced at Dstl), α-Ly6G-PE (Becton Dickinson™ [clone 1A8]), α-CD11b-PerCP-cy5.5 (Becton Dickinson™ [clone M1/70]), α-CD11c-PE-cy7 (Becton Dickinson™ [clone HL3]), α-F4/80-APC (Insight™ [clone CI:A3-1]) and α-CD45-APC-cy7 (Becton Dickinson™ [clone 30-F11]). The selection criteria for leukocytes comprised the following: (1) Cell-sized events selected on forward vs. sidescatter. (2) Doublet reduction was performed using forward scatter height vs. time. (3) Leukocytes were selected on the basis of CD45 expression on a sidescatter vs. CD45 plot. (4) Neutrophils ($Ly6G^+$, $CD11b^+$) and non-neutrophils were selected on the basis of Ly6G and CD11b expression. (5) From non-neutrophils, macrophages ($F4/80^+$ $sidescatter^{high}$) and non-macrophages were selected on a sidescatter vs. F4/80 plot. (6) Non-macrophages were split into dendrocytes ($CD11c^+$), monocytes ($CD11b^+$, $CD11c^-$) and "others" ($CD11b^-$, $CD11c^-$) on a CD11b vs. CD11c plot. The macrophage population was further characterised using a CD11c vs. CD11b plot. These cell selection criteria are described in previous work [16].

Actual cell counts were calculated by using the TRUcount system (Becton Dickinson™) where samples are placed in tubes containing a known number of beads. The proportion of the beads that were taken up when running the samples allowed the back calculation of the number of cells per organ. The cytometric bead arrays were run to determine cytokine concentration on the flow cytometer as described in manufacturers' instructions.

### 2.5. Statistical analysis

All graphs and all statistical analyses were generated using Graphpad PRISM V4.0. Data from cytometric bead arrays were analysed using PRISM, by fitting a quadratic regression to the standard curves and reading the samples as unknowns.

## 3. Results

A pathogenesis study was performed in BALB/c mice following infection with aerosolised *B. pseudomallei* strain K96243 (summarised in Fig. 1). Briefly, animals were culled at time points post infection, organs extracted, mashed and analysed. The bacteria were found to grow exponentially, colonising the spleen at detectable levels at between 2 h and 10 h post-challenge, and with the animals becoming measurably bacteraemic at between 10 h and 24 h. Furthermore, by using flow cytometry to measure cell populations in different tissues, *B. pseudomallei* infection was found to generate a rapid, significant influx of neutrophils ($Ly6G^+$, $CD11b^+$ cells) into lungs ($P < 0.001$, 2 way ANOVA), spleen ($P < 0.01$, 2 way ANOVA) and blood ($P < 0.001$, 2 way ANOVA), occurring at between 10 h and 24 h post infection. $CD11b^+$ $F4/80^-$ cell numbers increased in the lungs ($P < 0.01$, 2 way ANOVA) and blood ($P < 0.05$, 2 way ANOVA) but not in the spleen ($P > 0.05$, 2 way ANOVA). Numbers of $F4/80^+$ macrophages and $CD11c^+$ dendrocytes were also measured, but there was no observed difference in their numbers (data not shown). A rapid and significant (all $P < 0.05$, 1 way ANOVA) induction of the cytokines interleukin-6 (IL-6), monocyte chemo-attractant protein-1 (MCP-1), interferon-γ (IFN-γ) and tumour necrosis factor (TNF) in the lungs, spleen and blood was also detected.