Low-Dose Exposure of C57BL/6 Mice to *Burkholderia pseudomallei* Mimics Chronic Human Melioidosis

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***Burkholderia pseudomallei* is the etiological agent of human melioidosis, a disease with a broad spectrum of clinical manifestations ranging from fatal septicemia to chronic localized infection or asymptomatic latent infection. Most clinical and immunological studies to date have focused on the acute disease process; however, little is known about pathology and immune response in chronic melioidosis. Here, we have developed a murine model of chronic disease by challenging C57BL/6 mice intranasally with a low dose of *B. pseudomallei* and monitoring them up to 100 days postinfection. Bacterial burdens were heterogeneous in different animals at all time points, consistent with the spectrum of clinical severity observed in humans. Proinflammatory cytokines such as gamma interferon (IFN- γ), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor- α (TNF- α) were induced during chronic infection, and histopathological analysis showed features in common with human melioidosis. Interestingly, many of these features were similar to those induced by *Mycobacterium tuberculosis* in humans, such as development of a collagen cord that encapsulates the lesions, the presence of multinucleated giant cells, and granulomas with a caseous necrotic center, which may explain why chronic melioidosis is often misdiagnosed as tuberculosis. Our model now provides a relevant and practical tool to define the immunological features of**

chronic melioidosis and aid in the development of more effective treatment of this disease in humans. (Am J Pathol 2011, 179:270–280; DOI: 10.1016/j.ajpath.2011.03.031)

Burkholderia pseudomallei is a Gram-negative soil bacterium that is the causative agent of melioidosis. This pathogen is endemic in Southeast Asia and Northern Australia, but cases of melioidosis are now being reported in numerous other tropical areas, suggesting a more global distribution of the organism in the environment.^{1,2} The main routes of infection are believed to be via inhalation of aerosols during the rainy season or via cutaneous inoculation in people who have direct contact with wet soil.³ The consequences of infection with *B. pseudomallei* are remarkably diverse, ranging from an acute septicemia to a chronic localized disease or an asymptomatic latent infection. Acute infection is characterized by bacteremia, progression to septic shock with the induction of a massive but ineffective cytokine response, and a high mortality rate.^{3–5} Chronic infection is generally less severe and often characterized by persistent, localized infection that is difficult to eradicate.^{3,6} Recurrent disease is also common, and it is mostly due to relapse rather than reinfection.⁷ The disease can also reactivate after decades of clinical latency,⁸ suggesting that the bacteria may enter a dormant state where it can avoid immune surveillance.⁹ Despite the clear impact of this infection on public health in endemic regions, we do not understand how *B. pseudomallei* evades the immune response and causes a chronic infection. Chronic melioidosis is frequently misdiagnosed as tuberculosis, and the two diseases share several histological features,^{2,10} suggesting the possibility of common immunological mechanisms between these two infections.

Animal models represent powerful tools to study immunity and virulence factors, and both outbred and inbred strains have been previously described as models

Supported by National Institute of Health grant U01AI061363.

Accepted for publication March 29, 2011.

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to study human melioidosis.^{10–15} Typically, BALB/c mice, which are very susceptible, have been used to study acute melioidosis.^{14,16,17} In contrast, C57BL/6 mice show increased resistance to *B. pseudomallei* infection but are unable to clear infection, so they are better candidates to mimic chronic infection in humans.^{11,14} The mechanisms that regulate the development of either acute, chronic, or latent melioidosis remain unclear. However, it has been suggested that differential inflammatory responses in BALB/c mice versus C57BL/6 after *B. pseudomallei* infection may be responsible for different susceptibility to infection, suggesting that high levels of inflammatory cytokines in BALB/c may contribute to the immunopathogenesis.¹⁸ Other animals such as hamsters, guinea pigs, and rats have also been used,^{10,19} but there is little information on truly chronic models where disease progression is monitored over several months post-challenge.^{10,20}

No licensed vaccine exists for either prophylactic or therapeutic use against *B. pseudomallei*.^{21,22} Its high rate of infectivity via the aerosol route and its resistance to many common antibiotics makes this pathogen a potential candidate for bioterrorism.^{3,22,23} Different strategies have been used to develop an effective vaccine, including immunization with heat-killed bacteria,²⁴ with live attenuated mutants,^{25,26} or with immunogenic antigens such as lipopolysaccharide (LPS) or outer membrane proteins.^{27–31} Although most have shown some protection, none have reliably achieved sterilizing immunity, instead converting an otherwise overwhelming infection into a chronic disease that is eventually lethal. Thus, persistence in the face of immune or antibiotic pressure is a key feature of melioidosis, but the mechanisms underlying these events are not known.

To study the biology of chronic melioidosis, we infected C57BL/6 mice intranasally with a low dose of *B. pseudomallei* 576 and investigated the histological and inflammatory responses to infection over an extended time course. Our aim was to develop a murine model of chronic disease that reflects chronic disease in humans. In the longer term, this model would be invaluable for evaluating pretreatments and therapeutics that might be used to treat or eliminate chronic *B. pseudomallei* infections.

Materials and Methods

Bacteria Strain

B. pseudomallei strain 576 was originally isolated from a patient with a fatal case of human melioidosis in Thailand, and was kindly provided by Dr. Tyrone Pitt (Health Protection Agency, London, UK). Frozen stocks were prepared as previously described.³² All procedures involving live bacteria were performed under ACDP (Advisory Committee on Dangerous Pathogens) containment level 3 conditions.

Infection of Mice and Determination of Tissue and Blood Bacterial Load

Female C57BL/6 mice (6- to 8-week-old; Harlan Laboratories, Bicester, Oxon, UK) were used throughout the studies. All animal experiments were performed in accordance with the guidelines of the Animals (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee at the London School of Hygiene and Tropical Medicine.

A total number of 241 animals were used (191 chronically infected, 10 acutely infected, and 40 uninfected controls), distributed in 11 independent experiments. The number of animals used per experiment and the number of experiments are shown in the figure legends. For survival curves, a total of 76 mice were used. Bacterial load and cytokine production were analyzed at different time points postinfection (p.i.; days 22, 35, 45, and 50) involving 33 and 38 animals, respectively. Some samples used to analyze bacterial burdens were also assayed for cytokines. Histopathology and IHC studies were performed in a total of 79 animals, of which 48 were chronically infected.

For each infection, aliquots were thawed from frozen bacteria stocks and diluted in pyrogen-free saline (PFS). Before intranasal (i.n.) infection, mice were anesthetized intraperitoneally with ketamine (50 mg/kg; Ketaset; Fort Dodge Animal Health, Southampton, UK) and xylazine (10 mg/kg; Rompun; Bayer, Leverkusen, Germany) diluted in PFS. Challenge was performed by administering i.n. a total volume of 50 μ L containing 2500 colony-forming units (CFU; acute model) or 100 CFU (chronic model) of *B. pseudomallei* 576. Infection dose was confirmed as described elsewhere.³² Control uninfected mice received 50 μ L of PFS.

At different times p.i., mice were sacrificed, lungs and spleens aseptically removed in cold PBS, and blood collected in 100 μ L of heparin. Organs were homogenized through a 100- μ m cell strainer (BD Biosciences, Oxford, UK), serial 10-fold dilutions of blood and tissue in PBS homogenates were plated onto tryptone soy agar plates (Sigma, Dorset, UK), and bacterial colonies were counted after incubation for 24 to 48 hours at 37°C. The limits of detection were 50 CFU/organ or 20 CFU/mL of blood.

Measurement of Spleen and Serum Cytokine and Chemokine Production

Spleens were harvested in cold PBS containing Complete Mini Protease Inhibitor Cocktail (Roche, Sussex, UK) and homogenized through a cell strainer. Cells were lysed with 0.1% Triton X-100 (Sigma) before centrifugation at 500 $\times$ g for 10 minutes at 4°C to pellet cell debris. The homogenates were then assayed for presence of gamma interferon (IFN- γ), IL-6, IL-10, tumor necrosis factor- α (TNF- α), and monocyte chemotactic protein-1 (MCP-1) using Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Bioscience, Oxford, UK) following the manufacturer's instructions. Sera were collected after

centrifugation of blood at $500 \times g$ for 10 minutes at 4°C and IFN- γ , IL-6, IL-10, TNF- α , and MCP-1 measured using the same CBA Mouse Inflammation Kit as described above.

Histopathological Studies

Lung, spleen, and liver samples were collected in neutral sodium salt-buffered formalin (Prolabo, Briare, France) at day 3 (p.i.), when animals received an acute challenge (2500 CFU *B. pseudomallei*) or at days 20 to 60 p.i., when mice were infected with 100 CFU (chronic challenge). Samples were fixed for 1 month, embedded in paraffin wax, and 3- μm sections were cut, dewaxed, and rehydrated through xylene and alcohols and then washed in running tapwater for 10 minutes. Hematoxylin and eosin (H&E) and Sirius Red histochemical techniques were then performed. Microscopic tissue sections were analyzed by a veterinary pathologist.

Immunohistochemical Studies

For immunohistochemical detection of *B. pseudomallei*, a murine monoclonal antibody (3VIE5) that reacts with capsular polysaccharide of *B. pseudomallei* was used (1/2000 dilution). Deparaffinized sections had a purified water rinse, and 20 $\mu\text{mol/L}$ proteinase K (Novocastra Proteinase K, Newcastle, UK) was applied for 5 minutes at room temperature. The slides were then washed in Tris-buffered saline for 5 minutes and then endogenous peroxidase block was applied for 15 minutes (3% hydrogen peroxide in methanol). Sections were washed in running tapwater for 10 minutes and then clipped into Shandon Sequenza clips (Thermo Fisher Scientific, Loughborough, UK) and washed in Tris-buffered saline for 5 minutes. The Vector M.O.M. Immunodetection kit (Vector, Peterborough, UK) was used. Sections were counterstained with Mayer's hematoxylin and mounted with DPX (BDH, Poole, UK). A mouse IgG2b-negative control (Serotec, Kidlington, UK) was used as the isotype control. Samples were imaged with a Zeiss axioplan 2 microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with a Retiga 2000R Fast CCD camera (QImaging, Marlow, UK) using Volocity software from Improvision. Images were acquired at $\times 400$, $\times 200$, $\times 100$, or $\times 50$.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). To compare cytokine and chemokine responses among groups, Kruskal-Wallis test was used followed by Dunn's post test. Data were considered statistically significant when $P < 0.05$. The degree of significance was denoted as follows: * $P < 0.05$; ** $P < 0.01$.

Figure 1. Survival and bacterial burdens after intranasal infection with *Burkholderia pseudomallei*. C57BL/6 mice were intranasally infected with 100 colony-forming units (CFU) of *B. pseudomallei* strain 576. **A:** Mice were monitored for survival ($n = 30$). **B:** Bacterial loads in the lung, spleen, and blood at days 22, 35 ($n = 5$), and 45 ($n = 3$) postinfection (p.i.). Each symbol represents the number of CFU of the respective organ from an individual animal. The horizontal line represents the median. Survival data are representative of four independent experiments, and CFU data are representative of three independent experiments.

Results

Intranasal Challenge with a Low Dose of *B. pseudomallei* Induces Chronic Infection in Mice That Is Characterized by Differences in Bacterial Load

To develop a murine model of chronic melioidosis, C57BL/6 mice ($n = 30$) were i.n. challenged with a low dose (100 CFU) of *B. pseudomallei* 576, and monitored for survival (Figure 1A). A chronic infection was established in which mice started to die from day 10 p.i. onwards, with a median survival time of 58 days. However, 20% of mice still exhibited no clinical signs of illness 3 months post-challenge when the experiment was terminated. Analysis of lung bacterial loads at day 1 p.i. showed that all animals had detectable bacteria from the initial challenge (data not shown). Analysis of bacterial burdens in lung, spleen, and blood revealed differences in bacterial counts later in the study period between different animals and between organs of the same animal (Figure 1B and Table 1). In many infected animals, bacteria could not be detected after plating tissue samples onto agar from day 20 onwards. It is noteworthy that 35% of animals with detectable *B. pseudomallei* in lung and/or spleen, were not bacteremic, showing that although the pathogen is

Table 1. Bacterial Loads and Macroscopic Pathology of Chronically Infected C57BL/6 Mice

Time p.i. (day)	Lung CFU	Blood CFU	Spleen CFU	Splenomegaly	Visible lesion
22	—	—	—	Yes	No
22	—	—	—	No	No
22	8.5×10^5	6.8×10^4	1.3×10^7	Yes	Lung and Spleen
22	450	610	50	No	No
22	750	129	9.7×10^6	Yes	Spleen
22	—	—	1.2×10^8	Yes	Spleen
22	—	—	—	Slightly	No
22	1.8×10^6	72	2.9×10^8	Yes	Lung and Spleen
22	—	—	1.4×10^8	Yes	Spleen
22	3.4×10^4	950	1.9×10^5	Yes	Lung and Spleen
35	—	—	—	No	No
35	—	—	—	No	No
35	—	—	1.4×10^6	Yes	Spleen
35	—	—	—	No	No
35	7.2×10^6	56	1.1×10^8	Yes	Lung and Spleen
45	7800	2688	4.4×10^4	Yes	Spleen
45	100	—	50	No	No
45	2350	333	8.3×10^7	Yes	Spleen
50	—	—	2×10^8	Yes	Spleen
50	8300	—	4.7×10^8	Yes	Spleen
50	—	512	2.4×10^8	Yes	Spleen
50	—	—	2.8×10^5	Slightly	Spleen
50	—	1632	5.4×10^6	Slightly	Spleen

C57BL/6 mice were i.n. infected with 100 CFU *B. pseudomallei*. At different time points p.i., organs and blood were harvested, bacterial burdens analyzed, and macroscopic lesions recorded.

CFU, colony-forming units; —, not detected.

likely to be spread via the hematogenous route, focal growth of the organism can occur under physical containment. Surprisingly, although mice were challenged via the i.n. route, 41% of the infected mice in which bacteria could be detected in the spleen did not have culturable organisms in the lung from day 20 onwards.

Chronic Infection Induces the Expression of Proinflammatory Cytokines in Spleens with Lesions and Sera from Bacteremic Mice

To evaluate cytokine and chemokine responses in chronic infection, serum samples or whole-spleen homogenates were prepared on days 22, 35, or 45 p.i. and assayed *ex vivo* for IFN- γ , TNF- α , IL-6, MCP-1, and IL-10. Samples were allocated to three groups: uninfected mice, challenged mice without macroscopically visible lesions/splenomegaly or bacteremia, or challenged mice with visible lesions/splenomegaly or bacteremia. Levels of all of the inflammatory cytokines studied (IFN- γ , TNF- α , IL-6, MCP-1) were increased in spleens of challenged mice that had splenomegaly and lesions compared to challenged mice without splenomegaly and lesions, or uninfected mice ($P < 0.05$ and $P < 0.01$, respectively) (Figure 2, A to D). Increased IFN- γ and IL-6 production in the spleen directly correlated with bacterial burden in this organ ($P < 0.05$; $r = 0.89$ and $r = 0.94$, respectively; data not shown). No difference was detected in the production of IL-10 among groups (Figure 2E). Robust proinflammatory cytokine responses were also observed in sera in the presence of bacteremia but not in either uninfected mice or those infected without bacteremia (Figure 3).

Histopathological Analysis of Acute versus Chronic B. pseudomallei-Infected Tissues

Lung, spleen, and liver are the most common organs affected in human melioidosis.⁶ To analyze the histopathological features of chronic infection, mice were infected intranasally with 100 CFU of *B. pseudomallei*, organs harvested at day 20 to 60 p.i., and pathology compared to either uninfected mice or mice undergoing an acute septic infection 3 days after a high-dose challenge (2500 CFU).

Previous flow cytometry studies have demonstrated that activated neutrophils are rapidly recruited to the lungs after a high-dose pulmonary challenge with *B. pseudomallei*.^{15,33} Consistent with this, lungs from mice acutely infected showed pulmonary inflammation characterized by multifocal suppurative pneumonia with many neutrophils, both degenerated and viable, and a few lymphocytes and macrophages (Figure 4, B and E). Cell debris was present in the bronchial lumen and perivascular edema and occasional hemorrhages were also observed. In contrast, chronically infected mice developed solid lung lesions that were composed of infiltrating lymphocytes and epithelioid macrophages (Figure 4, C and F). In addition, multinucleated giant cells (MNGC) were detected in some lung lesions (Figure 4F).

We observed four main types of lesions in the lungs of chronically infected mice (Figure 5): Type I, characterized by discrete foci of macrophages and lymphocytes, usually close to blood vessels and the bronchial tree; Type II, round lesions with areas of granulomatous pneumonia with similar distribution to those in Type I, but containing epithelioid macrophages; Type III, large, solid

Figure 2. Effect of chronic *B. pseudomallei* infection on cytokine and chemokine responses in the spleen. At different time points following infection, cytokine protein concentrations from whole-spleen homogenates were measured by cytometric bead array (CBA) analysis. Samples from infected mice were allocated to two groups depending on the presence or absence of macroscopically visible lesions in the organ ($n = 9$ uninfected, $n = 13$ infected). **A:** TNF- α . **B:** IFN- γ . **C:** IL-6. **D:** MCP-1. **E:** IL-10. Each symbol represents an individual animal. The horizontal line represents the mean. * $P < 0.05$, ** $P < 0.01$. Data are representative of two independent experiments.

nonnecrotic granulomas consisting mainly of epithelioid cells and lymphocytes, although a few scattered MNGC were also observed; and Type IV, large granulomas characterized by a caseous necrotic center. Epithelioid macrophages were the main population surrounding the necrotic core in Type IV; however, neutrophils and some lymphocytes and plasma cells were also observed. A distinct fibroblast-rich layer was detected delimiting the necrotic core from nonnecrotic tissue. Thus, both necrotic and solid granulomas develop in the lungs of chronically infected mice although the latter are the most common.

The histology of acutely infected spleens obtained 72 hours p.i. consisted of small inflammatory foci with a central area of necrosis. General lymphoid architecture remained intact, with multifocal necrotic splenitis containing degenerated neutrophils (Figure 4, H and K). By

contrast, massive destruction of normal lymphoid structure was observed in the spleens of chronically infected animals (Figure 4, I and L). Splenomegaly and multifocal to coalescent pyogranulomatous splenitis containing a necrotic center were common although nonnecrotic microgranulomas composed of epithelioid macrophages were also observed (data not shown).

The acute inflammatory lesions in the liver were defined by necrotic areas containing degenerated neutrophils and macrophages (Figure 4, N and Q). Hepatic lesions detected in chronically infected mice were mainly large pyogranulomas with a necrotic center that were surrounded by macrophages, lymphocytes, and plasma cells (Figure 4, O and R). However, small pyogranulomas composed mainly of degenerated, but viable, neutrophils, and macrophages were also common. Inflammation

Figure 3. Effect of chronic *B. pseudomallei* infection on cytokine and chemokine response in the blood. At different time points following infection, cytokine protein concentrations from sera were measured by CBA. Samples ($n = 9$ uninfected, $n = 13$ infected) from infected mice were divided into 2 groups according to the presence or absence of bacteria in the blood. **A:** TNF- α . **B:** IFN- γ . **C:** IL-6. **D:** MCP-1. **E:** IL-10. Each symbol represents an individual animal. The horizontal line represents the mean. ** $P < 0.01$. Data are representative of two independent experiments.

Figure 4. Histopathological changes observed in lung, spleen, and liver of mice infected i.n. with *B. pseudomallei*. C57BL/6 mice were infected with 2500 CFU (acute model, organs collected day 3 p.i., **B, E, H, K, N, and Q**) or 100 CFU (chronic model, organs collected day 20 to 60 p.i., **C, F, I, L, O, and R**). Control mice received PFS (**A, D, G, J, M, and P**). **B** and **E**: Lungs collected at day 3 p.i. showed multifocal suppurative pneumonia, with areas of inflammatory infiltrates (ii and **arrows**) which consisted mainly of neutrophils and a few macrophages and lymphocytes, and necrotic areas (n) containing mainly degenerated and viable neutrophils. **C** and **F**: Lung lesions from chronically infected mice are characterized by nonnecrotic solid lesions, composed of epithelioid macrophages and lymphocytes. **F**: MNGC are observed in lung lesions (**arrows** and **insets**). **H** and **K**: Spleens collected at day 3 p.i. showed multifocal necrotic (n) areas (**arrows**) with degenerated neutrophils. **I** and **L**: Spleens from chronically infected mice showed multifocal to coalescent pyogranulomatous splenitis. **N** and **Q**: Livers collected at day 3 p.i. showed multifocal necrotic (n) areas with degenerated neutrophils. **O** and **R**: Chronic lesions in the liver are characterized by necrotic centers (**arrows**) containing mainly degenerated but also viable neutrophils; necrotic foci are surrounded by macrophages, lymphocytes, and plasma cells. Sections were stained with H&E. Scale bars: 140 μ m (**A, B, C, G, H, and I**); 70 μ m (**M, N, and O**); 36 μ m (**J, K, and L**); 18 μ m (**D, E, F, P, Q, and R**). MNGC, multinucleated giant cells; PFS, pyrogen-free saline; p.i., postinfection. Data are representative of three independent experiments ($n = 5$ mice, acute model; $n = 8$ to 28 mice/experiment, chronic model).

Figure 5. - Representative images of the four types of pulmonary lesions after *B. pseudomallei* infection. **A:** Type I is characterized by a discrete focus of macrophages and lymphocytes. **B:** Type II, area of focal pneumonia composed of numerous lymphocytes and epithelioid macrophages. **C:** Type III, solid nonnecrotic granuloma consists mainly of epithelioid macrophages and lymphocytes; MNGC are observed in this stage. **D:** Type IV, large granuloma with necrotic center surrounded by epithelioid macrophages, neutrophils, and some lymphocytes and plasma cells. **Arrows** point to epithelioid macrophages that are shown at higher magnification in **insets**. Sections were stained with H&E. Scale bar = 140 μ m. Data are representative of three independent experiments ($n = 8$ to 28 mice/experiment).

tory infiltrates surrounding the blood vessels in the liver were found in both acute and chronic infection (data not shown). In general, liver chronic lesions were very similar to those present in the spleen.

Although mice were challenged intranasally, only 17 of 45 (38%) chronically infected mice evaluated for histology showed lung lesions, whereas 41 of 44 (93%) mice had spleen lesions and 100% had liver lesions. Chronic infection induces splenomegaly, massive destruction of spleen tissue, and high bacterial loads in this organ, suggesting that the spleen, and to a lesser extent the liver, are the most permissive organs for bacterial growth in this chronic model. Furthermore, 17 of 47 (36%) animals had lost weight at the time of collecting the organs, which is a sign of systemic illness.

Chronic Infection with *B. pseudomallei* Induces a Fibrotic Response

The development of a fibrotic capsule containing the foci of bacterial replication is a common feature in other chronic bacterial infections.^{34–36} To see whether *B. pseudomallei* infection also induced this response, lung, liver, and spleen sections from acute or chronically infected mice were stained with Sirius Red to detect the deposition of collagen. Despite extensive tissue pathology and high bacterial burdens, no collagen was detected at day 3 p.i. in any of the organs studied when mice received an acute challenge (data not shown). However, collagen was readily detected in chronically infected mice, forming irregular cords of variable thickness surrounding numerous lesions. This fibrotic capsule was observed in necrotic lung granulomas surrounding the necrotic core but was not present in solid nonnecrotic lesions (Figure 6,

A, D, G, and J). In spleen and liver lesions, a fibrotic structure was also observed surrounding well-defined isolated lesions that contained a necrotic center (Figure 6, B, C, E, and F); however, most coalescent and fused pyogranulomas were not encapsulated (Figure 6, H, I, K, and L).

Most chronic lesions in the spleen and liver were characterized by a necrotic, highly acidophilic center, typically surrounded by a fibrotic structure composed of collagen (Figure 7). Fibroblasts were the main cell population found in this physical barrier that separated the necrotic tissue from nonnecrotic tissue where neutrophils and macrophages were the main population recruited. Epithelioid macrophages were also common within the fibroblast barrier. Finally, lymphocytes and plasma cells were found in abundance in the periphery of the lesions surrounding neutrophils and macrophages.

Location of *B. pseudomallei* in Chronic Infection

The number and size of tissue lesions in chronically infected mice were directly related to the bacterial loads in these tissues. To determine the location of *B. pseudomallei* within these lesions, lung, liver, and spleen sections were initially stained with Gram Twort, but no bacteria were detected (data not shown). However, immunohistochemical staining using the monoclonal antibody 3VIE5, which recognizes an exopolysaccharide antigen from *B. pseudomallei*, revealed large amounts of bacterial antigen in lung, liver, and spleen lesions. This material was located mainly within the cytoplasm of macrophages in lung lesions (Figure 8, D and G). It was also observed within dead and viable macrophages surrounding the areas of necrosis in liver and spleen pyogranulomas, within the fibrotic rim in those lesions that are encapsulated, although it was also observed in some recruited macrophages outside of, but close to, the lesions (Figure 8, E, F, H, and I). No antigen was detected in either uninfected controls, healthy tissue in any of the infected organs, or when compared to sections stained with the isotype control (Figure 8, A, B, and C).

Discussion

In humans and experimental animals, sterilizing immunity against *B. pseudomallei* is difficult to achieve, and persistence of bacteria either subclinically or as a chronic localized infection is common. However, the mechanisms that underlie persistence and the immune responses that develop during chronic melioidosis are not understood. To address these issues, we have developed a model of chronic experimental melioidosis by infecting C57BL/6 mice via the i.n. route to mimic pulmonary exposure in endemic regions. By combining a low challenge dose and a genetically resistant mouse strain, we were able to generate a chronic infection that persisted for over 3 months.

A key feature of this model was the heterogeneity of disease progression within the infected cohorts. At each

Figure 6. Effect of chronic *B. pseudomallei* infection on collagen deposition in lung, spleen, and liver. C57BL/6 mice were infected with 100 CFU of *B. pseudomallei* and organs collected day 20 to 60 p.i. **A** and **D**: Lung granulomas containing necrotic center were surrounded by a rim of collagen. **G** and **J**: Solid nonnecrotic lung lesions were not encapsulated by a fibrotic structure. **B** and **E**: Numerous spleen and liver (**C** and **F**) necrotic lesions were contained in a collagen matrix. However, most coalescent/fused lesions, both in the spleen (**H** and **K**) and the liver (**I** and **L**), were not surrounded by a rim of collagen. Sections were stained with H&E (**A**, **B**, **C**, **G**, **H**, and **I**) or Sirius Red (**D**, **E**, **F**, **J**, **K**, and **L**). Scale bars: 140 μ m (**A**, **B**, **D**, **E**, **H**, **I**, **K**, and **L**); 70 μ m (**C** and **F**); 36 μ m (**G** and **J**). Results shown are representative of three independent experiments ($n = 8$ to 28 mice/experiment).

evaluated time point, some animals had progressed to chronic disease, characterized by weight loss and demonstrable bacterial burdens in the blood, liver, lung, or spleen, whereas others showed no signs of illness and no bacteria could be detected in target organs. We believe the latter reflects a latent infection with persistence of low numbers of bacteria that eventually can reactivate. A variable pattern of disease progression has also been observed in immunized mice after challenge with virulent *B. pseudomallei*.^{25,26} Interestingly, although mice are much more susceptible to infection via the airways than

to intraperitoneal challenge,^{13,37} we found the spleen to be a more permissive site for bacterial replication than the lung in this model. The portal of entry and the *B. pseudomallei* strain used are important factors that may influence the cellular response, pathology, and onset of disease in experimental models.^{13–15,18,33} However, chronic infections have also been observed following intraperitoneal or subcutaneous challenge and with other *B. pseudomallei* strains, suggesting that development of chronic infection is an inherent feature of this pathogen

Figure 7. Structure of spleen lesions of mice chronically infected with *B. pseudomallei*. C57BL/6 mice were infected with 100 CFU of *B. pseudomallei* and spleens collected day 20 to 60 p.i. **A**: Spleen section shown represents a granulomatous reaction with necrotic center (n). **B** and **C**: A collagen barrier (co) delimits necrotic and nonnecrotic tissue. **D** and **E**: Necrotic center (1) is usually encapsulated by a fibrotic capsule (2) that is predominantly surrounded by neutrophils and macrophages (3). Lymphocytes (4) are the main population surrounding the lesions. Epithelioid macrophages are pointed in **A** and **B** with small arrows. Sections were stained with H&E (**A**, **B**, **D**, and **E**) or Sirius Red (**C**). Scale bars: 70 μ m (**A**); 18 μ m (**B**, **C**, **D**, and **E**). Co, collagen; n, necrotic center. Results are representative of three independent experiments ($n = 8$ to 20 mice/experiment).

Figure 8. Location of *B. pseudomallei* in tissues from chronically infected mice. C57BL/6 mice were infected with 100 CFU of *B. pseudomallei* and organs collected at day 20 to 60 p.i. A strong immunoreactivity indicating the presence of the exopolysaccharide antigen from *B. pseudomallei* (brown color) was observed in lung lesions within the cytoplasm of macrophages (**D** and **G**), and in liver (**E** and **H**) and spleen (**F** and **I**) pyogranulomas, mainly within macrophages (arrows; enlarged in insets **A**, **B** and **C**, isotype control. Scale bars: 140 μ m (**A**, **B**, **C**, **D**, **E**, and **F**); 18 μ m (**G**, **H**, and **I**). Data are representative of three different experiments ($n = 8$ to 15 mice/experiment).

(A. Easton, G.J. Bancroft, unpublished data using *B. pseudomallei* 708A and K96243).^{10,12}

Mice that show active disease at the time of harvest (ie, splenomegaly and pyogranulomas and bacteremia) produced proinflammatory cytokines, whereas infected but asymptomatic mice did not. This included IFN- γ , TNF- α , IL-6, and MCP-1, showing similarities to previous reports in acute infections of mice^{17,18} and humans.^{4,5} Previous data from our own and other laboratories have demonstrated the essential role of IFN- γ and TNF- α for the control of systemic *B. pseudomallei* infection,^{12,32,38} suggesting that the production of proinflammatory cytokines is a mechanism induced by the host to try to control the infection. Here, bacterial burdens in the spleen directly correlated with TNF- α levels in the sera ($P < 0.05$; data not shown). In addition, IFN- γ , TNF- α , and IL-6 were increased in sera from mice that were not bacteremic but had spleen lesions, compared to sera from uninfected mice ($P < 0.05$; data not shown). This indicates that detection of peripheral blood cytokines can reflect the presence of chronic tissue lesions even in the absence of bacteremia.

Although the cytokine responses of acutely (data not shown) and chronically infected mice were broadly similar, their histopathological features were clearly distinct. In humans, *B. pseudomallei* causes pyogenic or granulomatous inflammation at virtually any site, but lung, spleen, and liver are the main organs affected.³⁹ A combination of both acute necrotizing pneumonia and acute respiratory distress syndrome seem to be responsible for respiratory failure in melioidosis patients.³⁹ Here, intranasal challenge of C57BL/6 mice with a high dose of bacteria led to multifocal suppurative pneumonia with many neutrophils but also with macrophages and lymphocytes, similar to that observed in BALB/c mice infected via the aerosol route.¹³ No splenomegaly was observed when

mice were acutely infected, and macroscopic lesions could not be detected in either the liver or the spleen. However, microscopic necrotizing multifocal areas containing mainly neutrophils and macrophages were common both in liver and spleen, consistent with previous reports.^{13,14}

In contrast, chronic lung pathology was mainly characterized by solid nonnecrotic lesions containing infiltrating lymphocytes and epithelioid macrophages. MNGC, which are present in autopsy samples from humans who have died from melioidosis and *in vitro* following infection of macrophages with *B. pseudomallei*, were also observed in some lung lesions.^{40,41} MNGC have not been described in previous animal models of melioidosis, although Dannenberg et al reported the presence of large phagocytic cells containing vacuoles with dark staining phagocytized nuclear debris.⁴² Whether MNGC formation is induced by the pathogen to evade the immune system or whether it is a protective host response remains unclear.⁴³ Although not common, we also observed large lung granulomas containing a caseous necrotic center. These resemble the characteristic histological lesions of human tuberculosis and have also been described in human chronic melioidosis.^{44–46} The different types of lung lesions seen in our chronic melioidosis model may reflect progressive stages of granuloma development. Alternatively, as it is proposed for *M. tuberculosis*, initial exposure to the pathogen may induce a primary lesion characterized by an inflammatory focus with central necrosis. Subsequent dissemination from this site in the presence of an ongoing adaptive immune response may then generate secondary lesions composed of macrophages, epithelioid cells, and lymphocytes in the absence of necrosis.⁴⁷

Splenomegaly and massive destruction of lymphoid architecture were also common features of chronically infected animals.⁴² Spleen and liver lesions displayed

similar patterns, with multifocal to coalescent pyogranulomatous splenitis or hepatitis containing necrotic areas composed of degenerated and viable macrophages and neutrophils. Both macrophages and neutrophils play a role in early resistance against *B. pseudomallei* infection,^{15,33,48} although their role in the control of chronic disease is not known. These cells are also recruited to the spleen and liver in a systemic model of *B. pseudomallei* infection.¹⁴ Histological analysis of tissue from patients with melioidosis has demonstrated the presence of mainly neutrophils, but also macrophages at infected sites.^{45,49} Nevertheless, our data suggest that despite their recruitment to the site of infection, neutrophils are not sufficient to control the infection.

The presence of epithelioid macrophages, MNGC, and fibrosis in mice chronically infected with *B. pseudomallei* shows parallels to more established models of granuloma formation in other infections such as tuberculosis and schistosomiasis.^{50,51} Here, the organized deposition of collagen that surrounded many of the chronic lesions colocalized with areas of fibroblast recruitment and accurately delimited healthy tissue from areas of necrosis. Not all of the lesions were encapsulated, and these may represent sites of rapid bacterial dissemination that would result in overwhelming infection. Although limiting spread of the bacteria, the fibrotic response may also prevent access of new inflammatory cells, allowing the bacteria to replicate and persist, and potentially impairing the penetration of antibiotics.⁵² Intense immunostaining by a monoclonal antibody specific for the exopolysaccharide antigen of *B. pseudomallei* was observed within chronic necrotic lesions in all tissues, consistent with sustained bacterial growth at these sites. However, when macroscopic lesions were observed, they correlated with a high bacterial load in that organ, suggesting that viable bacteria are confined in granulomas in the lung and pyogranulomas in spleen and liver, and that these lesions provide a suitable niche for the pathogen to persist.⁵³

In conclusion, we have developed a murine model of infection with *B. pseudomallei* that shares features observed in chronic human melioidosis. The histological characteristics of this model are distinct from that observed in acute melioidosis with development of granulomas, caseous necrosis, multinucleated giant cells, and fibrosis that also resemble the pathology of tuberculosis. Van Schaik et al recently reported a novel model of chronic *B. pseudomallei* infection in rats using agar beads that also resulted in granulomatous pathology.¹⁰ Our murine model provides the additional advantages of allowing use of the wide range of immunological reagents available in this species, does not require the use of agar beads, and allows investigation of chronic infection over more extended periods of time. Testing the paradigms of bacterial persistence and granuloma biology established in tuberculosis for their validity in these models should provide important new insights into the biology of melioidosis.

Acknowledgments

We thank Robert Gilbert and all of the members of the London School of Hygiene and Tropical Medicine Biological Services Facility for animal husbandry and Dr. Tyrone Pitt (Health Protection Agency, London, UK) for the provision of *Burkholderia pseudomallei* 576.

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