A Critical Role for Neutrophils in Resistance to Experimental Infection with *Burkholderia pseudomallei*

Anna Easton,1 Ashraful Haque,1 Karen Chu,1 Roman Lukaszewski,2 and Gregory J. Bancroft1

1London School of Hygiene and Tropical Medicine, London, and 2Defence, Science, and Technology Laboratories, Porton Down, Salisbury, United Kingdom

Inhalation is an important route of infection with *Burkholderia pseudomallei*, the causative agent of melioidosis. In resistant C57BL/6 mice, activated neutrophils are rapidly recruited to the lungs after intranasal *B. pseudomallei* infection. Prevention of this response by use of the anti–Gr-1+ cell–depleting monoclonal antibody RB6-8C5 severely exacerbated disease, resulting in an acute lethal infection associated with a 1000-fold increase in lung bacterial loads within 4 days. C57BL/6 interferon (IFN)–γ−/− mice were also acutely susceptible to pulmonary *B. pseudomallei* infection, dying within 3 days of challenge; this suggests that IFN-γ is essential for control in the lungs and precedes the protective role of neutrophils in resistance. In neutrophil-depleted mice, lung concentrations of tumor necrosis factor (TNF)–α, IFN–γ, and interleukin-6 were decreased by up to 98%. Natural killer cells were the principle source of IFN-γ, and monocytes were the principle source of TNF-α, suggesting that neutrophils play an important indirect role in the generation of the early cytokine environment in the lungs.

The gram-negative bacterium *Burkholderia pseudomallei* is the causative agent of melioidosis, a major cause of morbidity and mortality in areas of endemicity in Southeast Asia and tropical Australia [1, 2]. *B. pseudomallei* exists in the soil and water, and infection is thought to occur through inhalation or subcutaneous inoculation of contaminated particles [1, 2]. Melioidosis has a broad spectrum of disease presentation, ranging from fatal septicemia to chronic abscess formation or asymptomatic latent infection. Reactivation of latent infection can also lead to acute disease up to 62 years after the initial exposure [3]. Although virtually any organ can be affected, abscess formation in the liver and spleen [4, 5] and pulmonary involvement are most common, with the latter being associated with the highest mortality rates [5–7]. This bacterium is listed as a category B potential bioterrorism agent by the US Centers for Disease Control and Prevention. At present, there is no vaccine for melioidosis, and antibiotic treatment is not fully effective, with frequent relapses despite long courses of drug therapy [1, 2].

Innate immune mechanisms are critical in determining the outcome of infection with many bacterial pathogens. This may also be the case in melioidosis, given that, in humans, disease can progress rapidly in cases of high exposure [1] and that, in mice, differences in susceptibility between mouse strains are evident within 24 h of infection [8–11]. We have previously demonstrated that rapid production of interferon (IFN)–γ is essential for resistance after intraperitoneal *B. pseudomallei* infection in mice [12, 13]. Macrophages activated by IFN-γ have been shown to kill *B. pseudomallei*, which can otherwise survive and replicate inside...
innate cellular response to B. pseudomallei of neutrophils in resistance to B. pseudomallei cytokines and chemokines [23, 24]. To date, the contribution activity [22] and through the production of immunoregulatory other pulmonary pathogenic bacteria [17–21]. Neutrophils play response and are important for protection against a variety of endemic and chronic renal failure [5, 28], are associated with im-

neutrophils play a critical early role in resistance, both by direct antimicrobial activity [22] and through the production of immunoregulatory cytokines and chemokines [23, 24]. To date, the contribution of neutrophils in resistance to B. pseudomallei infection has not been directly addressed, but several indirect lines of evidence suggest that they may play an important role. Studies in humans and mice have identified neutrophils and neutrophil-recruiting chemokines at sites of B. pseudomallei infection, particularly in association with abscess formation [4, 25–27]. Also, conditions that predispose individuals to melioidosis, such as diabetes mellitus and chronic renal failure [5, 28], are associated with impaired neutrophil function [29].

Here, we used a murine model of pulmonary B. pseudomallei infection, mimicking the natural route of exposure in areas of endemicity and in a potential biodefense scenario, to investigate the role played by neutrophils in host resistance. We show that activated neutrophils are rapidly recruited to the lungs after infection and that depletion of these cells exacerbated disease, resulting in an acute, lethal outcome. This increased susceptibility was associated with a decrease in the early proinflammatory cytokine responses in the lungs.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice (7–10 weeks of age; Charles River) were used. Age- and sex-matched C57BL/6 IFN-γ−/− mice were bred at the London School of Hygiene and Tropical Medicine (LSHTM). All animal experiments were performed under animal biohazard containment level 3 conditions, in accordance with the guidelines of the Animals (Scientific Procedures) Act of 1986 and the LSHTM Ethical Review Committee.

Bacteria, infection of mice, and in vivo treatments. B. pseudomallei strain 576, isolated from a patient with a fatal case of human melioidosis in Thailand, was obtained from T. Pitt (Health Protection Agency, London). Frozen stocks were prepared as described elsewhere [13]. For each infection, aliquots of B. pseudomallei were diluted in pyrogen-free saline (PFS). For intranasal infection, mice were anesthetized intraperitoneally with ketamine (50 mg/kg; Ketaset; Fort Dodge Animal Health) and xylazine (10 mg/kg; Rompum; Bayer) diluted in PFS, and the inoculum was delivered in a total volume of 50 μL. For in vivo depletion, 200 μg of anti–Gr-1 antibody, RB6-8C5 (hereafter, “RB6”), or IgG2b isotype control monoclonal antibody (MAB), Mac-5, was administered intraperitoneally 1 day before infection.

Determination of bacterial loads in tissues of infected mice. Mice were killed, and organs were aseptically removed in PBS. Tissues were homogenized through a cell strainer, and serial 10-fold dilutions were plated onto tryptophane soy agar (Oxoid). Colonies were enumerated after overnight incubation at 37°C; the limit of detection was 100 bacteria/organ.

Preparation of single cell suspensions from tissue for flow cytometry. Lungs were chopped to a fine slurry and were digested with 0.4 mg/mL Liberase Cl (Roche) and 10 μg/mL DNase ( Worthington Biochemicals) in RPMI 1640 (Gibco) for 30 min at 37°C. Digestion was stopped by the addition of R10 (RPMI 1640 and 10% fetal calf serum [FCS]; Sigma), 10 mmol/L L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin (Gibco), and 50 μmol/L 2-mercaptoethanol (Gibco), and a single cell suspension was obtained by passing tissue through a cell strainer. Red cell lysis buffer (Sigma) was added for 2–3 min, and cells were resuspended in R10.

Samples for intracellular cytokine staining were incubated with brefeldin A (10 μg/mL; Sigma) for 3 h at 37°C in 5% CO₂. Cells were washed in fluorescence-activated cell sorter buffer (PBS, 1% FCS, and 0.1% NaN₃), and nonspecific binding was blocked by use of anti-CD16/CD32 MAb (2.4G2; 1 μg/mL; BD Biosciences). For surface staining, samples were incubated with the following directly conjugated MAbss (from BD Biosciences, unless otherwise stated): phycoerythrin (PE)–anti–Gr-1 (RB6-8C5; 1 μg/mL); allophycocyanin (APC)–anti–Gr-1 (RB6-8C5; 1 μg/mL; Caltag Laboratories); fluorescein isothiocyanate (FITC)–anti-neutrophil (7/4; 0.5 μg/mL, Caltag Laboratories); Tri-Colour–anti-CD8 (CT-CD8a; 2 μg/mL, Caltag Laboratories); PE–anti-CD11c (HL3; 2 μg/mL; Alexis); and FITC–anti-CD3ε (145-2C11; 5 μg/mL). For intracellular cytokine staining, cells were permeabilized with 0.1% saponin and incubated with PE– or APC–anti–IFN-γ (XM1G1.2; 0.05 μg/mL) or with PE–anti–tumor necrosis factor (TNF)–α (MP6-XT22; 0.05 μg/mL). Cells were washed twice and fixed overnight in 2% paraformaldehyde. Acquisitions were performed on a BD Biosciences FACSCalibur device, with >100,000 events per sample, and were analyzed using FlowJo software (version 8.1.1; Tree Star) after gating on total live cells or lymphocytes by forward- and side-scatter values.

Measurement of reactive oxygen intermediate (ROI) production in neutrophils. Lung cell suspensions (1 × 10⁶ cells/100 μL) were stimulated in vitro with PMA or zymosan (both from Sigma) for 20 min at 37°C, and then 50 μL of 100 μmol/L dihydroorhodamine-123 (Calbiochem) was added for 10 min [30]. Cells were washed twice in PBS, and neutrophils were identified by flow cytometry with APC–anti–Gr-1.

Measurement of lung cytokine and chemokine responses. Lungs were harvested into PBS containing Complete Mini pro-
tease inhibitor cocktail (Roche) and homogenized. Cells were lysed with 0.1% Triton X-100 (Sigma) before centrifugation to pellet cell debris. Samples were assayed for IFN-γ, interleukin (IL)–12, TNF-α, IL-6, IL-10, and monocyte chemotactic protein (MCP)–1 by use of CBA Mouse Inflammation Kits (BD Biosciences), in accordance with manufacturer’s instructions.

**Statistical analysis.** Kaplan–Meier survival curves were compared by log rank analysis, and all other data were analyzed using Student’s unpaired t test. $P<.05$ was considered to be significant.

**RESULTS**

**Recruitment of activated neutrophils to the lungs after intranasal *Burkholderia pseudomallei* infection.** C57BL/6 mice (n = 5) were challenged intranasally with either saline or $1 \times 10^5$ cfu of *B. pseudomallei* strain 576, and the percentage of neutrophils in the lungs was determined by flow cytometry before infection (day 0) and at days 1, 3, and 8 after infection (A) as well as at day 2 after infection (B). Neutrophils were identified as Gr-1(high)/4(high). Data are mean ± SE values and are representative of 6 independent experiments of similar design. ***$P<.001$.

**Figure 1.** Kinetics of neutrophil recruitment to the lungs after intranasal *Burkholderia pseudomallei* infection. C57BL/6 mice (n = 5) were challenged intranasally with either saline or $1 \times 10^5$ cfu of *B. pseudomallei* strain 576, and the percentage of neutrophils in the lungs was determined by flow cytometry before infection (day 0) and at days 1, 3, and 8 after infection (A) as well as at day 2 after infection (B). Neutrophils were identified as Gr-1(high)/4(high). Data are mean ± SE values and are representative of 6 independent experiments of similar design. ***$P<.001$.

Neutrophils and *B. pseudomallei* Infection • JID 2007:195 (1 January) • 101

sions obtained from either the saline-treated or the *B. pseudomallei*-infected mice were stimulated in vitro, and neutrophil-derived ROIs were detected by flow cytometry. Neutrophils from the infected mice produced a significantly greater ROI burst on stimulation with a range of concentrations of PMA (0.03–1 μmol/L; figure 2B) or with zymosan (data not shown), compared with those from the uninfected control mice. Thus, pulmonary exposure to *B. pseudomallei* rapidly elicits the recruitment of activated neutrophils.

**Exacerbation of pulmonary *B. pseudomallei* infection following Gr-1+ cell depletion.** The contribution of this neutrophil response to resistance against *B. pseudomallei* infection was investigated by use of the cell-depleting MAb RB6, which is specific for the neutrophil surface marker Gr-1. Preliminary experiments showed that treatment with 200 μg of RB6 depleted >95% of neutrophils for at least 4 days, as verified by flow cytometry of lung and spleen homogenates (data not shown). C57BL/6 mice were treated with 200 μg of RB6 or isotype control 1 day before intranasal infection with *B. pseudomallei* and were monitored for survival. Depletion of Gr-1+ cells significantly increased susceptibility to infection, with an MST of 6.5 days in the RB6–treated mice, compared with 49.5 days in the control-treated mice ($P<.05$ (figure 3). The effect of RB6 treatment was dose dependent, with MSTs of 6, 8.5, and 35 days at RB6 doses of 100, 50, and 10 μg, respectively (data not shown).

Given we have previously shown that IFN-γ is critical for early control of intraperitoneal *B. pseudomallei* infection [12,
31–33]. Because RB6 treatment may also deplete these populations, the potential contribution of these cells to resistance was assessed after pulmonary B. pseudomallei infection. In addition to the neutrophil influx, there was a 1.5- and 4.8-fold increase in Gr-1+ monocytes at day 2 ($P<.05$; data not shown) and day 3 ($P<.005$) after infection, respectively (figure 5C). However, there was no change in the frequency of Gr-1+ CD8+ T cells and pDC populations, compared with that in the uninfected control mice (figure 5C). After RB6 treatment, lung neutrophils, monocytes, and Gr-1+ CD8+ T cells were depleted by 99%, 85%, and 83%, respectively, at the time of infection, without depletion of pDCs (figure 5B). Monocyte numbers recovered to control levels by day 1 after infection and were unaffected by RB6 treatment at day 2 (data not shown) and day 3 after infection (figure 5C). Neutrophils and Gr-1+ CD8+ T cells remained depleted for the first 3 days, with neutrophils depleted by 98%, 98%, and 97% and with CD8+ Gr-1+ T cells depleted by 91%, 74%, and 78% at days 1, 2, and 3 after infection, respectively (data summarized from 3 independent experiments; representative data from day 3 are shown in figure 5C). However, the mice treated with the depleting anti-CD8 MAb did not die of infection until day 10 onward, with an MST of 14 days—significantly later than the RB6-treated mice (MST of 5.5 days; $P<.005$; data not shown), compared with the 45 days in the isotype-treated mice. From this we conclude that neutrophils are the key Gr-1+ cell type mediating resistance to B. pseudomallei infection.

Identification of neutrophils as the critical Gr-1+ cell type mediating resistance to B. pseudomallei infection. In addition to the high level of expression on neutrophils, Gr-1 is also expressed at an intermediate level on a subset of monocytes (Gr-1$m^+/4^+$), a subset of Gr-1+ CD8+ T cells, and plasmacytoid dendritic cells (pDCs; Gr-1$^+$ CD11c$^+$ 120.G8+) (figure 5A) [31–33]. Because RB6 treatment may also deplete these populations, the potential contribution of these cells to resistance was assessed after pulmonary B. pseudomallei infection. In addition to the neutrophil influx, there was a 1.5- and 4.8-fold increase in Gr-1+ monocytes at day 2 ($P<.05$; data not shown) and day 3 ($P<.005$) after infection, respectively (figure 5C). However, there was no change in the frequency of Gr-1+ CD8+ T cells and pDC populations, compared with that in the uninfected control mice (figure 5C). After RB6 treatment, lung neutrophils, monocytes, and Gr-1+ CD8+ T cells were depleted by 99%, 85%, and 83%, respectively, at the time of infection, without depletion of pDCs (figure 5B). Monocyte numbers recovered to control levels by day 1 after infection and were unaffected by RB6 treatment at day 2 (data not shown) and day 3 after infection (figure 5C). Neutrophils and Gr-1+ CD8+ T cells remained depleted for the first 3 days, with neutrophils depleted by 98%, 98%, and 97% and with CD8+ Gr-1+ T cells depleted by 91%, 74%, and 78% at days 1, 2, and 3 after infection, respectively (data summarized from 3 independent experiments; representative data from day 3 are shown in figure 5C). However, the mice treated with the depleting anti-CD8 MAb did not die of infection until day 10 onward, with an MST of 14 days—significantly later than the RB6-treated mice (MST of 5.5 days; $P<.005$; data not shown), compared with the 45 days in the isotype-treated mice. From this we conclude that neutrophils are the key Gr-1+ cell type mediating resistance to B. pseudomallei infection.

**Figure 3.** Effect of Gr-1+ cell depletion vs. the deletion of interferon (IFN)$-\gamma$ on resistance to intranasal Burkholderia pseudomallei infection. C57BL/6 IFN$-\gamma^{-/-}$ mice or C57BL/6 mice treated with the monoclonal antibody (MAb) RB6-8C5 (RB6) or isotype control MAb (n=6) were infected intranasally with $1 \times 10^6$ cfu of B. pseudomallei strain 576 and were monitored for survival. The RB6 depletion data are representative of 5 independent experiments, and the IFN$-\gamma^{-/-}$ data are representative of 2 independent experiments. *$P<.05$; **$P<.005$.

In addition to contaminated aerosols, cutaneous inoculation is another likely route of exposure to B. pseudomallei in areas of endemicity [1, 2]. RB6 treatment also exacerbated subcutaneous B. pseudomallei infection, with 5 of 6 RB6-treated mice dying within 5 days, compared with 5 of 6 control mice surviving for >50 days ($P<.001$; data not shown). This was associated with increased bacterial loads in the liver and spleen, with up to 1,000,000-fold more bacteria in the livers of the RB6-treated mice than in the control mice.

Identification of neutrophils as the critical Gr-1+ cell type mediating resistance to B. pseudomallei infection. In addition to the high level of expression on neutrophils, Gr-1 is also expressed at an intermediate level on a subset of monocytes (Gr-1$m^+/4^+$), a subset of Gr-1+ CD8+ T cells, and plasmacytoid dendritic cells (pDCs; Gr-1$^+$ CD11c$^+$ 120.G8+) (figure 5A) [31–33]. Because RB6 treatment may also deplete these populations, the potential contribution of these cells to resistance was assessed after pulmonary B. pseudomallei infection. In addition to the neutrophil influx, there was a 1.5- and 4.8-fold increase in Gr-1+ monocytes at day 2 ($P<.05$; data not shown) and day 3 ($P<.005$) after infection, respectively (figure 5C). However, there was no change in the frequency of Gr-1+ CD8+ T cells and pDC populations, compared with that in the uninfected control mice (figure 5C). After RB6 treatment, lung neutrophils, monocytes, and Gr-1+ CD8+ T cells were depleted by 99%, 85%, and 83%, respectively, at the time of infection, without depletion of pDCs (figure 5B). Monocyte numbers recovered to control levels by day 1 after infection and were unaffected by RB6 treatment at day 2 (data not shown) and day 3 after infection (figure 5C). Neutrophils and Gr-1+ CD8+ T cells remained depleted for the first 3 days, with neutrophils depleted by 98%, 98%, and 97% and with CD8+ Gr-1+ T cells depleted by 91%, 74%, and 78% at days 1, 2, and 3 after infection, respectively (data summarized from 3 independent experiments; representative data from day 3 are shown in figure 5C). However, the mice treated with the depleting anti-CD8 MAb did not die of infection until day 10 onward, with an MST of 14 days—significantly later than the RB6-treated mice (MST of 5.5 days; $P<.005$; data not shown), compared with the 45 days in the isotype-treated mice. From this we conclude that neutrophils are the key Gr-1+ cell type mediating resistance to B. pseudomallei infection.

**Figure 4.** Effect of Gr-1+ cell depletion versus the deletion of interferon (IFN)$-\gamma$ on bacterial loads after infection with Burkholderia pseudomallei. C57BL/6 IFN$-\gamma^{-/-}$ mice or C57BL/6 mice treated intraperitoneally with the monoclonal antibody (MAb) RB6-8C5 (RB6) or isotype control MAb were infected intranasally with $1 \times 10^6$ cfu of B. pseudomallei strain 576. A, Bacterial loads in the lungs (n=3) over the first 4 days of infection. B, Bacterial loads in the lungs (n=5) at day 2 after infection. Data are mean ± SE values from 2 independent experiments. *$P<.05$; NS, not significant.
Neutrophils and *B. pseudomallei* Infection

**Figure 5.** Effect of RB6-8C5 (RB6) treatment on Gr-1⁺ cell populations in the lungs before and after intranasal *Burkholderia pseudomallei* infection.

A, Gating definitions of Gr-1⁺ cell populations in the lungs identified by flow cytometry in uninfected mice are shown as follows: (i) neutrophils (Gr-1⁺7/4⁺high); (ii) Gr-1⁺CD8⁺; (iii) plasmacytoid dendritic cell (pDCs; CD11c⁺120G8⁺); and (iv) pDC Gr-1 expression (gated on R1). B and C, Gr-1⁺ cell populations before and after infection. C57BL/6 mice (♀ = 5) were treated with the monoclonal antibody (MAb) RB6 or isotype control MAb, and lungs were harvested before intranasal infection (B) or at day 3 after infection (C) with 1 × 10⁵ cfu of *B. pseudomallei* strain 576. Gr-1⁺ cells from whole lung preparations were identified by flow cytometry using the markers described above. Data are mean ± SE values and are representative of 5 independent experiments of similar design. *; **; ***; NS, not significant.

**Change in the cytokine profile of the lungs induced by Gr-1⁺ cell depletion after pulmonary *B. pseudomallei* infection.**

To assess the effect of neutrophil depletion on pulmonary cytokine and chemokine responses after *B. pseudomallei* infection, whole lung homogenates from RB6- or isotype-treated mice were assayed for IL-12, IFN-γ, TNF-α, IL-6, IL-10, and MCP-1. Two days after infection, there were large increases in the concentrations of TNF-α, IL-6, and, to a lesser extent, IFN-γ in the lungs, whereas these cytokines were undetectable in the uninfected mice (figure 6). These responses were transient, with a ~60% decrease in the levels of all 3 cytokines by day 3 after infection (data not shown). The levels of MCP-1 increased 11-fold by day 2 and remained elevated at day 3 after infection (figure 6 and data not shown). IL-12 and IL-10 were undetectable at these times. RB6 treatment brought about dramatic changes in the pulmonary cytokine response at day 2 after infection, with a 98%, 88%, and 91% reduction in levels of TNF-α, IL-6, and IFN-γ, respectively, compared with those in the isotype-treated control mice (figure 6). MCP-1 levels were not affected by RB6 treatment.

To further characterize the nature of the pulmonary IFN-γ and TNF-α responses, the cellular sources of these cytokines were identified in the lungs after *B. pseudomallei* challenge by flow cytometry. IFN-γ was produced by NK cells (NK1.1⁺; ~80% of total IFN-γ) and T cells (CD3⁺) but not neutrophils (figure 7A), whereas TNF-α was produced by monocytes (Gr-1⁺7/4ⁿ⁴; ~90% of total TNF-α) and neutrophils (Gr-1ⁿ⁴⁴/⁴ⁿ⁷; figure 7B).

**DISCUSSION**

*B. pseudomallei* is an environmental saprophyte, and human infection primarily occurs through inhalation or cutaneous inoculation of contaminated particles [1, 2]. Here, we used murine models of intranasal and subcutaneous infection, which mimic these natural routes of exposure, to investigate the innate immune responses critical for resistance. Genetically resistant C57BL/6 mice survived for >2 weeks after intranasal challenge with 1 × 10⁵ cfu *B. pseudomallei*, suggesting that the pulmonary innate immune response can control infection and prevent the development of acute disease. Control of bacterial growth in the lungs correlated with the recruitment of large numbers of activated neutrophils and monocytes. Similar findings have been observed in a systemic model of *B. pseudomallei* infection with neutrophils and mononuclear cells recruited to the spleen and liver [26], whereas histological analysis of tissues from patients with melioidosis has shown predominantly neutrophilic lesions at infected sites [4, 25]. Neutrophil recruitment
B. pseudomallei with cfu of (MAb) RB6-8C5 (RB6) or isotype control MAb and infected intranasally metric bead array analysis. Concentrations from whole lung homogenates were measured by cytometry. C57BL/6 mice ( ) were treated with the monoclonal antibody Burkholderia pseudomallei responses in the lungs after intranasal infection. RB6 treatment prior to infection resulted in an acute, lethal outcome within a week of challenge and was associated with loss of control of bacterial replication at the primary site of infection after dissemination to distal organs such as the liver and spleen. However, a potential complication of using the RB6 MAb is that Gr-1 is also expressed at an intermediate level on other cells [31–33], of which both monocytes and Gr-1’CD8’ T cells were depleted from the lungs after MAb treatment. Previous studies have suggested that the involvement of other Gr-1’ cells can be excluded by adjusting the dose and timing of the RB6 treatment to restrict depletion to neutrophils [36]. However, in our hands this was not the case—lower RB6 doses (100 or 50 μg) removed appreciable numbers of Gr-1’m populations, and, although a selective neutrophil depletion was achieved with 10 μg of RB6, it was too transient to cover the period of neutrophil recruitment (data not shown). However, despite initial depletion, monocyte recruitment was unaffected by treatment with 200 μg of RB6, and total CD8’ cell depletion studies had no impact on the early stages of pulmonary infection, as has been shown previously in systemic infection [13]. Therefore, we believe that the increased susceptibility to infection after RB6 treatment is most likely due to the loss of neutrophils and not to the effects of other Gr-1’ cells. Taken together, these data demonstrate that neutrophils are an essential component of the protective immune response to B. pseudomallei at multiple sites of infection, independent of the route of exposure.

We have previously shown that IFN-γ is essential for control of systemic B. pseudomallei infection [12, 13], and here we demonstrate that IFN-γ is also critical for resistance to pulmonary B. pseudomallei infection. IFN-γ−/− mice were acutely susceptible to intranasal challenge, with increased lung bacterial loads as early as day 2 after infection—which correlated with the kinetics of pulmonary IFN-γ expression observed in this study and reported by others [11]. In the lungs, IFN-γ was produced mainly by NK cells with a small contribution by T cells, similar to our previous findings in the spleen after systemic infection [13]. IFN-γ−/− mice were more susceptible to infection than neutrophil-depleted mice, suggesting that production of IFN-γ is the first critical immune response to B. pseudomallei infection, preceding the protective effect of neutrophils.

Neutrophils are potent effectors of the innate immune response and in other infections contribute to protection through their direct antimicrobial capacity and the production of cytokines and chemokines that instruct the recruitment and activation of other immune cells [35, 37–39]. Here, we show that TNF-α, IFN-γ, IL-6, and MCP-1 are induced in the lungs after pulmonary B. pseudomallei infection, extending the findings of previous mRNA studies [10, 11], and that RB6 treatment dramatically decreases levels of TNF-α, IL-6, IFN-γ by as much as 98%. This indicates that neutrophils play a critical role in the generation of the early proinflammatory cytokine responses in the lungs. We have previously demonstrated that TNF-α neutralization renders mice more susceptible to systemic [12] and pulmonary B. pseudomallei infection (data not shown). Studies using the closely related bacterium B. mallei have also demonstrated a critical role for TNF-α in resistance (R.L., un-
Figure 7. Cellular sources of interferon (IFN)–γ and tumor necrosis factor (TNF)–α in the lungs after intranasal *Burkholderia pseudomallei* infection. C57BL/6 mice (*n* = 3) were challenged intranasally with either saline or *B. pseudomallei* strain 576, and cytokines detected in pooled whole lung homogenates by flow cytometry at day 2 after infection. **A**, IFN–γ production by total cells, NK cells (NK1.1+), and T cells (CD3+) after challenge with 1 × 10⁵ cfu. Data are representative of 2 independent experiments. **B**, TNF–α production by total cells, monocytes (Gr-1int7/4high), and neutrophils Gr-1²/21high7/4high after challenge with 1 × 10⁴ cfu. Isotype staining was included as control. Nos. represent the percentage of live cells. SSC-H, side-scatter height.
Neutrophils are known to produce TNF-α [40, 41], and decreases in TNF-α levels after RB6 treatment have been reported in other models of infection [38, 39]. However, here we have identified Gr-1+ monocytes as the predominant (>90%) source of TNF-α during infection, with only a small involvement of neutrophils. This reduction in TNF-α level cannot be explained by the transient depletion of monocytes by RB6 treatment and may suggest an indirect role for neutrophils in monocyte activation, as has been shown for DCs [42].

The pulmonary IFN-γ response, produced mainly by Gr-1+ NK cells, was also impaired by Gr-1+ cell depletion. The partial depletion of Gr-1+ CD8+ T cells is likely to have a minimal effect on the IFN-γ response, because this population comprises 5%–10% of lung T cells and would, therefore, contribute a maximum of 2% of the total IFN-γ. Neutrophil depletion did not affect the recruitment of NK cells (data not shown) [39, 43], and the mechanism by which neutrophils might instruct IFN-γ production is unclear at present. Neutrophils are known to produce the IFN-γ-inducing cytokine IL-12 in other infections [24], and, in models of Mycobacterium tuberculosis and L. pneumophilia infection, RB6 treatment also resulted in decreased IFN-γ production mediated by the loss of neutrophil-derived IL-12 [17, 37].

Although our data supports a cytokine-mediated immunomodulatory role for neutrophils in B. pseudomallei infection, it is also possible that they contribute to protection through direct bactericidal effector functions. Several reports have demonstrated that neutrophils are capable of killing B. pseudomallei [44–46], but others have shown that neutrophils can phagocytose B. pseudomallei and trigger a respiratory burst without significant bactericidal activity [47, 48]. Also, similar to B. cepacia, B. pseudomallei is resistant to purified human defensin, a potent neutrophil antimicrobial peptide [14, 49]. In vitro studies have shown that B. pseudomallei is sensitive to both ROS and reactive nitrogen intermediates [15], but their importance in vivo is not known.

In conclusion, by use of antibody-depletion studies, we have clearly demonstrated that resistance against B. pseudomallei infection via several clinically important routes of inoculation and in multiple organs is critically dependent on Gr-1+ inflammatory cells. In light of the extent and duration of the depletion, we believe that this is primarily a result of the depletion of neutrophils. We have shown that neutrophils are critical to the generation of the early proinflammatory cytokine environment in the lungs and have suggested that they play an important immunomodulatory role during infection. In humans, clinical susceptibility and the development of severe bacteremic disease are often associated with diabetes mellitus [5, 28], a condition in which neutrophil function is known to be impaired [29]. Neutrophil function in diabetic patients with melioidosis has not been examined, but, in other studies, neutrophils from patients with diabetes have been shown to be less responsive to chemotactic stimuli and to have a reduced capacity for phagocytosis and the generation of the oxidative burst [50]. These observations in humans and the findings from the experimental models presented here support the concept that neutrophil function is an important determinant of resistance to melioidosis.

Acknowledgments

We thank the members of the London School of Hygiene and Tropical Medicine Biological Services Facility, for animal husbandry, and Heidi Alderton and Dr. Debbie Smith, for supervision of work done at biohazard containment level 3.

References


106 • JID 2007:195 (1 January) • Easton et al.


42. Bennouna S, Denkers EY. Microbial antigen triggers rapid mobilization of TNF-alpha to the surface of mouse neutrophils transforming them into inducers of high-level dendritic cell TNF-alpha production. J Immunol 2005; 174:4845–51.


