Defect in early lung defence against *Pseudomonas aeruginosa* in DBA/2 mice is associated with acute inflammatory lung injury and reduced bactericidal activity in naïve macrophages

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*Pseudomonas aeruginosa* is an opportunistic pathogen that causes serious respiratory disease in the immune-compromised host. Using an aerosol infection model, 11 inbred mouse strains (129/Sv, A/J, BALB/c, C3H/HeN, C57BL/6, DBA/2, FVB, B10.D2/oSnJ, B10.D2/nSnJ, AKR/J and SWR/J) were tested for increased susceptibility to *P. aeruginosa* lung colonization. DBA/2 was the only mouse strain that had increased bacterial counts in the lung within 6 h post-infection. This deficiency incited a marked inflammatory response with reduced bacterial lung clearance and a mortality rate of 96.7 %. DBA/2 mice displayed progressive deterioration of lung pathology with extensive alveolar exudate and oedema formation at 48–72 h post-infection. The neutrophil-specific myeloperoxidase activity remained elevated throughout infection, suggesting that the increased leukocyte infiltration into alveoli caused acute inflammatory lung injury. DBA/2 mice lack the haemolytic complement; however, three additional mouse strains (AKR/J, SWR/J and A/J) with the same defect effectively cleared the infection, indicating that other host factors are involved in defence. Bone marrow-derived macrophages of DBA/2 showed an initial increase in phagocytosis, while their bactericidal activity was reduced compared to that of C57BL/6 macrophages. Comparison of pulmonary cytokine profiles of DBA/2 versus C57BL/6 or C3H/HeN indicated that DBA/2 had similar increases in tumour necrosis factor (TNF)-α, KC and interleukin (IL)-1α as C3H/HeN, but showed specific induction of IL-17, monocyte chemotactic protein (MCP)-1 and vascular endothelial growth factor (VEGF). Together, DBA/2 mice have a defect in the initial lung defence against *P. aeruginosa* colonization, which causes the host to produce a greater, but damaging, inflammatory response. Such a response may originate from the reduced antimicrobial activity of DBA/2 macrophages.

INTRODUCTION

Immunocompromised patients, including cancer and burn patients, those hospitalized in intensive care units, and those with human immunodeficiency virus (HIV) are at a higher risk for respiratory colonization and developing pneumonia with various bacterial pathogens. Respiratory diseases accounted for 11 % of patients hospitalized in the USA in 2003, with a 37 % diagnosis of pneumonia (DeFrances et al., 2005). A recent survey of nosocomial pneumonia identified *Pseudomonas aeruginosa* as the number one isolated bacterium, with the second-highest mortality rate (Lee et al., 2005). *P. aeruginosa* is a ubiquitous micro-organism in nature that rarely causes disease in healthy individuals. However, it is capable of targeting immune-compromised individuals, and is a predominant airway pathogen in bacterial pneumonia. It remains unclear how this pathogen establishes its initial colonization within its hosts. There are also host factors that play significant roles in susceptibility. Acute pneumonia often begins with the inhalation and/or aspiration of the bacteria-laden droplets produced from the upper respiratory tracts (Ramphal, 2001). Subsequent colonization of these micro-organisms deep into the lungs results in the clinical manifestations of lung disease.

To simulate a natural route for acquiring deep-lung infections in mice, we used a bacterial aerosol delivery
technology (Orme & Collins, 1994) for the deposition of airborne pathogens into the alveoli of the mouse lungs, causing acute airway colonization (Yu et al., 1998a, 2000; Yu & Head, 2002). To better understand the aetiology of bacterial lung infections in conjunction with host genetic factors, we examined 11 inbred mouse strains for the initial phase of lung colonization caused by P. aeruginosa aerosols. We report that an inbred mouse strain, DBA/2, is highly susceptible to bacterial airway colonization by P. aeruginosa compared to other inbred strains. Also, we characterize the infection-specific phenotype in DBA/2 mice in terms of lung histopathology, neutrophil recruitment as measured with a neutrophil-specific marker (myeloperoxidase, MPO), macrophage phagocytosis and killing, and cytokine production. The infection mouse model described here displays many symptoms similar to the acute inflammatory lung injury seen in humans. We conclude that the increased susceptibility in DBA/2 mice is associated with exaggerated neutrophilic inflammation and defects in the killing ability of naïve macrophages.

METHODS

Mice. All mice in this study were 6–8 weeks old at the inception of the experiment. They were purchased from Charles River Laboratories and The Jackson Laboratory. All mice used throughout this study were males, except for the lung colonization, which was determined with both DBA/2 males and females (Fig. 1). The mice were housed under the conventional facility within the Division of Animal Resources (DAR) at the Marshall University School of Medicine, which is Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited. The DAR routinely monitors for bacteriological and viral contamination, which were found negative throughout this study. All manipulations with live animals were performed in compliance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The following mouse strain abbreviations are used in this paper: DBA/2, D2; C57BL/6, B6; and C3H/HeN, C3.

Bacterial strains and lung infection model. The reference strains of P. aeruginosa PAO1 (Stover et al., 2000) and P. aeruginosa pMRP-1, a GFP-labelled isogenic strain of PAO1, were used throughout this study, unless stated otherwise. P. aeruginosa pMRP-1 contains the GFP expression vector pMRP9-1 (Davies et al., 1998) and constitutively expresses GFP. P. aeruginosa strains CF149 and 311058, both of clinical origin, were as previously described (Head & Yu, 2002). To better understand the aetiology of P. aeruginosa infections, we examined 11 inbred mouse strains for the initial phase of lung colonization caused by P. aeruginosa aerosols. We report that an inbred mouse strain, DBA/2, is highly susceptible to bacterial airway colonization by P. aeruginosa compared to other inbred strains. Also, we characterize the infection-specific phenotype in DBA/2 mice in terms of lung histopathology, neutrophil recruitment as measured with a neutrophil-specific marker (myeloperoxidase, MPO), macrophage phagocytosis and killing, and cytokine production. The infection mouse model described here displays many symptoms similar to the acute inflammatory lung injury seen in humans. We conclude that the increased susceptibility in DBA/2 mice is associated with exaggerated neutrophilic inflammation and defects in the killing ability of naïve macrophages.

The model was based on a bacterial aerosol-induced lung infection (Head & Yu, 2004; Yu et al., 1998b; Yu & Head, 2002) with the following modifications. To prepare the infection dose for nebulization, 125 ml Lennox broth (LB; BD Difco) culture for each bacterial strain was grown for 13–15 h with aeration at 37°C. The cells were pelleted by centrifugation at 5800 g for 15 min at 4°C and resuspended in 7–10 ml PBS, pH 7.4, in 1% proteose peptone (PPBS; BD Difco). The wet cell mass of the bacterial pellet was weighed, and viable counts of the suspension were determined to ensure that a uniform amount of bacteria was used for nebulization. Five millilitres of bacterial suspension was dispersed into the nebulizer-venturi unit of the Inhalation Exposure System (Glas-Col) and aerosolized for 30 min (compressed air control = 15–20, vacuum control = 50) followed by cloud decay for 25 min and UV light decontamination for 5 min. Five B6 mice were sacrificed immediately after exposure at time = 0 to determine the initial deposition dose in the lung. Each group of tested mice was terminated at time = 6, 12, 24, 48 or 72 h for the kinetics of lung clearance. Mice were terminated by carbon dioxide inhalation. Whole lungs were removed en bloc, and the right lung was homogenized in 1 ml PPBS using a motorized Ultra-Turrax T8 homogenizer (IKM-Werke). The lung homogenates were assayed for viable bacteria via 10-fold serial dilutions in PPBS with plate counts on LB agar. The viable bacterial counts recovered from the mouse lungs were normalized based on the lung mass. The remaining lung homogenate samples were stored at −80°C for cytokine and MPO activity analysis. The left lungs were used for histology.

Histopathology. Left lungs were inflated in situ with 2% paraformaldehyde in PBS, fixed at room temperature for at least 1 week before changing to a fresh paraformaldehyde solution, and stored at 4°C. The tissue was transferred to 10% neutral formalin and processed in a Tissue-Tek II tissue processor (Sakura Finetechical) for histology. After the sample was processed, it was embedded in paraffin, sectioned at 4 μm, and stained with haematoxylin and eosin (H&E). The tissue slides were examined under an Olympus BX51 System microscope attached to a DP70 microscope digital camera. The captured images were analysed with Image-Pro Plus software (version 5.1, Media Cybernetics) on a Dell Optiplex GX280 computer.

MPO activity assay. The lung tissue-associated MPO activity was measured using an assay based on the oxidation of o-dianisidine hydrochloride (Bradley et al., 1982). The homogenized lung samples were diluted in 10 mM EDTA and 10% hexadecyltrimethylammonium bromide in 100 mM potassium phosphate (pH 6.0). Samples were vortexed and centrifuged at 14 000 g for 10 min at room temperature. Diluents were allowed to react with a solution of o-dianisidine hydrochloride (0.167 mg ml−1) in 50 mM potassium phosphate, pH 6.0, and 0.006% H2O2. Samples were assayed in triplicate. Changes in A450 were recorded for the initial 5 min of reaction. The extinction coefficient of the oxidized product of o-dianisidine is 11.3 mM−1 cm−1, and this was used to calculate the number of moles of H2O2 degraded. One unit of MPO activity was defined as the degradation of 1 μmol H2O2 min−1 (mg lung tissue)−1 at room temperature. Purified human MPO (Biodesign International) was used as a positive and internal control for assay accuracy (> 99%).

Isolation of naïve macrophages. Bone marrow-derived macrophages were produced from progenitor cells as described elsewhere (Fortier & Falk, 2005) with some adjustments (Davies & Gordon, 2005). Briefly, bone marrow plugs were flushed from femurs and tibias with RPMI 1640. Cells (1 × 105–3 × 105) were cultured in 10 ml RPMI medium supplemented with 10% fetal bovine serum (FBS)/15% L-cell-conditioned medium/20% WEHI-3B conditioned medium in a 25 ml flask and kept at 37°C and 5% CO2. The following day, the cell suspension was transferred to a 75 ml flask, and on days 2, and 4, 10 ml supplemented medium was added. Cells were harvested after a total of 7 days using a cell scraper and plated on untreated plates in RPMI medium containing 10% FBS/15% L-cell-conditioned medium. Cells were used the following day for the killing assay, and within 7 days for flow cytometry and phagocytosis assays. This protocol yielded macrophages ≥ 80% enriched with contaminating monocyte precursors.

Epifluorescent microscopy. Macrophages were cultured on cell culture-treated coverslips which were transferred to fresh macrophage culture medium and incubated with a saturated overnight culture of P. aeruginosa pMRP-1 (1 : 75) and incubated at 37°C for 20 min. Cells were washed twice in RPMI and incubated in 5.0 μM ER-Tracker Blue-White DPX (Invitrogen) in macrophage culture medium at 37°C for 30 min. Cells were then placed in 1 : 1 PBS and RPMI medium containing 20 μg ethidium bromide ml−1 at room temperature for 5 min, washed twice in RPMI and wet-mounted in
PBS. Cells were immediately visualized using the same system as in the phagocytosis assay, with appropriate filters for epifluorescent analysis.

**Phagocytosis assay.** Macrophages were cultured from bone marrow isolated from the femurs and tibias of B6 and D2 mice, and were used to determine the degree of phagocytosis by each mouse strain. The assay was adapted from a protocol described elsewhere (Campbell et al., 2005). Briefly, $5 \times 10^5$ macrophages were mixed together with $1 \times 10^7$ P. aeruginosa pMRP-1, a 1:20 ratio, in 500 ml RPMI and 5% FBS. The mixture was tumbled end over end in a Labquake shaker model 400110 (Barnstead International) for 20 min at 37°C. Next, the macrophages were pelleted by centrifugation at 250 g for 8 min at 4°C, washed twice with 500 ml RPMI and 5% FBS to remove the majority of the extracellular bacteria, and then resuspended in 150 ml PBS. To distinguish between internalized and any remaining extracellular bacteria, ethidium bromide was added to a working concentration of 50 µg ml⁻¹. Ethidium bromide will turn any extracellular bacteria red while internalized bacteria will remain green. Macrophages were visualized using a X-Cite 120 fluorescence illumination system, Olympus DP70 camera and associated software (DP Controller and DP Manager) version 1.2.1.108. The phagocytosed bacteria were counted in 50 individual macrophages per mouse strain within 15 min of the addition of ethidium bromide. The phagocytotic index was calculated from the mean number of bacteria, in macrophages containing bacteria, multiplied by the percentage of macrophages positive for bacteria.

**Flow cytometry.** Macrophages suspended in 500 µl 5% FBS/RPMI medium were inoculated with $3 \times 10^6$ P. aeruginosa pMRP9-1 (1:20) and incubated for 20, 60 or 120 min at 37°C while being rotated end over end on a Labquake shaker. Each time point was performed in triplicate with a mock infected control. After exposure to bacteria, cells were immediately placed on ice and analysed on a Beckman Coulter Epics Altra cytometer. Fifty thousand events were recorded and the data were analysed using EXPO32 version 1.2 software.

**Bacterial killing assay.** B6 or D2 macrophages were mixed in a 1:5 ratio ($1.0 \times 10^7$ macrophages per 100 µl RPMI and 5% FBS with a total volume of 1 ml) with P. aeruginosa PAO1 grown overnight at 37°C from a single isolated colony. A control without macrophages was used to account for extracellular bacterial growth. Aliquots from the test samples and control were taken immediately after (time = 0) and at time = 20, 60, 90, 120, 150, 180, 240 and 300 min. The samples were tumbled at 37°C between time points. c.f.u. were determined by serial dilution and plating on LB agar plates. The aliquots for c.f.u.

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**Fig. 1.** Differential susceptibility to lung colonization by P. aeruginosa in inbred mouse strains. (a) Mice were exposed to P. aeruginosa aerosols. Shown is the box plot of viable counts 6 h post exposure from the right lung (line within each box is the median; upper and lower lines of the box are 75 and 25 percentiles, respectively; upper and lower black circles are 95 and 5 percentiles, respectively). Three strains of P. aeruginosa were used: PAO1, CF149 and 311058. The number below each bacterial strain is the log of the mean combined initial delivery dosage to the mouse lungs from eight separate trials for P. aeruginosa PAO1, and one trial each for CF149 and 311058, normalized based on the lung weight. The number below each box indicates the number of mice used. D2M and D2F indicate D2 males and females, respectively. *$P<0.01$ (one-way ANOVA and Mann–Whitney rank sum test). (b) Lung clearance kinetics after respiratory exposure to P. aeruginosa PAO1. The initial deposition of PAO1 is shown on the y axis at time 0, which was determined with five B6 mice sacrificed immediately after exposure. c.f.u. were determined at each time point using five exposed mice per strain. Due to mortality from the exposed D2 mice, only four mice were used for c.f.u. counts at 48 and 72 h (*$P<0.05$ D2 versus B6; #$P<0.05$ D2 versus C3).
determination at each time point were diluted in water and incubated at room temperature for 5 min to lyse the macrophages. The bacteria were pelleted by centrifugation at 10,000 g for 1 min and resuspended in PPBS to inhibit bacteria binding to plastics. All of the remaining dilutions used PPBS. The amount of killing was determined by plate count of surviving bacteria.

Cytokine assays. Assays were performed using the Beadlyte Mouse 21-Plex Cytokine Detection System (Upstate Biotechnology) according to the manufacturer’s instructions. Briefly, 50 μl lung homogenate was incubated for 2 h with the Beadlyte Mouse 21-Plex Multi-Cytokine beads in individual wells of a 96-well plate. Each sample was plated in duplicate. Next, 25 μl Beadlyte Mouse 21-Plex Multi-Cytokine biotin was added to each well and the plate was incubated for 1.5 h. Diluted Beadlyte Streptavidin–Phycocerythrin was added to the wells (25 μl per well) and the plate was incubated for 30 min. The plate was then read on a LumineX xMAP 100 analyser (Upstate Biotechnology). The concentration of cytokine was then extrapolated from a standard curve using BeadView Data Analysis software (Upstate Biotechnology). For construction of the dendrograms, log ratios of D2:B6 or D2:C3 at each time point for each cytokine were exported into Multiple Array Viewer software (The Institute for Genomic Research). Hierarchical clustering was performed on the cytokines using average linkage and euclidean distance metric. Clusters were cut at a distance threshold of 2.8 to produce the clusters.

Statistical analysis. Analysis of lung colonization (Fig. 1a) was done with one-way ANOVA followed by all pairwise multiple comparisons with the Tukey test. The Mann–Whitney rank sum test was used to compare the medians of the percentage viable bacterial counts remaining in the lungs 6 h post-infection. Analysis of the lung kinetic clearance (Fig. 1b) and the MPO activity (Fig. 3) was carried out with one-way repeated measure ANOVA and with Fisher’s protected least significant difference. Survival (Fig. 4) was analysed by a log rank test followed by all pairwise multiple comparisons using the Holm–Sidak method. The data from the phagocytosis assay (Fig. 5c) were determined to be not normally distributed according to the empirical distribution function. The Kolmogorov–Smirnov non-parametric test was used to determine P values, since log transformation was not possible given that elements with values of 0 were present. The confidence level for all significance was set at 95%. For flow cytometry data (Fig. 5g), Overton analysis was performed on GFP + events using a mock infected control as the reference, followed by a Student’s t test. P values were corrected for multiple comparisons using the Tukey–Kramer method. ANOVA was performed on the datasets for each time point for the killing assay (Fig. 5h). Those showing significance were tested using a Student’s t test assuming unequal variances and corrected for multiple comparisons using the Holm’s method. Single-tailed t tests were used in comparisons with the control, while two-tailed t tests were used in comparing D2 to B6. Significance between cytokine expression levels (Fig. 6) was calculated using a two-tailed t test assuming unequal variances at each time point. All analyses were conducted with SigmaStat (version 3.1, Systat Software) and SigmaPlot (version 9.0, Systat Software) software.

RESULTS

D2 lungs are deficient in the initial defence against P. aeruginosa colonization

Using an aerosol infection mouse model (Yu et al., 1998a; Yu & Head, 2002), we compared 11 inbred mouse strains, 129/Sv, A/J, BALB/c, C3, B6, D2, FVB, B10.D2/oSnJ, B10.D2/nSnJ, AKR/J and SWR/J, for their susceptibility status to lung colonization by P. aeruginosa PAO1. Six hours after the exposure, viable counts from the right lungs were determined (Fig. 1a). Increased P. aeruginosa PAO1 loads were noted in D2 mice in comparison to the other inbred strains and to the initial deposition. The percentage of viable bacterial counts in the lungs was calculated using the ratio of c.f.u. 6 h post-infection to the mean initial input of bacteria. At 6 h, the median of P. aeruginosa PAO1 recovered from B6 lungs was 6.1% of the initial input (range 2.6–31%, n = 39), while that from D2 lungs was 287% (range 61–1146%, n = 26) (P < 0.001). Male and female D2 mice showed equivalent levels of bacterial counts. These results suggested that in our aerosol model, among the strains of mice studied, D2 mice are uniquely susceptible to infection, as has been shown earlier in other models (Cerquetti et al., 1986; Larsen et al., 1982; Morissette et al., 1995).

A similar pattern of increased bacterial retention in D2 lungs was seen 6 h post-infection with two clinical isolates, P. aeruginosa CF149 and 311058 (Head & Yu, 2004) compared to the resistant B6 and C3 mice (Fig. 1a). The genome sizes of these two clinical strains are known to be significantly larger than that of PAO1 (Head & Yu, 2004). At 6 h post-infection with CF149, the number of c.f.u. in B6 and D2 lungs was 3% (n = 10) and 250% (n = 11) of the initial input (P < 0.001), respectively. Similarly, 6 h post-infection with 311058, the number of c.f.u. in B6 and D2 lungs was 8% (n = 11) and 171% (n = 5) of the initial input (P = 0.02), respectively. Furthermore, D2 lungs were more sensitive to colonization with these two clinical isolates than C3 lungs (P < 0.001). While the resistant mice, including B6 and C3, showed a decrease in colonization compared to the initial delivery dose, D2 lungs displayed an increase of c.f.u. within the first 6 h after respiratory colonization, indicating that D2 lungs are deficient in removing the initial colonizing clinical isolates of P. aeruginosa.

Next, we compared the kinetic lung clearance of P. aeruginosa PAO1 in B6, C3 and D2 mice. After exposure, viable counts were measured over a period of 72 h (Fig. 1b). Overall, all three mouse strains displayed a reduction in bacterial counts throughout the 72 h. However, bacterial loads in D2 lungs remained significantly higher than those in B6 or C3 lungs at each time point (P < 0.001). To ensure that the bacterial droplets inhaled by mice were distributed evenly through the entire lung, we compared viable counts and lung histopathology between the left and right lungs over a period of 24 h after infection with P. aeruginosa PAO1. No difference was seen in c.f.u. between left and right lungs, and histopathology was also similar (data not shown), indicating that this aerosol procedure can be used to simulate whole-lung infections. Taken together, these data indicate that D2 mice are unable to resolve P. aeruginosa lung colonization as well as other inbred mouse strains.

Lung infection with P. aeruginosa PAO1 causes high and rapid mortality in D2 mice

While testing the kinetic lung clearance of P. aeruginosa PAO1, we noted that a few D2 mice succumbed to infection...
after 24 h, while none of the B6 or C3 mice died. To examine the mortality more fully, the mortality of D2 mice relative to other mice was then monitored over a period of 144 h. A mortality of 96.7% was noted in D2 (number dead/number tested = 58/60) and 5.6% in B6 (2/36). The mean survival times for D2 females and males were 36.5 ± 2.2 (mean ± SEM) and 53.7 ± 3.7 h, respectively (P < 0.0001) (data not shown). The mean survival times for the other inbred strains of mice were greater than 138 h, and there were no differences among them (P = 0.858).

**P. aeruginosa PAO1 causes extensive lung inflammation and injury in D2 mice**

To examine pathological changes in the mouse lungs, the left lobes were processed for histology at each time point (Fig. 2). Perivascular and peribronchiolar infiltrates were noted in all lung sections of the three mouse strains. At 24 h, D2 mice showed more diffuse and patchy accumulations of inflammatory cells within the alveolar space than B6 mice. At 48 h, while the infiltrates appeared to subside in B6 mice, they remained abundantly present within the alveoli of D2 mice coupled with oedema. At 72 h, improved lung histology was seen in B6 and C3 lungs, while D2 lungs were still heavily inflamed. An exudate was noted in the alveoli of D2 lungs at 24 h, which increased over time along with infiltrations of inflammatory cells. At 72 h, the alveolar septae in D2 mice were disrupted. This and the extensive exudate formation caused a significant reduction in alveoli space. Along with interstitial and alveolar oedema formation, this response is similar to that seen with acute inflammatory lung injury. While the exudate was also detected in the early sections of B6 mice (up to 24 h post-infection), it disappeared after 48 h. Overall, the lung histopathology showed that D2 mice have a more robust, but detrimental, response to *P. aeruginosa* PAO1 compared to B6 and C3 mice. This response is coupled with a greater influx of inflammatory cells, extensive production of exudate, and tissue damage.

To see whether bacteraemia was playing a role in the death of D2 mice, we measured the viable bacterial counts in blood

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**Fig. 2.** Progressive, increased lung inflammation and injury in D2 mice. B6, C3 and D2 mice were exposed at the same time to *P. aeruginosa* PAO1 aerosols as in Fig. 1(b), and lung tissues were harvested at 0, 6, 12, 24, 48 and 72 h. Processing of mouse lungs for histology and light microscopy of H&E-stained infected lung sections were carried out as described in Methods. Magnification was ×40 or ×600, as indicated. Sections are representative of five mice studied at each time point per group. Bars, ×40, 1600 μm; ×600, 100 μm.
and spleens of D2 and B6 mice. Viable bacteria were detected within 24 h of exposure, but there was no difference in the blood and spleen samples between the mouse strains (data not shown). No bacteria were isolated from the blood or spleen of either mouse strain at 48 or 72 h (data not shown).

While there was a transient presence of bacteria in the blood of B6 and D2 mice, the death of D2 mice appeared to be primarily due to the complication in the lungs, indicating that the D2 mice, compared to the other strains of mice tested, are unable to overcome the increased and prolonged inflammatory response.

Since neutrophils are directly involved in killing invading bacteria and histopathology indicates that there is recruitment of inflammatory cells, we examined the activity of neutrophils in lung tissues by measuring MPO levels in B6, C3 and D2 mice after exposure to *P. aeruginosa* PAO1 (Fig. 3). MPO is abundantly present in neutrophils, and is an accepted marker for neutrophil infiltration (Haddad et al., 2002). At 6 h post exposure, there was no significant difference in MPO activity among the three strains of mice. From 6 to 12 h, MPO activity continued to rise in C3 and D2 mice, while activity in B6 mice started to decrease. From 12 to 48 h, MPO activity declined in all three mouse strains, but the MPO activity of C3 and D2 was significantly higher at each time point than that of B6 (*P* = 0.01). At 72 h, MPO activity in B6 and C3 mice returned to the same level as that of uninfected or saline-exposed controls (data not shown), but it remained elevated in D2 mice (*P* = 0.01). These results indicate that D2 mice have increased neutrophil activity after exposure to PAO1 throughout the infection.

**Other host factors besides complement 5 are involved in the increased susceptibility to lung infection**

Due to a mutation, D2 mice are deficient in the production of complement 5 (C5). The split product C5a acts as an anaphylatoxin to recruit neutrophils to sites of infection. Several papers have cited this defect as a cause for D2 susceptibility to *P. aeruginosa* (Cerquetti et al., 1986; Larsen et al., 1982; Morissette et al., 1995). To determine if the C5 deficiency is responsible for the susceptibility of D2 mice in our aerosol model, several C5-deficient strains were compared. A varied response of susceptibility was found when these C5-deficient strains were exposed to aerosolized *P. aeruginosa* PAO1 (Fig. 4). While B6, C3 and B10.D2/nSnJ are C5 sufficient, D2, SWR/J, AKR/J, B10.D2/oSnJ, C3 and D2 strains were infected with PAO1 to monitor the mortality. A/J, SWR/J, B10.D2/oSnJ and D2 mice are C5 deficient.
Fig. 5. D2 naïve macrophages have different phagocytic and bactericidal activity from B6 macrophages. Epifluorescent images of naïve macrophages produced from bone marrow progenitor cells harvested from (a) B6 and (b) D2 mice after 20 min exposure to *P. aeruginosa* pMRP-1. The nuclei are stained red with ethidium bromide, while the endoplasmic reticulum is stained blue with ER-Tracker Blue-White DPX and the bacteria appear green due to GFP expression (white arrows). Bars, 25 μm. (c) After a 20 min exposure, there was a qualitative difference between D2 (pink bar) and B6 (blue bar) macrophage phagocytosis. This difference was quantified by determination of the phagocytic index (percentage of macrophages with bacteria multiplied by the mean number of bacteria per macrophage containing bacteria) by microscopic examination. The experimental data were statistically different on two replicates (*P* < 0.05, Kolmogorov–Smirnov), with the values shown being a representative trial (*n* = 50). (d–f) Naïve macrophages were first separated from monocyte precursors and multiple cell aggregates by forward-scatter (FSC) and side-scatter (SSC) characteristics (monocytes have lower SSC values) and analysed for GFP levels. (d) FSC and SSC of cultured naïve macrophages are shown with the population on the right being the subject of analysis. (e) Unexposed macrophages were used as a negative control in gating. (f) GFP+ macrophages exposed to GFP-expressing *P. aeruginosa*. (g) The proportion of macrophages that were GFP+ is shown after 20, 60 and 120 min exposure to bacteria. Comparisons between strains at each time point were significantly different (*P* < 0.05, Overton analysis, Student’s *t* test, Holm’s method). Data shown represent mean ± SD (*n* = 6). Blue bars, B6; pink bars, D2. (h) Data from bacterial killing assays plotted on a logarithmic scale indicate that B6 macrophages are able to control *P. aeruginosa* growth significantly better (*P* < 0.05, ANOVA, Student’s *t* test, Holm’s method) than D2 macrophages for up to 5 h exposure. The control represents medium inoculated with bacteria in the absence of macrophages. Data plotted are the mean of three experiments.
D2 macrophages have reduced ability to eliminate P. aeruginosa

In addition to neutrophils, macrophages are a major immune cell involved in innate immunity. Studies have shown that there are four distinct types of monocyte lineage in the lung that have different biological functions (Lohmann-Matthes et al., 1994; Sibille & Reynolds, 1990). In addition, macrophages display complex activation characteristics and heterogeneity due to micro-environmental influences (Gordon, 2003; Hume et al., 2002; Rauh et al., 2005). These influences can have large effects on the
Examination of live cultured macrophages exposed for 20 min to *P. aeruginosa* pMRP-1 revealed that D2 macrophages have increased initial phagocytosis ability (*P* <0.05) (Fig. 5a–c). The phagocytosis index of D2 macrophages was twofold higher than that of B6 macrophages (based on two independent trials). To verify these results and monitor phagocytosis over an extended time, flow cytometry was conducted to analyse the phagocytosis of *P. aeruginosa* pMRP-1 by macrophages. D2 and B6 naïve macrophages were found to be significantly different (*P* <0.05) in the percentage of macrophages that were GFP⁺ after 20, 60 and 120 min exposure (Fig. 5g). Results at 20 min were consistent with microscopy data, in that D2 macrophages had a higher level of phagocytosis. However, the difference (5%) was more subtle than the twofold difference seen with microscopy (Fig. 5c), most likely due to the sensitivity of flow cytometry compared to microscopy in these assays. After 60 min exposure, B6 macrophages had a significant increase (*P* <0.05) in phagocytosis compared to D2 macrophages. This increase was maintained up to 120 min after exposure. Therefore, while the initial phagocytosis of bacteria by the susceptible D2 strain may be superior, B6 macrophages are able to respond over time with even higher phagocytosis activity. Conversely, D2 macrophage phagocytosis remains constant, without an increase in response, for up to 2 h after exposure.

To determine if the difference in phagocytosis is relevant to the ability of these macrophages to control bacterial growth, an *ex vivo* *P. aeruginosa* killing assay was performed (Fig. 5h). In this assay the number of surviving bacteria was significantly lower (*P* <0.05) when exposed to B6 macrophages in comparison to D2 macrophages 4 and 5 h after exposure. There was no significant difference between bacterial proliferation in the control (no macrophages) versus D2 macrophages at any time point. However, after 2 h, the B6 macrophages displayed significantly reduced bacterial proliferation compared to both control and D2 macrophages. Variation increased in the data when the bacterial population entered the exponential-growth phase (time = 150 and 180 min), such that after correction for multiple comparisons, differences were no longer significant. However, all time points after 180 min showed significant differences between D2 and B6 macrophages. Thus, D2 macrophages are not able to control *P. aeruginosa* PAO1 proliferation as well as B6 macrophages. This time frame is consistent with the 6 h time point identified in our exposure assay that shows a difference between these two strains. Since PA14 is known to be more virulent than PAO1, with the presence of pathogenicity islands containing various types of novel virulence factors (Lee *et al.*, 2006), we tested whether D2 macrophages are also deficient in killing PA14. Similar to what was seen with PAO1 (Fig. 5h), D2 macrophages were less efficient than B6 macrophages in containing the growth of PA14 (data not shown). This suggests that D2 macrophages lack the necessary elements to control *Pseudomonas* bacterial growth after internalization, and both *P. aeruginosa* PAO1 and *P. aeruginosa* PA14 have common genetic elements which allow them to survive intracellularly.

**Elevation of proinflammatory and immunoregulatory markers in D2 lungs**

Cytokines and chemokines are important mediators and signalling molecules in the innate immune system. After exposure to *P. aeruginosa* PAO1, we measured the levels of pulmonary cytokines from B6, C3 and D2 mice. Fig. 6(a, b) shows two comparisons of 21 cytokines from aerosol exposures over a 72 h time frame. The cluster tree indicates cytokines that have similar expression profiles based on the log ratio of D2 versus B6 or D2 versus C3. Overall, comparison between D2 and B6 revealed that D2 had higher levels of inflammatory cytokines, including tumour necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1β, KC, interleukin (IL)-1z and monocyte chemotactic protein (MCP)-1. IL-17 and RANTES peaked at 12 h, and vascular endothelial growth factor (VEGF) had a higher level from 12 to 24 h in D2 mice. Comparison between D2 and C3 mice indicated that the levels of TNF-α, MIP-1β, KC and IL-1z were similarly elevated up to 48 h post-infection. However, these cytokines in C3 mice returned to the uninfected level at 72 h. D2 mice had increased levels of MCP-1 and VEGF at 12 and 12–48 h, respectively. IL-17 was elevated in D2 mice throughout the infection. Based on these proinflammatory cytokine profiles, D2 mice have a persistent, increased inflammatory response to lung colonization with *P. aeruginosa* PAO1 compared to B6 mice, and unlike C3 mice, the lung inflammation in D2 mice cannot be completely resolved.

**DISCUSSION**

The overall finding of this study is that D2 mice are not effective in mounting a robust lung clearance against *P. aeruginosa*. Attempts to correct this deficiency result in a larger influx of inflammatory cells to the lung tissues. Although an increase in the number of recruited neutrophils helps to reduce bacterial loads, an excessive inflammatory response results, as was verified by lung pathology, MPO activity and the elevated production of pro-inflammatory chemokines and cytokines. The acute inflammatory lung injury was the chief cause of the rapid mortality, achieving a rate of 96.7% in D2 mice. Naïve D2 macrophages showed an initial increase in bacterial phagocytosis, but were unable to restrict *P. aeruginosa* growth compared to those of the resistant B6 mice. This inability to rapidly clear respiratory colonization with *P. aeruginosa* may stem from the reduced
antimicrobial activity of macrophages in D2 mice. This loss of antimicrobial activity is also accompanied by the failure of D2 macrophages to elevate phagocytic activity relative to resistant B6 macrophages. These results suggest that there are multiple deficiencies in D2 mice that affect the innate resistance to lung infection.

Innate immunity against respiratory colonization with P. aeruginosa is a dynamic process which requires the participation of many host factors. B6, C3 and D2 mice showed a clear difference in the initial colonization in the lungs that was neutrophil independent. Although at the 12 h time point the correlation of increased MPO activities with a decrease in bacterial counts suggests that neutrophils help eliminate P. aeruginosa from the D2 lungs (Figs 1b and 3), this early counter-colonization defence in D2 mice is not sufficient to withstand the bacterial insults, which leads to an intense inflammatory response in the host. While the lung inflammation resolved in resistant B6 mice, increased inflammation was seen with a slower decline in MPO activity and increased lung pathology in D2 mice (Figs 2 and 3). The enhanced MPO activity in D2 and C3 mice suggests that the role of neutrophils is important in the lung defence against P. aeruginosa. It has been shown that neutropenic mice are highly susceptible to P. aeruginosa lung infection (Priebe et al., 2003). However, the complete lack of early bactERICIdal activity before the peak of MPO activity (Figs 1b and 3) suggests that the resident defence factors, such as macrophages, in the lungs are also important in the defence. Additionally, D2 macrophages are deficient in their ability to control bacterial growth, and D2 mice produce an enhanced proinflammatory cytokine profile compared to B6 mice (Figs 5h and 6). This supports the notion that a defect in macrophages and/or deregulation of chemokine and cytokine networks in D2 mice may affect the effective removal of the invading bacteria. The resulting intense neutrophilic inflammation causes damage to the alveolar septa with the production of oedema fluid during the later stages of infection (Fig. 2). The extensive exudate formation within alveoli could further exacerbate the activity of neutrophils and macrophages (Keicho & Kudoh, 2002; Tsuda et al., 2004). The significant pathology characterized by excessive and directed neutrophil infiltration as well as interstitial and alveolar oedema indicates that this acute lung infection model with D2 mice displays symptoms similar to those of acute lung injury (ALI), a major debilitating illness that has a high incidence in hospital settings (Rubenfeld et al., 2005).

Increased susceptibility to lung infection in D2 mice has been previously seen with intratracheal instillation of P. aeruginosa embedded in agar beads (Morissette et al., 1995, 1996). This defect has also been seen with other bacterial species: Staphylococcus aureus (Cerquetti et al., 1983; K. R. Wilson & H. D. Yu, unpublished results), Klebsiella pneumoniae (K. R. Wilson & H. D. Yu, unpublished results) and Streptococcus pneumoniae (K. R. Wilson & H. D. Yu, unpublished results). The early lung-defence defect with D2 mice seems not to be bacterium specific, while the underlying mechanisms remain unknown. An earlier report has shown that lung macrophages display similar in vitro phagocytic and bactericidal activities in BALB/c and D2 mice (Morissette et al., 1996). Lung macrophages are known to be heterogeneous. Each population of lung macrophages has distinct patterns of gene expression and function (Laskin et al., 2001). Because of the macrophage heterogeneity, the role of alveolar macrophages in defence against P. aeruginosa is still unclear. The depletion of alveolar macrophages has been shown to cause a decrease in cytokine production (Fujimoto et al., 2002), while alveolar macrophages have been shown to have only a marginal role in the defence of the lung against P. aeruginosa (Cheung et al., 2000). Part of the reason that no early difference has been seen in the earlier reports could be the macrophage heterogeneity in the lungs. However, our system accounts for possible micro-environmental influences by eliminating them as a variable, instead concentrating on cell-autonomous factors. In the current study, naïve D2 macrophages are initially more phagocytic than B6 macrophages; however, they are defective in clearing or controlling bacterial colonization as effectively as the B6 macrophages. This finding suggests that the initial bacterial killing defect between the resistant and susceptible mice could be attributed to subsets of lung macrophages, some of which may have reduced antibacterial activity. The increased phagocytosis could be due to increased expression of P. aeruginosa receptors on D2 naïve macrophages. Alternatively, it could be caused by attenuated activation of macrophages with the reduced production of interferon (INF)-γ and/or the decreased production of reactive oxygen species (Daniel et al., 2006). Increased phagocytosis in macrophages could also evoke signals in the form of increased cytokine and chemokine production to set off a cascade of increased inflammatory host responses.

D2 mice are one of several C5-deficient inbred mouse strains (Cinader et al., 1966; Wetsel et al., 1990). The absence of C5a would predict a reduction in neutrophil recruitment to the lung tissues. However, a larger-than-normal influx of inflammatory leukocytes to D2 lungs suggests that the loss of C5a may be compensated by the host by increasing the production of TNF-α and KC as redundant/alternative neutrophil chemotactic factors. An increased infiltration of leukocytes in D2 mice is consistent with persistently high levels of IL-1, TNF-α, MIP-1β, MCP-1, RANTES and KC (Fig. 6). KC is the functional equivalent of IL-8, which has strong activity for neutrophil recruitment and activation. IL-1, TNF-α and LPS are all known inducers of this chemokine. MCP-1, RANTES and MIP-1β also possess specific chemotactic activity for neutrophils and monocytes. These results are congruent with the severe lung pathology seen during the later stages of infection in D2 mice.

The classical method to induce ALI in the mouse is the intratracheal injection of IgG immune complex. Recently it has been demonstrated that the immune complex alone.
cannot effectively induce ALI in C5-deficient mice (Huber-Lang et al., 2006). However, in the current study, the use of live *P. aeruginosa* caused a massive influx of neutrophils in D2 lungs, causing inflammatory damage to alveoli. Therefore, it is possible that ALI is caused by different inducing agents, such as immune complex and/or bacteria. In the presence of C5, the immune complex plays a key role in the formation of ALI. In the absence of C5, as in the case of our present study, *P. aeruginosa* may activate an alternative host-defensive mechanism that leads to ALI. In particular, two cytokines, MIP-1β and IL-17 (Fig. 6) had a marked increase after exposure to *P. aeruginosa*. MIP-1β has been linked with the development of ALI in rats (Bless et al., 2000), while IL-17 has recently been implicated as a major biomarker for a distinct lineage of CD4 T cells that upregulates tissue inflammation (Park et al., 2005). Furthermore, IL-23 and IL-17 have been shown to be critical for maintaining normal granulopoiesis in the tissues (Stark et al., 2005). The divergence in the production of these biomarkers may be linked with deregulation of neutrophils trafficking into tissues.

In summary, we have demonstrated that D2 mice are hypersusceptible to lung colonization by *P. aeruginosa*, leading to rapid development of fatal pneumonia. The inability to mount an effective early lung defence correlates with the lack of initial bactericidal activity in D2 macrophages, indicating that lung macrophages are an important factor in the first line of defence against the initial colonization. Additionally, in response to *P. aeruginosa* lung infection, D2 mice are capable of recruiting an increased number of neutrophils to the lungs, but fail to resolve the neutrophilic inflammation. These combining factors lead to an increased susceptibility, as seen from the increased lung colonization, neutrophil recruitment and mortality in D2 mice. The infection model presented here shows similarities to what is seen with bacterial pneumonia and acute inflammatory lung injury in humans. This model could possibly be used to study host genetic factors that predispose certain individuals to ALI or lung colonization.

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