Role of Inducible Nitric Oxide Synthase and NADPH Oxidase in Early Control of *Burkholderia pseudomallei* Infection in Mice[▽]

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Infection with the soil bacterium *Burkholderia pseudomallei* can result in a variety of clinical outcomes, including asymptomatic infection. The initial immune defense mechanisms which might contribute to the various outcomes after environmental contact with *B. pseudomallei* are largely unknown. We have previously shown that relatively resistant C57BL/6 mice can restrict bacterial *B. pseudomallei* growth more efficiently within 1 day after infection than highly susceptible BALB/c mice. By using this model, our study aimed to investigate the role of macrophage-mediated effector mechanisms during early *B. pseudomallei* infection. Depletion of macrophages revealed an essential role of these cells in the early control of infection in BALB/c and C57BL/6 mice. Strikingly, the comparison of the anti-*B. pseudomallei* activity of bone marrow-derived macrophages (BMM) from C57BL/6 and BALB/c mice revealed an enhanced bactericidal activity of C57BL/6 BMM, particularly after gamma interferon (IFN-γ) stimulation. In vitro experiments with C57BL/6 $gp91phox^{-/-}$ BMM showed an impaired intracellular killing of *B. pseudomallei* compared to experiments with wild-type cells, although C57BL/6 $gp91phox^{-/-}$ cells still exhibited substantial killing activity. The anti-*B. pseudomallei* activity of C57BL/6 $iNOS^{-/-}$ BMM was not impaired. C57BL/6 $gp91phox^{-/-}$ mice lacking a functional NADPH oxidase were more susceptible to infection, whereas C57BL/6 mice lacking inducible nitric oxide synthase (iNOS) did not show increased susceptibility but were slightly more resistant during the early phase of infection. Thus, our data suggest that IFN-γ-mediated but iNOS-independent anti-*B. pseudomallei* mechanisms of macrophages might contribute to the enhanced resistance of C57BL/6 mice compared to that of BALB/c mice in the early phase of infection.

The gram-negative saprophyte *Burkholderia pseudomallei is* the causative agent of melioidosis, an infectious disease which has emerged as an important cause of severe community-acquired infections in certain areas of the tropics (52). Infections of humans and animals are thought to be acquired by inoculation of environmental organisms into minor cuts or abrasions or by inhalation after contact with water or soil (7). The clinical manifestations of melioidosis are extremely variable, being either localized or disseminated and either chronic or acute infections with fulminant septicemias. Long periods of latency and persisting infections after antibiotic treatment are characteristic of melioidosis (25). *B. pseudomallei* is a serum-resistant bacterium (8, 36) that invades host cells, multiplies intracellularly within the cytosol, and induces the formation of actin tails and membrane protrusions, leading to direct cell-to-cell spreading (4, 20, 22). Previous studies on the molecular basis of *B. pseudomallei* virulence have identified a number of virulence determinants in various animal models, including surface polysaccharides (8, 35) and components of a *B. pseudomallei* type III protein secretion system (1, 44). In a recent study, we

identified a number of *B. pseudomallei* virulence and biosynthetic pathway genes which are essential for the intracellular life cycle and which are important for causing disease in a murine model of infection (34).

Although there is considerable genetic variability between *B. pseudomallei* strains (15, 33) and differences in virulence among wild-type strains could be demonstrated in mouse models of infection (42), it is most likely that host factors are also important determinants for the development of disease. Some risk factors (e.g., diabetes mellitus) for severe systemic infection are known (52), but the initial immunological mechanisms which might contribute to the variable outcome after environmental contact with *B. pseudomallei* still have to be defined.

We and others have shown that C57BL/6 and BALB/c mice, although possessing the same mutated, nonfunctional natural resistance-associated macrophage protein 1 (Nramp1), differ dramatically in their susceptibilities to *B. pseudomallei* infection (16, 24, 27). In this model, the more resistant C57BL/6 mice can restrict bacterial growth more efficiently in various organs within 1 day postinfection than highly susceptible BALB/c mice (16). These results indicate that innate immune effector mechanisms might contribute to the resistance pattern observed (16). A recent study found gamma interferon (IFN-γ) to be essential for the early control of *B. pseudomallei* infection in C57BL/6 mice, since C57BL/6 mice lacking either IFN-γ or the IFN-γ-inducing cytokines interleukin-12 and in-
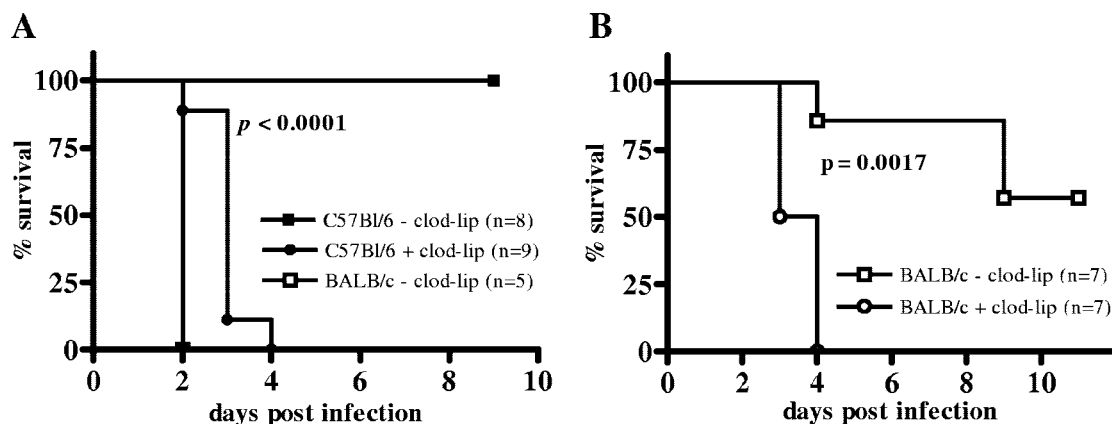
A

B

FIG. 1. Survival curves of macrophage-depleted mice after i.v. infection with *B. pseudomallei*. The 50% lethal dose for *B. pseudomallei* strain E8 at 4 weeks postinfection (i.v.) is in the range of $>5 \times 10^4$ CFU in C57BL/6 mice and >10 to <100 CFU in BALB/c mice. (A) Clodronate (clod-lip)-treated and control C57BL/6 mice received 2.8×10^3 to 5.7×10^3 CFU. To compare the survival of macrophage-depleted C57BL/6 mice with that of nondepleted BALB/c mice, control BALB/c mice were infected with 5.7×10^3 CFU. (B) Clodronate-treated and control BALB/c mice received 40 to 100 CFU. Pooled data from two independent experiments are shown.

terleukin-18 became highly susceptible to *B. pseudomallei* (14). T cells, NK cells, and, to a lesser extent, macrophages have been shown to be a source for IFN-γ production in the first hours after *B. pseudomallei* infection (14, 26). Neither cytokine-activated "bystander" T cells nor NK cells were shown to be essential for early resistance of C57BL/6 mice, since IFN-γ produced by macrophages appeared to be sufficient to control infection in vivo (14).

In vitro experiments demonstrated a pivotal role for IFN-γ to eliminate intracellular *B. pseudomallei* in macrophage cell lines (31, 49). From the inhibition of NO generation in *B. pseudomallei*-infected and IFN-γ-stimulated macrophage cell lines, it was concluded that reactive nitrogen species are important effector molecules in anti-*B. pseudomallei* killing activity (31, 48, 49). To our knowledge, no phagocyte-mediated anti-*B. pseudomallei* effector mechanisms have been investigated in vivo. This study aimed to investigate the role of macrophages and corresponding effector mechanisms in the early control of murine *B. pseudomallei* infection.

MATERIALS AND METHODS

Bacteria. *B. pseudomallei* strain E8 comprises an L-arabinose nonassimilating soil isolate from the surrounding area of Ubon Ratchathani, Northeast Thailand (54), and was obtained from N. J. White (Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). Bacteria were grown on Columbia agar at 37°C for 24 h. Colonies were harvested using tryptic soy broth containing 10% glycerol and frozen immediately in 0.2-ml aliquots at a concentration of 10^9 to 10^{10} CFU per ml at -70°C.

Mice. Female 10- to 20-week-old C57BL/6 and BALB/c mice were obtained from Charles River (Wiga Sulzfeld, Germany). C57BL/6 $gp91phox^{-/-}$ and C57BL/6 $iNOS^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Animals were provided with food and water ad libitum. All animal studies were approved by the local authorities.

In vivo macrophage depletion. To selectively deplete macrophages systemically in vivo, liposomes containing dichloromethylene-biphosphonate (clodronate; gift from Roche Diagnostics GmbH, Mannheim, Germany) were used as previously described (50). Mice receiving phosphate-buffered saline (PBS) containing liposomes served as controls. Mice were treated intravenously (i.v.) with 0.1 ml/10 g of body weight of the liposome clodronate suspension, which has been shown to induce the complete depletion of splenic and hepatic macrophages within 24 h (50).

IFN-γ neutralization in vivo. IFN-γ neutralization was performed via intraperitoneal application of 300 μg anti-murine IFN-γ monoclonal antibody R4-6A2 6 h prior to infection. Control mice received 300 μg of rat immunoglobulin G intraperitoneally.

Infection of mice and determination of organ bacterial burden. For each infection experiment, an aliquot of the *B. pseudomallei* suspension was freshly thawed from the stock. Bacterial cells were diluted in PBS to obtain the appropriate concentration. For i.v. injection route, a bacterial suspension of 200 μl was injected into the lateral tail vein. For intranasal (i.n.) infection, mice were anesthetized by a mixture of ketamin hydrochloride and xylazin hydrochloride. Bacterial cells were applied in 30 μl per animal and given into both nostrils. The mortality of animals was monitored daily. To enumerate bacteria in spleen, liver, and lung, organs were aseptically removed and homogenized in 0.5 to 1 ml sterile PBS containing 0.5% Tergitol and 1% bovine serum albumin and the number of CFU was determined. The detection limit of this procedure was 5 to 15 CFU per organ, depending on the organ weight.

Culture of bone marrow-derived macrophages. To cultivate bone marrow-derived macrophages (BMM), tibias and femurs from either BALB/c or C57BL/6 mice were aseptically removed from euthanized mice and dissected free of adherent tissue. The bone ends were cut, and marrow was flushed with sterile PBS. A single-cell suspension was produced by pipetting cells rigorously; cells were then centrifuged at $150 \times$ g for 10 min and resuspended in RPMI 1640 (Biochrom) containing 10% fetal calf serum, 50 μmol mercaptoethanol, and 33 ng granulocyte-macrophage colony-stimulating factor per ml (kindly provided by Siegfried Weiss, GBF, Braunschweig, Germany). Cells were maintained in 250-ml cell culture flasks, fed every 2 or 3 days with fresh medium, and harvested at days 8 to 12. Staining for macrophage-specific antigens Mac-1, F4/80, and BM-8 revealed approximately 95% positive cells for each marker after 8 days of cultivation from both mouse strains.

Infection of BMM. Twenty hours prior to infection experiments, 1.8×10^5 BMM were seeded in 48-well plates. In some experiments, cells were stimulated with recombinant murine IFN-γ (Roche) in a concentration as indicated. Bacteria were thawed from the stock and grown on Columbia agar plates at 37°C for 20 h, harvested, and diluted using the culture medium used for BMM cultivation. Prior to infection, cells were washed twice with PBS and then infected with different multiplicities of infection (MOI) for 30 min at 37°C as indicated. Then, the medium was removed and exchanged for medium containing 250 μg kanamycin per ml and incubated for 20 min to eliminate extracellular bacteria. This point was taken as time zero, and cells were further incubated for several hours to determine the number of intracellular CFU. At indicated time points, cells were washed twice with PBS and subsequently lysed using 150 μl PBS containing 0.5% Tergitol TMN (Fluka, Buchs, Switzerland) and 1% bovine serum albumin per well. After 20 min of incubation, appropriate dilutions of these suspensions were plated onto Columbia agar or Mueller-Hinton plates and incubated at 37°C for 48 h before colony counts were performed.
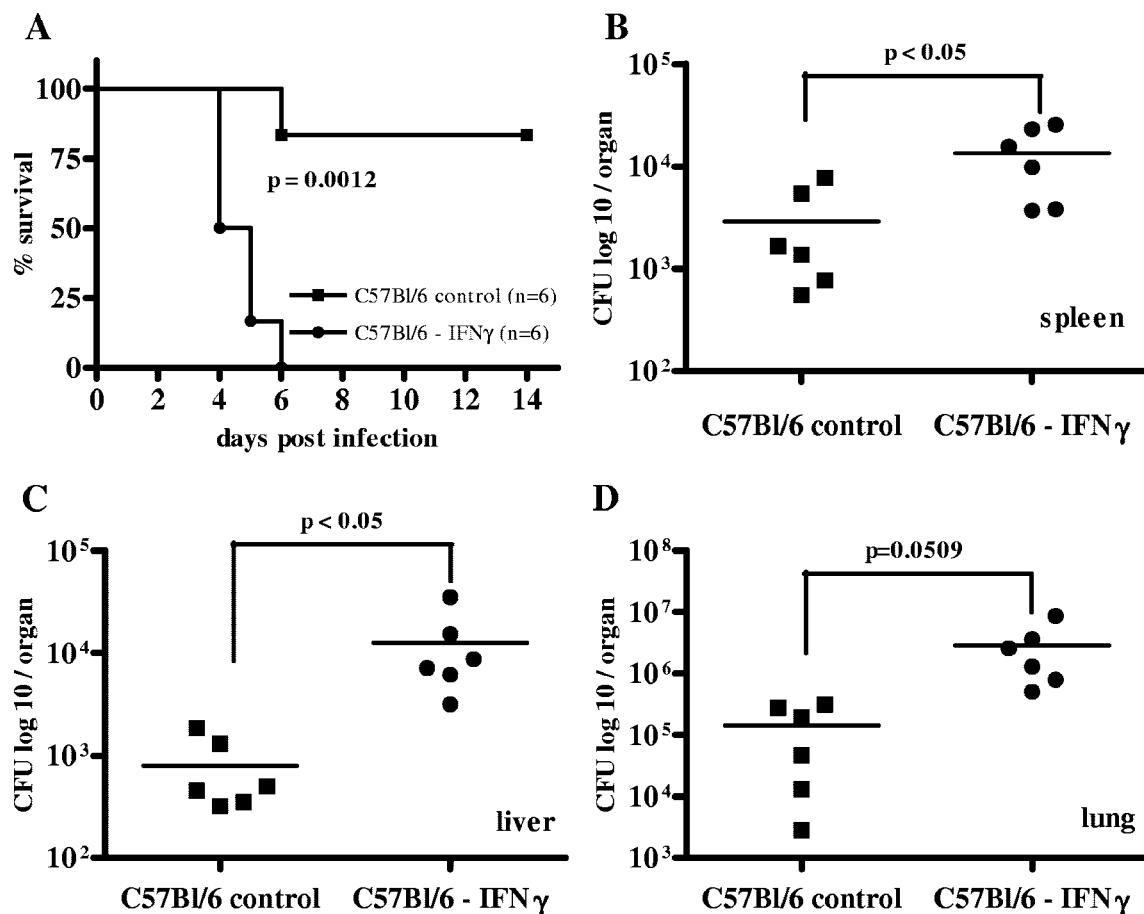
FIG. 2. Survival curve (A) and bacterial burden in spleen, liver, and lung (B to D) 48 h after i.n. *B. pseudomallei* infection of IFN-γ-depleted C57BL/6 mice with the respective control animals. The 50% lethal dose for *B. pseudomallei* strain E8 at 4 weeks postinfection (i.n.) is in the range of >10^2 to <10^3 CFU in C57BL/6 mice and <10 CFU in BALB/c mice. Mice received approximately 200 CFU i.n. Single dots represent the number of CFU of the respective organ from single animals. The horizontal line represents the mean. Pooled data from two independent experiments are shown.

Statistical analyses. Survival curves were compared using log rank Kaplan-Meier tests. For all other statistical analyses, Student's *t* test was used. *P* values of <0.05 were considered to be statistically significant. Statistical analyses were performed using GraphPad Prism version 4.0.

RESULTS

Macrophages are essential to control early *B. pseudomallei* infection in C57BL/6 and BALB/c mice. To test the hypothesis of an essential role of macrophages in the early control of *B. pseudomallei* infection, we treated both susceptible BALB/c and resistant C57BL/6 mice via i.v. administration with clodronate-containing liposomes in order to eliminate macrophages in spleen and liver, the organs known to harbor *B. pseudomallei* shortly after i.v. or i.n. infection (16, 19, 27). As illustrated in Fig. 1A, macrophage-depleted C57BL/6 mice died within 4 days after i.v. infection whereas all animals of the control group survived and did not show any clinical symptoms during the observation period. In fact, macrophage depletion rendered C57BL/6 mice almost as susceptible to *B. pseudomallei* as nondepleted BALB/c mice (Fig. 1A). However, macrophage depletion in BALB/c mice also led to increased mortality (Fig.

1B). These results indicate a key role of macrophages in controlling the early phase of *B. pseudomallei* infection.

IFN-γ is important for restricting *B. pseudomallei* growth during the early phase of infection in C57BL/6 mice. For the subsequent experiments, the i.n. infection route was used because in this model, very low infectious doses cause disease and, moreover, the respiratory tract route is very likely to be a natural route of infection in humans (6). In this model, *B. pseudomallei* disseminates into liver and spleen shortly after infection (19, 27). Since IFN-γ is an important activator of macrophages, we investigated and compared the relevance of this cytokine via antibody-mediated neutralization in both susceptible BALB/c and resistant C57BL/6 mice. As already observed by others (39), IFN-γ neutralization led to a dramatic increase in mortality within the first week postinfection in C57BL/6 mice compared to that in untreated animals (Fig. 2A). The increased susceptibility correlated with an increased bacterial burden in spleen, liver, and lung of IFN-γ-depleted animals at 48 h postinfection compared to bacterial burden in those of controls (Fig. 2B to D). Contrary to C57BL/6 mice, IFN-γ neutralization in BALB/c mice did not result in an
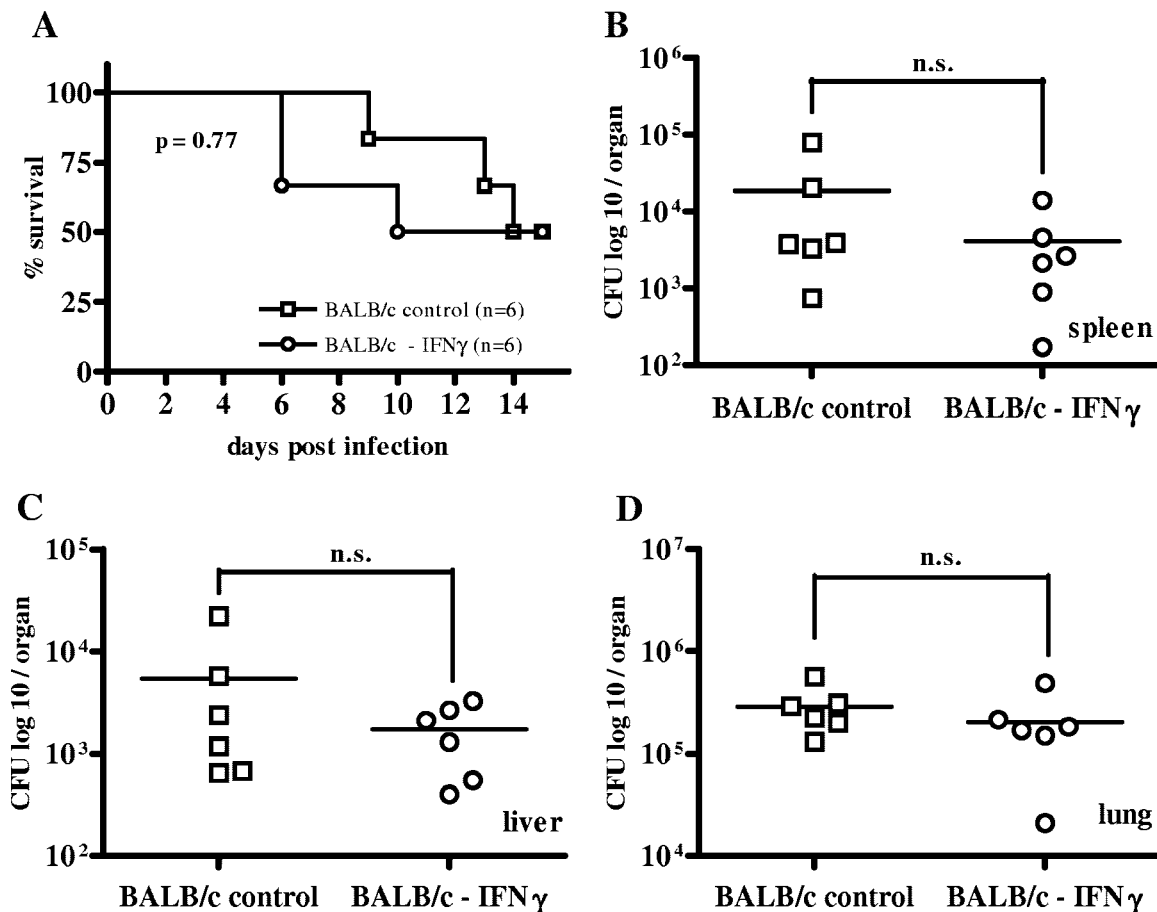
FIG. 3. Survival curve (A) and bacterial burden in spleen, liver, and lung (B to D) 48 h after i.n. *B. pseudomallei* infection of IFN-γ-depleted BALB/c mice with the respective control animals. Mice received 5 to 15 CFU i.n. Single dots represent the number of CFU of the respective organ from single animals. The horizontal line represents the mean. Pooled data from two independent experiments are shown. n.s., $P > 0.05$.

increased mortality rate compared to that in control mice (Fig. 3A). Furthermore, there was no significant difference in the bacterial burden in spleen, liver, and lung between IFN-γ-depleted and nondepleted BALB/c mice at 48 h postinfection (Fig. 3B to D), but there was a tendency for lower bacterial counts of IFN-γ-depleted animals.

Bone marrow-derived macrophages from BALB/c and C57BL/6 mice differ in their anti-*B. pseudomallei*-activities. In the following experiments, we tested the hypothesis that the early difference in resistance against *B. pseudomallei* between BALB/c and C57BL/6 mice is at least partly due to differences in direct anti-*B. pseudomallei* macrophage activities. Infection of either C57BL/6 or BALB/c BMM with *B. pseudomallei* resulted in the same primary uptake in both cell populations. There was also no difference in primary uptake between IFN-γ-stimulated and nonstimulated cells (Fig. 4A and B). Without IFN-γ stimulation, we observed a slightly better intracellular bacterial elimination in C57BL/6 BMM in the first hours after low-dose infection (MOI, 1:1) than in BALB/c BMM (Fig. 4A), although the pathogen was able to replicate in both cell types within 24 h under these conditions. We could also observe a slightly more effective intracellular killing in IFN-γ-stimulated C57BL/6 BMM after a low-dose infection (MOI, 1:1) (Fig. 4B). However, this effect was much more pro-

nounced at a higher infectious dose (MOI, 50:1) (Fig. 4C). Under these conditions, BALB/c BMM could not completely eliminate the pathogen whereas C57BL/6 BMM were able to clear the infection within 24 h. IFN-γ-stimulated BMM of either BALB/c or C57BL/6 origin had a significantly higher bactericidal capacity than the IFN-γ-stimulated BALB/c-derived macrophage cell line J774A.1 (data not shown). Further experiments showed that a positive correlation exists between the difference in killing activity between BMM from BALB/c and C57BL/6 mice and the amount of exogenously added IFN-γ (Fig. 4D). We therefore suggest that differences in the IFN-γ-induced bactericidal responses on the macrophage level contribute to the distinct susceptibilities of BALB/c and C57BL/6 mice for *B. pseudomallei* infection.

NADPH oxidase contributes to intracellular anti-*B. pseudomallei* activity of C57BL/6 bone marrow-derived macrophages. IFN-γ is known to increase bactericidal effector functions in macrophages via activation of NADPH oxidase. To investigate the contribution of NADPH oxidase in the intracellular *B. pseudomallei* elimination of C57BL/6 macrophages, we infected BMM of C57BL/6 wild-type mice and C57BL/6 *gp91phox*$^{-/-}$ mice, lacking *gp91phox* encoding for an NADPH oxidase subunit. Our experiments showed that bactericidal activity was slightly impaired in IFN-γ-stimulated *gp91phox*$^{-/-}$
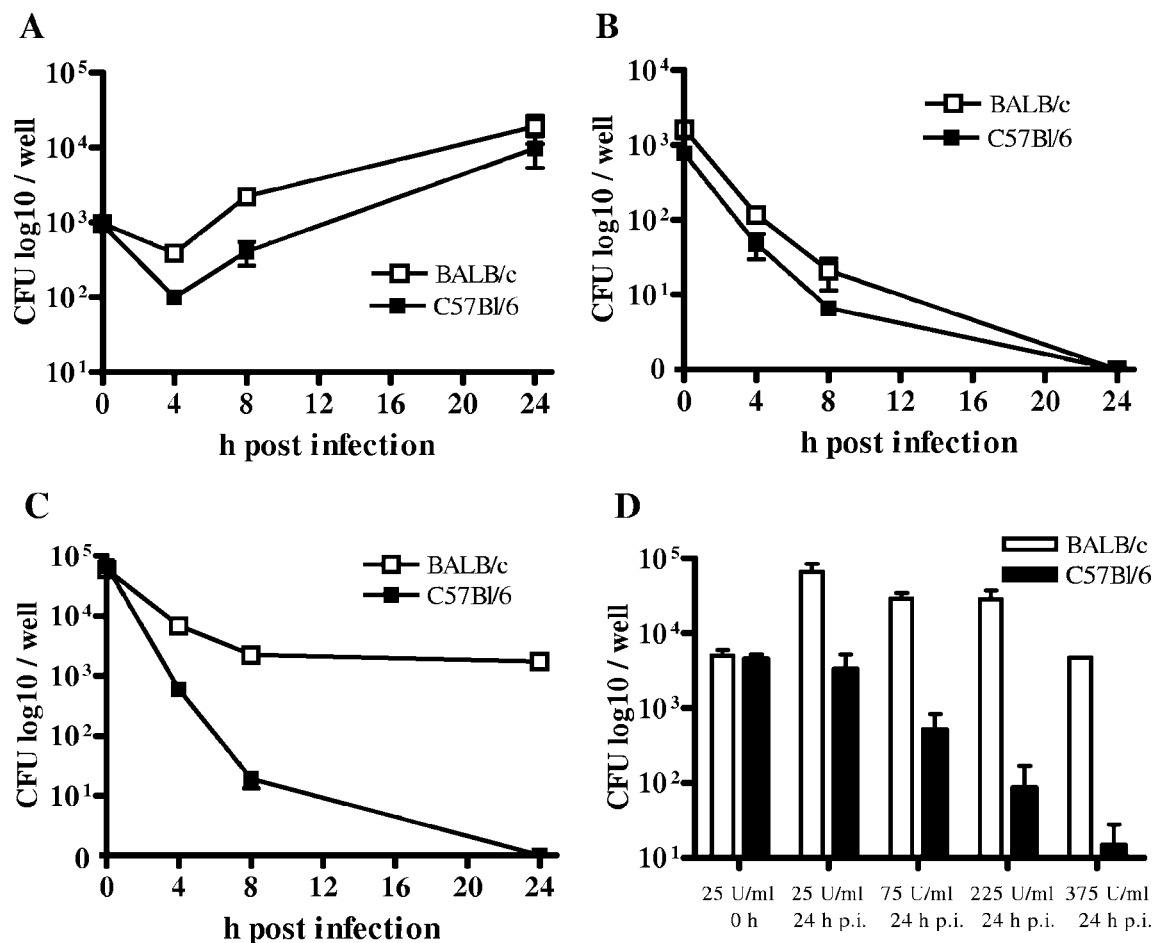
FIG. 4. Intracellular survival of *B. pseudomallei* in BMM from BALB/c and C57BL/6 mice. (A) Unstimulated macrophages infected with an MOI of 1:1. (B) IFN-γ-stimulated (375 U/ml) macrophages infected with an MOI of 1:1. (C) IFN-γ-stimulated (375 U/ml) macrophages infected with an MOI of 50:1. (D) Survival of *B. pseudomallei* 24 h after infection (MOI, 50:1) of macrophages stimulated with different doses of IFN-γ. There was no significant difference between the values of cells stimulated with either 25 U or 375 U per ml at 0 h (data not shown). Values are the means ± standard deviations from at least duplicate determinations. Representative experiments are shown. p.i., postinfection.

BMM compared to that in wild-type macrophages (Fig. 5A). However, IFN-γ-stimulated C57BL/6 *gp91phox*^{−/−} BMM were still able to reduce intracellular bacteria in the first hours after infection.

iNOS is not essential in C57BL/6 BMM to eliminate *B. pseudomallei*. Inducible NO synthesis represents another important IFN-γ-mediated antibacterial effector mechanism in macrophages. We therefore investigated the role of inducible nitric oxide synthase (iNOS) with respect to intracellular elimination of *B. pseudomallei* in C57BL/6 macrophages. After infection of IFN-γ-stimulated C57BL/6 *iNOS*^{−/−} and wild-type BMM, we did not observe any significant difference in intracellular killing (Fig. 5B). The same observation was made when NO production was inhibited by using aminoguanidine-treated C57BL/6 BMM (data not shown). Our results suggest that inducible NO production does not contribute to the improved killing activity of C57BL/6 BMM compared to that of BALB/c BMM. In contrast, NADPH oxidase might play a role in the enhanced resistance of C57BL/6 BMM. However, our results also indicate a significant role of IFN-γ-dependent but iNOS-

and NADPH oxidase-independent mechanisms in the intracellular elimination of *B. pseudomallei* in C57BL/6 BMM.

NADPH oxidase contributes to early resistance of C57BL/6 mice against *B. pseudomallei*. We then used *gp91phox*^{−/−} mice to address the role of NADPH oxidase in *B. pseudomallei* infection of C57BL/6 mice. Our experiments clearly showed a higher mortality rate for *gp91phox*^{−/−} mice after *B. pseudomallei* infection than for wild-type animals (Fig. 6A and B). Moreover, bacterial load in internal organs (Fig. 6C to E) was significantly higher in the liver of *gp91phox*^{−/−} mice after infection (Fig. 6D). A significantly higher mortality was also observed in C57BL/6 mice lacking the *p47* subunit of NADPH oxidase (data not shown). Together, our results substantiate a role for NADPH oxidase in reducing bacterial load in the early phase of *B. pseudomallei* infection.

iNOS is not essential to protect C57BL/6 mice during early *B. pseudomallei* infection. To assess the in vivo role of inducible NO in C57BL/6 mice resistance to *B. pseudomallei*, we infected C57BL/6 *iNOS*^{−/−} mice and surprisingly found them to be more resistant than wild-type animals (Fig. 7). *iNOS*^{−/−} mice
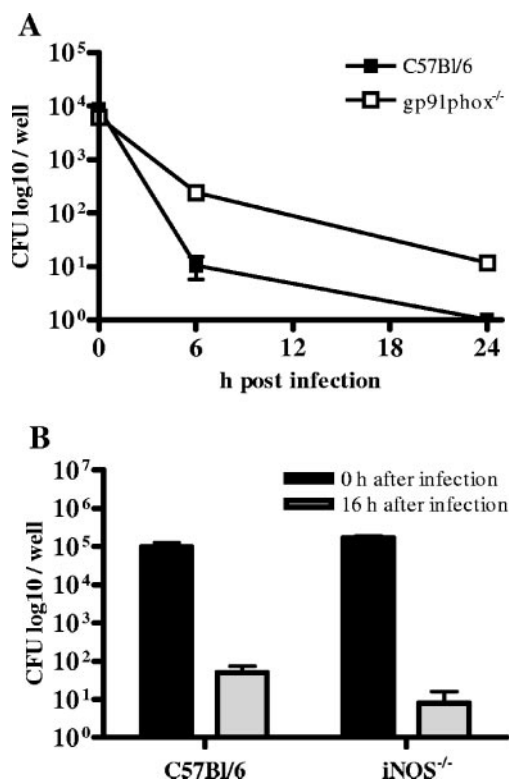
FIG. 5. Intracellular survival of *B. pseudomallei* in C57BL/6 *gp91phox*$^{-/-}$ BMM, *iNOS*$^{-/-}$ BMM, and the respective control cells. (A) IFN-γ-stimulated (100 U/ml) *gp91phox*$^{-/-}$ BMM infected with an MOI of 80:1. (B) IFN-γ-stimulated C57BL/6 *iNOS*$^{-/-}$ BMM infected with an MOI of 170:1. Values are the means ± standard deviations from triplicate determinations. Representative experiments are shown.

showed significantly lower bacterial burden in spleen, liver, and lung (Fig. 7B to D) and also revealed a tendency for prolonged survival after *B. pseudomallei* infection (Fig. 7A). Our results suggest that inducible NO production is not important for protection during the early phase of *B. pseudomallei* infection in C57BL/6 mice but that abolishment of inducible NO production has a protective effect.

DISCUSSION

In the present study, we investigated the role of macrophages and oxidative killing mechanisms in the early control of murine *B. pseudomallei* infection. Moreover, we addressed a possible contribution of macrophages and corresponding effector mechanisms to determine early differences in resistance between C57BL/6 and BALB/c mice. In previous work, we could demonstrate that the bacterial burden of *B. pseudomallei* is significantly higher in susceptible BALB/c mice within 1 day after infection than in more-resistant C57BL/6 mice (16), indicating that early innate immune mechanisms might contribute to the observed resistance pattern.

Our macrophage depletion experiments revealed an essential function of macrophages in the early control of *B. pseudomallei* infection in both BALB/c and C57BL/6 mice. Moreover, macrophage-depleted C57BL/6 mice became almost as susceptible as untreated BALB/c mice. Since IFN-γ is

an important activator of macrophages, we analyzed the role of this cytokine in the early course of infection in BALB/c and C57BL/6 mice. Our experiments showed that the dramatic increase in susceptibility of C57BL/6 mice after IFN-γ neutralization, as already shown by others (14, 39), is accompanied by an early increase in bacterial burden in internal organs. Interestingly, neutralization of IFN-γ in BALB/c mice neither increased early mortality nor led to an increase in early bacterial burden. Similar observations were made with the *Yersinia enterocolitica* and *Brucella abortus* mouse models, showing that anti-IFN-γ treatment abrogated resistance in C57BL/6 mice but only marginally modulated the course of infection in susceptible BALB/c mice (2, 32). One could hypothesize that the lacking effect of IFN-γ neutralization in BALB/c mice is due to a per se insufficient IFN-γ production during the early course of *B. pseudomallei* infection in BALB/c mice. However, in C57BL/6 mice, it has been shown that although rapid IFN-γ production is essential to prevent rapid mortality, as little as 5% of this response is sufficient to provide initial control of *B. pseudomallei* infection (14).

The view that differences in the rapid antigen-independent IFN-γ production are responsible for the early resistance pattern of the two strains of mice is also challenged by reports that IFN-γ mRNA expression in the liver and spleen of BALB/c mice is increased compared to that of C57BL/6 mice at 24 h after infection with *B. pseudomallei* (46). *B. pseudomallei* was recently shown to induce the expression of the suppressor of cytokine signaling 3 and cytokine-inducible Src homology 2-containing protein in RAW 264.7 macrophages which interfere with IFN-γ signaling (10). It remains to be determined whether these factors contribute to the observed differences in anti-*B. pseudomallei* activities of BALB/c and C57BL/6 macrophages. Taken together, our experimental results and data from the literature support the hypothesis that differences in the responsiveness to IFN-γ and, consequently, differences in IFN-γ-induced anti-*B. pseudomallei* effector functions between BALB/c and C57BL/6 macrophages rather than early differences in IFN-γ production might contribute to the early resistance pattern observed.

A previous ex vivo study showed that peritoneal exudate cells from C57BL/6 mice exhibit an enhanced anti-*B. pseudomallei* activity compared to cells from BALB/c mice (47). However, no pure macrophage preparations were examined and a different in vivo priming of the peritoneal cells from the two strains of mice prior to the in vitro testing could not be excluded. Furthermore, the role of IFN-γ was not investigated. We therefore compared the anti-*B. pseudomallei* activities of BMM from C57BL/6 and BALB/c mice raised under identical culture conditions. Interestingly, we found an increased potential of C57BL/6 BMM to eliminate intracellular *B. pseudomallei* in vitro. The fact that the observed difference in the killing potential between both cell populations increased with increasing doses of exogenous IFN-γ (Fig. 4D) further supports the fact that differences in the response to IFN-γ are responsible for the observed effect.

The bactericidal activities of macrophages derived from distinct susceptible mouse strains have also been investigated in the *Brucella abortus* and *Coxiella burnetii* mouse models (40, 55). *Brucella abortus* infection of peritoneal macrophages from susceptible BALB/c and relatively resistant C57BL/10 mice
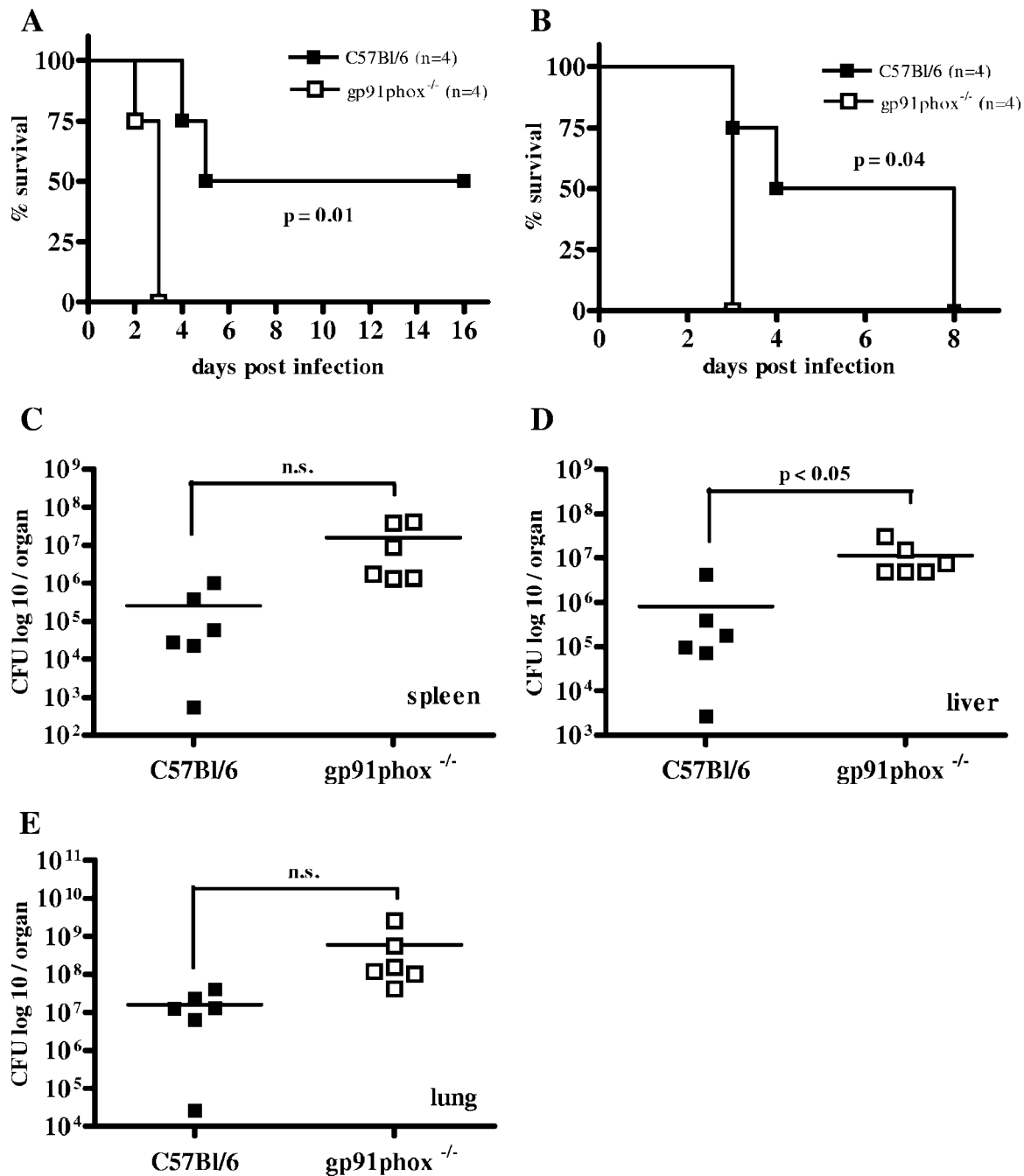
FIG. 6. Survival curves (A and B) and bacterial burden in spleen, liver, and lung (C to E) 48 h after i.n. *B. pseudomallei* infection of *gp91phox*$^{-/-}$ mice with the respective control animals. Mice received 6.1 × 10^2 CFU (A), 5.3 × 10^3 CFU (B), and 2 × 10^3 CFU (C to E) i.n. Single dots represent the number of CFU of the respective organ from single animals. The horizontal line represents the mean. (A and B) Single experiments are shown. (C to E) Pooled data from two independent experiments are shown. n.s., $P > 0.05$.

revealed no difference in killing activities of these cells. Furthermore, the killing potential after IFN-γ stimulation was enhanced to the same extent in both types of macrophages (40). Infection of BMM from BALB/c and C57BL/6 mice with *Coxiella burnetii* revealed enlarged replication vacuoles in macrophages from susceptible BALB/c mice compared to those in macrophages from C57BL/6 mice; however, the bacterial loads in BALB/c and C57BL/6 macrophages were not found to be different (55). Thus, to our knowledge, this is the first report describing a model in which increased BMM killing activity from resistant mice, compared to a less sufficient control of intracellular bacteria in macrophages from susceptible mice, could be detected.

To evaluate possible IFN-γ-mediated oxidative killing mechanisms responsible for the enhanced anti-*B. pseudomallei* activity of C57BL/6 BMM, we first investigated the contribution
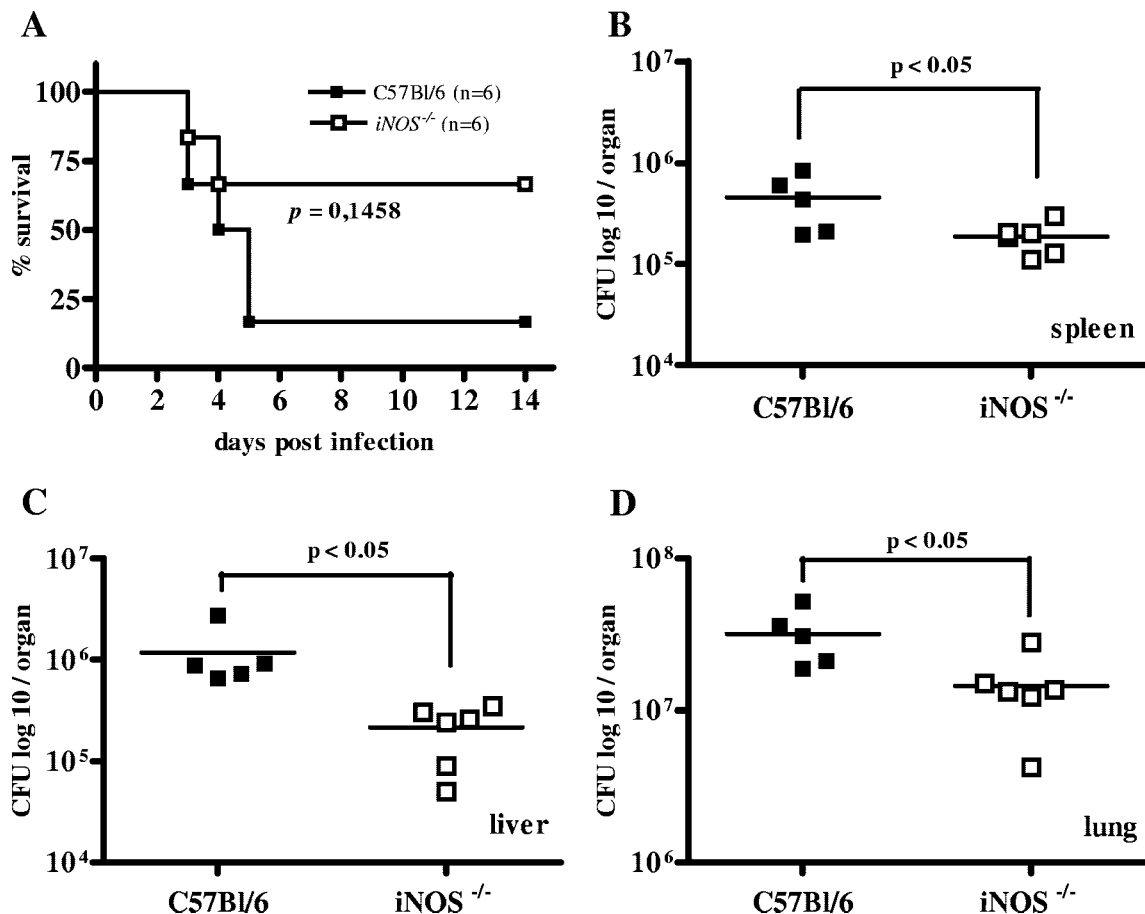
FIG. 7. Survival curve (A) and bacterial burden in spleen, liver, and lung (B to D) 48 h after i.n. *B. pseudomallei* infection of *iNOS$^{-/-}$* mice with the respective control animals. Mice received approximately 3×10^3 CFU i.n. Single dots represent the number of CFU of the respective organ from single animals. The horizontal line represents the mean. Pooled data from two independent experiments are shown.

of NADPH oxidase, which has been intensively studied with various experimental infections (3, 29, 51). NADPH oxidase comprises an important enzyme complex in macrophages as well as in neutrophils responsible for the production of reactive oxygen species (ROS). The fact that IFN-γ-activated *gp91phox$^{-/-}$* BMM exhibited only a slightly impaired capacity to combat intracellular bacteria but were still able to reduce the number of bacteria significantly indicated that ROS-independent mechanisms in C57BL/6 BMM are pivotal to eliminate the pathogen. These results are in clear contrast to the situation found with *Salmonella enterica* serovar Typhimurium, where C57BL/6 *gp91phox$^{-/-}$* macrophages were not able to eliminate the pathogen at all (51). However, at this stage of knowledge, we cannot exclude the fact that a differential NADPH oxidase activity might contribute to the enhanced anti-*B. pseudomallei* activity observed in C57BL/6 compared to BALB/c BMM.

Mice lacking subunits of NADPH oxidase comprise murine models to investigate chronic granulomatous disease (CGD), an inherited disorder of NADPH oxidase in which phagocytes are defective in generating ROS. CGD patients suffer from life-threatening infections, including bacterial infections caused by *Burkholderia cepacia* complex species and other *Burkholderia* species, which are highly virulent in these patients

(23, 38, 53). In line with these observations are clinical reports of CGD patients suffering from melioidosis (9, 37). Our in vivo experiments with C57BL/6 *gp91phox$^{-/-}$* mice revealed an important role of NADPH oxidase in resistance of this mouse strain against *B. pseudomallei* infection. Since neutrophils might also play a role in resistance against this bacterium, the increased susceptibility of C57BL/6 *gp91phox$^{-/-}$* mice might also be the result of a decreased bactericidal activity of neutrophils. In addition, NADPH oxidase has been shown to exhibit immune regulatory functions (13), which might contribute to its role in resistance against *B. pseudomallei* in vivo. A recent study with the related pathogen *Burkholderia cepacia* also revealed an essential role of NADPH oxidase for the early control of infection in C57BL/6 mice (41).

Besides the production of ROS, the generation of reactive nitrogen species has also been shown to be important in the control of various pathogens (5, 17, 43). A better outcome for C57BL/6 mice than for BALB/c mice after infection with high numbers of *Chlamydia* was shown to be highly dependent on enhanced NO production in C57BL/6 mice (17). The fact that macrophages from C57BL/6 mice were shown to produce enhanced levels of NO compared to BALB/c macrophages after infection with *Trypanosoma congolense* or lipopolysaccharide stimulation (21, 30) seemed to be a plausible hypothesis for

their increased anti-*B. pseudomallei* activity. Indeed, we could also observe increased NO production in C57BL/6 macrophages compared to that in BALB/c macrophages after infection with *B. pseudomallei* (data not shown), and previous studies described an increased intracellular survival of the pathogen in the NO-inhibited macrophage cell lines J774A.1 and RAW 264.7 (31, 49). Moreover, it has been shown that unlike other bacteria, *B. pseudomallei* can invade unstimulated RAW 264.7 cells without inducing the expression of iNOS, and it was suggested that this phenomenon represents a possible mechanism of *B. pseudomallei* to evade macrophage killing (49). Thus, surprisingly, our results obtained with C57BL/6 $iNOS^{-/-}$ BMM clearly show no essential role for iNOS-mediated mechanisms in eliminating *B. pseudomallei* in these cells over a period of 16 h. In addition, inhibition of NO release in C57BL/6 $gp91phox^{-/-}$ BMM by using aminoguanidine in order to create a reactive nitrogen intermediate and ROS double knockout did also not further impair intracellular elimination of *B. pseudomallei* (data not shown). We therefore suggest a major role for both reactive nitrogen intermediate- and ROS-independent killing mechanisms in C57BL/6 macrophages to combat infection with *B. pseudomallei*. The absence of any measurable reduction of *B. pseudomallei* killing in C57BL/6 $iNOS^{-/-}$ macrophages is different from the situation seen after infection of such cells with *S. enterica* serovar Typhimurium, where the ability to kill the pathogens was strongly reduced within 14 h (51). The discrepancy of our results obtained with C57BL/6 $iNOS^{-/-}$ BMM and results from the literature using *B. pseudomallei*-infected BALB/c-derived cell lines might be explained by differences in cell functions between primary cells and tumor cell lines or could be due to the existence of redundant resistance mechanisms in C57BL/6 BMM which can compensate for the loss of iNOS and do not exist in BALB/c macrophages. In agreement with results from the in vitro experiments, our in vivo results showed that there is no role for iNOS in early resistance to *B. pseudomallei* in C57BL/6 mice but indicate a detrimental role of this enzyme in the early phase of infection. C57BL/6 $iNOS^{-/-}$ mice seemed to be slightly protected from infection, since bacterial burden in spleen, liver, and lung after 48 h was significantly lower in the knockout mice. A similar phenomenon was also observed in the *Chlamydia* mouse model when NO production in C57BL/6 mice was inhibited and in C57BL/6 $iNOS^{-/-}$ mice infected with *Mycobacterium avium* (12, 17). The fact that NO beside direct bactericidal functions was shown to have an inhibitory effect on IFN-γ production (45), can have damaging effects on tissues (28), and might also directly inhibit NADPH oxidase (11, 18) could be an explanation for the observed phenotype. There is also no role for iNOS in the early control of *B. cepacia* infection in C57BL/6 mice (41). However, we cannot exclude a crucial function for iNOS at later time points during the chronic stage of murine melioidosis.

In conclusion, we provided evidence for an essential function of macrophages in the early phase of *B. pseudomallei* infection in mice. We could further show that NADPH oxidase-mediated mechanisms contribute to early resistance in C57BL/6 mice whereas iNOS is not essential. Finally, our results suggest that differences in bactericidal functions between BALB/c and C57BL/6 macrophages in the response to IFN-γ might contribute to the resistance pattern observed during the early course of *B. pseudomallei* infection. Future studies comparing the early gene expression patterns of BALB/c and C57BL/6 BMM after infection with *B. pseudomallei*, followed by the knockdown of selected genes, will possibly reveal previously unknown IFN-γ-mediated innate immune mechanisms against this intracellular pathogen.

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