Inactivation of [Fe-S] Metalloproteins Mediates Nitric Oxide-Dependent Killing of *Burkholderia mallei*

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Abstract

**Background:** Much remains to be known about the mechanisms by which O₂-dependent host defenses mediate broad antimicrobial activity.

**Methodology/Principal Findings:** We show herein that reactive nitrogen species (RNS) generated by inducible nitric oxide (NO) synthase (iNOS) account for the anti-*Burkholderia mallei* activity of IFNγ-primed macrophages. Inducible NOS-mediated intracellular killing may represent direct bactericidal activity, because *B. mallei* showed an exquisite sensitivity to NO generated chemically. Exposure of *B. mallei* to sublethal concentrations of NO upregulated transcription of [Fe-S] cluster repair genes, while damaging the enzymatic activity of the [Fe-S] protein aconitase. To test whether [Fe-S] clusters are critical targets for RNS-dependent killing of *B. mallei*, a mutation was constructed in the NO-induced, [Fe-S] cluster repair regulator *iscR*. Not only was the *iscR* mutant hypersusceptible to iNOS-mediated killing, but its aconitase pool was readily oxidized by NO donors as compared to wild-type controls. Although killed by authentic H₂O₂, which also oxidizes [Fe-S] clusters, *B. mallei* appear to be resilient to NADPH oxidase-mediated cytotoxicity. The poor respiratory burst elicited by this bacterium likely explains why the NADPH oxidase is nonessential to the killing of *B. mallei* while it is still confined within phagosomes.

**Conclusions/Significance:** Collectively, these findings have revealed a disparate role for NADPH oxidase and iNOS in the innate macrophage response against the strict aerobe *B. mallei*. To the best of our knowledge, this is the first instance in which disruption of [Fe-S] clusters is demonstrated as cause of the bactericidal activity of NO congeners.

Introduction

The gram negative, nonmotile *B. mallei* bacillus is the causative agent of glanders, a disease that can be transmitted to humans upon cutaneous, mucosal or aerosol exposure to mucopurulent discharge from the eyes, nose and lips of infected solipeds [1]. The clinical presentation of glanders is characterized by an acute or chronic suppurative syndrome involving the upper and lower respiratory tract. If untreated, the local signs of glanders often evolve into full-blown sepsis, multiorgan system failure and shock. The poor understanding of the pathogenesis of glanders, the severity and clinical diversity of the infection and a lack of vaccines against these bacteria [8–12]. The importance that the NADPH oxidase may also contribute to the host cell arsenal by promoting the release of cationic proteases from the proteoglycan matrix into the phagosomal lumen [4]. Professional phagocytes independently use O₂ and NADPH in the oxidation of L-arginine with the consequent generation of L-

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To the best of our knowledge, this is the first instance in which disruption of [Fe-S] clusters is demonstrated as cause of the bactericidal activity of NO congeners.

Recent investigations have demonstrated that members of the genus *Burkholderia* survive within mononuclear phagocytes [7], prompting an interest in the role of ROS and RNS in defense against these bacteria [8–12]. The importance that the NADPH oxidase plays in resistance to some *Burkholderia* spp. is made evident by the fact that *B. cepacia* is the second most lethal infection in chronic granulomatous disease patients carrying mutations in
membrane-bound or cytosolic components of the NADPH oxidase [13]. The clinical importance of NADPH oxidase in resistance to *B. cepacia* has been recreated in p47phox-deficient mice [14]. The NADPH oxidase has also been linked to the anti-*B. pseudomallei* arsenal of macrophages [12]. In contrast to ROS, current investigations on NO-related anti-*Burkholderia* activity have been rather controversial. Experimental animal models and macrophage cell cultures have shown that iNOS is dispensable for innate host defense against *B. cepacia* and *B. pseudomallei* [12,14]. Moreover, others have shown that the greatest *Burkholderia* growth occurs during maximal NO synthesis [8]. Inhibition of iNOS mRNA transcription appears to underlie the lack of a role of RNS against *B. pseudomallei* [9,10]. On the other hand, IFNγ can enhance NO-mediated intracellular killing of *B. pseudomallei* [11]. Recently, it has been reported that *B. mallei* is susceptible to NO generated by macrophages [15], although the mechanisms of antimicrobial activity remain unclear. Similarly, it remains unclear whether the NADPH oxidase plays a role in resistance to the intracellular pathogen *B. mallei*. The major goal of this study was to characterize the contribution of NADPH oxidase and iNOS hemoproteins to the macrophage antimicrobial arsenal against *B. mallei*.

**Methods**

**Bacterial Strains and genetic manipulations**

*B. mallei* strain ATCC 23344 was used in these studies (table 1).

| Strains/Plasmids | Relevant properties | Description | Source |
|------------------|---------------------|-------------|--------|
| *B. mallei* ATCC 23344 | Wild type | Isolated in 1944 from a human case of glanders: Gm\(^{+}\), Pb\(^{+}\) | [50] |
| *B. mallei* Mo126.2 | iscr mutant | bma1709-pMO126 | This study |
| S. Typhimurium BC696 | flic\(^{-}\) fjb\(^{-}\) | SL1344 Δflic Δfjb | Laboratory stock |
| DH5\(\alpha\) | DH5\(\alpha\) harboring pRK2013 | F\(^{+}\) phoA1 lacZΔM15 endA1 recA1 hsdS68 (K\(^{-}\) mK\(^{+}\)) supE44 thi-1 Δ(gyrA96 relA13) lacZΔM15 | Stratagene |
| JM109 | Cloning strain harboring pMO79 & pMO126 | e14 (McrA\(^{+}\) recA1 endA1 gryA96 thi-1 hsdR17 (R\(^{k}\) mK\(^{+}\)) supE44 relA1 Δ(lac-proAB) [p18tr d36 proAB lacF27 damC15] | Stratagene |
| pRK2013 | Helper plasmid | RK2 derivative, Kan\(^{+}\) mob\(^{+}\) tra\(^{+}\) ColE1 | [16] |
| pMO79 | Mobilizable replicative plasmid | oriT\(_{Bma1709}\) Kan\(^{+}\) mob\(^{+}\) gfp rfp | Hamad & Voskuil |
| pMO90 | Mobilizable suicide plasmid | ColE1 Kan\(^{+}\) mob\(^{+}\) gfp xylE | Hamad & Voskuil |
| pMO126 | Mobilizable disruption plasmid | pMO90 carrying an internal fragment of the *B. mallei* iscr homologue | This study |

Gm\(^{+}\) = gentamicin sensitive. 
Pb\(^{+}\) = polymixin B resistant. 
Km\(^{+}\) = kanamycin resistant.

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Macrophages

C57BL/6 and congenic iNOS\(^{-/-}\) [18] or gp91phox\(^{-/-}\) [19] mice were bred in our animal facility according to Institutional Animal Care and Use Committee guidelines. Peritoneal macrophages were harvested from mice 4 days after intraperitoneal inoculation of 1 mg/ml sodium periodate as described [20]. The peritoneal exudate cells were resuspended in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD), 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich), and 100 U/ml penicillin/streptomycin (Cellgro) (RPMI\(^{+}\) medium). The RPMI\(^{+}\) medium used to culture the THP-1 cell line was supplemented with 50 \(\mu\)M 2-mercaptoethanol. The peritoneal exudate cells were seeded in flat-bottom 96-well plates for macrophage killing assays at a density of 2\(\times\)10\(^{5}\) cells per well. The macrophages were selected by adherence after 24 h of culture at 37°C in a 5% CO\(_2\) incubator. Murine J774 (clone ATCC TIB-67) and AMJ2-C11 (clone ATCC CRL-2456) and human U937 (clone ATCC CRL-1593.2) and THP1 (clone ATCC TIB-202) macrophage-like cells grown in RPMI\(^{+}\) medium supplemented as described above were used as additional sources of mononuclear phagocytes. Selected groups of macrophages were treated with 200 U/ml of IFNγ (Life Technologies, St. Paul, MN) overnight prior to *Burkholderia* infection. Just prior to infection the macrophages were washed with pre-warmed RPMI without antibiotics.

**Macrophage killing assays**

The macrophage killing capacity was quantified by a gentamicin protection assay following a modified protocol described for
Salmonella [21]. B. mallei were grown overnight in LBG broth and sub-cultured to OD_{600} of 0.6 as described above. The bacteria were spun down for 5 min at 1500 RPM onto the macrophages at an MOI of 200 after opsonization in 10% normal mouse serum in RPMI medium. Unless specified, extracellular bacteria were removed from the monolayers after a 2 h incubation by washing with pre-warmed RPMI medium containing 6 μg/mL of gentamicin (Sigma-Aldrich). After washing the noninternalized bacteria, the MOI was determined to be 10. Burkholderia-infected macrophages were lysed with 1% Triton X-100 in phosphate buffered saline (PBS) at the indicated times after challenge. The intracellular bacteria recovered at various points after infection were enumerated on LB agar plates. The % survival was calculated as (cfu_tn/cfu_t0)×100.

Superoxide anion determination

O_{2}^{-} was quantified by the superoxide dismutase–inhibitable reduction of ferricytochrome c [22]. The macrophages were infected at an MOI of 40 with Burkholderia resuspended in phenol red–free Earle’s balanced salt solution containing 60 μM ferricytochrome c. Because B. mallei is aflagellated, a strain of Salmonella enterica serovar Typhimurium deficient in fliC, fljB was used as a positive control. After 1 h incubation in 5% CO_{2} at 37°C, the OD of the supernatants was determined spectrophotometrically at 550 nm. The concentration of O_{2}^{-} was calculated by using an ε_{550} of 2.1×10^{3} M^{-1} cm^{-1}. All reagents were purchased from Sigma-Aldrich.

Nitrite measurement

The concentration of nitrite (NO_{2}^{-}) produced by Burkholderia-infected macrophages 6 h post-challenge was estimated spectrophotometrically at 550 nm in a Biotek Synergy HT-2 reader after mixing culture supernatants with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphthylethylenediamine hydrochloride in 2.5% phosphoric acid). The NO_{2}^{-} concentration was calculated by regression analysis using a NaNO_{2} standard curve.

Susceptibility to RNS and ROS in vitro

Overnight cultures of B. mallei diluted 1:100 in LBG broth were grown at 37°C with shaking to OD_{600} of 0.6. Bacteria were concentrated by centrifugation and the pellets resuspended in PBS. The bacteria were diluted 1:100 in PBS and incubated with spermine NONOate (Cayman Chemical, Ann Arbor, MI), H_{2}O_{2} (ThermoFisher Scientific, Waltham, MA), or spermine (Sigma-Aldrich, St. Louis, MO) at 37°C for 2 min, cooled 5 min at 25°C and plated on pre-hybridized Burkholderia array slides (Colorado State University RM-RCE Genomics Core). Hybridization was conducted in standard hybridization chambers overnight in a 42°C water bath and washed with 1× SSC and 0.05% SDS, followed by two washes in 0.06× SSC. The slides were scanned using a GenePix 4000B scanner. Initial data analysis was conducted using GenePix Pro software, followed by analysis using Microsoft Excel as described [23].

Electron microscopy

The phagocytes were plated as described above for killing assays at a density of 4×10^{5} cells per chamber of a 8-well Permanox Labtek chamber slide system (Nalgene Nunc International, Rochester, NY). The macrophages were challenged with B. mallei at an MOI of 10 as described above. Between 2 and 3.5 h after infection, the cells were fixed in 2.5% glutaraldehyde in phosphate buffer, pH 7.4. The specimens were postfixed in 1% osmium tetroxide, treated with uranyl acetate, dehydrated in ascending ethanol series, and infiltrated with Embed 812. Ultrathin sections were examined in a FEI Tecnai 62 electron microscope operated at 80 kV.

Aconitase enzymatic assay

Aconitase activity is expressed as ΔOD_{240}/min/μg protein.

Statistical analysis

Data are presented as mean ± SEM or in box-and-whiskers plots as median, intraquartile and total ranges. A two-tailed,
Student’s t-test was used for statistical analysis and the data considered statistically significant when $p<0.05$.

Results

Survival of *B. mallei* in human and murine cell lines

The number of log phase *B. mallei* internalized by J774 cells increased as a function of time, reaching approximately $10^6$ CFU/10^5 macrophages 2 h after infection. Because of this delay in phagocytosis, all subsequent *Burkholderia* infections, unless indicated, were carried out for 2 h before gentamicin was added to the medium. Mice offer a wide variety of genetic reagents that facilitate investigations of O$_2$-dependent host defenses in host-pathogen interactions. Therefore, we deemed it important to compare the intracellular growth of *B. mallei* in a number of murine (J774 and alveolar AMJ-C11 macrophages) and human (i.e., THP1 and U937) cell lines (fig. 1). Remarkably, all murine and human macrophage-like cell lines tested killed *B. mallei* with very similar kinetics. These findings indicate that murine macrophages are a relevant model in which to study *B. mallei*-phagocyte interactions.

**IFN-$
\gamma$ enhances killing of *B. mallei* by macrophages**

It has been shown that IFN-$
\gamma$ enhances the antimicrobial activity of macrophages against the opportunist pathogen *B. pseudomallei*. However, to our knowledge, no data are available on the role that IFN-$
\gamma$ plays in intracellular resistance to *B. mallei*. Because IFN-$
\gamma$ has recently been shown to be critical for resistance of mice to *B. mallei* [24], we tested the effect of IFN-$
\gamma$ treatment on the anti-*Burkholderia* activity of J774 cells (fig. 2). IFN-$
\gamma$-primed macrophages were significantly ($p<0.01$) more efficient at killing *B. mallei* than unstimulated controls. The number of *B. mallei* recovered from IFN-$
\gamma$-treated macrophages was at least 10-fold lower than controls (fig. 2). In some cases, IFN-$
\gamma$-primed J774 cells completely eliminated the bacteria from the cell cultures. These data demonstrate that IFN-$
\gamma$ enhances the innate resistance of murine macrophages to *B. mallei*.

**Reactive oxygen and nitrogen species differentially contribute to the anti-*B. mallei* activity of IFN-$
\gamma$-primed macrophages**

ROS and RNS are key components of the antimicrobial arsenal of the innate and the activated response of macrophages against a variety of intracellular pathogens [25]. To test the importance of ROS and RNS in the killing of *B. mallei*, macrophages were obtained from C57BL/6 mice and their congenic gp91-phox$^{-/-}$, iNOS$^{-/-}$ and gp91-phox$^{-/-}$/iNOS$^{-/-}$ immunodeficient strains. Unstimulated macrophages isolated from C57BL/6 mice killed over 99% of *B. mallei* within 5 h of infection (fig. 3A). Strikingly, singly or doubly immunodeficient macrophages lacking gp91-phox and/or iNOS hemoproteins exhibited similar bactericidal activity as that of unstimulated wild-type controls. These findings suggest that neither ROS nor RNS appear to play a significant role in the early killing of *B. mallei* by unstimulated macrophages. Similar to the improved killing seen in the J774 cell line, IFN-$
\gamma$ increased ($p<0.001$) the anti-*B. mallei* activity of primary macrophages isolated from C57BL/6 mice. It should be noted that some of the IFN-$
\gamma$-primed macrophages completely eliminated *B. mallei* from the cultures, a phenomenon that was seen in all of the IFN-$
\gamma$-primed macrophages. Conversely, RNS contributed to the anti-*B. mallei* arsenal of IFN-$
\gamma$-activated macrophages (fig. 3B). In fact, this intracellular pathogen was recovered in significantly higher numbers from both IFN-$
\gamma$-primed, iNOS$^{-/-}$ and iNOS$^{-/-}$/gp91-phox$^{-/-}$ macrophages than from wild-type or gp91-phox$^{-/-}$-controls. The killing activity of IFN-$
\gamma$-treated, iNOS- or iNOS/ gp91-phox-deficient macrophages was similar to that of unstimulated wild-type controls. Collectively, these data indicate that RNS but not ROS mediate the anti-*Burkholderia* activity of IFN-$
\gamma$-primed macrophages.

**Anti-*Burkholderia* activity of IFN-$
\gamma$-primed macrophages**

The intracellular survival of *B. mallei* was assessed in control and IFN-$
\gamma$-primed J774 cells. Selected groups of macrophages were treated with 200 U/ml IFN-$
\gamma$ 16 h before infection. Data from 6 independent observations gathered on 2 separate experiments are represented in box-and-whiskers plots as median, intraquartile and total ranges.

*P*<0.01 compared to unstimulated control macrophages.

![](image-url)

**Figure 1. Antimicrobial activity of murine and human macrophage-like cell lines against *B. mallei*** Intracellular survival of *B. mallei* was studied over a 20 h period in murine (i.e., J774 and AMJ) and human (i.e., THP1 and U937) macrophage-like cell lines. Data from 8–14 independent observations gathered in 3 separate experiments are represented in box-and-whiskers plots as median, intraquartile and total ranges.

**Figure 2. Anti-*Burkholderia* activity of IFN-$
\gamma$-activated macrophages.** The intracellular survival of *B. mallei* was assessed in control and IFN-$
\gamma$-primed J774 cells. Selected groups of macrophages were treated with 200 U/ml IFN-$
\gamma$ 16 h before infection. Data from 6 independent observations gathered on 2 separate experiments are represented in box-and-whiskers plots as median, intraquartile and total ranges. *P*<0.01 compared to unstimulated control macrophages.
infection was tested by measuring the accumulation of NO$_2^-$. Supernatants of  B. mallei-infected J774 cells contained about 200 µM NO$_2^-$ after 5 h of infection (fig. 4A). The concentration of NO$_2^-$ seen in B. mallei-infected cells closely paralleled the NO$_2^-$ levels contained in RPMI medium, raising the possibility that a bacterial nitrate reductase activity is responsible for the observed NO$_2^-$ burst. This idea was substantiated by the fact that the putative  B. mallei nitrate reductase activity could be inhibited by the flavoprotein inhibitor diphenyleneiodonium (fig. 4A). To determine whether host cell iNOS activity can be stimulated upon  B. mallei challenge, the capacity of macrophages to generate NO$_2^-$ was therefore estimated in NO$_3^-$-free medium. As shown in figure 4B, IFN$_γ$-primed macrophages, but not unstimulated controls, generated copious amounts of NO congeners in response to  B. mallei. Accumulation of NO$_2^-$ in the supernatants reflects a functional iNOS enzymatic complex, since the amount of NO$_2^-$ generated by iNOS-deficient macrophages was negligible. Figure 4C shows that  B. mallei are extraordinarily susceptible to RNS. Spermine NONOate reduced the viability of  B. mallei in a dose and time dependent manner (fig. 4C & D). The viability of  B. mallei was significantly reduced after a 4 h exposure to as low as 10 µM of the NO donor spermine NONOate (fig. 4C). A time course indicated that the killing of  B. mallei by 100 µM spermine NONOate was already evident after 30 min and continued unabated for at least 2 h (fig. 4D). The anti-  B. mallei effects associated with spermine NONOate are specific to RNS generated by this NO donor, since the viability of  B. mallei was similarly unaffected upon culture in PBS or exposure to the spermine base control.

**NO enhances transcription of genes associated with [Fe-S] cluster assembly**

To gain insight into the targets of RNS antimicrobial activity, global transcriptional profiles were compared between  B. mallei cultures untreated or treated with sublethal NO concentrations. Microarray expression analyses showed the transcriptional up-regulation of gene products involved in iron acquisition and [Fe-S] cluster assembly and repair (table 2). This pattern of transcription suggests that [Fe-S] clusters of dehydratases are critical  B. mallei targets of RNS-mediated cytotoxicity. Accordingly, the enzymatic

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**Figure 3. Contribution of reactive oxygen and nitrogen species to the anti-Burkholderia activity of macrophages.** The contribution of ROS and RNS to host defense against  B. mallei was assessed by comparing the antimicrobial activity of primary macrophages isolated from immunocompetent C57BL/6 mice (control) or congenic phox$^{-/-}$, iNOS$^{-/-}$ or phox$^{-/-}$/iNOS$^{-/-}$-immunodeficient mice. Selected groups of macrophages were treated with 200 U/ml IFN$_γ$ 16 h before infection. The number of  B. mallei recovered from the macrophages was estimated 5 h after the infected macrophages were treated with gentamicin. Data from 16 independent observations gathered on 3 separate experiments are represented in box-and-whiskers plots as median, intraquartile and total ranges. *P<0.05 compared to unstimulated control macrophages. **P<0.05 compared to IFN$_γ$-primed macrophages from C57BL/6 mice.

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**B. mallei is susceptible to RNS**

The poor anti-Burkholderia activity of IFN$_γ$-primed macrophages lacking iNOS suggest that RNS generated by activated phagocytes exert early antimicrobial activity against  B. mallei. Consequently, the ability of macrophages to produce NO in response to  B. mallei was assessed in low NO$_3^-$-producing capacity of macrophages responding to  B. mallei was assessed in low NO$_3^-$ DMEM medium (B). The survival of  B. mallei to increasing concentrations of the NO donor spermine NONOate is shown in C. The effects of 100 µM spermine NONOate on the viability of  B. mallei was studied overtime (D). Untreated or spermine-treated  B. mallei were used as controls. Data in panel A are represented in box-and-whiskers plots as median, intraquartile and total ranges. The rest of the data represent the mean ± sem.

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**Figure 4. Antimicrobial activity of reactive nitrogen species generated by Burkholderia-infected macrophages.** The ability of  B. mallei to generate NO$_2^-$ in RPMI medium is shown in A. Selected samples were treated with 10 µM of the flavin inhibitor diphenyleneiodonium (DPI). The NO$_2^-$-producing capacity of macrophages responding to  B. mallei was assessed in low NO$_3^-$ DMEM medium (B). The survival of  B. mallei to spermine NONOate on the viability of  B. mallei was studied overtime (D). untreated or spermine-treated  B. mallei were used as controls. Data in panel A are represented in box-and-whiskers plots as median, intraquartile and total ranges. The rest of the data represent the mean ± sem.

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activity of the [Fe-S] cluster protein aconitase was markedly reduced in B. mallei cultures treated with spermine NONOate. For instance, about 100 pmoles of NO per 10^6 bacteria completely inactivated the activity of aconitase (fig. 5A). To test more directly whether [Fe-S] clusters are a key target of the anti-B. mallei activity of RNS, an insertion mutant was constructed in the iscR transcriptional factor that regulates [Fe-S] cluster assembly. The lack of iscR increased the susceptibility of B. mallei to RNS generated by the NO donor spermine NONOate (fig. 5B). We next tested whether the iscR mutant is susceptible to NO produced by activated macrophages. Because wild-type B. mallei is already highly susceptible to the iNOS-mediated antibacterial activity of IFNγ-primed macrophages after 5 h of infection (fig. 3B), the survival of wild-type and iscR-deficient B. mallei was compared after 2 h of challenge. As anticipated (fig. 5B), at this early time the iscR mutant was more susceptible than wild-type controls to the antimicrobial activity of IFNγ-primed macrophages (fig. 5C). The difference in intracellular survival of wild-type and iscR-deficient bacteria was not as great in IFNγ-primed macrophages lacking iNOS compared to those containing iNOS (fig. 5C). These data suggest that iscR mutant bacteria are hypersusceptible to the RNS generated by the host cells. The aconitase activity of the iscR mutant grown overnight in LBG medium was similar to that of wild-type cultures (fig. 5D). However, the enzymatic activity of aconitase was more vulnerable to RNS-mediated inactivation in the absence of a functional iscR. Together, these findings are consistent with the notion that [Fe-S] clusters are key targets of the RNS-dependent bactericidal activity against B. mallei.

Figure 5. *iscR*-deficient B. mallei is hypersusceptible to NO-mediated cytotoxicity. The aconitase activity of B. mallei exposed to spermine NONOate (NO) for 30 min can be seen in panel A. Panel B shows the cytotoxicity of 10 μM spermine NONOate against wild-type (WT) or *iscR*-deficient B. mallei. The intracellular survival of WT and isogenic *iscR*-deficient B. mallei was recorded in IFNγ-primed macrophages isolated from wild-type C57BL/6 mice (B6) or iNOS-deficient congenic controls (C). The % survival after 2 h of infection was estimated according to the number of bacteria originally added to the macrophages. The aconitase activity present in overnight cultures of wild-type or *iscR*-deficient B. mallei is shown in D. Selected groups of bacteria were treated with 500 μM spermine NONOate for 30 min before the aconitase activity present in cytoplasmic extracts was recorded. Panels A & D represent data of two independent experiments. Data in panel B & C are the mean ± sem from 4–8 independent observations.

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Table 2. Iron-sulfur cluster assembly genes induced by nitric oxide.

| Gene No. | Gene | Ratio | St.Dev. | Protein Function | Pathway  |
|----------|------|-------|---------|------------------|----------|
| BMA2033  |      | 2.7   | 1.4     | iron compound ABC transporter | iron assimilation |
| BMA0021  |      | 4.6   | 1.3     | siderophore-interacting protein | iron assimilation |
| BMAA0883 |      | 4.5   | 2.7     | iron permease, FTTR1 family | iron assimilation |
| BMAA1800 |      | 14.1  | 3.0     | putative bacterioferritin-associated ferredoxin | iron assimilation |
| BMAA0666 | cystD-1 | 3.5   | 0.7     | sulfate adenylyltransferase, subunit 2 | sulfur assimilation |
| BMAA0667 | cystN  | 4.3   | 0.5     | sulfate adenylyltransferase, subunit 1 | sulfur assimilation |
| BMAA2729 |      | 2.2   | 0.4     | sulfur carrier protein | sulfur assimilation |
| BMAA0610 |      | 3.8   | 1.7     | cysteine desulfurase | [Fe-S] assembly |
| BMAA1706 |      | 3.3   | 0.1     | [Fe-S] assembly accessory protein | [Fe-S] assembly |
| BMAA1707 |      | 2.3   | 0.7     | [Fe-S] assembly scaffold | [Fe-S] assembly |
| BMAA1708 | isc-1 | 3.9   | 0.5     | cysteine desulfurase | [Fe-S] assembly |
| BMAA1709 | iscR  | 6.9   | 2.9     | [Fe-S] assembly transcription factor | [Fe-S] assembly |
| BMAA1430 |      | 2.0   | 0.5     | [Fe-S] binding protein | [Fe-S] assembly |

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NO killing of *B. mallei*
*B. mallei* is resistant to the oxidative burst of macrophages despite its marked susceptibility to ROS

We investigated the possible mechanism for the apparent dispensability of the NADPH oxidase in the intracellular killing of *B. mallei*. *B. mallei* was found to be susceptible to authentic H$_2$O$_2$ (fig. 6A). As little as 100 μM H$_2$O$_2$ killed over 90% of *B. mallei* after 2 h. A kinetic study revealed that the viability of *B. mallei* was markedly reduced 90 min upon exposure to 200 μM H$_2$O$_2$, declining further thereafter (fig. 6B). These findings demonstrate that *B. mallei* are extraordinarily susceptible to ROS-mediated killing. The ability of *Burkholderia* to escape into the cytosol [26,27] could rationalize the apparent dispensability of NADPH oxidase in the anti-*B. mallei* arsenal of macrophages.

To test the intracellular localization of *B. mallei*, the ability of the bacterium to form actin-tails was evaluated by fluorescence microscopy. As shown in figure 6C–E, *B. mallei* do not appear to polymerize actin tails at the early times examined here, although polymerization of actin was evident in about 10% of the *B. mallei*-containing phagosomes (figure 6E). Electron microscopy studies revealed that the totality of *B. mallei* were found within phagosomes in both unstimulated and IFNγ-primed macrophages (fig. 6F & G). Collectively, the fluorescence and electron microscopy studies indicate that *B. mallei* remain within the confines of phagosomal membranes during the early stages of infection studied herein, irrespective of whether the phagocytes are stimulated with IFNγ (fig. 6F & G). We tested whether the seeming dispensability of the NADPH oxidase in the anti-*B. mallei* activity of macrophages was due to a poor respiratory burst. Estimates of O$_2^{-}$ production by the superoxide dismutase-inhibitable reduction of cytochrome c revealed that *B. mallei* was a poor stimulant of the respiratory burst as compared to an aflagellated *B. mallei* Salmonella control (fig. 6H).

**Discussion**

O$_2$-dependent host defenses are the best-characterized components of the antimicrobial arsenal of professional phagocytes. O$_2$ is used by NADPH oxidase and iNOS hemoproteins for the generation of O$_2^{-}$ and NO, which are the precursors of a plethora of ROS and RNS. Pathogenic microorganisms show a spectrum of susceptibilities to the antimicrobial actions of RNS. For example, RNS are essential for resistance to *Mycobacterium tuberculosis* and *Leishmania major* [12,28], but appear to be dispensable in host defense against *Legionella pneumophila* and *Pseudomonas aeruginosa* [25]. Similar to *Mycobacterium* and *Leishmania*, our investigations have shown that the intracellular pathogen *B. mallei* is highly susceptible to RNS-mediated host defenses. We find

Figure 6. Susceptibility of *Burkholderia* to ROS generated by macrophages. The susceptibility of *B. mallei* to increasing concentrations of H$_2$O$_2$ is shown in A. Kinetic analysis of the viability of *B. mallei* in response to 200 μM H$_2$O$_2$ is shown in B. The cellular localization of phallloidin-labeled actin filaments (C) and GFP-expressing *B. mallei* (D) was examined by fluorescence microscopy. The host cell nucleus labeled with DAPI (blue), *B. mallei* (green) and actin (red) are visualized in the merge panel (E). The micrograph is representative of a total of 285 bacteria examined from 33 cells collected in 2 independent experiments. The location of *B. mallei* in the cytoplasm of control (F) and IFNγ-primed (G) macrophages from C57BL/6 mice was visualized by transmission electron microscopy 3.5 h after infection. Bars in F and G denote 2 and 0.5 μm, respectively. The micrographs are representative of 97 *Burkholderia*-containing phagosomes from 5 independent experiments. Production of superoxide by *B. mallei*-infected macrophages isolated from C57BL/6 mice was assessed spectrophotometrically as the superoxide dismutase-inhibitable reduction of cytochrome c (H). Data in panels A, B and G represent the mean ± sem from 6 independent observations.

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that the inactivation of [Fe-S] clusters is a major component of the anti-\textit{B. mallei} activity of RNS.

Poor killing of \textit{B. mallei} by IFN-\gamma-treated macrophages lacking iNOS, irrespective of the NADPH oxidase status of the cell, suggests that the RNS-mediated killing of \textit{B. mallei} does not depend on host-derived ONOO− synthesis. NO itself, nitrogen oxides derived from the autoxidation of NO in the presence of O2, or ONOO− generated in the bacterial cytoplasm from the reaction of NO with endogenous O2− represent a few of the RNS that may be responsible for the iNOS-dependent killing of \textit{B. mallei}. \textit{B. mallei} are extraordinarily susceptible to RNS as shown by the profound killing seen after treatment with 10 \textmu{}M spermine NONOate. NO donors also exert antimicrobial activity against phylogenetically diverse microbes such as \textit{Candida albicans}, \textit{Salmonella enterica} and \textit{Escherichia coli} [29–31]. However, NO donors, even when used at high micromolar or low millimolar concentrations, are cytostatic for these microbial pathogens. Therefore, our studies identify \textit{B. mallei} as one of the few pathogens for which host-derived RNS are microbicidal.

Aconitase is inactivated upon incubation of \textit{B. mallei} with NO congeners, possibly reflecting the RNS-dependent oxidation of the solvent exposed Fea in the [Fe-S] cluster [32,33]. It is therefore not surprising that genes encoding for iron-sulfur cluster assembly are highly transcribed in NO-treated \textit{B. mallei}. RNS-mediated oxidation of [Fe-S] clusters of dehydratases [33–36] has for a long time been seen as a concomitant event of cytotoxic, NO-producing macrophages [32]. However, a causative relationship between the oxidation of [Fe-S] clusters and killing by RNS has not been previously demonstrated. The rapid loss in viability and aconitase activity of an \textit{iscR} mutant exposed to sublethal NO concentrations demonstrate a direct relationship between [Fe-S] cluster damage and the bactericidal activity of RNS against \textit{B. mallei}. Oxidation of [Fe-S] prosthetic groups of dehydratases critical for intermediate metabolism may explain the bactericidal activity of RNS against the obligate aerobe \textit{B. mallei}. According to our model, the overt resistance of facultative anaerobes to NO reflects the ability of these microbes to generate energy by switching from oxidative phosphorylation heavily dependent on RNS-modifiable dehydratases to fermentative pathways resilient to nitrosative stress. Future experiments will be needed to test this hypothesis.

Our data indicate that the NADPH oxidase is dispensable for intracellular killing of \textit{B. mallei}. These findings might seem striking since \textit{B. mallei} are readily killed upon exposure to H2O2. Microbes have devised an assortment of strategies to avoid NADPH oxidase-dependent cytotoxicity. For example, detoxification of oxyradicals by superoxide dismutases, catalases and hydroperoxydases or by superoxide dismutases, catalases and hydroperoxydases or scavengers such as low molecular weight thiols are efficient ways to diminish oxidative stress [6]. The antimicrobial activity of the NADPH oxidase is best manifested against intracellular pathogens confined within endocytic membranes [37,30]. At the early times examined here, \textit{B. mallei} do not escape into the cytosol as has been shown for various \textit{Burkholderia} spp. at later time points after infection [27,39]. The poor respiratory burst elicited by \textit{B. mallei} represents a mechanism for minimizing exposure to the NADPH oxidase during the time that this intracellular pathogen remains in the phagosome. Analogous to other microorganisms [40], the putative polysaccharide extracellular capsule [41] may help \textit{B. mallei} avoid signaling cascades that stimulate a productive respiratory burst. The resistance of \textit{B. mallei} to the NADPH oxidase-mediated antimicrobial activity of macrophages does not, however, eliminate the possibility that this hemoprotein may play a role in the purulent phase of the host response as has been shown for \textit{B. cepacia} and \textit{B. pseudomallei} [12,14].

O2−-independent mechanisms are a significant component of the innate killing of \textit{B. mallei} as indicated by the fact that macrophages from doubly immunodeficient mice lacking both NADPH oxidase and iNOS hemoproteins efficiently kill this bacterium. Innate activation of the autophagic pathway as described for the respiratory pathogen \textit{Legionella pneumophila} [42–44] is one possible mechanism for the O2−-independent killing of \textit{B. mallei} seen in macrophages lacking both gp91phox and iNOS. Recent studies have shown that small GTPases induced in response to IFN\gamma stimulate killing of \textit{M. tuberculosis} by promoting autophagosome formation [45,46]. Most of the enhanced antimicrobial activity stimulated by IFN\gamma against \textit{M. tuberculosis} and \textit{Salmonella enterica} is nonetheless associated with the production of RNS [21]. In contrast to these two intracellular pathogens, the enhanced killing of \textit{B. mallei} by IFN\gamma-primed macrophages is exclusively dependent on the expression of functional iNOS. This role for RNS in the bactericidal activity of IFN\gamma-primed macrophages against \textit{B. mallei} contrasts with the apparent dispensability of iNOS to the early innate host defenses of unstimulated macrophages. The short time course of the infection studied herein likely prevented innate production of nitrogen oxides in \textit{B. mallei}-infected macrophages. Given the extraordinary susceptibility of \textit{B. mallei} to RNS (studies herein) and the stimulation of TLR4 signaling by LPS purified from \textit{B. mallei} [47], it is not surprising that NO produced at later times of the innate response contributes to the killing of this intracellular pathogen [15].

In summary, our studies indicate that despite its susceptibility to ROS and RNS, \textit{B. mallei} are selectively killed by RNS generated by IFN\gamma-activated macrophages. These investigations place \textit{B. mallei} among the limited number of microorganisms for which NO congeners are microbicidal. Our biochemical and genetic studies indicate that [Fe-S] clusters are critical targets of RNS-mediated killing of \textit{B. mallei}. The extraordinary hypersusceptibility of \textit{B. mallei} to RNS raises the intriguing possibility that NO donors could be explored as antibiotics to treat glanders, as shown in the experimental treatment of cutaneous mycosis and leishmaniasis [40,49]. However, analogous to herpes simplex virus and toxoplasma (reviewed in 49), the possibility exists that despite exerting potent antimicrobial activity, NO could still exacerbate pathology. Future experiments will be needed to determine the value of NO donors in the treatment of glanders.

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Author Contributions

Conceived and designed the experiments: AV JJ. Performed the experiments: JJ JL AS. Analyzed the data: AV JJ MV. Contributed reagents/materials/analysis tools: MH. Wrote the paper: AV JJ MV JL MH.

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