The Intracellular Environment of Human Macrophages That Produce Nitric Oxide Promotes Growth of Mycobacteria

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Nitric oxide (NO) is a diffusible radical gas produced from the activity of nitric oxide synthase (NOS). NOS activity in murine macrophages has a protective role against mycobacteria through generation of reactive nitrogen intermediates (RNIs). However, the production of NO by human macrophages has remained unclear due to the lack of sensitive reagents to detect NO directly. The purpose of this study was to investigate NO production and the consequence to mycobacteria in primary human macrophages. We found that Mycobacterium bovis BCG or Mycobacterium tuberculosis infection of human macrophages induced expression of NOS2 and NOS3 that resulted in detectable production of NO. Treatment with gamma interferon (IFN-γ), l-arginine, and tetrahydrobiopterin enhanced expression of NOS2 and NOS3 isoforms, as well as NO production. Both of these enzymes were shown to contribute to NO production. The maximal level of NO produced by human macrophages was not bactericidal or bacteriostatic to M. tuberculosis or BCG. The number of viable mycobacteria was increased in macrophages that produced NO, and this requires expression of nitrate reductase. An narG mutant of M. tuberculosis persisted but was unable to grow in human macrophages. Taken together, these data (i) enhance our understanding of primary human macrophage potential to produce NO, (ii) demonstrate that the level of RNIs produced in response to IFN-γ in vitro is not sufficient to limit intracellular mycobacterial growth, and (iii) suggest that mycobacteria may use RNIs to enhance their survival in human macrophages.

Mycobacterium tuberculosis is one of the most successful human pathogens. There were approximately 8.8 million incident cases of tuberculosis worldwide in 2010, and nearly one-third of the world population has been infected (1). M. tuberculosis infection is typically acquired by inhalation of aerosolized bacteria. The bacteria then infect, survive, and proliferate in macrophages of the lung. The mycobacterial phagosome can resist maturation into phagolysosomes in unstimulated macrophages, allowing the bacteria to avoid the intracellular stress of this compartment (reviewed in references 2, 3, and 4). However, a greater understanding of the pathogenesis of mycobacteria in human macrophages is still necessary to improve current strategies to reduce the global incidence of tuberculosis.

Nitric oxide (NO) is a diffusible radical gas that is a product of nitric oxide synthase (NOS) activity that has antimicrobial action against a variety of pathogens, including viruses, bacteria, fungi, and protozoa (reviewed in references 5 and 6). Reactive nitrogen intermediates (RNIs) produced by murine cells have been established to provide protection against M. tuberculosis (7, 8). Induction of RNIs generated from l-arginine (l-Arg) in murine macrophages kills M. tuberculosis; this mycobactericidal activity is blocked by inhibitors of NOS2 (9). Similarly, murine macrophages that do not express a nos2 allele are unable to kill M. tuberculosis (10). Mice deficient for a nos2 allele or treated with a NOS inhibitor are impaired in the control of M. tuberculosis growth and succumb to infection (7, 11).

Three NOS isoforms have been described: neuronal (nNOS, or NOS1), endothelial (eNOS, or NOS3), and inducible (iNOS, or NOS2) (5). NOS2 is widely distributed in various cell types but was first cloned from human macrophages (5). Historically, it was thought that primary macrophages express only NOS2. However, NOS3 has been shown to be another source for NO in human macrophages; some have demonstrated constitutive NOS3 activity (12, 13, 14). All three isoforms of NOS catalyze the production of NO by the same biochemical pathway (5). One molecule of l-Arg is oxidized to produce the intermediate N⁶-oH-l-arginine that is further oxidized to yield NO and l-citrulline. NOSs must homodimerize to gain enzymatic activity, and the cofactor tetrahydrobiopterin (BH₄) is essential for this process (15).

There has been controversy regarding the production of NO by human macrophages. However, numerous references describe NOS expression (NOS2 and NOS3) and evidence of NO production in human macrophages (16, 17, 18, 19, 20). Nicholson and colleagues demonstrated that NOS2 was more frequently expressed in the lungs of patients with tuberculosis than in those of healthy controls (18). This observation is in line with increased levels of NO that are exhaled by patients with active tuberculosis compared with levels in healthy subjects (21). NO activity, indicated by nitrite and nitrate detection or conversion of l-Arg to l-citrulline in human peritoneal macrophages, was demonstrated but at substantially lower levels than that in murine J774 macrophages (20). Another study revealed that NOS2, NOS3, and nitro-
tyrosine (marker for NO production) were markedly increased in surgically resected human tuberculous lungs (22). Therefore, the human macrophage potential to produce NO as well as the influence during mycobacterial infection needs to be further clarified with reagents that can sensitively detect NO directly.

Bacterial nitrate reductases are important for nitrogen metabolism since all reductive branches of nitrogen cycles involve the conversion of nitrate to nitrite (23). Recently, it has been shown that Salmonella enterica serovar Typhimurium uses nitrate reductase to limit NO toxicity (24). Nitrate reduction by M. tuberculosis has been implicated in promoting survival during the interruption of aerobic respiration by hypoxic, acidic environment, and exposure to NO (25, 26, 27). M. tuberculosis and Mycobacterium bovis BCG (BCG) share 99.9% genetic identity (28). BCG also produces a nitrate reductase with high homology to that of M. tuberculosis. However, differences in reductase activity have been reported that are at least in part the result of differences in expression levels arising from promoter mutations (29). The mycobacterial nitrate reductase may be instrumental to adaption and survival in a harsh host environment.

Although there is evidence that supports NO production in human macrophages (19, 22), more detailed mechanistic information regarding the production of NO and its relation to mycobacteria is still needed. Here, we show that M. tuberculosis and BCG infection induced the expression of NOS2 and NOS3 in human macrophages. NOS2 expression was further increased by supplemental gamma interferon (IFN-γ) and BH4. These enzymes colocalized with the mycobacterium-containing phagosome. NO production was directly detected and dependent on NOS2 and NOS3 activity. Furthermore, NOS2, NOS3, and NO production in response to a nitrate reductase mutant of M. tuberculosis (ΔnarG strain) was markedly diminished.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Mycobacterium bovis Calmette-Guérin (BCG) was purchased from ATCC (Manassas, VA). Mycobacterium tuberculosis H37Rv and the corresponding narG mutant strain RVW1 (30) along with the complemented strain have been described previously (31). BCG was maintained in Middlebrook 7H9 broth supplemented with ADC (albumin, dextrose, and catalase) enrichment medium at 37°C with 5% CO2. M. tuberculosis and Mycobacterium bovis BCG (BCG) share 99.9% genetic identity (28). BCG also produces a nitrate reductase with high homology to that of M. tuberculosis. However, differences in reductase activity have been reported that are at least in part the result of differences in expression levels arising from promoter mutations (29). The mycobacterial nitrate reductase may be instrumental to adaption and survival in a harsh host environment.

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pattern recognition. Pearson’s correlation was employed to analyze colocalization between NOs and auramine O-stained M. tuberculosis or BCG cells since this analysis considers similarity between shapes. This ensures that colocalization is not the product of higher signal intensities (33).

**Quantitative real-time PCR.** Human macrophages (1.5 × 10^7/well) cultivated in 24-well dishes were treated as indicated in the figures and legends. At appropriate time points, medium was removed from cultures, the cells were lysed with PureZol (Bio-Rad), and RNA was isolated according to a commercial product protocol. First-strand cDNA synthesis was performed using iScript cDNA synthesis reagents (Bio-Rad) according to the manufacturer’s protocol, and iQ Supermix (Bio-Rad) was performed in triplicate using an iQ5 cycler (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. For narG gene expression analysis, primers were synthesized by Integrated DNA Technologies, Inc. The following primer sets were used for amplification of narG, narK2, or 16S rRNA transcripts with SsoFast EvaGreen supermix: NarG, 5′-TC CAAGTTGCTGTTTTCAC-3′ (forward) and 5′-CCCCCCATTTGTCGCGCA-3′ (reverse); NarK2, 5′-TGGTTCTGATGACCCACCTATTTGT-3′ (forward) and 5′-CCGCCGAAACAGATCGCCGTACAGAAA CGAC (reverse); BCG 16S RNA, 5′-GTTGGGGGAGCCGAACAGAT-3′ (forward) and 5′-TGGTTGCGGGGCGCCTGCA-3′ (reverse); MTB 16S rRNA, 5′-GGATTGACCGTAGTGAGGAGA-3′ (forward) and 5′-G TGAGATTTCCAGAAACCGGAC-3′ (reverse).

**Mycobacterial enumeration by 16S rRNA analysis.** To generate a standard curve for enumeration of bacteria, 2 × 10^2 cultures proliferated in 24-well plates were infected with known MOIs of 0, 0.1, 1, 10, and 100 of M. tuberculosis or BCG for 4 h. RNA was collected by lysis cells in PureZol from standards at 4 h or test samples from macrophage cultures treated and infected as indicated in the figures and legends. RNA concentrations were adjusted to 10 ng/μL and cDNA was synthesized as described above. Real-time PCR was performed for amplification of the 16S rRNA transcript corresponding to M. tuberculosis or BCG and human GAPDH as an internal reference. Changes in 16S rRNA were expressed relative to GAPDH and then normalized to medium alone. The amplification data for standards were plotted against the known number of bacteria. The amplification data for test samples were then extrapolated from the standard curve to determine actual CFU numbers.

**Mycobacterial enumeration by auramine O staining.** Macrophages were seeded at 2 × 10^5/well in 24-well plates. Infected and control cultures were treated as described in the figures and legends for the indicated time periods. Cells were fixed with 4% PFA for 30 min, washed with PBS three times, and air dried completely. A modified auramine O staining kit (SDL, IL) was used as described above. Fluorescence (Aex of 420 and Aem of 508 nm) was measured with a Synergy HT Multi-Mode Microplate Reader (Biotek, VT).

**Statistical analysis.** A Wilcoxon signed-rank test was used to compare sample groups in the experiment that analyzed the percent NO production. A Student’s t test was used to compare sample groups in all other experiments. P values smaller than 0.05 were considered statistically significant.

**RESULTS**

NO is produced by monocyte-derived human macrophages. Although murine macrophages are capable of high-output NO production (34), a greater controversy has surrounded demonstration of this ability in human macrophages. Since NO is highly diffusible and has a very short half-life, most of the methods that have been used to detect NO are indirect and insensitive. The compound DAF-2 DA is a membrane-permeable reagent, has a low detection limit (2 to 5 nM), and provides the opportunity for direct detection of intracellular NO (35). DAF-2 DA reaction with NO yields a green fluorescent signal that can be detected both qualitatively and quantitatively (16, 36). We wanted to determine NO production during infection and in response to a classical macrophage activation signal important in the host response to intracellular pathogens. Thus, human monocyte-derived macrophages were infected with BCG or treated with IFN-γ alone. After 48 h, samples were stained with DAF-2 DA and visualized by confocal microscopy (Fig. 1A). The mean fluorescence intensity (MFI) of each image was obtained by Zeiss confocal analysis software (Fig. 1B). Infection by BCG alone resulted in the production of detectable but low levels of NO (Fig. 1). Similarly, IFN-γ treatment alone induced detectable NO production (Fig. 1). The combination of IFN-γ treatment and BCG infection resulted in additive levels of DAF-2 DA staining indicative of NO (Fig. 1). Overall, these results suggest that NO is produced during mycobacterial infection and that the addition of IFN-γ significantly augments production.

Next, we wanted to address if there were limitations in human macrophages that prevent high-level synthesis of NO. We previously demonstrated constitutive arginase-1 protein expression in unstimulated human macrophages (37). A competition for pools of available substrate could curtail NO production. To address this possibility, 1-Arg (1 mM) was supplemented in macrophage cultures stimulated with IFN-γ and infected with BCG. The addition of 1-Arg did not further increase DAF-2 DA staining (Fig. 1A and B), suggesting that available substrate is not a limitation in the production of NO by human macrophages under these conditions. It has been suggested that human macrophages are limited in the ability to generate NO as a consequence of the inability to synthesize BH4 (38, 39). Therefore, BH4 (10 μM) was added to human macrophage cultures. This led to an increase in DAF-2 DA staining and an approximate 2-fold increase in MFI (Fig. 1A and B). This result suggests that there is an endogenous source of BH4 sufficient to generate NO. However, the availability of this essential cofactor is a limiting factor in the ability of human macrophages to generate high levels of NO.

The requirement for BH4 suggests the involvement of NOS isoforms in NO production. The expression of NOS2 and NOS3 has been reported in human macrophages (16, 17, 19, 20). To further define the involvement of NOS isoforms in NO production by human macrophages, chemical inhibitors of these enzymes were used. The macrophage cultures were treated with IFN-γ, infected with BCG, and supplemented with 1-Arg and BH4 for 48 h to maximally induce NO. The amount of fluorescent DAF-2 DA signal obtained with this treatment was set as a 100% control. The amount of signal obtained in the presence of 1-NAME (NOS2 and NOS3 inhibitor; 1 to 100 μM), S-MET (NOS2-specific inhibitor; 30 μM), Akt inhibitor (NOS3-specific inhibitor; 10 μM) (40), or a combination of these was expressed relative to the control. The presence of the NOS2 inhibitor 1-NAME dose-dependently decreased NO production (see Fig. S1 in the supplemental material), and 100 μM achieved approximately 70% inhibition (Fig. 1C and D). Similar results were achieved with S-MET, whereas the Akt inhibitor led to a 34% reduction (Fig. 1C and D). The combination of all inhibitors reduced NO production by 93% (Fig. 1D). There was no cytotoxicity toward macrophages by the inhibitors alone or in combination as determined by an MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] assay (data not shown). These data indicate that NOS2 and NOS3 are responsible for the production of NO, with a greater contribution by NOS2.
To further confirm NO production, we measured nitrite accumulation in response to mycobacterial infection and other inducers of NO. Following 48 h of infection with BCG or *M. tuberculosis*, nitrite was detected in culture supernatants (Fig. 2A). The nucleus was also stained with 4',6'-diamidino-2-phenylindole (blue). The images shown are from an individual experiment repeated three times and are representative of typical results. (B) Mean fluorescent intensity (MFI) analysis of DAF-2 DA staining was performed as described in Materials and Methods. The data shown are the combined results of three experiments (error bar, ± standard error from three combined experiments). Student’s t test was used to establish statistical significance in the 95% confidence interval between individual sample groups as indicated. (C) DAF-2 DA staining for NO at 48 h. The control indicates BCG-infected macrophages that were treated with IFN-γ, L-Arg, and BH₄ for maximal production of NO. All other samples were treated equally with the addition of L-NAME, S-MET, and/or Akt inhibitor as indicated. The confocal micrographs presented are representative of typical results from two independent experiments. (D) The percent NO production was calculated based on MFI analysis. The MFI value obtained from the control was set to 100%. All other conditions were expressed relative to this value. Data plotted here are the combined results from three independent experiments. A Wilcoxon signed-rank test was used for the indicated statistical comparisons in the 95% confidence interval between control and sample groups.

FIG 1 Monocyte-derived human macrophages produce NO. Macrophages were treated with medium alone (MED), infected with BCG, and/or treated with IFN-γ, L-Arg, BH₄, or a combination of these as indicated for 48 h. (A) DAF-2 DA staining (green, NO) was performed as described in Materials and Methods. The nucleus was also stained with 4',6'-diamidino-2-phenylindole (blue). The images shown are from an individual experiment repeated three times and are representative of typical results. (B) Mean fluorescent intensity (MFI) analysis of DAF-2 DA staining was performed as is described in Materials and Methods. The data shown are the combined results of three experiments (error bar, ± standard error from three combined experiments). Student’s t test was used to establish statistical significance in the 95% confidence interval between individual sample groups as indicated. (C) DAF-2 DA staining for NO at 48 h. The control indicates BCG-infected macrophages that were treated with IFN-γ, L-Arg, and BH₄ for maximal production of NO. All other samples were treated equally with the addition of L-NAME, S-MET, and/or Akt inhibitor as indicated. The confocal micrographs presented are representative of typical results from two independent experiments. (D) The percent NO production was calculated based on MFI analysis. The MFI value obtained from the control was set to 100%. All other conditions were expressed relative to this value. Data plotted here are the combined results from three independent experiments. A Wilcoxon signed-rank test was used for the indicated statistical comparisons in the 95% confidence interval between control and sample groups.

To further confirm NO production, we measured nitrite accumulation in response to mycobacterial infection and other inducers of NO. Following 48 h of infection with BCG or *M. tuberculosis*, nitrite was detected in culture supernatants (Fig. 2A). The addition of IFN-γ, supplemental L-Arg, and BH₄ further augmented nitrite levels (Fig. 2A). Although the levels were comparable with infection alone, *M. tuberculosis* infection led to a greater magnitude of nitrite accumulation than BCG in the presence of additional stimulators (Fig. 2A). We next evaluated whether the gene expression profiles for NOS2 and NOS3 matched their contributions to the NO output shown in Fig. 1. Therefore, gene expression of NOS2 and NOS3 was analyzed by quantitative real-time PCR. Both BCG and *M. tuberculosis* induced NOS2 and NOS3 gene expression at comparable levels.
were infected with BCG or *M. tuberculosis* and treated with IFN-γ, L-Arg, and BH_4 or medium alone. After 48 h, the macrophages were fixed and permeabilized, and NOS2 protein was immunolabeled with monoclonal antibodies. GAPDH was also labeled in uninfected and infected macrophages as a localization control (see Fig. S2 in the supplemental material). Each confocal micrograph was subsequently analyzed with Zeiss confocal analysis software for quantitative colocalization analysis as described in Materials and Methods. In uninfected macrophages NOS2 was not detected (Fig. 3A and B). When infected with BCG, macrophages expressed NOS2 protein that was more abundant during treatment with the combination of IFN-γ, L-Arg, and BH_4 (Fig. 3A and C). A very similar pattern of results was observed during *M. tuberculosis* infection (Fig. 3B and D). These protein expression profiles are consistent with the NOS2 transcriptional analysis (Fig. 2B). Localization of NOS2 was also determined in these experimental groups. The colocalization coefficient analysis showed that 18.5% of BCG and 21.29% of *M. tuberculosis* bacteria colocalized with NOS2 (Fig. 3A to D). These localization patterns were more pronounced when infected cells were treated with the combination of IFN-γ, L-Arg, and BH_4 (Fig. 3A to D). This increase in colocalization could be the result of greater NOS2 expression; the patterns of NOS2 expression parallel the patterns of colocalization (Fig. 3C and D). In contrast, GAPDH was well expressed in macrophages but did not colocalize with BCG (0% colocalization) (see Fig. S2). These data indicate that the NOS isoforms do associate with the bacteria and that this interaction increases with protein levels.

The effect of NO on bacterial growth. Since NOS isoforms colocalized with mycobacteria in macrophages that produce NO, we wanted to investigate the consequence to bacterial viability. To address this, human macrophages were infected with *M. tuberculosis* (Fig. 4A, open squares) or infected and treated as before to induce maximal production of NO (Fig. 4A, filled squares). To enumerate viable mycobacteria, a method that measures 16S transcripts and yields results consistent with traditional CFU enumeration was employed (see Fig. S3A to C in the supplemental material). Strikingly, by 24 h of infection, treatment with the combination of IFN-γ, L-Arg, and BH_4 increased the number of viable *M. tuberculosis* bacteria compared with medium alone, and this trend continued through 48 h (Fig. 4A and B). This was a consistent and reproducible finding over four independent experiments, all performed with different blood donors (Fig. 4B). Similar results were observed with BCG (see Fig. S3A to C). In addition, the presence of a slow-releasing NO donor dose-dependently augmented BCG growth measured at 48 h (see Fig. S3D). To further confirm these results by a second method, *M. tuberculosis* cells were stained with auramine O at 48 h, and green fluorescence was quantified. This analysis over four experiments with different blood donors also supports an increase in *M. tuberculosis* bacteria growth that is consistent with NO production (Fig. 4C). These results indicate that even at maximum output, the levels of NO produced by human macrophages are not sufficient to restrict mycobacterial growth. In fact, the bacteria thrive in the intracellular environment during NO production.

**NarG is required for optimal NOS expression and bacterial localization.** Since the number of mycobacteria recovered from macrophages was higher in the presence of NO, we hypothesized that *M. tuberculosis* and BCG may use NO or oxidized products as a source of nitrogen for metabolism or respiration. A previous study showed that the *M. tuberculosis* and BCG genomes contain...
genes homologous to the anaerobic nitrate reductase of *Bacillus subtilis* (42). The genes are clustered in the *narGHJI* operon (28, 42). *M. tuberculosis* expresses a functional nitrate reductase from these genes that mediates growth on nitrate under aerobic conditions and persistence under anaerobic conditions (27, 43). NarG is the catalytic subunit of the enzyme complex. BCG bacteria that lack *narG* do not reduce nitrate and are unable to survive in mice (44). Expression of *narG* and the nitrate transporter *narK2* is induced in BCG (data not shown) and *M. tuberculosis* following infection of macrophages (Fig. 4D). If NO is oxidized to nitrate and used metabolically by the bacteria, then this may explain why intracellular growth of mycobacteria was increased in the presence of NO (Fig. 4A). To address this possibility, human macrophages were treated to induce NO production and infected with an *narG* deletion mutant (Fig. 4A, open and filled triangles) or the corresponding complemented strain (Fig. 4A, open and filled circles). Recovered bacteria were enumerated at time points indicated on the figure. In contrast to growth of wild-type *M. tuberculosis*, the growth of the *narG* mutant strain was not increased when the macrophages produced NO (Fig. 4A and B). However, complementation with a functional copy of the deleted gene restored growth in the presence of NO to nearly wild-type levels (Fig. 4A and B). This is not the result of differences in the inocula; similar MOIs were used, and recovery levels at 4 h were comparable between strains (Fig. 4A). Cumulatively, these data suggest that the intracellular environment of macrophages that produce NO is favorable and not deleterious to mycobacterial growth. This requires *narG* and may involve direct utilization of NO or oxidized products.

**NarG is important for NOS2 levels and production of NO.** The *narG* mutant was not able to grow comparably to wild-type bacteria in macrophages stimulated to produce NO. Thus, we
wanted to address whether NO reached the mutant bacteria and was produced at the same level. To do this, we investigated the localization of NOS2 in the \( H_9004 \) narG-infected macrophages. For the localization studies, we employed Pearson’s correlation coefficient. This analysis considers the shapes between two channels (blue, NOS2; green, auramine O-stained bacteria). Macrophages were infected and treated as described earlier. NOS2 protein levels did not increase as much as they did with wild-type \( M. \) tuberculosis when macrophages were infected with the narG mutant and stimulated for NO production (Fig. 5A and B, upper panel). This finding is further consistent with reduced gene expression levels (Fig. 5C). The colocalization pattern was also different (Fig. 5A, light blue, and B, lower panel). In the narG mutant-infected macrophages NOS2 did not colocalize with the bacteria at a level comparable to that of the wild-type (Fig. 5A and B). The Pearson’s correlation coefficient analysis shows that the percent colocalization between \( H_9004 \) narG and NOS2 was reduced by 65% compared with the wild type when infected macrophages were stimulated to produce NO (Fig. 5B, lower panel). This is in part the result of less protein being present (Fig. 5A and B, upper panel). Consistent with less protein, reduced levels of NOS2 gene expression were observed in response to the mutant bacteria (Fig. 5C). This may be the result of the lower numbers of bacteria present at 48 h rather than a direct effect mediated by NarG. NOS2 gene expression, protein levels, and percent colocalization returned to nearly wild-type levels in the complemented mutant (Fig. 5A to C). Similarly, NOS3 protein levels and colocalization were reduced during infection with the narG mutant compared with levels of wild-type bacteria (data not shown). This analysis suggests that NOSs are localized to the \( M. \) tuberculosis bacteria-containing phagosomes.

Next, we evaluated the level of nitrite as a marker of NO production in response to wild-type or narG mutant \( M. \) tuberculosis. In either case, the bacteria alone induced only low levels of nitrite as before (Fig. 5D). However, when treated to induce higher levels of NO, \( H_9004 \) narG-infected macrophages did not increase nitrite levels (Fig. 5D). In contrast, the wild-type \( M. \) tuberculosis-infected macrophages increased NO output by 73% (Fig. 5D). Nitrite levels returned to nearly wild-type levels during infection with the complemented mutant (Fig. 5D).

**DISCUSSION**

NO is an important mediator of murine macrophage-mediated cytotoxicity against microbes. However, much controversy has surrounded the expression of NOS isoforms and the production of NO by human macrophages. Studies in human macrophages have largely been done using indirect assays that measure metabolites such as nitrite that are indicative of NO production. Historically, indirect methods have not been sensitive to small amounts...
of NO. Here, we used the sensitive probe DAF-2 DA to detect NO. Other fluorogenic reagents to measure nitrite support the detection of NO. We characterized the cellular localization of NOS and determined the consequences of NO production to mycobacterial growth.

We showed that mycobacterial infection or IFN-γ treatment induced expression of NOS isoforms and increased levels of NO (Fig. 1). The combination of IFN-γ treatment during infection yielded additive levels of NO. NOSs only produce NO when homodimeric. Each subunit associates with at least five other molecules, including heme, BH₄, calmodulin (CaM), and flavins (5). It has been suggested that the addition of BH₄ is necessary to generate NO from human macrophages since they are unable to synthesize the essential cofactor (45). In contrast, another group determined that the intracellular level of BH₄ is not a factor for the production of NO by human macrophages (46). Bertholet et al. showed that U937 monocytes were able to produce NO when transfected with a construct that allowed for expression NOS2. However, this expression required the addition of BH₄ (38). Our results showed that supplemental BH₄ significantly increased NO levels. This suggests that sensitive reagents allow for the detection of NO in the absence of added BH₄, but this molecule is a limiting factor for higher levels of NO production. Treatment with NOS inhibitors blocked NO production (Fig. 1; see also Fig. S1 in the supplemental material), further confirming the involvement of these enzymes. NOS2 appears to be the primary producer of NO in human macrophages, based on selective inhibition (Fig. 1) and greater expression of NOS2 protein (Fig. 3) relative to NOS3 (data not shown).

FIG 5 NarG is important for expression and localization of NOS2. Macrophages were infected with wild-type M. tuberculosis (WT), ΔnarG bacteria, or the complemented mutant (Comp). Some infected macrophages were treated with IFN-γ, L-Arg, and BH₄. (A) Immunostaining for NOS2 (blue) relative to auramine O-stained M. tuberculosis (green) was performed as described in Materials and Methods. Colocalized NOS2 and M. tuberculosis appeared as light blue in the merged images. Representative images from four independent experiments are shown. (B) Mean fluorescence intensity (MFI) of NOS2 (blue) was calculated by ImageJ software (upper panel). The percent colocalization was calculated based on Pearson’s correlation coefficient (lower panel). Data shown here are the combined results from four independent experiments. (C) Quantitative analysis of NOS2 or NOS3 transcripts at 48 h postinfection is presented as the mean log₂ change in gene expression of duplicate samples ± standard error for two combined experiments. Values were normalized to the mean expression of GAPDH within a sample group and are expressed relative to the value of medium alone. (D) Nitrite production ± standard error for three experiments with separate donors is shown. Student’s t test was used for the statistical comparisons between the indicated sample groups in the 95% confidence interval (B, C, and D).
not shown) in infected macrophages (Fig. 3B). However, NOS3 should not be ignored; it is expressed in infected macrophages (Fig. 2B), and selective inhibition indicates a contribution to the total NO output (Fig. 1C and D).

NOS2-derived RNI plays an essential role in killing M. tuberculosis by murine macrophages (6, 7, 8, 9). Since NOS2 colocalizes to BCG and M. tuberculosis in human macrophages (Fig. 3), suggesting that NO is released directly into the phagosome, we investigated whether there was an effect on mycobacterial growth. NO failed to reduce the bacterial load in human macrophages (Fig. 4). It is apparent that the levels of NO produced in monocytic-derived human macrophages under our culture conditions are not toxic to BCG or M. tuberculosis. We acknowledge that many factors such as the source of cells, isolation technique, and manner of stimulation may influence the magnitude of NO production that is achieved. In addition, the granuloma is a complex and dynamic environment that we do not contend we have replicated here. Indeed, in human tuberculous lungs, NOS expression and the level of nitrosylation are increased compared with levels in normal lungs (22). Similarly, recent work done in cynomolgus macaques demonstrated increased expression and activity of NOS in granulomatous tissue relative to infected controls or even uninjured lung tissue from an infected monkey (47). Additionally in the same report, strong nitrosylation of tyrosines was shown at the epithelioid macrophage-caseum interface, further evidence of NOS activity (47). Taken together, these reports suggest that in the context of a granuloma, NO production may be increased relative to the level in healthy tissue. Nonetheless, the cumulative body of work discussed here warrants that in each model system and in human disease, NO production and the corresponding levels be carefully considered with regard to mycobacterial toxicity. That there was an increase in intracellular mycobacterial growth in the presence of NO in our system (Fig. 4; see also Fig. S3 in the supplemental material) may suggest that mycobacteria can use NO or, more likely, the oxidized products, nitrite or nitrate, to their benefit. This possibility is consistent with the requirement for nitrate reductase and the expression of narK2, as well as direct augmentation of intracellular BCG growth by a direct source of NO (see Fig. S3D). However, we cannot rule out an influence of NO on the macrophages that changes the intracellular environment to favor mycobacterial growth and survival. NO serves a role as a signaling agent (48). In line with this idea, NO was recently shown to have an important role in limiting inflammation to the benefit of the murine immune response (49). It has become more appreciated that excessive levels of inflammation are harmful during tuberculosis. In our system, it is possible that NO acts on the macrophage in a manner that changes the inflammatory environment. Other evidence that NO is not bactericidal can be derived from studies in which M. tuberculosis growth in broth culture was monitored over time in the presence of NO donors (50, 51). An initial bacteriostatic effect that paralleled oxygen depletion was observed with an intermediate concentration (500 μM) of slow-releasing NO donor (51). Over longer periods of time (through 96 h) the bacteria still replicated although growth was slowed compared to that of an untreated control (50). A 5 mM concentration of fast-release NO donor did not kill M. tuberculosis independent of the starting bacterial density (50).

The M. tuberculosis and BCG genomes contain genes clustered in the narGHJI operon that are homologous to the anaerobic nitrate reductase of Bacillus subtilis (42). M. tuberculosis uses a variety of mechanisms to detoxify reactive nitrogen species, such as NADPH-dependent peroxidase, peroxynitrite reductase, and truncated hemoglobins (2). The bacteria may also use nitrate reductase to adapt to a changing intracellular environment. NO inhibits aerobic respiration in M. tuberculosis (51). In other systems this has been reported to result from NO by competition with oxygen for availability to cytochrome c oxidase (52). However, NO does not inhibit nitrate reductase (53). Therefore, in the presence of NO, nitrate reduction may allow M. tuberculosis to use nitrate for energy. Thus, nitrate reduction may play an important role in intracellular growth and survival in human macrophages. Our data strengthen this notion. M. tuberculosis bacteria containing the narG deletion were unable to grow in human macrophages, and this was not augmented by conditions that induced NO during infection by wild-type bacteria (Fig. 4). In narG mutant-infected macrophages, NOS2 isoform expression was reduced and not localized to the bacteria (Fig. 5). During BCG infection of immunodeficient SCID mice, reduced numbers of ΔnarG cells were found in the lungs compared with levels of wild-type bacteria (54). Likewise, BCG cells deficient for nitrate reduction do not grow or persist well in BALB/c mice (44, 54). In contrast to these reports, a narG mutant of M. tuberculosis did not exhibit impaired growth or persistence in C57BL/6 mice (55). Another primary conclusion from this report was that M. tuberculosis-induced granulomas in mice are not anoxic, consistent with limited tissue necrosis (55). The availability of oxygen may be an important factor in interpreting our results relative to these reports. Similarly, the inherent differences in granulomas and other potentially unique stressors that exist between murine and human cells may be important. Although we cannot rule out a direct influence of NarG on induction of NOS isoforms to generate NO that can be oxidized to nitrate and imported into the bacterial cell, it seems more likely that a lower bacterial burden fails to strongly stimulate NOS2 expression in narG-deficient bacteria. Bacterial lipoproteins trigger Toll-like receptor 2 (TLR2), and M. tuberculosis strongly enhances IFN-γ-induced NOS2 expression in murine macrophages (10, 56). To maximize use of NO-derived products would require that NOS be localized near the bacteria. Similarly to an influence on NO2 expression, we cannot exclude an involvement of NarG with localization of NOS to the bacteria but expect that decreased colocalization with the narG mutant is a reflection of reduced bacterial numbers. We hypothesize that the bacteria have adapted to the intracellular environment but do not actively recruit NOS2 or RNI. The detailed mechanisms involved with the impact of NarG on NOS expression and localization to mycobacteria remain to be elucidated.

A number of studies indicate that anaerobic respiration may be important during murine or human infection by mycobacteria (41, 44, 53, 54, 57, 58). The expression of M. tuberculosis genes for aerobic and anaerobic respiration during active tuberculosis supports the idea of a microaerobic environment (57). In addition, narG was expressed in the distant lung of a tuberculosis (TB) patient (57, 59). During chronic murine infection, transcripts for the nitrate transporter NarK2 are upregulated (53). Furthermore, the transcriptional profiles in response to oxygen depletion and exposure to NO exhibit strong similarities (51, 53, 60). In this study, we demonstrate that narG as well as narK2 gene expression is induced during M. tuberculosis infection of macrophages (Fig. 4D). This suggests that an anaerobic respiratory pathway is turned on within the macrophage and is perhaps critical for growth. The
expression of *narK2* is an indicator of exposure to NO, hypoxia, IFN-γ-activated macrophages from NOS2-expressing mice, and infection of the murine lung (41, 51, 53, 60, 61). IFN-γ-activated murine macrophages that produce NO block aerobic respiratory pathways and induce nitrate respiration in *M. tuberculosis* bacteria (62, 63). *NarG* may fulfill a respiratory role in infected macrophages by reducing nitrate that has been transported via *NarK2* (Fig. 6). When NO levels fall below a threshold (50 μM diethylenetriamine-nitric oxide [DETA]-NO) but are not absent, a bacteriostatic effect is overcome, and *M. tuberculosis* growth resumes (51). It is possible that in our system the levels of NO are below this threshold, and thus a replicating state exists. In the presence of another stress within the macrophage that is not present in broth culture, the availability of nitrate derived from NO may confer a growth advantage. Since growth is dependent on oxygen, this advantage may be realized by aerobic and nitrate respiration occurring in parallel. Indeed, parallel operation of aerobic and nitrate respiration has been postulated during the second of three respiratory states that occur during *M. tuberculosis* infection of the mouse lung (53). It has been described in other microorganisms (64). This respiratory flexibility could become possible with subinhibitory levels of NO that lead to available nitrate.

One of the most well described IFN-γ-mediated antimycobacterial mechanisms in murine macrophages is the action of NOS isoforms. However, our findings regarding NO production in human macrophages did not result in the clearance of bacteria. This may suggest that human macrophages require NOS-independent mechanisms to restrict mycobacterial growth. A recent study showed that autophagy is necessary for restriction of both *M. tuberculosis* and BCG (65). Overall, the data presented in this report provide a greater understanding of the potential to produce NO in human macrophages and a new consideration for how mycobacteria may adapt in the intramacrophage environment.

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