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Chlamydia trachomatis Inhibits Inducible NO Synthase in Human Mesenchymal Stem Cells by Stimulating Polyamine Synthesis

Mohammad Abu-Lubad, Thomas F. Meyer, and Munir A. Al-Zeer

Chlamydia trachomatis is considered the most common agent of sexually transmitted disease worldwide. As an obligate intracellular bacterium, it relies on the host for survival. Production of NO is an effective antimicrobial defense mechanism of the innate immune system. However, whether NO is able to arrest chlamydial growth remains unclear. Similarly, little is known about the mechanisms underlying subversion of cellular innate immunity by C. trachomatis. By analyzing protein and mRNA expression in infected human mesenchymal stem cells, combined with RNA interference and biochemical assays, we observed that infection with C. trachomatis led to downregulated expression of inducible NO synthase (iNOS) in human mesenchymal stem cells in vitro. Furthermore, infection upregulated the expression of the rate-limiting enzyme in the polyamine biosynthetic pathway, ornithine decarboxylase, diverting the iNOS substrate L-arginine toward the synthesis of polyamines. Inhibition of ornithine decarboxylase activity using small interfering RNA or the competitive inhibitor difluoromethylornithine restored iNOS protein expression and activity in infected cells and inhibited chlamydial growth. This inhibition was mediated through tyrosine nitration of chlamydial protein by peroxynitrite, an NO metabolite. Thus, Chlamydia evades innate immunity by inhibiting NO production through induction of the alternative polyamine pathway.

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Materials and Methods

Chemicals and Abs

The following Abs were used for immunofluorescence analysis and Western immunoblotting: mouse monoclonal anti-nitrotyrosine (Clone: HM11; Novus Biologicals), mouse monoclonal anti-arginase 2 (Abcam), mouse monoclonal anti–ornithine decarboxylase (ODC; Abcam), mouse monoclonal anti–C. trachomatis hsp60 (Enzo Life Sciences) and mouse monoclonal anti–iNOS (Sigma-Aldrich), rabbit monoclonal anti-Chla-
mydia genus-specific Ab (Milan Analytica AG, La Roche, Switzerland), rabbit polyclonal anti-iNOS (Abcam), rabbit polyclonal anti-IDO (Cell Signaling), and rabbit polyclonal anti-arginase 1 (Abcam). Appropriate secondary-labeled Abs were purchased from Jackson ImmunoResearch Laboratories for immunofluorescence and from Amersham Biosciences for Western blot analyses. Difluoromethylornithine and L-NG-nitroarginine methyl ester (L-NAME) were purchased from Sigma-Aldrich; Nor-Hydroxy-nor-\textit{\textalpha}–arginine (norNOHA) was purchased from Calbiochem. Human IFN-\gamma and proteasome inhibitor, MG132, were purchased from Sigma-Aldrich.

Ethics statement

Fallopian tube samples were collected from human subjects by the Department of Gynecology, Charité University Hospital, Berlin, Germany. Scientific use of the tissue, in accordance with EU and national law and the Declaration of Helsinki, was approved by the Ethics Commission of the Charité University Hospital, Berlin, Germany, approval EA1/002/07. Samples were anonymized, and written informed consent was obtained from all donors.

Isolation of MSCs from fallopian tube

Normal pieces from human fallopian tube tissue samples were used to isolate and culture MSCs as described previously (26).

C. trachomatis propagation and infectivity titration assay

\textit{C. trachomatis} lymphogranuloma venereum serovar L2 (ATCC-VR902B) and D (ATCC-VR888) were routinely propagated in HeLa cells grown in cell growth medium consisting of RPMI 1640 medium (Invitrogen) supplemented with glutamine and 5% FBS. 

\textit{Chlamydia} culturing, preparation of EB stock, and estimation of inclusion forming units (IFU) per milliliter were conducted as described previously (27).

Host cells were infected statically with \textit{C. trachomatis} serovar L2 diluted in cell growth medium at a multiplicity of infection (MOI) of 1 and incubated at 35°C and 5% CO\textsubscript{2}. Host cells were similarly infected with \textit{C. trachomatis} serovar D at MOI of 5 followed by centrifugation for 10 min at 1000 rpm. Two hours postinfection (p.i.), cells were washed, medium was replaced, and cultures were incubated for the indicated times. Formation of infectious \textit{C. trachomatis} progeny in a secondary infection was assessed as described previously (27).

Difluoromethylornithine treatment

Monolayers of MSCs were incubated with medium alone or containing various concentrations of difluoromethylornithine (DFMO) at 35°C from 2 h p.i. until the end of the incubation time. The cells were then washed with DMEM and tested for infectious progeny as described above.

Fluorescence confocal microscopy

Cells were plated onto coverslips in 12-well plates at a density of 5 \times 10\textsuperscript{4} cells per well and incubated overnight at 37°C and 5% CO\textsubscript{2}. Cells were then inoculated with \textit{C. trachomatis} and incubated at 35°C and 5% CO\textsubscript{2}. At the indicated time points, cells were fixed with 4% PFA for 30 min at room temperature, permeabilized with 0.3% Triton-100\times and blocked with 2.5% BSA-PBS. Cells were then incubated with primary Abs (overnight at 4°C) followed by secondary Abs (1 h at room temperature) in 2.5% BSA. Coverslips were mounted onto glass slides using Mowiol and examined with a Leica TCS-SP laser scanning confocal microscope. Photomicrographs were processed using Adobe Photoshop CS3.

Transmission electron microscopy

Infected host cells plated in six-well plates were washed twice with cold PBS and fixed with 2.5% glutaraldehyde and detached by rubber policeman. The cells were postfixed with 1% osmium tetroxide (OsO\textsubscript{4}) and contrasted with 1% osmium tetroxide and tannic acid. Ultrathin sections were subsequently dehydrated in a graded ethanol series (50–100%) and embedded in agar 100. Ultrathin sections (70 nm) were produced, contrasted with lead citrate, and examined with a Zeiss EM 10 electron microscope.

Immunoblotting

Cell monolayers were lysed directly with SDS lysis buffer (3% 2-ME, 20% glycerin, 0.05% bromophenol blue, 3% SDS). Whole cell lysates were prepared by direct lysis to avoid proteolysis during sample preparation. Cell lysates were then harvested and boiled at 95°C for 10 min. Equal amounts of protein were separated using SDS-PAGE and immunoblotting as described previously (27).

Immunoprecipitation

Host cells were lysed in 1× Cell Lysis Chaps Buffer (150 mM NaCl, 10 mM HEPES [pH 7.4], 1% Chaps [w/v]) containing Complete Protease Inhibitor (Roche Diagnostics). The lysates were incubated with 1 µg anti-chlamydial Hsp60 Ab overnight at 4°C. Protein G-agarose beads were subsequently added for 4 h to precipitate Ag-Ab complexes. After removing the supernatant, the precipitate was eluted by heating to 95°C in SDS loading buffer and the individual proteins separated by SDS-PAGE. Western blotting was used to assess the precipitate using anti-nitro-
yrosine Ab.

Arginase activity assay

Cells were harvested and lysed using arginase activity lysis buffer, and the assay was performed as described (28). The colored products were measured using a spectrophotometer at 540 nm.

Measurement of NO\textsubscript{2} concentration

Concentration of nitrite produced by MSCs was determined using the Griess reagent as described by Munder et al. (29). The supernatant was removed from each culture well at 48 h p.i., filtered using a 0.22-µm Millex-GP Syringe Filter Unit (Millipore) to remove cell debris and bacteria, and absorbance at 540 nm measured using a spectrophotometer.

Polyamine measurements

Extracts of basic amino acids and polyamines were prepared as described by Gilbert et al. (30). Extracted samples were derivatized with p-nitrobenzoylcarbonyl chloride as described by Brückner and Lipke (31). Derivatized samples were analyzed by reverse-phase chromatography on a BEH C18 column (Waters) with a linear gradient from 100 mM NaOAc (pH 4.4) to 100% acetonitrile. Polyamines were detected at 260 nm.

RNAi

The small interfering RNA (siRNA) targeting ODC (ON-TARGET Plus SMART pool containing the following siRNAs: 5′-AACGATTTGTAGC- TTGTAACA-3′, 5′-TAGCTGTGAACTGCAAGTTTA-3′, 5′-AACCCAGCGTGTGACAAATA-3′, and 5′-CAGAGAGGATATTCTATTCGCAA-3′) were synthesized by Dharmacon Research (Lafayette, CO) and firefly luciferase (5′-AAUCUACGCGUGACUUCUGA-3′) purchased from Qiagen (Hilden, Germany). The transfection of siRNAs was performed using Hyperfect transfection reagent (Qiagen) according to the manufacturer’s instructions. Two rounds of transfections were used: 24 h prior to infection and 2 h p.i. Cells were harvested to determine the knockdown efficiency and infectious progeny using Western blot and infectivity titration assay, respectively.

Quantitative PCR

Total RNA was isolated from host cells using the RNeasy Kit (Qiagen) according to the manufacturer’s protocol. The OneStep RT-PCR Kit (Qiagen) was used for CDNA preparation and amplification. Primer sequences were as follows: iNOS forward 5′-ACCATTCTGGTGTTGAGTCTGAC-3′, iNOS reverse 5′-TCGCAGAQACCTGGACCAG-3′, GAPDH forward 5′-GGT ATG GTG GAA GGA GCT ATG AC-3′, GAPDH reverse 5′-ATG CCA GTG AGC TTC CCG TTC AG-3′.

Flow cytometry

Host cells were washed with warm D-PBS, trypsinized, and centrifuged at 1200 rpm, followed by resuspension in warm 4% PFA for fixation. After washing, cells were incubated for 30 min at 4°C with fluorescently labeled iNOS Ab. The population of intact cells equivalent to 10,000 events was gated, and fluorescence intensity of the gated population determined with FACS analysis.

Statistical analysis

Data were analyzed using nonparametric one-way ANOVA and Bonferroni multiple comparison test or Student t test.
Results
C. trachomatis infection downregulates iNOS protein expression and activity

To assess whether chlamydial infection induces or inhibits iNOS expression, we used human MSCs as a model. The cells were derived from fallopian tubes—a rich source of MSCs (26). Although the basal layer of the endometrium, which contains MSCs, is normally shielded from Chlamydia by the functional layer, this is sloughed off during menses, leaving the regenerative basal layer exposed (32, 33). In particular, MSCs are found around blood vessels spanning both layers, and they have been recovered from menstrual blood (34). Because Chlamydiaceae exhibit a broad spectrum of natural host infection tropism (35), MSCs are likely to be subject to chlamydial infection. Different infection models have been used to study host—C. trachomatis interactions; therefore, we successfully established primary human MSCs as a favorable alternative to the widely used tumorigenic cell lines. The isolated MSCs expressed the MSC surface markers CD44, CD73, and CD90 (26), as well as vimentin, a hallmark of mesenchymal cells and the stemness markers Oct4 and Sox2. The MSCs also lacked epithelial markers such as E-cadherin and EpCAM (data not shown), confirming that the cultures did not contain any contaminating epithelial cells.

In our hands, MSCs were similarly susceptible to Chlamydia infection as epithelial cell lines. We compared infection in HeLa cells and MSCs with or without addition of IFN-γ. Quantification of infectious EBs at 72 h p.i. revealed a similar decrease in infectivity for C. trachomatis serovar D in response to IFN-γ in both cell types (Supplemental Fig. 1A, 1B). To define the IFN-γ-mediated factor that inhibits C. trachomatis serovar D in each case, we analyzed iNOS and IDO protein expression using immunoblotting. In both HeLa cells and MSCs, infection alone reduced iNOS protein expression, whereas IFN-γ treatment alone or combined with C. trachomatis infection did not (Supplemental Fig. 1C, 1D). IFN-γ treatment of HeLa cells and MSCs did induce expression of the IDO protein that inhibits Chlamydia replication by depleting tryptophan pools (Supplemental Fig. 1C, 1D). These results strongly suggest that the IFN-γ-mediated inhibition of C. trachomatis in MSCs and HeLa cells was caused by IDO expression and tryptophan depletion, confirming that primary fallopian tube MSCs represent a useful model system for chlamydial infection, which recapitulates the host-pathogen interactions of established cell lines.

MSCs were infected with C. trachomatis at different MOIs for 48 h and the levels of iNOS determined by Western blotting. With increasing MOIs, iNOS levels were progressively reduced compared with uninfected cells (Fig. 1A, Supplemental Fig. 2A). To examine the time-course of this downregulation, total cell lysates were collected at 6, 12, 24 and 48 h post infection (p.i.). There were no changes in iNOS protein levels at 6 h p.i., but a noticeable decrease at 24 h p.i. to almost undetectable levels by 48 h p.i. (Fig. 1B, Supplemental Fig. 2B). Treating the cells with the proteasome inhibitor MG132 was not sufficient to rescue the iNOS degradation phenotype after C. trachomatis infection in MSCs, showing that the reduction in iNOS protein was not due to proteosomal degradation (Supplemental Fig. 2C). To investigate this reduction further, we used immunofluorescence to assess the effect of C. trachomatis infection (MOI = 5) on iNOS from 12–48 h. iNOS expression was reduced at 48 and 24 but not at 12 h p.i. (Fig. 1C), confirming the results obtained with Western blotting. Despite this observed decrease in protein levels, quantitative real-time polymerase chain reaction showed that the levels of iNOS mRNA remained unchanged from 6 to 48 h p.i. (Supplemental Fig. 2D).

Next, we investigated the activity of iNOS in C. trachomatis–infected cells using the Griess assay to measure nitrite production. Nitrite ion concentrations were reduced in infected cell supernatants compared with uninfected controls (Fig. 1D). Thus, while iNOS mRNA expression remains unchanged in C. trachomatis–infected cells, the levels of iNOS protein and its reaction products are progressively reduced with time after infection and with increasing chlamydial load.

We then determined the effect of exogenous supplementation of L-Arg on iNOS expression in C. trachomatis–infected cells, because it has been demonstrated that in some pathogenic infections, such as Helicobacter pylori, the intracellular L-Arg pool plays a major role in regulating iNOS protein expression (36). Infected cells were treated with different concentrations of L-Arg (0.1–12 mM) for 72 h before total cell lysates were subjected to Western blotting. The levels of iNOS protein in control C. trachomatis–infected cells was similar to that in infected cells supplemented with varying concentrations of L-Arg (Supplemental Fig. 2E). In contrast, after L-Arg treatment alone iNOS levels were comparable to those in uninfected control cells (Supplemental Fig. 2E). In conclusion, although C. trachomatis infection does not downregulate the expression of the iNOS gene, it does lead to downregulation of iNOS protein, independent of the intracellular L-Arg pool.

C. trachomatis stimulates ornithine decarboxylase activity
NO is synthesized from L-Arg by NO synthase. Pathogens like H. pylori (37) and Salmonella (38) are known to interfere with NO synthesis by inducing an alternative pathway of L-Arg metabolism in host cells. Thus, we sought to determine the effect of C. trachomatis infection on the activity of two enzymes involved in L-Arg metabolism—arginase and ODC—in C. trachomatis–infected MSCs by immunohistochemistry and Western blotting. After infection with C. trachomatis for 24 h, cells showed strong vesicular ODC staining (red) that did not localize to the inclusion (green), whereas in uninfected cells only a faint, diffuse signal for ODC was visible, without evidence of vesicular staining (Fig. 2A). Western blot analysis confirmed that infection of MSCs with C. trachomatis (MOI = 5) for 6, 12, 24, and 48 h led to a drastic increase in levels of ODC protein over time compared with uninfected cells (Fig. 2B, Supplemental Fig. 3A). In contrast, there was no significant increase in the protein levels of arginase 1 and 2 (Fig. 2B). Furthermore, spectrophotometry assay indicated that arginase activity was also unaffected after chlamydial infection compared with control uninfected cells at the same time points (Supplemental Fig. 3B). The growth of C. trachomatis in MSCs was investigated in the presence of the arginase inhibitor norNOHA. There was no significant difference in infectious progeny between cells treated with various norNOHA concentrations (25-400 μM) and untreated cells (Supplemental Fig. 3C), indicating that norNOHA did not inhibit chlamydial growth. Thus, infection of MSCs by C. trachomatis results in upregulation of the enzyme ODC, which catalyzes the first step in the conversion of l-ornithine, downstream of arginase, into the polyamines.

We next used HPLC to examine whether C. trachomatis infection has any effect on host cell polyamine production. We found that at 12 h p.i., both spermine and spermidine levels were significantly elevated in C. trachomatis–infected cell lysates compared with uninfected cells (Fig. 2C). Taken together, these results indicate that Chlamydia infection boosts host ODC expression, leading to enhanced polyamine production. This upregulation of polyamine biosynthesis might be associated with simultaneous inhibition of the alternative metabolic pathway that uses L-Arg as a substrate, leading to the production of reactive nitrogen species via the activity of iNOS.
C. trachomatis growth is inhibited by DFMO

It has recently been reported that treatment with DFMO, an irreversible inhibitor of ODC, the rate-limiting enzyme in the polyamine synthesis pathway, suppresses colonization and growth of other bacteria (39, 40). Thus, we next investigated whether various concentrations (1.25–20 mM) of DFMO have any effect on C. trachomatis growth. DFMO on its own had no apparent toxic effect on MSCs, as measured with LDH assay (data not shown). Quantification of infectious EBs at 48 and 72 h p.i. revealed a dose-dependent decrease in infectivity for both L2 and D C. trachomatis serovars (Fig. 3A). DFMO also inhibited growth of both C. trachomatis serovars in HeLa cells (Supplemental Fig. 4A).

The gene coding for the chlamydial 60-kDa heat shock protein (Hsp60) is expressed during active chlamydial infection under normal conditions (41). During the persistent state, which is induced in response to the external stresses, Hsp60 is upregulated, in contrast to genes that are involved in replication and energy generation (42). Because we found that DFMO treatment impaired chlamydial replication, we went on to determine whether DFMO treatment could affect the production of Hsp60. We used Western immunoblotting to quantify the amount of bacterial Hsp60 in infected cells cultured for 72 h in the presence of increasing DFMO concentrations. We observed a dose-dependent reduction in levels of Hsp60 protein compared with untreated cells (Fig. 3B, Supplemental Fig. 4B), correlating with the inhibitory effect of DFMO on bacterial growth and replication (Fig. 3A). Thus, the reduction in Hsp60 expression in response to DFMO was due to bacterial eradication, not persistence (Fig. 3B).
To examine the effect of DFMO treatment at higher resolution, we used transmission electron microscopy to visualize the ultrastructural features of chlamydial inclusions. In untreated cells inclusions developed normally at 48 h p.i. (Fig. 3C). DFMO treatment induced the formation of aberrant chlamydial bodies and enlarged, abnormal reticulate bodies (Fig. 3C). Furthermore, DFMO treatment reduced the number of mature EBs and IBs and induced numerous RBs that were morphologically abnormal (Fig. 3C). DFMO supplementation induced aberrant chlamydial growth.

Knockdown of ODC restores iNOS expression in C. trachomatis–infected cells

In addition to the chemical inhibitor DFMO, we next used siRNA to knock down ODC in MSCs. Knockdown efficiency was determined and compared with luciferase-specific control knockdown (Fig. 4A). Cell viability was unaffected by knockdown with the siRNA used (data not shown). C. trachomatis–infected cells transfected with ODC siRNA displayed a significant decrease in infectious progeny compared with control siRNA against luciferase along with restoration of iNOS expression (Fig. 4A). This was accompanied by a significant decrease in infectious progeny (Fig. 4B). Chlamydial growth was reduced by 80% in ODC knockdown cells (Fig. 4B). Next, we used the Griess assay to measure nitrite production. Nitrite concentrations were increased...
in infected ODC knockdown cells compared with control knockdown cells (Fig. 4C). Using siRNA to knock down ODC in MSCs, we found that doing so significantly inhibited the rise in both spermine and spermidine in *C. trachomatis*–infected cell lysates compared with control knockdown, as determined by HPLC (Fig. 4D).

**DFMO restores iNOS expression in C. trachomatis–infected cells**

To analyze whether DFMO treatment restores iNOS in *C. trachomatis*–infected cells, we used immunoblotting to monitor levels of iNOS following treatment with different concentrations of DFMO. In uninfected cells, levels of iNOS cells were comparable in control and DFMO conditions (Fig. 5A, 5B). Following infection with *C. trachomatis* (MOI = 5), levels of iNOS decreased significantly (Fig. 5A). DFMO treatment, however, significantly increased iNOS protein expression in response to infection to above the level observed in uninfected cells, in a dose-dependent manner (Fig. 5A, 5B).

To confirm these results, we used immunofluorescence to analyze iNOS expression at the single-cell level in *C. trachomatis*–infected cells in the presence or absence of DFMO. Infected cells (MOI = 5) were treated with 20 mM DFMO 2 h p.i. and processed for indirect immunofluorescence at 48 h p.i. (Fig. 5C). In uninfected control cells, iNOS protein was detected in all cells. In infected cultures, cells that contained bacteria, as evidenced by the presence of green inclusions, did not show staining for iNOS, whereas cells within the same monolayer that had remained uninfected were immunopositive for iNOS. Thus, as observed with the immunoblot, DFMO restored iNOS immunoreactivity in infected cells (Fig. 5C). In contrast, when analyzing iNOS at the level of gene expression, neither infection nor DFMO treatment altered the levels of iNOS mRNA in cells, as measured by quantitative real-time polymerase chain reaction (Supplemental Fig. 4C). Thus, iNOS downregulation upon *C. trachomatis* infection occurs at the protein level and is dependent on ODC activity, indicating that the specific antichlamydial activity of DFMO is mediated through restoring iNOS protein levels in infected cells.

**iNOS activity is increased in C. trachomatis–infected cells treated with DFMO**

The production of NO in living cells can be detected with microscopy, with spectrofluorometry, or by measuring nitrite production. To confirm the increased iNOS enzymatic activity, we measured nitrite production 12, 24, and 48 h after *C. trachomatis* infection in the presence or absence of DFMO using the Griess assay (29). Uninfected control cells produced low levels of nitrite, and DFMO treatment alone did not significantly affect basal nitrite production (Fig. 6A). *C. trachomatis* infection (MOI = 5) alone reduced the amount of nitrite production significantly. However, in the presence of DFMO (1.25–20 mM), nitrite production was
FIGURE 5. iNOS expression restoration upon DFMO treatment in C. trachomatis–infected MSCs. Host cell monolayers were infected with C. trachomatis D (MOI = 5) and treated with increasing concentrations of DFMO for 48 h or left untreated or infected as controls. (A) Western immunoblot analysis revealed that C. trachomatis infection reduces iNOS expression compared with uninfected cells. In contrast, DFMO treatment resulted in a progressive recovery of iNOS protein levels with increasing concentrations. Host β-actin was used as loading control. (B) Quantification of iNOS band densities confirms that iNOS levels increase significantly even at the lowest DFMO concentration tested (1.25 mM). Results shown are the mean values of three independent experiments. Error bars: ± SD. * p < 0.05. (C) Immunofluorescence micrographs of C. trachomatis–infected cells treated with DFMO (20 mM) for 48 h or left untreated as control. Cells were labeled with Abs against Chlamydia (green) and iNOS (red); nuclei were stained with DRAQ5 (blue). The middle panel shows an infected cell containing an inclusion, but without notable iNOS staining. Surrounding cells that have remained uninfected display strong iNOS labeling. DFMO treatment restored iNOS expression in C. trachomatis–infected cells (right panel). Scale bar, 10 μm.

DFMO treatment induces nitration of chlamydial proteins

Our data clearly show that DFMO exerts a toxic effect on C. trachomatis, which is accompanied by increased NO production. The bactericidal activity of NO appears to be mediated by the formation of the highly toxic peroxynitrite (ONOO−) that causes protein tyrosine nitration (11, 12). To assess the effect of DFMO on chlamydial protein nitration, cells were infected with C. trachomatis (MOI = 5) for 2 h. After 12 h incubation in the presence of DFMO, cells were immunostained for nitrotirosine (red) and early chlamydial inclusions (green). No colocalization of nitrotyrosine inclusions was detected in untreated cells (Fig. 7A). In DFMO–treated cells, however, C. trachomatis inclusions colocalized to a high degree with nitrotyrosine (Fig. 7A). Thus, DFMO treatment leads to a specific modification of C. trachomatis inclusions characteristic of peroxynitrite–induced nitration.

To examine the physical association between nitrotyrosine and bacterial protein, we performed immunoprecipitation. Upon DFMO stimulation, the chlamydial Hsp60 protein coprecipitated with host cell–derived nitrotirosine in C. trachomatis (Fig. 7B). In contrast, in the absence of DFMO there was no interaction between the chlamydial Hsp60 and the host nitrotirosine. Together, these data indicate that NO produced by iNOS is converted to reactive nitrogen species, such as peroxynitrite, which are known to have direct antimicrobial activities as shown before against pathogens like Salmonella (44).

Discussion

Autonomous innate immunity, including production of NO, plays a central role in the protection of cells against pathogens in vivo and in vitro. In this study, we demonstrated that C. trachomatis actively reduces NO synthesis in host cells, allowing the establishment of an acute infection. This inhibition is mediated by increased levels of polyamines (45), which result from the induction of ODC ex-

significantly increased in a dose-dependent manner up to sevenfold the levels seen in uninfected controls levels (Fig. 6A).

We next used 4-amino-5-methylamino-2',7'-difluororescein diacetate (DAF-FM) (43) to image NO accumulation in C. trachomatis–infected MSCs in situ. The dye was added to the cells for 30 min before fixation and analysis by confocal microscopy. Low basal levels of NO were detected in both uninfected control and DFMO-treated cells. Infection reduced NO production, as indicated by the absence of green DAFM product (Fig. 6B). In contrast, infected cells treated with DFMO showed increased levels of green product, which was concentrated in vesicles (Fig. 6B).

Although imaging allows analysis of NO production, FACS analysis combined with a DAF-FM probe was used to confirm the reduction of iNOS activity in infected cells in the absence of DFMO. We observed three distinct populations with low, medium, and high fluorescence yield (Fig. 6C). NO levels in 20 mM DFMO-treated cells were low and comparable to uninfected cells. Infected cells showed medium fluorescence yield. The cell population with the highest fluorescence yield was infected cells treated with DFMO (Fig. 6C). This increase in NO-mediated fluorescence intensity was prevented by treatment with L-NMMA, a nonselective inhibitor of all NOS isoforms. Growth of C. trachomatis inclusions was rescued and formation of infectious EBs increased upon L-NMMA treatment in the presence of DFMO (Supplemental Fig. 4D), confirming that L-NMMA treatment counteracted the inhibitory effect of DFMO on C. trachomatis replication. Moreover, the increased nitrate production observed upon DFMO treatment in infected cells was prevented by L-NMMA supplementation (Supplemental Fig. 4E). Taken together, our results show that the constitutive levels of NO production are low in uninfected MSCs, and C. trachomatis infection reduces these levels even further. In the presence of DFMO, however, infected cells increase the production of NO, reducing chlamydial replication.
Inhibiting ODC activity, either with a chemical inhibitor or by RNA interference, restores the cells’ ability to upregulate iNOS in response to infection. NO in turn, was found to induce disruption of elementary body formation and a significant reduction in chlamydial infectivity. To our knowledge, the present results are the first to demonstrate the role of peroxynitrite formation in the acute defense against intracellular chlamydial infection.

NO is a highly reactive molecule with innate immune functions against different pathogens. NO is produced in mammalian macrophages by NO synthase, which is strongly upregulated following infection and believed to have an important role in destroying invading microorganisms (15, 16). The antimicrobial potential of NO during chlamydial infection in humans and mice appears to be variable (15, 16, 46). Furthermore, innate immunity against Chlamydia in human epithelial cells is predominantly mediated by the induction of the IDO that starves Chlamydia of the essential nutrient tryptophan (47), whereas in human macrophages the induction of iNOS has been found to be the first line of defense against chlamydial infection (48).

It has been shown that both intracellular and extracellular L-Arg availability are rate-limiting factors in NO production (49). L-Arg is metabolized either by NOS or arginase (50). Arginase metabolizes L-Arg into urea and l-ornithine, which is further metabolized by ODC into the polyamines (51). Therefore, the consequences of several pathologic conditions are critically determined by the interplay between arginase and iNOS because induction of one usually leads to inhibition of the other. Several other pathogens are known to downregulate iNOS via arginase activity. In H. pylori infection, this occurs either by induction of its rocF gene, which encodes arginase, or by the host cell’s arginase 2 (39). Trypanosoma cruzi induces both arginase 1 and arginase 2 in BALB/c mice (52). Salmonella enterica serovar Typhimurium (38) and Mycobacterium bovis (53) also use arginase as a means to escape the host NO defense. In these cases, iNOS downregulation can be reversed by NOHA treatment (38, 54), arginine supplementation or the arginase inhibitor BEC (39).

In contrast, other bacteria, such as pathogenic Escherichia coli, evade NO toxicity by means other than arginase induction, indicating that the specific phenotype varies between pathogens (54). Similarly, in our model, arginase expression and activity remained unaltered, indicating that C. trachomatis does not induce host cell arginase. A direct effect via secretion of bacterial protein is un-

**FIGURE 6.** DFMO increases iNOS activity. (A) Nitrite quantification using Griess assay shows that infected cells produce less nitrite compared with uninfected cells, even at 48 h p.i. DFMO treatment caused a dose-dependent increase in nitrite production in infected cells to several times the levels found in uninfected cells. Results shown are the mean values of three independent experiments. Error bars: ± SD. *p < 0.05. (B) Immunofluorescence micrographs of NO production visualized with DAF-FM diacetate. C. trachomatis–infected cells in the presence or absence of DFMO (20 mM) were incubated with the dye for 30 min and visualized using confocal microscopy. DFMO treatment resulted in increased vesicular staining for DAF-DM (green) in infected cells compared with control infected cells. Scale bars, 6 μm. (C) Cell monolayers were infected and treated with DFMO (20 mM), L-NMMA (0.4 μM), or both or left untreated. Cells were harvested, stained with DAF-FM, and analyzed using FACS. A population of intact cells equivalent to 10,000 events were gated and their fluorescence intensity determined by FACS plot. DFMO treatment resulted in increased numbers of infected cells positive for NO compared with untreated cells. In contrast, L-NMMA treatment counteracted the effect of DFMO and inhibited the restoration of NO in infected cells. These results are representative of two independent experiments.
likely, as no chlamydial gene encoding for arginase has been reported so far. Rather, it appears that arginine is not the rate-limiting factor in iNOS regulation in our system, as supplementation with L-Arg or norNOHA did not restore iNOS expression in infected cells.

In contrast, we found that ODC, the rate-limiting enzyme in the polyamines biosynthetic pathway, is highly induced upon chlamydial infection and results in the production of the polyamines spermidine and spermine. The induction of ODC expression preceded the downregulation of iNOS, strongly indicating that the resultant upregulation of the polyamine pathway reduces the iNOS protein level. Polyamines are known to interfere with NO production and are required for the growth of several pathogens, such as *Leishmania donovani* (55, 56), *Toxoplasma gondii* (57), *Trypanosoma brucei* brucei (13), and *Leishmania major* (58). In *H. pylori* infection, induction of ODC activity and spermine production were found to mediate conformational changes in the cationic amino acid transporter 2, which ultimately reduce arginine uptake and iNOS expression (36). We found that the ODC inhibitor DFMO has antichlamydial activity in human MSCs and HeLa cells, and it is known to eradicate parasites effectively, including *Leishmania promastigotes* (59) and *Leishmania infantum* in mice (60). In our study, the induction of ODC and polyamine synthesis was associated with iNOS downregulation, whereas ODC inhibition or knockdown led to recovery of iNOS levels and activity.

In general, polyamine induction has been found to interfere with iNOS expression through several different mechanisms. Briefly, polyamine induction alters the 3-d structure of CAT2, which reduces L-Arg uptake, eventually suppressing NO production (36). In addition, spermine metabolites, such as spermine dialdehyde, inhibit iNOS induction (61). Similarly, it has been shown that spermine itself does not affect the levels of iNOS mRNA but attenuates iNOS protein levels and NO production. This reduction in iNOS protein is due to inhibition of iNOS translation but not iNOS protein degradation induction (45). ODC knockdown also

**FIGURE 7.** DFMO induces nitrotyrosinylation of chlamydial proteins. Cells were infected with *C. trachomatis* D (MOI = 5) or left uninfected and then treated with DFMO (20 mM) for 12 h or left untreated. (A) Immunofluorescence micrographs of infected cells labeled for nitrotyrosine (red) and *Chlamydia* (green). Stained with rabbit polyclonal *Chlamydia* genus–specific Ab. The interaction of nitrotyrosine with bacterial inclusions was minimal in untreated cells. By contrast, DFMO treatment induced nitrotyrosine colocalization with early chlamydial inclusions. Scale bar, 10 μm. (B) Whole cell lysates were prepared using a protocol that maintains the integrity of protein-protein complexes. Lysates were immunoprecipitated with anti-nitrotyrosine Ab and then immunoblotted with Ab against chlamydial Hsp60. Hsp60 is coprecipitated with nitrotyrosine from lysates infected with *C. trachomatis* only after DFMO treatment.
results in increased iNOS protein expression and NO production in response to H. pylori, without altering iNOS mRNA levels (45).

In our experiments, we also failed to observe regulation of iNOS mRNA levels after C. trachomatis infection. Instead there was potent inhibition of iNOS protein translation. Consistent with this result, knockdown of ODC by siRNA increased iNOS protein expression and NO production in response to C. trachomatis, which enhanced killing of the bacterium. In addition, knockdown of ODC could lead to increased iNOS and NO levels by directing L-Arg back toward iNOS, which leads to increase iNOS translation, as shown by Lee et al. (62) and El-Gayar et al. (63).

C. trachomatis and other intracellular pathogens, including Mycobacterium, share the presence of a particular tyrosyl radical site in the bacterial ribonucleotide reductase, which can neutralize the toxic effect of NO (64). Thus, C. trachomatis shows variable sensitivity to the normally powerful bactericidal effects of NO during the course of infection. On one hand, the toxic effect of NO appears significant in C. trachomatis serovars H and D in stimulated McCoy and RAW264.7 cells, respectively (20, 21, 65). In our study, however, C. trachomatis serovars D and L2 in MSCs and HeLa cells were not subject to NO toxicity under control conditions. However, the increased NO synthesis in response to C. trachomatis infection that was seen after DFMO treatment caused a significant reduction in chlamydial replication. We hypothesize that the NO reaction products react rapidly with reactive oxygen species, culminating in the formation of peroxynitrite, which induces nitrative and oxidative stresses (11, 12, 66). Nitrosative stress includes protein nitration, which is associated mainly with a loss of protein functions (67). Our model clearly shows that DFMO treatment leads to induction of nitrotyrosine in infected cells that colocalizes with early chlamydial inclusions. Furthermore, our immunoprecipitation data demonstrate the physical interaction between chlamydial protein Hsp60 and nitrotyrosine upon DFMO treatment.

The growth of other pathogens is reported to be similarly affected by inhibition of the polyamine biosynthetic pathway. In Salmonella infection, the inhibition of arginase 2 activity by NOHA decreased the bacterial burden because of increased bacterial protein nitrogen (38). Bacterial chaperones (e.g., Hsp60) are induced during infection to overcome innate immunity. If this inhibition is reversed by DFMO, then C. trachomatis growth is impaired, at least partly, by chlamydial protein nitration. Therefore, innate immunity modulation by Chlamydia is a necessary step in the production of an intracellular environment conducive to chlamydial pathogenesis.

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Disclosures

The authors have no financial conflicts of interest.

References

Figure S1. IFNγ treatment inhibits chlamydial growth in HeLa cells and human MSCs.

HeLa cells (A) and MSCs (B) were infected with Ctr serovar D (MOI 5) for 2 h and then treated with 100 U/ml IFNγ or left untreated as control. The yield of infectious progeny decreased upon IFNγ treatment. Infectivity expressed as a percentage of control cells + s.d. from three biologically independent experiments. (C-D) Anti-IDO and iNOS immunoblots analyses of total lysates from HeLa cells (C) and MSCs (D) from cultures mock-infected or infected with Ctr for 48 h and exposed to 100 U/ml IFNγ for 2 - 48 h p.i. or left untreated as control. IFNγ treatment induced IDO expression in both cell types similar to the infected cells treated with IFNγ. Infection alone reduced iNOS expression level compared to control cells in HeLa and MSCs. β-actin was used as loading control.
**Suppl. Figure 2**

(A) Quantification of iNOS band densities from Fig 1A confirms that iNOS levels decrease significantly after Ctr infection at MOI 3, 4, and 5 compared to control uninfected cells. Results shown are the mean values of 2 independent experiments. Error bars: ± s.d.; * p < 0.05. (B) Quantification of iNOS band densities from Fig 1B confirms that iNOS levels decrease significantly upon Ctr infection (48 hpi) compared with control uninfected cells. Results shown are the mean values of 2 independent experiments. Error bars: ± s.d.; * p < 0.05. (C) Proteosomal inhibition did not rescue iNOS from degradation in Chlamydia-infected cells. Western blotting showed no rescue of total iNOS in whole cell lysates prepared from cells infected with Ctr serovar D (MOI 5) for 48 h and treated with the proteasome inhibitor MG132 (100 nM) from 47 hpi. β-actin was used to control equal loading of proteins. (D) qRT-PCR analysis of iNOS mRNA from Ctr D-infected cell monolayers between 6 and 48 h p.i., normalized to uninfected control. Results are expressed as the mean values of 3 independent experiments. (E) Infected cells were treated with different arginine concentrations as indicated or left untreated (control). Western blot analysis of total cell lysates from control or infected cells shows that iNOS expression is reduced in infected cells despite arginine treatment. Host β-actin was used as loading control.

**Figure S2.** Chlamydia infection inhibits iNOS translation in MSCs

(A) Quantification of iNOS band densities from Fig 1A confirms that iNOS levels decrease significantly after Ctr infection at MOI 3, 4, and 5 compared to control uninfected cells. Results shown are the mean values of 2 independent experiments. Error bars: ± s.d.; * p < 0.05. (B) Quantification of iNOS band densities from Fig 1B confirms that iNOS levels decrease significantly upon Ctr infection (48 hpi) compared with control uninfected cells. Results shown are the mean values of 2 independent experiments. Error bars: ± s.d.; * p < 0.05. (C) Proteosomal inhibition did not rescue iNOS from degradation in Chlamydia-infected cells. Western blotting showed no rescue of total iNOS in whole cell lysates prepared from cells infected with Ctr serovar D (MOI 5) for 48 h and treated with the proteasome inhibitor MG132 (100 nM) from 47 hpi. β-actin was used to control equal loading of proteins. (D) qRT-PCR analysis of iNOS mRNA from Ctr D-infected cell monolayers between 6 and 48 h p.i., normalized to uninfected control. Results are expressed as the mean values of 3 independent experiments. (E) Infected cells were treated with different arginine concentrations as indicated or left untreated (control). Western blot analysis of total cell lysates from control or infected cells shows that iNOS expression is reduced in infected cells despite arginine treatment. Host β-actin was used as loading control.
Figure S3. Arginase activity is not induced after chlamydial infection and inhibition of arginase does not affect chlamydial growth in MSCs.

(A) Quantification of ODC band densities from Fig 2B confirms that ODC levels increase significantly upon Ctr infection compared with control uninfected cells. Results shown are the mean values of 2 independent experiments. Error bars: ± s.d.; * p < 0.05 (B) Arginase activity as measured by spectrophotometry of cells infected for 6-48 h with Ctr D (MOI 5) or left uninfected. Infection did not alter arginase activity levels at any of the time points examined. (C) MSCs infected with Ctr D (MOI 5) were treated with different NOHA concentrations as indicated, or left untreated (control). The formation of infectious progeny of Ctr was assessed by infectivity titration assays. The yield of Ctr infectious progeny was not changed upon NOHA treatment. Infectivity was calculated as IFU/ml and expressed as percentage of control. Results shown are the mean values of 3 independent experiments. Error bars: ± s.d.
Figure S4. DFMO inhibits Ctr growth but does not alter iNOS mRNA expression

(A) HeLa cells were infected with Ctr serovar D (MOI 5) or L2 (MOI 1) and simultaneously treated with increasing concentrations of DFMO as indicated or left untreated (control). The yield of Ctr infectious progeny decreased significantly upon DFMO treatment, in a dose-dependent manner. Infectivity was calculated as IFU/ml and expressed as percentage of control. Results are the mean values of 3 independent experiments (B) Quantification of Hsp60 band densities from Fig 3B confirms that Hsp60 levels decrease significantly upon treatment with 20 mM DFMO compared to control infected cells. Results are the mean values of 2 independent experiments. (C) qRT-PCR analysis of iNOS mRNA from cells infected with Ctr D (MOI 5) treated with increasing DFMO concentrations as indicated, normalized to uninfected control cells. DFMO supplementation had no significant effect on iNOS mRNA expression in infected cells. Results are the mean values of 3 independent experiments. (D, E) MSC monolayers were infected with Ctr D (MOI 5) and treated with DFMO (20 mM) and/or L-NMMA (0.4 µM) or left untreated as control. (D) Resulting cell lysates were used for secondary infectivity assay. The yield of infectious progeny decreased significantly upon DFMO treatment but was rescued by simultaneous L-NMMA treatment, indicating rescued bacterial growth. Infectivity was calculated as IFU/ml and expressed as percentage of control. (E) Nitrite production as quantified by Griess assay of infected and control cells treated as indicated. The induction of nitrite production in infected cells in the presence of DFMO was prevented by simultaneous L-NMMA treatment. Results are the mean values of 3 independent experiments. Error bars: ± s.d.; * = p<0.05.