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Chlamydia muridarum Infection of Macrophages Elicits Bactericidal Nitric Oxide Production via Reactive Oxygen Species and Cathepsin B

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The ability of certain species of *Chlamydia* to inhibit the biogenesis of phagolysosomes permits their survival and replication within macrophages. The survival of macrophage-adapted chlamydiae correlates with the multiplicity of infection (MOI), and optimal chlamydial growth occurs in macrophages infected at an MOI of ≤1. In this study, we examined the replicative capacity of *Chlamydia muridarum* in the RAW 264.7 murine macrophage cell line at different MOIs. *C. muridarum* productively infected these macrophages at low MOIs but yielded few viable elementary bodies (EBs) when macrophages were infected at a moderate (10) or high (100) MOI. While high MOIs caused cytotoxicity and irreversible host cell death, macrophages infected at a moderate MOI did not show signs of cytotoxicity until late in the infectious cycle. Inhibition of host protein synthesis rescued *C. muridarum* in macrophages infected at a moderate MOI, implying that chlamydial growth was blocked by activated defense mechanisms. Conditioned medium from these macrophages was antichlamydial and contained elevated levels of interleukin 1β (IL-1β), IL-6, IL-10, and beta interferon (IFN-β). Macrophage activation depended on Toll-like receptor 2 (TLR2) signaling, and cytokine production required live, transcriptionally active chlamydiae. A hydroxyl radical scavenger and inhibitors of inducible nitric oxide synthase (iNOS) and cathepsin B also reversed chlamydial killing. High levels of reactive oxygen species (ROS) led to an increase in cathepsin B activity, and pharmacological inhibition of ROS and cathepsin B reduced iNOS expression. Our data demonstrate that MOI-dependent TLR2 activation of macrophages results in iNOS induction via a novel ROS- and cathepsin-dependent mechanism to facilitate *C. muridarum* clearance.

Infection of host epithelial cells by *Chlamydia* spp. sets in motion a cascade of signaling events that recruit multiple innate immune effectors to the infected site. Upon recognition of chlamydial pathogen-associated molecular patterns (PAMPs) and host danger signals, infiltrating leukocytes undergo transcriptional reprogramming to amplify the immune response by producing several cytokines and antimicrobial factors. The subsequent inflammatory process aids in bacterial clearance and primes elements of adaptive immunity while also contributing to the damaging pathology associated with chlamydial disease (1, 2).

Cells of the monocyte-macrophage lineage play critical roles in innate and adaptive immunity against chlamydial infections. Depletion of macrophages from mice prior to infection with *Chlamydia muridarum* and *Chlamydia psittaci* results in increased morbidity and pathogen burden (3, 4). Adoptive transfer of macrophages to RAG-1$^{-/-}$/gamma interferon (IFN-γ)$^{-/-}$ mice has been shown to be sufficient for the control of *Chlamydia pneumoniae* in lung models of infection (5). *Chlamydia trachomatis* is rapidly rerouted to lysosomes in RAW macrophages, where the pathogen is killed (6). Macrophage killing and subsequent presentation of elementary body (EB) antigens plays a key role in priming CD4^{+} T-cell responses to chlamydial infection (7). However, macrophage activities may not lead to immediate pathogen clearance, since some species of *Chlamydia* can survive and undergo limited replication in these cells. For instance, lymphogranuloma venereum (LGV) biovar, but not oculogenital biovar, strains of *C. trachomatis* can productively infect macrophages (8–10). *C. pneumoniae* and *C. psittaci* have been similarly shown to replicate in macrophages by preventing maturation of the phagosome (11, 12). Chlamydial persistence or replication within phagocytic cells correlates with infection resolution and disease outcome. LGV strains are highly invasive and cause systemic infections by dissemination through the lymphatic system (13). These strains also require a longer antibiotic regimen for effective treatment than non-LGV strains (14). Persistent infection of macrophages by *C. pneumoniae* in several tissues has been associated with chronic inflammatory conditions, including atherosclerosis, reactive arthritis, and asthma (15, 16).

Intracellular replication of chlamydiae within macrophages *in vitro* is less efficient than in epithelial cells and is limited by the constitutive expression of perforin-2 in macrophages (17). Productive infection also appears to be contingent upon the macrophage activation state and multiplicity of infection (MOI). Macrophages classically activated by IFN-γ and lipopolysaccharide (LPS) or other microbial PAMPs switch to an M1 polarized state. This correlates with increased bacterial killing via proinflammatory cytokines, production of reactive oxygen species (ROS) and

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upregulation of inducible nitric oxide synthase (iNOS) (18). Several types of immune cells secrete IFN-γ in response to chlamydial infection, likely favoring M1 polarization (19). M2 anti-inflammatory macrophages are activated by interleukin 4 (IL-4) or IL-10 and participate in wound healing and fibrosis (18). None of the evaluated chlamydial species survive in M1 macrophages *in vitro*, but whether they selectively replicate in M2 or resting-macrophage (M0) reservoirs as opposed to M1 *in vivo* is unknown (20–23). Interestingly, a virulent strain of *C. psittaci*, 6BC, recruits M0 instead of activated macrophages in mice, and this correlates with increased morbidity (3). Other chlamydial species may also modulate the macrophage activation state or possess alternative macrophage subversion strategies.

Early work with macrophages isolated from various hosts and tissues indicated that recovery of chlamydial infectious particles is also dictated by the MOI (8, 12, 24). Optimal recoveries were obtained when macrophages were infected at an MOI of 1 or less. MOIs on the order of 10 to 250 of *C. psittaci* and *C. trachomatis* L2/434/Bu led to decreased EB recovery, and this was attributed to host cell cytotoxicity occurring within the first 6 to 10 h of infection.

In this study, we examined chlamydial recoveries from cells of the murine RAW 264.7 macrophage line (RAW macrophages) infected with the mouse pathogen *C. muridarum* at MOIs ranging from 0.5 to 100. We observed a decrease in chlamydial survival with increasing MOI, consistent with previous studies (12, 24). Interestingly, a majority of cells infected at an MOI of 10 remained healthy but failed to support chlamydial development. However, these cells produced high levels of proinflammatory cytokines, iNOS-derived nitric oxide (NO), and ROS, indicative of M1 polarization. Induction of iNOS expression depended on an increase in cathepsin B activity and ROS accumulation. Blocking iNOS, ROS, or cathepsin B restored chlamydial growth in moderately infected macrophages. Together, our results indicate that a novel ROS- and cathepsin B-dependent pathway for NO production controls the growth of *C. muridarum* in RAW macrophages. Importantly, our results also suggest that differing MOIs might explain some seemingly contradictory results concerning the relative importance of iNOS in the control of chlamydial infections reported previously.

MATERIALS AND METHODS

Cell lines and chlamydial propagation. The murine RAW 264.7 macrophage line was a kind gift from Cheng Kao (Indiana University, Bloomington, IN). RAW cells were maintained in low-adhesion 10-cm bacterial petri dishes (VWR) in RPMI 1640 medium containing 2 mM L-glutamine (Life Technologies) supplemented with 10% fetal bovine serum albumin (FBS) (Atlanta Biologicals), 10 mM HEPES (Gibco), and 1 mM sodium pyruvate. Only low-passage-number (≤4) macrophages were used for experiments. *C. muridarum* (a generous gift from Harlan Caldwell, Rocky Mountain Laboratories, NIAID, NIH) (62) was propagated in McCoy mouse fibroblasts (American Type Culture Collection CRL-1696), and EBs were purified as previously described (25). The McCoy cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–high-glucose medium with 4 mM L-glutamine, 110 mg/liter sodium pyruvate (HyClone), 10% FBS, 10 mM HEPES, and 100 μM nonessential amino acids (Gibco).

Infection of RAW cells and inclusion-forming unit (IFU) assays. RAW cells were seeded in 24-well cell culture plates (Thermo Scientific) 48 h prior to infection. Chlamydial infections were performed on confluent monolayers in sucrose-phosphate-glutamic acid (SPG) buffer by centrifugation at 168 × *g* at room temperature (RT) for 1 h. Following infection, the SPG buffer was replaced with fresh culture medium, and the plates were incubated at 37°C in 5% CO_2. In some experiments, RAW cells were treated 30 min prior to infection with one or more of the following reagents: 1 mM L-NG-monomethyl arginine citrate (L-NMMA), 2 mM L-NG-nitroarginine methyl ester (L-NAME) (Cayman Chemical), 15 mM *N*,*N*′-dimethylthiourea (DMTU) (Acros Organics), 50 or 100 ng/ml IL-1Ra, 25 μM acetyl (Ac)-YVAD-CHO (Peprotech), 25 μM Z-WEHD-FMK, and 25 μM CA-074 Me (kind gifts of Stanley Spinola, Indiana University School of Medicine, Indianapolis, IN). Treatment continued throughout the course of infection. The infected monolayers were frozen in 500 μl SPG buffer at 24 h postinfection (p.i.). Upon thawing, the cells were scraped from the wells and agitated with 3-mm glass beads to harvest EBs.

IFU assays were then performed by infecting McCoy cells in 96-well plates (Thermo Fisher) with serial dilutions of the harvests of experimental infections. At 24 h p.i., the cells were fixed with methanol and stained with mouse anti-chlamydial LPS monoclonal antibody (MAb) (EVIH1), followed by a secondary Alexa Fluor 488-conjugated MAb (Life Technologies). Chlamydial inclusions were imaged and counted using an Evos FL Auto Cell Imaging System (Life Technologies).

Cytotoxicity assay. Lactate dehydrogenase (LDH) assays were performed according to the manufacturer's instructions (OPS Diagnostics). RAW cells were infected with *C. muridarum* at an MOI of 0.5, 10, or 100. Maximal LDH release was determined by treating cells in control wells with 10% Triton X-100 10 min prior to the assay. Supernatants were removed from the wells at the indicated time points after a brief spin to remove debris. In a 96-well plate, 25 μl of the supernatants was mixed with 75 μl of the dye-buffer solution. Following incubation at 37°C for 15 min, absorbance was measured at 490 nm.

Immunofluorescence microscopy. RAW macrophages were grown on glass coverslips in 24-well plates. The cells were infected by centrifugation, fixed at 2 h p.i. with 4% formaldehyde, and permeabilized with 0.05% saponin. The cells were blocked in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) for 1 h, and then the chlamydial inclusions were stained with the primary MAb EVIH1, followed by an anti-mouse Alexa Fluor 488-conjugated secondary antibody (Life Technologies). The coverslips were mounted on glass slides using ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Life Technologies), and images were captured using a Leica DMI6000 B inverted microscope with a 63× oil immersion objective.

UV inactivation of *C. muridarum* EBs. EB stocks were diluted in SPG buffer at a 1:10 ratio, pipetted onto a petri dish, and exposed to 1,200 J/cm^2 twice in a UV-cross-linking cabinet (Spectralinker; Spectronics Corporation) (26, 27). The efficacy of the UV treatment was confirmed by IFU assays.

Cytokine analysis. Culture supernatants were collected from infected RAW macrophages at the indicated times and centrifuged briefly to remove debris. The supernatants were assayed for IL-1β, IL-6, 1L-10, IL-12p40, tumor necrosis factor alpha (TNF-α), and IFN-γ (Milliplex) at the Bio-Plex core (IUPUI, Indianapolis, IN) according to the manufacturer's instructions. IFN-β levels were determined using a mouse IFN-β enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (BioLegend, San Diego, CA).

IFNAR1, TLR2, and TLR4 neutralization experiments. RAW macrophages were incubated for 1 h with antibody (10 to 30 μg/ml) and then stimulated with *C. muridarum* (MOI = 10), conditioned medium, or *C. muridarum* (MOI = 0.5) with either 500 U/ml recombinant mouse IFN-β (R&D Systems) or 0.5 ng/ml *E. coli* LPS. Anti-IFNAR1 (clone MAR1.5A3), anti-Toll-like receptor 4 (TLR4)-MD2 (clone MTS510), anti-TLR2 (clone T2.5), and isotype-matched IgG1 and IgG2A antibodies were purchased from BioLegend (San Diego, CA). Cells were harvested for IFU assays at 24 h p.i., and supernatants from anti-TLR2-treated macrophages were assayed for IFN-β levels using an ELISA kit (BioLegend).

Griess assay. Nitrite concentrations in culture media were measured with a commercial Griess assay kit (Biotium), and 150 μl of culture su-

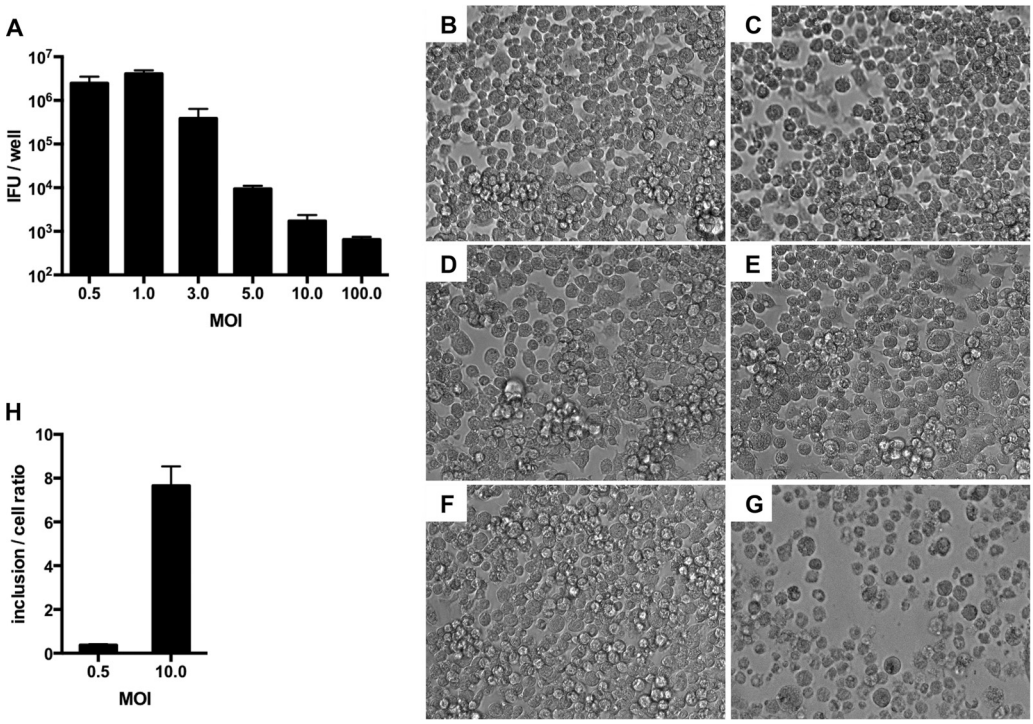

FIG 1 *C. muridarum* is inhibited in RAW macrophages infected at a high MOI. (A) Macrophages were infected with *C. muridarum* at MOIs from 0.5 to 100. IFU assays were performed at 24 h p.i. (B to G) Light microscopy of macrophages either mock infected (B) or infected with *C. muridarum* at various MOIs (0.5 [C], 3 [D], 5 [E], 10 [F], or 100 [G]) at 18 h p.i. (H) Numbers of inclusions counted per cell in macrophages infected at different MOIs at 2 h p.i. The error bars represent standard deviations (SD).

pernatant was removed from infected monolayers at 6, 12, and 24 h p.i. The samples were incubated with 20 μl Griess reagent [0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 0.5% sulfanilic acid, 2.5% phosphoric acid] in a 96-well plate at room temperature for 30 min. Absorbance was measured at 540 nm, and nitrite levels were calculated from a sodium nitrite standard curve.

Western blot analysis. Infected RAW cells were treated with various reagents; washed with PBS at 6, 12, or 24 h p.i.; and then incubated in 50 μl lysis buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 62 mM Tris, pH 6.8) containing a protease inhibitor minitablet (Pierce) for 10 min on ice. The cell lysates were boiled for 5 min, and protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated at 4°C overnight with rabbit iNOS MAb or rabbit GAPDH (glyceraldehyde-3-phosphate dehydrogenase) MAb and then incubated for 1 h with a secondary anti-rabbit–horseradish peroxidase conjugate. Antibodies were purchased from Cell Signaling Technology (Danvers, MA). Proteins were visualized using SuperSignal West Dura Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions.

ROS assay. ROS production of infected RAW cells in 96-well clear-bottom black plates (Corning) was assayed using the fluorogenic dye 2′,7′-dichlorofluorescein diacetate (DCFDA) (Abcam). Twenty micromolar DCFDA was mixed with culture media 45 min prior to measurement of fluorescence intensity (excitation and emission wavelengths, 485 nm and 535 nm).

Cathepsin B activity assay. RAW macrophages were cultured on coverslips in 24-well plates. Infections and pharmacological treatments of RAW macrophages were performed. At 10 h p.i., reconstituted Magic Red cathepsin B substrate reagent MR-(RR)2 (Immunochemistry Technologies) was added directly to the culture media in the wells. After 30 min of treatment, nuclei were counterstained with Hoechst 33342 for 10 min. Coverslips were analyzed for cathepsin B activity by fluorescence microscopy (Leica DMI6000 B; 63× oil immersion objective). Fluorescence intensities were quantified from 10 images obtained from each of three independent experiments using ImageJ software (28).

Statistics. Data were analyzed using Prism 6.0 software (GraphPad). For comparisons of multiple groups with two or more variables, data were subjected to log transformation and two-way analysis of variance (ANOVA) with Bonferroni posttest. Multiple comparisons for data with a single variable were analyzed by one-way ANOVA with Dunnett's posttest. Differences in values between two groups were determined using Student's *t* test. Differences were considered statistically significant when the *P* value was <0.05.

RESULTS

Productive *C. muridarum* infection of RAW macrophages is dependent on the multiplicity of infection. The effects of the MOI on *C. muridarum* IFU production were examined in RAW macrophages (Fig. 1A). At the lowest MOI (0.5), we observed a 2-fold increase in IFU recovery over input, which is in agreement with previous reports (24, 29). Maximal IFU recovery occurred when macrophages were infected at an MOI of 1. IFU recovery decreased at MOIs of 3 and higher. At moderate (10) and high (100) MOIs, IFU recoveries were approximately 0.001% and 0.0001% of input, respectively.

To determine if reduced IFU recovery at moderate and high MOIs could be explained by host cell death, infected macrophages were examined by light microscopy (Fig. 1B to G). Cells infected at a high MOI exhibited rounding and cytotoxicity (Fig. 1G). However, most cells infected at a moderate MOI had normal morphologies (Fig. 1F) and released basal levels of LDH until late in the chlamydial developmental cycle (see Fig. S1 in the supplemental

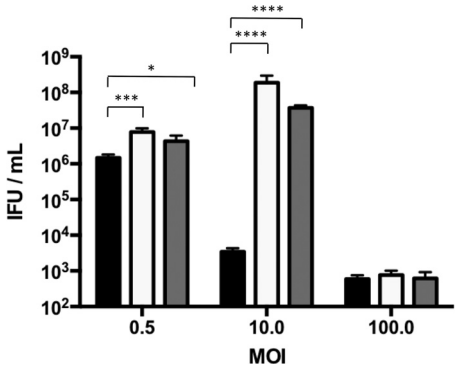

FIG 2 Inhibition of host protein synthesis rescues chlamydial growth in moderately infected macrophages. RAW macrophages were treated with 0.5 μg/ml cycloheximide 4 h before or at the time of infection at various MOIs. IFU assays were performed at 24 h p.i. Black bars, untreated; white bars, cycloheximide addition at 4 h prior to infection; gray bars, cycloheximide addition at 0 h p.i. ****, $P < 0.0001$; ***, $P < 0.001$; *, $P < 0.05$. Shown are means and SD.

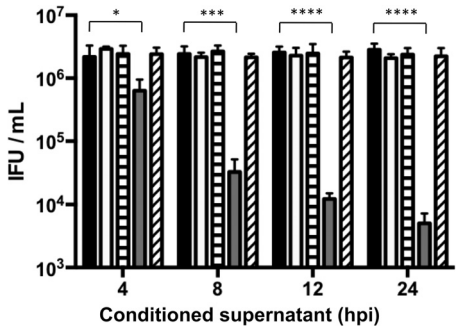

FIG 3 Supernatants from moderately infected macrophages contain antichlamydial factors. RAW macrophages were infected with *C. muridarum* at an MOI of 0.5 or 10. Supernatants from these infections were removed at 4, 8, 12, or 24 h p.i. and transferred onto macrophages that had been freshly infected at an MOI of 0.5. Some supernatants were heated at 95°C for 5 min prior to transfer. IFU assays were performed on supernatant-treated macrophages at 24 h p.i. Black bars, mock treated; white bars, MOI = 0.5; horizontally hatched bars, MOI = 0.5 and heated; gray bars, MOI = 10; obliquely hatched bars, MOI = 10 and heated). ****, $P < 0.0001$; ***, $P < 0.001$; *, $P < 0.05$. Shown are means and SD.

material). To test if low IFU recovery could be explained by reduced EB entry into macrophages, internalized EBs were quantified by fluorescence microscopy at 2 h p.i. The numbers of cytosolic EBs correlated with the input MOI (Fig. 1H). Overall, these findings suggested that moderate MOI infection triggered macrophage activation to inhibit chlamydial EB production.

Chlamydial inhibition in RAW macrophages requires *de novo* host protein synthesis. To elucidate the mechanism of *C. muridarum* growth inhibition in RAW macrophages, we initially tested if the antichlamydial factors were preformed. Infected macrophages were treated with cycloheximide to block protein synthesis either 4 h prior to or at the time of infection. Cycloheximide did not increase IFU production of macrophages infected at an MOI of 100 (Fig. 2). In contrast, cycloheximide treatment prior to and at the time of infection increased IFU production of macrophages infected at an MOI of 0.5 (low) by 3-fold and of macrophages infected at an MOI of 10 by more than 10,000-fold. These results implied that at low and moderate MOIs, *C. muridarum* induced macrophage defenses, whereas higher MOIs caused macrophage death.

Supernatants from RAW macrophages contain heat-sensitive antichlamydial factors. Activated macrophages release cytokines that can stimulate neighboring cells (30), so we tested if conditioned supernatants from infected macrophages could inhibit *C. muridarum* infection of newly infected macrophages. Conditioned supernatants from RAW cells infected at low or moderate MOIs were collected at various intervals postinfection. The conditioned supernatants were then transferred onto macrophages that had just been infected with *C. muridarum* at an MOI of 0.5 by centrifugation. Supernatants from low-MOI infections failed to inhibit chlamydial growth (Fig. 3). In contrast, supernatants from moderate-MOI infections strongly inhibited IFU production. The degree of this inhibition varied from 4-fold with 4-h p.i. supernatants to 1,000-fold with 24-h p.i. supernatants and was completely abolished if the supernatants were heated at 95°C for 5 min.

Cytokine secretion from RAW macrophages varies with the *C. muridarum* EB dose and treatment. To attempt to identify the *trans*-acting inhibitory factor(s), we assayed cytokines in the conditioned supernatants of macrophages that had been infected at various MOIs. The levels of most of the cytokines produced by the moderately infected macrophages peaked by 3 h p.i. and were higher than those produced by the macrophages infected at low levels (Fig. 4A). High levels of TNF-α were detected at both MOIs, but the cytokine peaked earlier in supernatants from the macrophages infected at the higher MOI (Fig. 4A).

Since killed and viable EBs elicit dissimilar cytokine responses (31, 32), cytokines in supernatants from macrophages that were infected with nonreplicating chlamydiae at an MOI of 10 (wild-type EBs with rifampin or UV-inactivated EBs) were assayed. With the exception of TNF-α, cytokine levels were substantially lower than those observed in experiments with viable *C. muridarum* (Fig. 4B). Overall, the results of these experiments suggested that most cytokine secretion required an antigen not present in EBs and which was synthesized *de novo* by *C. muridarum* in the host macrophage.

IFN-β secreted by RAW macrophages induces antichlamydial responses. IFN-β has been previously shown to have an antichlamydial role in macrophages (33), so we tested if IFN-β secreted by moderately infected RAW macrophages stimulated host defense pathways. IFN-β signaling was blocked by treating RAW macrophages with an IFNAR1-neutralizing antibody (anti-IFNAR1), and these macrophages were then treated with conditioned medium from moderately infected macrophages. The anti-IFNAR1 antibody, but not an isotype control, increased the chlamydial yield 75-fold (Fig. 5A). Chlamydial inhibition mediated by the addition of exogenous IFN-β to macrophages infected at a low MOI was reversed by anti-IFNAR1, confirming the specificity of the antibody (Fig. 5B). Thus, our results indicated that IFN-β secretion in response to a moderate level of *C. muridarum* infection contributed to the paracrine upregulation of antichlamydial macrophage defense mechanisms.

Antichlamydial activity of RAW macrophages is mediated by TLR2, but not TLR4. Chlamydial cell wall antigens and heat shock proteins induce TLR2 and, to a lesser extent, TLR4 signaling in phagocytes and epithelial cells (34, 35). To determine if TLR2 or TLR4 activation stimulated macrophage antichlamydial responses, we used antibodies to specifically block TLR2 (anti-

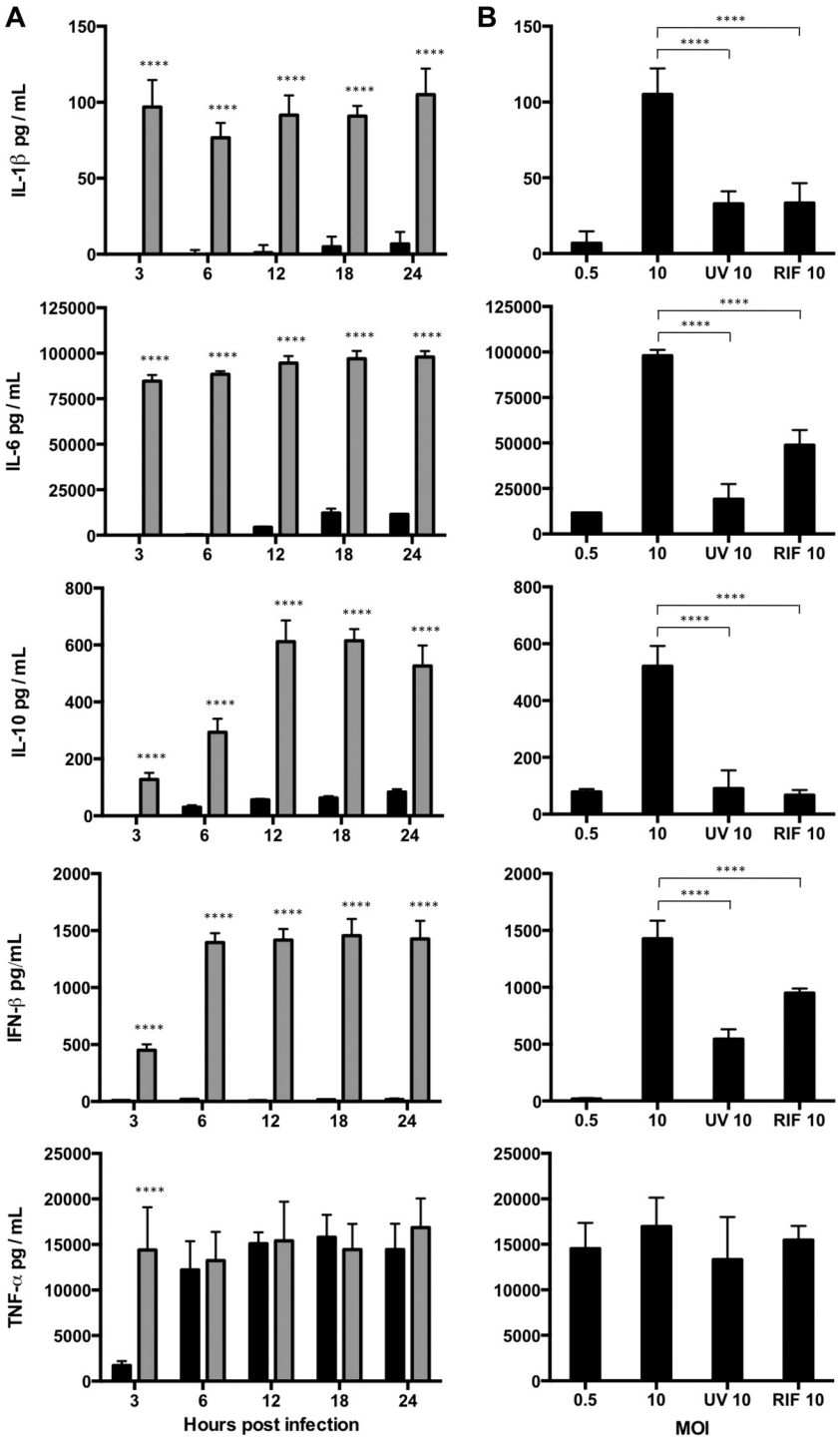

FIG 4 Cytokine profiles of infected macrophages. (A) RAW macrophages were infected at an MOI of 0.5 (black bars) or 10 (gray bars). Supernatants were collected at 3, 6, 12, 18, and 24 h p.i. and assayed for cytokines. (B) Supernatants were collected at 24 h p.i. from RAW cells infected at an MOI of 0.5 or 10 with live EBs in the absence or presence of rifampin (RIF 10) or UV EBs (UV 10). ****, $P < 0.0001$. Shown are means and SD.

TLR2) and TLR4 (anti-TLR4) responses. Control experiments were performed to establish that both antibodies effectively blocked cognate TLR function in RAW cells. Anti-TLR2 treatment of moderately infected macrophages reduced IFN-β production in a dose-dependent manner (Fig. 6A). Anti-TLR4 abolished chlamydial inhibition that was elicited by adding *E. coli* LPS to RAW macrophages infected with *C. muridarum* at a low MOI (Fig. 6B). However, only anti-TLR2 partially restored *C. muridarum* IFU production in macrophages infected at a moderate MOI (Fig. 6C and D). This indicated that the antichlamydial response of macrophages infected at moderate MOIs is primarily mediated by TLR2.

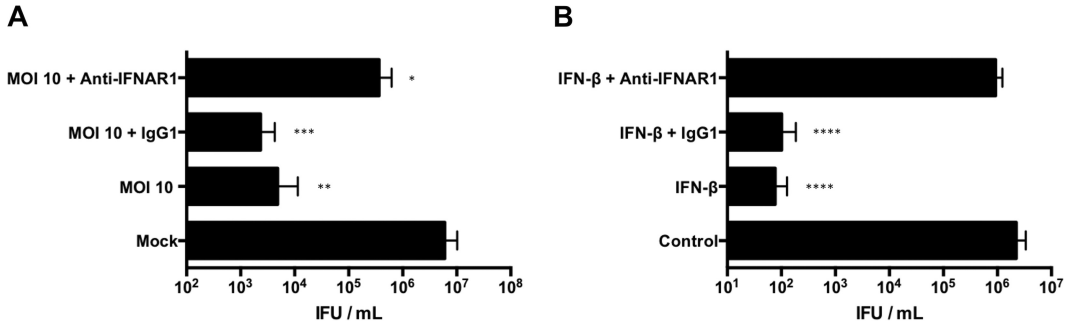

FIG 5 Secreted IFN-β inhibits *C. muridarum* in RAW macrophages. (A) IFU from macrophages infected with *C. muridarum* at an MOI of 0.5 and incubated with conditioned media from uninfected (mock) or moderately infected (MOI = 10) cells. Some wells were pretreated with anti-IFNAR1 or isotype control IgG1 antibody (Ab) (10 μg/ml). (B) IFU from macrophages infected with *C. muridarum* at an MOI of 0.5. Some wells treated with 500 U/ml IFN-β were preincubated with anti-IFNAR1 or isotype control IgG1 Ab. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Shown are means and SD.

Chlamydial inhibition at moderate MOIs is mediated by iNOS and ROS. Macrophages activated by chlamydial infection upregulate expression of iNOS, an enzyme that catalyzes NO formation (29, 33, 36, 37). They can also accumulate high levels of ROS via the phagocyte NADPH oxidase complex (phox) or damaged mitochondria (38). To determine if iNOS was induced in RAW macrophages by *C. muridarum* infection, we measured the quantity of nitrites in culture supernatants. Higher nitrite levels were observed in supernatants from macrophages infected at a moderate MOI than from macrophages infected at a low MOI (Fig. 7A). The levels of iNOS protein measured by Western blotting correlated with supernatant nitrite levels (Fig. 7B). While iNOS was not detected in cells infected at a low MOI, iNOS was detected by 6 h p.i. in macrophages infected at a moderate MOI.

The macrophage ROS response was assessed by incubating infected cells with the fluorogenic DCFDA. ROS production of macrophages infected at an MOI of 0.5 was low at all measured time intervals (Fig. 7C). However, macrophages infected at a moderate MOI produced strong fluorescence, which was almost entirely reversed by the addition of the hydroxyl radical scavenger DMTU.

To evaluate the chlamydicidal potential of macrophage-derived NO and ROS, infected macrophages were treated with the iNOS inhibitors L-NMMA and L-NAME or the hydroxyl radical scavenger DMTU. None of these compounds affected chlamydial recovery in low-MOI infections (Fig. 7D). However, both iNOS inhibitors and DMTU increased IFU production in the macrophages infected at a moderate MOI (39, 40). These results indicated that moderately infected RAW macrophages stimulated production of NO and reactive oxygen species that inhibited *C. muridarum*.

Ca-074Me, a cathepsin B inhibitor, rescues *C. muridarum* from antichlamydial macrophage responses. Many cytokines can stimulate macrophage iNOS expression, including caspase-1-dependent IL-1β (41–43). Since infected RAW macrophages secreted IL-1β, we evaluated its role, as well as that of caspase-1, in chlamydial inhibition using various inhibitors. Pretreatment of

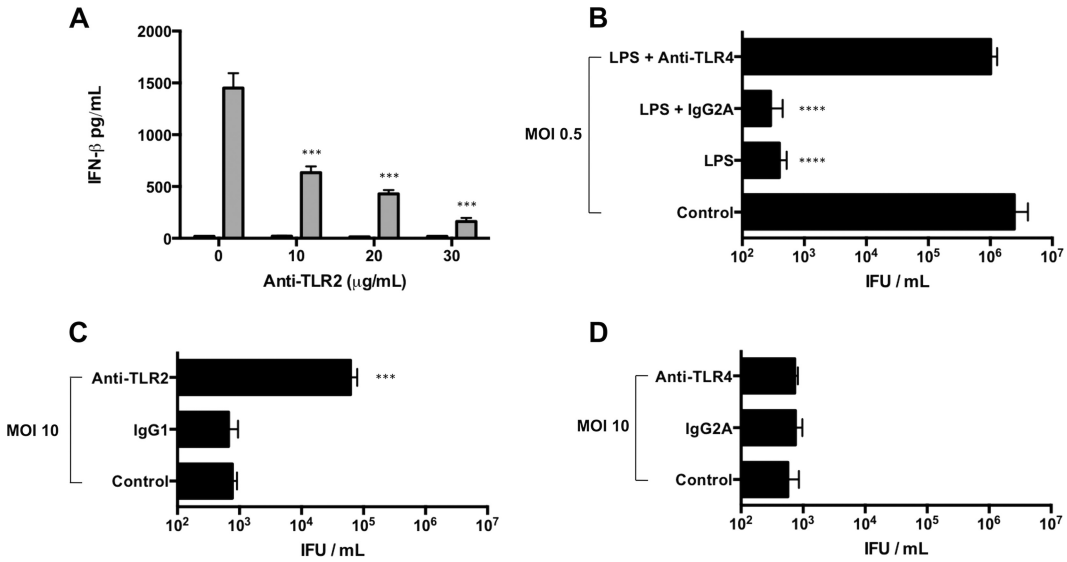

FIG 6 TLR2, but not TLR4, mediates macrophage inhibition of *C. muridarum* at intermediate MOIs. (A) Macrophages pretreated with anti-TLR2 were infected at an MOI of 0.5 (black bars) or 10 (gray bars). IFN-β levels in supernatants at 24 h p.i. were measured by ELISA. (B) IFU from macrophages infected with *C. muridarum* at an MOI of 0.5 at 24 h p.i. Some wells exposed to *E. coli* LPS were treated with anti-TLR4 or IgG2A isotype control Ab (10 μg/ml). (C and D) IFU at 24 h p.i. from macrophages infected at an MOI of 10 treated with anti-TLR2 or IgG1 isotype control Ab (30 μg/ml) (C) and anti-TLR4 or IgG2A (D). ****, $P < 0.0001$; ***, $P < 0.001$. Shown are means and SD.

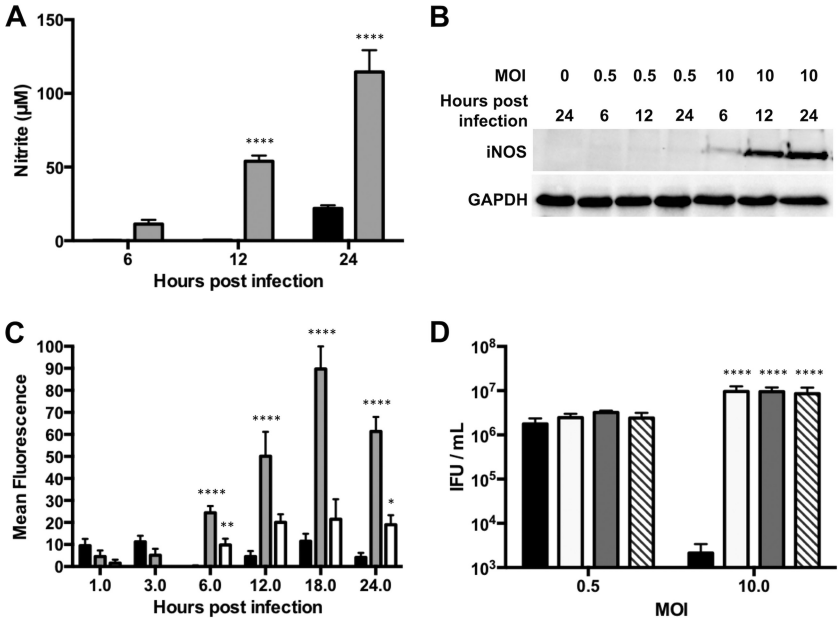

FIG 7 Moderately infected RAW macrophages inhibit *C. muridarum* by producing nitric oxide and reactive oxygen species. (A) Supernatants and protein were collected at 6, 12, or 24 h p.i. from RAW cells infected at an MOI of 0.5 (black bars) or 10 (gray bars). Nitrite levels were quantified by Griess assay. (B) Western blotting for iNOS levels. (C) RAW cells infected at an MOI of 0.5 (black bars), 10 (gray bars), or 10 with DMTU (white bars) were assayed for ROS production at 1, 3, 6, 12, 18, and 24 h p.i. (D) RAW cells infected at an MOI of 0.5 or 10 were left untreated (black bars) or were treated with L-NMMA (white bars), L-NAME (gray bars), or DMTU (hatched bars). IFU assays were performed 24 h p.i. ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$. Shown are means and SD.

the macrophages with IL-1Ra, an IL-1β antagonist, did not influence chlamydial recovery (Fig. 8A). The caspase-1 inhibitor Ac-YVAD-CHO also failed to alter IFU production. However, the less specific caspase-1 inhibitor Z-WEHD-FMK increased IFU recovery in moderately infected macrophages approximately 10-fold. Z-WEHD-FMK can also inhibit lysosomal cathepsins (44), so we treated infected macrophages with the selective cathepsin B inhibitor Ca-074Me. Ca-074Me increased IFU production by 1,000-fold in moderately infected macrophages (Fig. 8B). Analysis of cathepsin B activity at 10 h p.i. using the fluorogenic substrate CV-(RR)2 indicated no difference in fluorescence intensity between mock-infected cells and cells infected at a low MOI (Fig. 8C and D; see Fig. S2 in the supplemental material). In contrast, cathepsin B activity was significantly increased in the macrophages infected at a moderate MOI, and this was reversed by the addition of Ca-074Me (Fig. 8E and F; see Fig. S2 in the supplemental material). Together, these results indicated that increased cathepsin B activity in RAW macrophages influences chlamydial IFU production.

ROS and cathepsin B activities are necessary for maximal iNOS induction in *C. muridarum*-infected RAW macrophages. Pharmacological inhibition of ROS, iNOS, and cathepsin B led to similar chlamydial recoveries. We asked if these effector mechanisms exerted additive effects, but combined inhibition did not improve chlamydial recovery beyond that observed with single-drug treatments (Fig. 9A). This suggested that they functioned in the same inhibitory pathway or that they played cooperative roles in chlamydial killing. To distinguish between these possibilities, we first analyzed iNOS protein and nitrite levels in moderately infected macrophages treated with L-NAME, DMTU, or Ca-074Me. L-NAME interferes with iNOS activity, but not expression. Western blots showed equal quantities of iNOS protein in untreated and L-NAME-treated cells, but nitrite levels were reduced in samples treated with L-NAME (Fig. 9B; see Fig. S3 in the supplemental material). However, both iNOS and nitrite levels were substantially lower in cells treated with DMTU or Ca-074Me. This indicated that ROS and cathepsin B controlled iNOS expression.

We next investigated if ROS modulated cathepsin B activity or vice versa. Addition of DMTU to macrophages infected at a moderate MOI decreased cathepsin B activity to levels lower than those in mock-infected cells (Fig. 9C to E; see Fig. S4 in the supplemental material). Conversely, inhibition of cathepsin B with Ca-074Me in similarly infected macrophages caused a similar and small decrease in DCFDA fluorescence (see Fig. S5 in the supplemental material). However, since NO production was also inhibited in macrophages treated with Ca-074Me, we reasoned that the slight reduction in DCFDA intensity might be due to an absence of peroxynitrite. Indeed, DCFDA fluorescence levels did not differ between Ca-074Me- and L-NAME-treated macrophages (see Fig. S5 in the supplemental material). In summary, these results suggested that ROS production increased cathepsin B activity, which upregulated iNOS expression.

DISCUSSION

Members of the family *Chlamydiaceae* differ in their capacities to survive within macrophages, and this correlates with their ability to avoid fusion with lysosomes inside these cells (12). The ability to survive and replicate in macrophages has been linked to increased dissemination of *C. trachomatis* LGV strains, leading to systemic disease (13). Chlamydial persistence in macrophages can result in chronic inflammation and a delayed response to antibiotic treatment (14, 45). Thus, understanding the mechanisms that

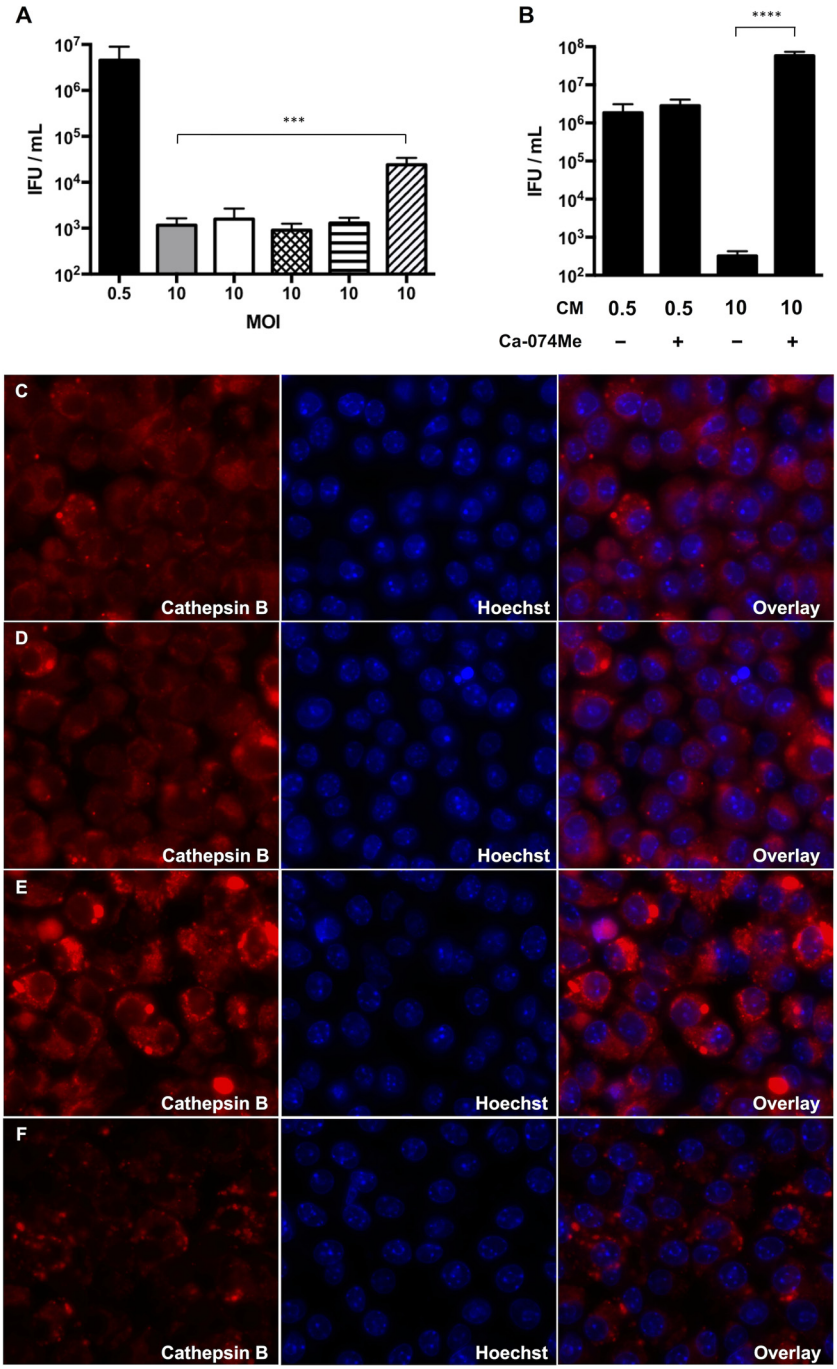

FIG 8 Ca-074Me, a cathepsin B inhibitor, rescues *C. muridarum* from macrophage inhibition. (A) RAW macrophages were infected at an MOI of 0.5 (black bar) or 10 (gray bar). Some cells infected at an MOI of 10 were treated with IL-1Ra at 50 μg/ml (white bar) or 100 μg/ml (crosshatched bar), Ac-YVAD-CHO (horizontally hatched bar), or Z-WEHD-FMK (obliquely hatched bar). (B) Treatment of macrophages infected at an MOI of 10 with CA-074Me. Shown are means and SD. (C to F) Representative live-microscopy images of cells at 10 h p.i. treated with the cathepsin B indicator dye MR-(RR)2 (red channel) and the nuclear stain Hoechst 33342 (blue channel). (C) Mock infected. (D) MOI = 0.5. (E) MOI = 10. (F) MOI = 10 with Ca-074Me. ****, $P < 0.0001$; ***, $P < 0.001$.

promote chlamydial survival in mononuclear phagocytes could have implications for the development of therapeutic strategies.

Chlamydial species that can evade phagolysosomal fusion within macrophages appear to do so only when host cells are infected at an optimal MOI. Several groups have reported reduced chlamydial recoveries from macrophages infected at MOIs of 100 or greater (12, 24). This correlated with immediate damage to the host cells and fusion of lysosomes with EB-containing phagosomes. In this study, we characterized the interactions of RAW 264.7 macrophages with *C. muridarum* and demonstrated that three different outcomes could be achieved in response to different MOIs. At an MOI of 1 or lower, RAW cells supported chla-

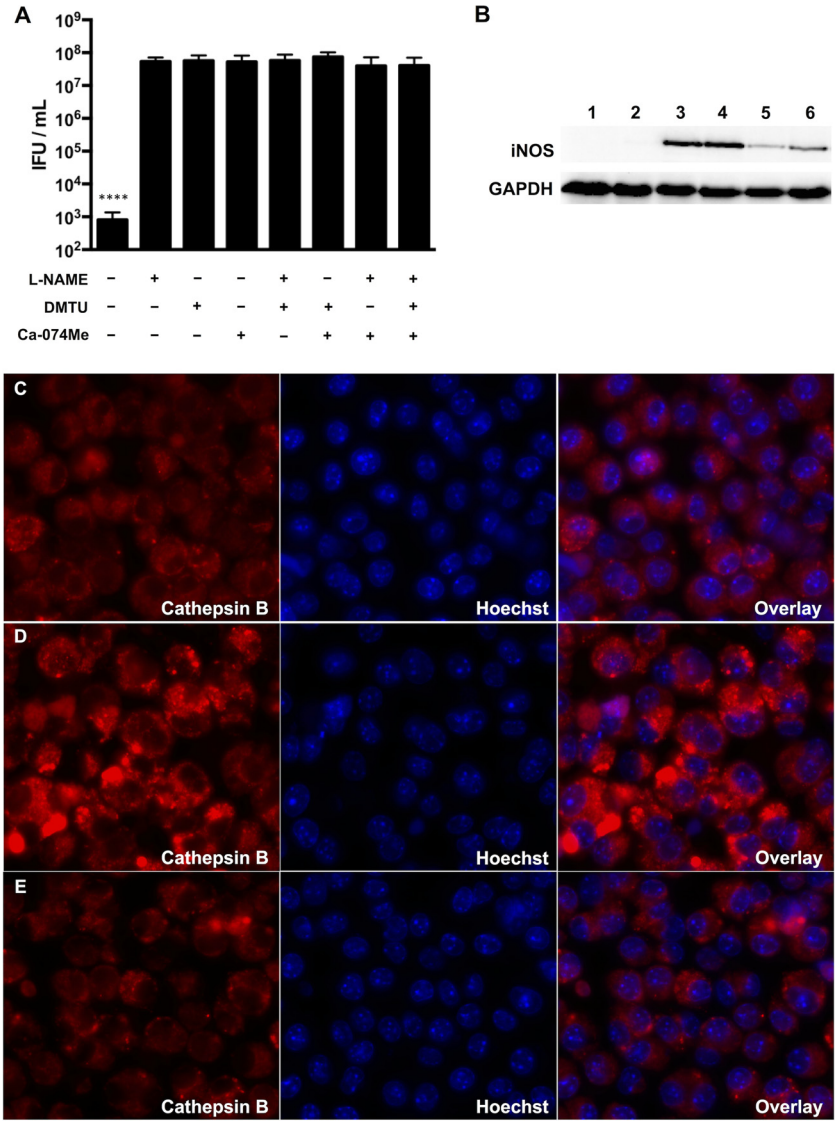

FIG 9 iNOS expression in moderately infected macrophages is regulated by ROS and cathepsin B. (A) RAW macrophages infected at an MOI of 10 were treated with one of more of the following chemicals: L-NAME, DMTU, and Ca-074Me. Shown are means and SD. (B) Western blot for iNOS at 24 h p.i. from mock-infected cells (lane 1); cells infected at an MOI of 0.5 (lane 2), 10 (lane 3), or 10 with L-NAME (lane 4); cells infected at an MOI of 10 with DMTU (lane 5); and cells infected at an MOI of 10 with Ca-074Me (lane 6). (C to E) Representative live-microscopy images of cells at 10 h p.i. treated with the cathepsin B indicator dye MR-(RR)2 (red channel) and the nuclear stain Hoechst 33342 (blue channel). (C) Mock infected. (D) MOI = 10. (E) MOI = 10 with DMTU. ****, $P < 0.0001$.

mydial replication. An MOI of 100 conferred immediate cytotoxicity, and few infectious chlamydial particles were recovered. Inhibition of host protein synthesis did not prevent chlamydial or host cell death. However, when RAW macrophages were infected at an intermediate MOI of 10, they did not succumb to early death and cleared *C. muridarum* infection by a process that could be reversed by cycloheximide.

Consistent with previous studies, we found that macrophage activation and subsequent clearance of *C. muridarum* were predominantly mediated by TLR2 (46, 47). The cognate chlamydial ligands for TLR2 have not been identified, but several candidates have been proposed in recent years. Recombinant chlamydial MIP-like protein can induce cytokine production via TLR2/TLR1/TLR6 and CD14 in human macrophages (48). Chlamydial LPS is less active than classic endotoxins but can elicit TLR2 signaling *in vitro* (49). Chlamydial Hsp60 induces inflammatory responses in mice in a TLR2- and TLR4-dependent fashion (34). Plasmid-deficient strains of *C. trachomatis* and *C. muridarum* exhibit impaired TLR2 responses, implying that one or more TLR2 antigens are either encoded or regulated by the plasmid (50, 51). Cytokine production via TLR2 signaling in epithelial cells also requires infection with live, replicating chlamydiae, indicating that the antigen is not present in EBs (32). Culture supernatants of RAW macrophages infected at an MOI of 10 were inhibitory and contained elevated levels of several cytokines, including IFN-β, which induced a paracrine antichlamydial pathway. Cytokine secretion by RAW cells required TLR2, as well as transcriptionally active chlamydiae.

Binding of microbial PAMPs to TLRs or other cytosolic pattern recognition receptors (PRRs) triggers cellular signaling events that culminate in the activation of NF-κB, mitogen-activated protein kinase (MAPK) or IRF3 pathways (52, 53). These in turn regulate the expression of genes that encode proinflammatory cytokines and iNOS. Aside from the direct induction of iNOS by PRR signaling, several cytokines can also regulate iNOS expression. Protective effects of nitric oxide in chlamydial infections have been well documented. Nitric oxide promotes IFN-γ-mediated eradication of chlamydiae and protects mice from the development of hydrosalpinx and infertility (54). Interestingly, TLR2/TLR4 double-deficient mice do not resolve *C. pneumoniae* lung infections because they do not induce iNOS, in spite of elevated IFN-γ secretion in these animals (55). This suggests that nitric oxide production relies on signaling via TLR2/TLR4 in the murine lung model of infection. We observed that RAW macrophages infected with *C. muridarum* at an MOI of 10 produced more iNOS and nitrite than cells infected an MOI of 0.5 and that this negatively impacted chlamydial survival. Our results are reminiscent of a study by Huang et al. in which the outcomes of low and high doses of intranasally inoculated chlamydiae in C57BL/6 mice were evaluated (56). Animals infected with higher numbers of *C. psittaci* remained healthier than those that were infected at low doses, and the accelerated bacterial clearance in heavily infected mice was linked to an increased NO production by macrophages. Comparable observations have been reported with *C. muridarum* infections of the murine genital tract, where high infectious doses result in less disease severity and fewer viable organisms in the oviducts (57). We speculate that similar macrophage activation programs control NO production in response to higher doses of infectious chlamydiae in both mice and cell culture.

We determined that iNOS induction in RAW macrophages was mediated by an increase in ROS production. Modulation of iNOS expression by ROS is not without precedent. High-MOI infection of macrophages with *Propionibacterium acnes* increases ROS levels, which in turn upregulate iNOS via NF-κB/AP-1 activation (58). Similar dependence on ROS for nitric oxide production has also been observed in mouse endothelial cells stimulated with IFN-γ and LPS (59). Multiple ROS sources exist within mammalian cells, including the NADPH oxidase complex and mitochondria. Whether ROS generated by RAW macrophages in our study was mitochondrion or NADPH oxidase derived was not determined. Several groups have reported a loss of mitochondrial membrane potential and integrity in cells invaded by pathogens (60). The subsequent mitochondrial ROS release can cause lysosomal membrane permeabilization (LMP), leading to the leakage of active lysosomal cathepsins into the cytosol. In agreement with these studies, we noted increased cathepsin B activity in RAW cells that was abolished by an ROS scavenger. However, a significant proportion of this cathepsin B activity at 10 h p.i. appeared to localize to large vesicular structures. Labeling with specific markers will be required to determine if these vesicles are lysosomes or also include EB-containing phagosomes that fused with lysosomes. Whether lysosomal cathepsin B leaks into the cytosol at later times due to ROS-mediated LMP requires further investigation. What is certain is that ROS affects cathepsin B activity in RAW macrophages. Surprisingly, the inhibition of cathepsin B led to a substantial decrease in iNOS and nitrite levels while also enabling chlamydial rescue. Pharmacological inhibition of iNOS did not greatly affect ROS levels or cathepsin B (unpublished observations) (see Fig. S5 in the supplemental material), indicating that chlamydial survival in RAW cells was dictated by the presence or absence of NO. However, we cannot rule out a direct role for ROS and cathepsin B in chlamydial inhibition, because chlamydiae damaged by reactive oxygen and nitrogen species might become more susceptible to lysosomal fusion and processing by cathepsin B.

Cathepsin B and ROS act in concert as innate immune effectors to activate NLRP3 inflammasome complexes, leading to IL-1β production (61). Inflammasome-derived IL-1β can stimulate iNOS-mediated nitric oxide production in macrophages to inhibit *Leishmania* spp. (42). Blocking IL-1β signaling in RAW macrophages did not reverse chlamydial killing or affect iNOS and nitrite levels (Fig. 8A and unpublished observations), so precisely how ROS and cathepsin B regulate iNOS expression in these cells remains unclear. A protective role for cathepsin B in immunity to *C. muridarum* disease in mice has not been described, and thus, future experiments will involve confirming our observations in primary macrophages and determining *C. muridarum* infection outcome in cathepsin $B^{-/-}$ mice.

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