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Differential signaling pathways are initiated in macrophages during infection depending on the intracellular fate of *Chlamydia* spp.

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Abstract

Chlamydia muridarum and *Chlamydia caviae* have equivalent growth rates in mouse epithelial cells but only *C. muridarum* replicates inside mouse macrophages, while *C. caviae* does not. Macrophages infected with *C. muridarum* or *C. caviae* were used to address the hypothesis that the early signaling pathways initiated during infection depend on the fate of chlamydiae in the host cell. Transmission electron microscopy of *C. muridarum*-infected macrophages showed intact chlamydial elementary bodies and reticulate bodies 2 h post-infection in compact vacuoles. Conversely, in macrophages infected with *C. caviae*, chlamydiae were observed in large phagocytic vacuoles. Furthermore, *C. caviae* infections failed to develop into inclusions or produce viable bacteria. Expression of pro-inflammatory cytokines TNF- α , IL-1 β , and MMP13 was similar in *C. caviae*- or *C. muridarum*-infected macrophages at 3 hours post-infection, indicating that chlamydial survival is not required for initiation of these responses. IL-1 β secretion, dependent on inflammasome activation, occurred in *C. caviae*-infected macrophages despite no chlamydial growth. Conversely, IFN β mRNA was observed only in *C. muridarum*- but not in *C. caviae*-infected macrophages. These data demonstrate that differential signaling events are initiated during a productive vs. non-productive chlamydial infection in a macrophage.

Keywords

Chlamydia; IFN β ; IL-1 β ; macrophages

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CONFLICT OF INTEREST

All authors have no conflict of interests

INTRODUCTION

Chlamydia trachomatis is the major cause of genital infection in women leading to pelvic inflammatory disease. Although many host cell types are susceptible to *Chlamydia* infection in tissue culture, the primary target cells in vivo are the columnar epithelial cells of the genital tract, the conjunctiva or the gut epithelia. Interestingly, some of the *Chlamydia spp.* have the ability to infect and grow efficiently inside macrophages both in vivo and in cell culture [reviewed in¹]. This includes the mouse *C. muridarum* and the human strains LGV and *C. pneumoniae*. Further, there are a few reports of *C. muridarum* infection at remote tissue sites, suggesting that macrophages could serve as vehicles for dissemination [reviewed in²]. Macrophages are professional phagocytes that normally internalize and degrade bacterial pathogens in their lysosomes. However, a number of bacterial pathogens, including *C. muridarum*, have evolved strategies to subvert phagocytosis by macrophages. Macrophages are recruited to the infection site and produce a number of cytokine/chemokines at the site of infection. Cytokine/chemokine responses to infection drive tissue pathology and the mechanism of initiation of these cytokine responses in immune cells has been an intense area of study.

We have previously shown that IFN β expression during *Chlamydia* infection requires chlamydial growth and DNA-sensing by the host DNA sensor cGAS-STING pathway^{3, 4}. We have also shown that NLRP3 and ASC are required for IL-1 β secretion in resting macrophages during *C. muridarum* infection, while only ASC is required for high levels of IL-1 β secretion during infection in LPS pre-stimulated/activated macrophages⁵. Finnerty et al⁶ have also shown the requirement of DNA-sensing AIM2 inflammasome for IL-1 β secretion in activated macrophages. Inhibition of bacterial growth by inhibitors of bacterial transcription and translation do not inhibit IL-1 β secretion⁵. These studies have indicated that successful inclusion formation by chlamydiae vs. their engulfment by lysosomes could initiate differential host cytokine responses.

We hypothesize that the early intracellular fate of chlamydiae determines the initiation of specific innate responses. To address this hypothesis, two species of chlamydiae, the guinea pig strain, *C. caviae*, and the mouse strain, *C. muridarum*, which display differential growth properties in mouse macrophages, were used. *C. caviae* and *C. muridarum* grow at almost equal rates in mouse L929 cells but only *C. muridarum* grows in primary macrophages to form mature inclusions, while *C. caviae* fails to do so. Epithelial cells support growth of multiple chlamydial strains at all times under resting conditions. Therefore, differential growth of chlamydial strains in macrophages provides a better model system for studying the mechanism of initiation of cytokine responses to successful infection vs. just phagocytosis and killing of live chlamydiae. Our results show that induction of pro-inflammatory cytokine mRNAs, such as TNF α and IL-1 β , occur independent of chlamydial growth in macrophages although their sustained expression required chlamydial growth. ASC-dependent inflammasome activation was observed independent of chlamydial growth. Conversely, as previously reported³, IFN β and IFN response genes are dependent on chlamydial survival and growth in macrophages. Our data demonstrate that the intracellular niche of *Chlamydia* contributes to differential sensing and signaling in the host.

RESULTS

***Chlamydia muridarum* can replicate in mouse macrophages while *C. caviae* fails to do so.**

C. muridarum and *C. caviae* stocks were titrated to give equal percentage of infection in McCoy cells by IFU and FACS, and used for infecting primary mouse macrophages at 1 MOI of infection. *C. muridarum* and *C. caviae* grow in mouse L929 cells forming inclusions (yellow) (Fig 1A). However, in primary mouse macrophages, only *C. muridarum* forms mature inclusions at 24 h post-infection, with about a 40–60% infection rate, while *C. caviae* fails to do so (Fig 1A). Cell lysates from infected macrophages were harvested and numbers of infectious elementary bodies (EB) were measured by infecting L929 cells. Although macrophages were infected with the same numbers of EB, a three log less infectious yield was obtained from *C. caviae* relative to *C. muridarum* (data not shown). The percentage of recovered IFU showed a 200% recovery of IFU for *C. muridarum* at 24 h, suggesting formation of new EBs, while only 0.2% *C. caviae* were recovered, suggesting active killing and no growth of *C. caviae* in macrophages. Consistent with the IFU results, TEM images of *C. muridarum*-infected macrophages at 24 hours post-infection showed mature inclusions containing both the metabolically active reticulate body (RB) forms and the infectious EB forms (Fig 1C and Supplementary Fig S1). The RBs line up on the inner side of the inclusion membrane in some inclusions as predicted by Wilson et al model⁷. At 24 h post-infection, *C. caviae*-infected macrophages were indistinguishable from uninfected macrophages (data not shown).

To observe the chlamydial fate and changes in host cell morphology during early stages of infection, TEM of macrophages were obtained at 2 h post-infection. *C. muridarum* EBs were secured inside a closed membrane bound vacuole, with chlamydial membrane in close proximity to the vacuolar membrane (Fig 2). Conversely, *C. caviae* EBs/RBs were found in the large mono-layered phagocytic vacuoles with no proximity of chlamydial membrane to the vacuolar membrane (Fig 2). Further, *C. muridarum*-infected macrophages were unperturbed with their pseudopods, while *C. caviae*-infected macrophages contained large vacuoles and lost their pseudopods (Figure S2). To determine the nature of the vacuoles where the chlamydiae were localized, macrophages were stained for an early endosomal marker EEA-1 and the lysosomal marker LAMP-1. Both *C. muridarum* and *C. caviae* did not co-localize with the EEA-1 suggesting that the chlamydia-containing vacuoles had already lost the characteristics of early endosomes (Fig 3 and Fig S3). At 3 h post-infection, *C. muridarum* did not co-localize with LAMP-1, suggesting escape from lysosomes. However, a larger proportion of *C. caviae* co-localized with LAMP-1, suggesting fusion of *C. caviae* containing vacuoles with lysosomes (Fig 3 and Fig S3). UV-killed chlamydiae did not co-localize to lysosomes at this time, suggesting differential kinetics for live vs. killed bacteria (data not shown).

Differential gene expression in *C. muridarum*- and *C. caviae*-infected macrophages.

To address the hypothesis that early host signaling events are determined by chlamydial fate in the host cells, RNA was extracted from infected macrophages and mRNA from two groups of genes were analyzed, based on the activation of the essential transcription factor(s). The genes in the first group were *Tnfa*, *Scya4*, *Il1 β* , and *Mmp13*, all of which

require NF κ B/MAPK activation for their expression. The second group included *Ifn β* , and type I IFN dependent genes; *Egr-1*, *Cxcl10*, and *Mx-1*, which require activation of transcription factor IRF3 in addition to NF- κ B. We have previously shown that CXCL-10 expression during *Chlamydia* infection is dependent on IFN α/β signaling⁸. The IFN β -inducible nature of these genes has been reported by microarray analysis⁹ and was further validated at <http://systemsimmunity.org/tools/shiny/IntegratedBrowser/>. Infection with both *C. muridarum* and *C. caviae* led to similar induction of mRNA for *Tnfa*, *Scya4*, *Il1 β* , and *Mmp13* (Fig 4A–4D) at 3 h post-infection, although mRNA levels were significantly reduced by 8 h post-infection only in *C. caviae*-infected cells. Similar results were observed for *Il-6* and *Tnfs9* (data not shown). Additionally, infection with UV-killed chlamydiae was also able to initiate these responses at 3 h, although sustained expression was not observed at 8 h pi. These results suggest that uptake of chlamydiae by macrophages was sufficient to initiate these responses. Conversely, *Ifn β* , *Egr-1*, *Cxcl10*, and *Mx-1* were expressed at 3 h post-infection only in *C. muridarum*-infected but not in *C. caviae*-infected macrophages (Fig 4E–4H) and their expression increased with time in *C. muridarum*-infected macrophages. Similar results were also obtained with *Ccl5* and *GemGTPase* (data not shown). UV-killed bacteria failed to initiate these responses, as previously observed for IFN β ¹⁰. These data suggest differential initiation of gene expression depend on early chlamydial fate, before the initiation of chlamydial replication.

Early NF κ B activation occurs independent of chlamydial growth.

Induction of inflammatory cytokines through NF κ B activation requires activation of the p65 subunit of NF κ B, its nuclear localization and binding to the promoter of several inflammatory genes. Immunofluorescence staining of NF- κ B p65 showed that p65 was localized to the nucleus at 3 h post-infection in macrophages infected with *C. muridarum* or *C. caviae* (Fig 5A). Infection with UV-killed bacteria also resulted in some p65 nuclear localization (data not shown). By 8 h post-infection, a reduction in nuclear localization of p65 was observed in macrophages infected with *C. caviae* relative to those infected with *C. muridarum* (Fig 5B). Consistent with nuclear localization, equivalent levels of p65-DNA binding activity was observed at 3 h post-infection with both strains, although the activation level declined by 8 h in *C. caviae*-infected macrophages (Fig 5C).

Chlamydia caviae infection results in equivalent levels of IL-1 β secretion as *C. muridarum* infection in resting macrophages.

IL-1 β secretion needs two signals. In the first signal, IL-1 β mRNA is expressed and inactive pro-IL-1 β protein is made. In the second, activation of inflammasome results in caspase-1 activation, which cleaves pro-IL-1 β to mature IL-1 β allowing the protein to be secreted out of the cells to initiate inflammation. Since, NF κ B activation and IL-1 β mRNA levels were similar in *C. caviae*-infected and *C. muridarum*-infected macrophages at 3 h post-infection, we tested inflammasome-dependent IL-1 β protein secretion in the culture supernatants. Surprisingly, *C. caviae* infection resulted in equivalent or more IL-1 β secretion, than *C. muridarum* infection, suggesting no alteration in inflammasome activation (Fig 6A). During *C. muridarum* infection, IL-1 β secretion was fully dependent on the inflammasome adaptor ASC (Fig 6A) as shown before⁵. Although not completely abolished, IL-1 β secretion was significantly reduced in ASC KO macrophages during *C. caviae* infection. *C. caviae* failed to

grow in ASC macrophages, with similar numbers of IFU recovered as observed for WT macrophages (data not shown). *C. caviae* also co-localized to LAMP-1 at 3 h post-infection (data not shown), suggesting a minimal role for inflammasome in growth inhibition. These results reveal that *C. caviae* infection was able to provide both signals for IL-1 β production despite no chlamydial growth. To address if an inhibitor of vacuolar ATPase, which prevents acidification of late endosomes, will alter chlamydial growth and inflammasome activation, macrophages were exposed to Bafilomycin A, a known inhibitors of endosomal acidification. Bafilomycin exposure had minimal effect on *C. muridarum* inclusion formation or IL-1 β secretion at lower concentrations. Bafilomycin exposure did not rescue *C. caviae* growth (data not shown), but a significant increase in IL-1 β secretion was observed for *C. caviae* infection, suggesting blocking this pathway increased inflammasome activation, specifically in the absence of chlamydial growth.

DISCUSSION

Chlamydia is predominantly an epithelial pathogen and its growth is initiated at the mucosal surface. However, dissemination to other tissue sites has been observed in the mouse model in the absence of IFN γ R signaling¹¹ or in the absence of B cells¹². This ability to disseminate has been attributed to the growth of *C. muridarum* in macrophages^{11, 13}. Growth of human chlamydial isolates in primary macrophages is variable^{1, 14}. With the exception of LGV, most human *C. trachomatis* isolates do not grow well in macrophages¹. Restrictive growth has been attributed to perforin-2¹⁵, early phago-lysosome fusion, and late autophagy¹⁶. *C. muridarum* and *C. caviae* have different host requirements *in vivo*, but in *in vitro* cell culture both grow in epithelial cells at equal rates. However, while *C. muridarum* can replicate successfully in an inclusion in macrophages, *C. caviae* fails to do so. In this study, we have used primary mouse macrophages as a model system to study early host response to chlamydial survival or failure to do so, using *C. muridarum* and *C. caviae*. Our results indicate that IFN β and IFN-response gene expression are initiated only when chlamydiae escape phago-lysosome fusion and survive. These responses further require chlamydial growth/replication inside an inclusion, since UV-killed *C. muridarum* fail to induce these responses. In contrast, early NF κ B-dependent signaling in the host is initiated independent of chlamydial survival and whether or not chlamydiae are inside lysosomal vacuoles. Further, our data demonstrate that inflammasome activation in macrophages does not require chlamydial growth. These findings suggest that intracellular niches for bacterial ligands determine initiation of host-signaling events.

DNA sensing by cGAS⁴ and activation of STING³ are essential for IFN β induction during infection with multiple *C. trachomatis* isolates⁴. It has also been shown that direct sensing of chlamydial second messenger cdi-AMP by STING can induce IFN β ¹⁷. IFN β expression requires actively replicating bacteria and inhibition of bacterial growth by chloramphenicol abrogates this response¹⁸. During chlamydial entry, followed by localization to early endosomes that resist fusion with lysosomes, chlamydial DNA is unavailable for recognition by cytosolic host DNA sensors. Therefore, initiation of an IFN β response could be a result of direct sensing of the chlamydial second messenger cdi-AMP by STING, in the host cytosol¹⁷. As *C. muridarum* replicates inside the inclusion, it is possible that DNA fragments released inside the inclusion escape into the cytosol and are detected by the cytosolic DNA

sensor cGAS. Conversely, during *C. caviae* infection, fusion of early endosomes with lysosomes leads to digestion of bacteria by lysosomal enzymes preventing any cdi-AMP production. However, bacterial digestion in the lysosomes could release fragmented DNA fragments. But these are likely not sensed by cGAS as it is unable to access DNA at this niche. This could explain why no IFN β transcripts are made during *C. caviae* infection versus during *C. muridarum* infection at 3 h post-infection. Therefore, even though exogenous DNA in the host cells is a danger signal, it may not be seen as a “viral” threat to generate an IFN β response if the DNA is inside the lysosomes.

Previously we have shown that resting macrophages induce very low levels of IL-1 β during *C. muridarum* infection⁵. One possible explanation for the poor inflammasome activation in resting macrophages during *C. muridarum* infection could be due to the pathogen’s ability to inhibit fusion with lysosomes rapidly and secure itself in endosomes. Consequently, the macrophage experiences minimal mitochondrial stress as observed by cell morphology with intact pseudopodia (Supplementary Fig S2) or DNA-release, required for NLRP3 or AIM2-mediated inflammasome activation¹⁹. It is interesting to note that different chlamydial preparations induce differential levels of IL-1 β at similar MOI, suggesting that less pure preparation may activate phagocytic pathway and increased signal. Macrophages exposed to LPS before infection secrete high levels of IL-1 β secretion⁵, although the numbers of *C. muridarum* inclusions are significantly reduced, with several trapped in lysosomes (data not shown), suggesting a minimal role of chlamydial growth in this response. Entry of *C. caviae* into macrophages does not inhibit lysosomal fusion and bacteria appear in several large vacuoles in macrophages 2 h post-infection (Fig 1 and Supplementary Fig S2). Since *C. caviae* is very effective in inducing TLR2 responses²⁰, there is significant first signal leading to NF κ B activation to generate pro-IL-1 β . *C. caviae*-infected resting macrophages thereby secrete high levels of IL-1 β without pre-stimulation. Inflammasome activation was dependent on ASC, suggesting contribution from NLRP3 and AIM2, which have been previously shown to contribute to this response. UV-killed *C. muridarum* fail to activate inflammasome, despite production of high levels of IL-1 β mRNA, suggesting the requirement of viable bacteria for this process. However, UV-killed *C. caviae* infection result in low levels of IL-1 β secretion, which was surprisingly ASC independent.

Considering both AIM2 and cGAS sense exogenous DNA, it is interesting that cGAS sensing occurs only when inclusions are observed as in *C. muridarum* infection, while inflammasome-mediated sensing is chlamydial growth-independent. Further studies are required to determine the compartmentalization of these two sensors during infection which results in differential cytokine responses. It also remains unclear why *C. muridarum* is successful in establishing infection in macrophages while most other *C. trachomatis* strains are not. Likewise, human isolate LGV has been shown to grow in macrophages¹⁴. Whether these strains have additional effectors that subvert macrophage phagocytic pathways giving them an edge remains unclear and requires independent investigation.

The antagonistic nature of IFN β and IL-1 β pathways has been described, with type I IFN inhibiting IL-1 β production²¹ and, conversely caspase-1-mediated cleavage of cGAS²², suggesting preferential signaling in one direction versus the other. Interestingly, both IFNAR signaling²³ and IL-1R signaling²⁴ promote oviduct pathology during genital *C. muridarum*

infection in the mouse model, suggesting that signaling pathways initiated by differential host responses does not ensure protection from disease. IFN β expression is an antiviral response, but it is ineffective in eradicating intracellular bacteria. IFN β has been shown to down-modulate IFN γ R signaling during *Listeria* infection²⁵ and *Chlamydia* infection²³. IL-1R signaling has some contribution in controlling bacterial numbers but also contributes to neutrophil recruitment and oviduct pathology²⁴. These findings underscore how *Chlamydia* is a successful pathogen despite engaging several host responses. Furthermore, our results demonstrate that sustained chlamydial growth is not necessary for certain cytokine responses such as TNF α - and IL-1- signaling, suggesting antibiotics to stop chlamydial growth or replication are not sufficient to inhibit the damage caused by some cytokine responses in the female genital tract.

METHODS

Chlamydial stocks:

C. muridarum strain “Nigg” was originally obtained in 1977 as a yolk sac preparation from the ATCC and has been passaged continually in our laboratory since that time. *C. caviae* was originally obtained from the late Edward Murray in the early 1970’s and has been continually passaged in Dr. Rank’s laboratory since that time. Both isolates were grown in mycoplasma-free McCoy cells as described previously^{26, 27}. Elementary bodies were harvested from infected cells, resuspended in SPG buffer (250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid, pH 7.2), and quantified as inclusion forming units (IFU) on McCoy cell monolayers as described previously.

Isolation of murine macrophages, in vitro infection and Bafilomycin treatment:

C57BL/6J (WT) or ASC^{-/-} mice were injected with 3% thioglycollate and peritoneal macrophages were harvested after 3 days. Animal protocols were approved by UAMS Little Rock and UNC Chapel Hill. Macrophages were plated in complete media (RPMI media supplemented with 10% FBS, 100 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μ M nonessential amino acids, 100 U/ml penicillin/100 μ g/ml streptomycin, 50 mM β -mercaptoethanol) for 48–72 h prior to infection at 8×10^5 cell/well in a 24 well culture plate. In vitro infection using chlamydiae were carried out as described⁸. To obtain similar levels of infection with *C. muridarum* and *C. caviae* in macrophages, the chlamydial stocks were used to infect McCoy cells at different MOI of the 2 chlamydial strains. The host cells were treated with 0.3% saponin and intracellular staining for chlamydial inclusions was carried out using the “Pathfinder” FITC-conjugated murine anti-chlamydial mAb (Bio-Rad). McCoy cells were analyzed by flow cytometry to determine the percentage of infected cells. MOIs that gave an equal percentage of infection in McCoy cells were used to infect macrophages. Infected macrophages on cover slips were fixed with methanol 24 hours post-infection and stained for chlamydial inclusions using the “Pathfinder” FITC-conjugated murine anti-chlamydial mAb (Bio-Rad) according to the manufacturer’s instructions. Inclusions were viewed using an Olympus fluorescent microscope. For Bafilomycin A1 exposure, the peritoneal macrophages from WT mice infected with 1MOI of *C. caviae* or *C. muridarum* were either kept in media or exposed to 30 nM, 100 nM or 300 nM of

Bafilomycin A1 (Sigma Aldrich) during the period of infection. At 18 hours post-infection, supernatants were collected and assayed for IL-1 β by ELISA.

Electron microscopy:

Macrophages (10⁷) were infected with *C. caviae* or *C. muridarum* as described above in plates coated with poly-L-lysine. The infected macrophages were detached at indicated times with 10 $\times$ trypsin containing EDTA, by gently scraping cells with a rubber policeman. Macrophages were washed and centrifuged at 1200 rpm for 5 min; the cell pellets were then suspended in EPON fixative and processed for transmission electron microscopy (TEM) as described earlier²⁸.

Quantitative real time RT-PCR:

RNA was prepared using the RNeasy kit (Qiagen). Reverse transcription reaction and quantitative real time PCR (RT-PCR) were carried out as described previously⁸. Primers used are described in supplementary Table 1. The results are presented as fold increase in expression over uninfected samples, by comparing the threshold cycle of gene of interest to β -actin gene expression, using the CT method²⁹.

NF κ B activity and p65 nuclear translocation:

Nuclear extracts from infected cells were prepared using Nuclear extract kit (Active Motif). TransAM NF κ B kits (Active Motif) were used to determine p65 activity. For NF κ B p65 staining, mouse primary macrophages grown on coverslips were infected at indicated times and fixed in methanol-free 4% paraformaldehyde (EM Bioscience) for 15 min at room temperature. Cells were rinsed with PBS three times and blocked for 1 h at room temperature in 5% normal goat serum diluted in 1 $\times$ PBS and 0.3% Triton X-100. NF κ B p65 Ab (C22B4, rabbit mAb #4764 Cell-signaling) was used at 1:25 in 1 $\times$ PBS with 1% BSA and 0.3% Triton X-100 and incubated overnight at 4°C on a shaker. Mouse immune sera containing anti-chlamydial antibody was used to stain for Chlamydia. Coverslips were rinsed thrice in PBS for 5 min each. Secondary antibodies (anti-rabbit AF488 and anti-mouse AF568-Invitrogen) were used at 1:1500 dilution in 1% BSA and 0.3% Triton X-100 and incubated for 2 h at room temperature. Coverslips were rinsed three times in PBS for 5 min each and mounted with Prolong gold anti-fade reagent (Invitrogen), with or without DAPI for nuclear staining.

EEA1 and LAMP-1 staining:

Mouse macrophages infected with 1 MOI of *C. muridarum* or *C. caviae* were fixed with 4% PFA (Electron Microscopy Sciences, Hatfield, PA) at 3 h and 18 h post-infection. Cells were stained for either early endosomal marker EEA1 using anti-EEA1, Cat # MA5-14794 (Invitrogen, Thermo Fisher Scientific) or lysosomal marker LAMP-1 using anti-LAMP1, Cat# 21997-1-AP (Proteintech) at 1:200 dilution, and with Chlamydial LPS (mouse monoclonal Anti-LPS, cat# MCA2718) (BioRAD) used at 1:300 dilution. The secondary antibody combination contained goat anti-mouse Alexa 488 (cat# A11017) and goat anti-rabbit Alexa 594 (cat# A11037) (Invitrogen, Thermo Fisher Scientific) at 1:1000 dilution. Cells were washed and mounted using Prolong anti-fade containing DAPI (Invitrogen).

Confocal images were acquired with the 63× oil 0.8 numerical aperture objective using Zeiss confocal microscope (LSM 880) and images were analyzed using image J or Zen lite software.

IL-1 β ELISA:

Macrophages from C57BL/6 and ASC knock out mice were plated (8×10^5) and incubated for 48–72 h prior to infection. Macrophages were infected at the same infection rate with *C. muridarum* and *C. caviae* as explained in above methods section. ASC KO mice peritoneal macrophages were included in the study to determine inflammasome involvement in IL-1 β release during *C. caviae* infection. Supernatants were collected at indicated times for IL-1 β protein quantitation. IL-1 β ELISA was carried out as described in manufacturer's protocol (R&D systems).

Statistics:

Infection phenotype, IFU, RNA and protein analysis were performed using at least three independent times, EEA/LAMP1 staining and p65 study were done twice and TEM was done once. Significance determined using the analysis component of graph pad software. Unpaired student T test was used for determining significance between two individual groups. For more than two treatment group with one variable, a one-way ANOVA with pairwise multiple comparison (Holm-Sidak method) was performed to determine statistically significant differences. For more than two treatment group with two variables, a 2-way ANOVA with the post-hoc Tukey test as a multiple or pair wise comparison procedure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1: *C. muridarum* survives and forms mature inclusion in primary mouse macrophages while *C. caviae* does not.

L929 cells and primary mouse macrophages were infected with *C. muridarum* or *C. caviae* for 24 h at 1 MOI and stained using Pathfinder FITC-conjugated murine anti-chlamydial mAb (yellow) with evans blue counter stain (red) (A). Cell lysates from infected macrophages (done in triplicate) were collected and used to infect L929 cells for IFU counts. Data represent means plotted to show input and recovered EB from macrophages (B). Error bars represent SD and significance determined by one-way ANOVA. TEM image of macrophages infected with *C. muridarum* at 24 h post-infection (C and inset). Darker smaller forms inside the inclusions are chlamydial EBs and larger less dense forms are chlamydial RBs.

Fig 2: At 2 h post-infection, *C. muridarum* survives in small compact vacuoles, while *C. caviae* are in large vacuoles.

TEM images of macrophages infected at 10 MOI for 2 h with *C. muridarum* (A) or *C. caviae* (B). Inset in each panel shows chlamydiae in small compact vacuoles (*C. muridarum*) or in large vacuoles (*C. caviae*). Arrows point to chlamydiae inside vacuoles.

Fig 3: At 2 h post-infection, both *C. muridarum* and *C. caviae* do not co-localize with early endosomes, but more numbers of *C. caviae* co-localize with lysosomes. Macrophages infected with *C. muridarum* or *C. caviae* at 1 MOI infection for 3 h p.i were fixed and immuno-stained for an early endosomal marker EEA-1 (A) or lysosomal marker LAMP-1 (B) with chlamydial LPS staining. Arrows indicate co-localization of LAMP-1 and LPS. Image shows a single cell for clarity. Larger field is shown in Fig S3. A representative image from one of two experiments is shown.

Fig 4: Pro-inflammatory gene transcripts are induced following both *C. muridarum* and *C. caviae* infection, while IFN β and IFN response gene transcripts are induced only during *C. muridarum* infection.

Quantitative RT-PCR data are expressed as fold changes compared to uninfected (UI) controls from RNA extracted from macrophages at 3, 8, and 24 h post-infection. RNA was extracted at 3 h post-infection in cells infected with UV inactivated (UV-in) *Chlamydia*. Individual gene expression was normalized to actin expression. TNF α (A), Scya4 (B), IL-1 β (C) and Mmp13 (D) are shown on the left panel and data for IFN β (E) and IFN-response genes, Egr-1 (F), CXCL10 (G) and Mx-1 (H) are shown on the right panel. Samples were run in triplicates and data are representative of 3 independent experiments. Mean values are shown and error bars represent SD. * represents significant changes ($P < 0.05$) by multiple t tests.

Fig 5: p65 nuclear translocation and its promoter binding activity were similar between *C. muridarum*- and *C. caviae*- infected macrophages.

Macrophages were infected with *C. muridarum* or *C. caviae*, and stained for p65, chlamydial inclusion (LPS) and DAPI for nuclear staining at 3 and 8 h post-infection (A). Cells were fixed and visualized using a Zeiss confocal microscope. Top panel shows staining for p65 (red), chlamydial inclusion (green) and DAPI (blue), while lower panel shows the same image for p65 staining only. Red signal inside the nucleus and similar area in the cytosol for each group was quantified using Image J software. Data represented as nuclei/cytoplasm ratio (B). Data shows mean and SEM from 8 cells. Significance determined by one way ANOVA with multiple comparison tests. Nuclear extracts were prepared from infected cells at 3 h and 8 h post-infection and p65 DNA-binding activity (C) was determined as described in Methods. Data represent mean of triplicate values from one of two experiments, error bars represent SD, and significance determined by one way ANOVA. * represents <0.05, ** represents <0.01, *** represents <0.001 and ns represents “not significant”.

Fig 6: *C. caviae* induces inflammasome activation in resting macrophages.

(A) Supernatants from WT and ASC KO-infected macrophages were analyzed for IL-1 β protein by ELISA 24 h post-infection. Data represents averages from 3 experimental replicates (B) WT macrophages treated with three different concentrations of Bafilomycin were infected with *C. muridarum* or *C. caviae* and supernatants analyzed for IL-1 β by ELISA. Mean values are shown and error bars represent SD. *** indicates $p < 0.001$ and ** indicates $p < 0.01$ by one way ANOVA. ND=Not detected.