Inflammation and Fibrosis during *Chlamydia pneumoniae* Infection Is Regulated by IL-1 and the NLRP3/ASC Inflammasome

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*Chlamydia pneumoniae* is a common respiratory pathogen associated with atypical pneumonia, and it has been suggested as a trigger or promoter of several chronic inflammatory conditions, such as asthma and atherosclerosis. The β form of IL-1 (IL-1β) is a pro-inflammatory cytokine released by many cell types and is an important mediator of inflammation during infection. IL-1β production is a tightly controlled process that includes regulation at multiple levels and typically requires two distinct signals for activation and release. In this study, we investigated the ability of *C. pneumoniae* to induce IL-1β secretion. We found that *C. pneumoniae* is unique among the other *Chlamydia* species tested in its ability to potently induce secretion of mature IL-1β from unprimed bone marrow-derived macrophages during a productive infection. TL2 was required for induction of pro–IL-1β, whereas the NLRP3/ASC was required for caspase-1 activation and pro–IL-1β cleavage to produce mature IL-1β. Caspase-1 cleavage was independent of endogenous ATP release, but required potassium flux, lysosomal acidification, and cathepsin B release. We further investigated the role of IL-1 in host defense against *C. pneumoniae*-induced pneumonia using mice deficient in the type I IL-1R. Although the IL-1R−/− mice developed an inflammatory infiltrate, the number of infiltrating neutrophils was lower, whereas there was evidence of increased infiltrating fibroblasts and mesenchymal cells and more lung fibrosis. We conclude that *C. pneumoniae* directly activates the NLRP3/ASC inflammasome, leading to the release of biologically active IL-1β, and that concurrent IL-1 signaling is required for optimal host defense against acute bacterial pneumonia.  

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*Chlamydia pneumoniae* is a common respiratory pathogen associated with atypical pneumonia, pharyngitis, bronchitis, and sinusitis. The prevalence of Ab seropositivity increases with age so that 80% of men and 70% of women show evidence of prior infection by the age of 65 y (1). Although 50% of young adults have serological evidence of infection, most infections are asymptomatic or mild. The disease is most common in military bases and college campuses where crowding is felt to be a risk factor. Beyond the acute clinical syndromes associated with *C. pneumoniae*, it has also been suggested as a trigger or promoter of chronic inflammatory states and has been linked to a variety of syndromes including bronchial hyperresponsiveness characteristic of asthma and inflammatory vascular lesions, such as those associated with atherosclerosis. However, the mechanism behind pathogen-induced chronic inflammation is essentially unknown. Chlamydiae exhibit a complex, obligate intracellular dimorphic lifestyle that sets them apart from other bacterial species (reviewed in Ref. 2). There are two major developmental forms that are recognized: the infectious, but metabolically inert form, known as the elementary body (EB); and the replicative form known as the reticulate body (RB). The developmental cycle begins with attachment and entry by the EB, although the precise mechanism by which this occurs remains unclear and likely varies with the cell type. Once it has entered the cell, the EB is surrounded by the plasma membrane that will form the intracellular inclusion membrane; the EB then converts to the RB form and replicates by binary fission. Like the intracellular compartment of many parasitic organisms, the chlamydial inclusion fails to undergo lysosomal fusion (3). However, infection does not result in global arrest of vesicular fusion. At some point late in the infectious process, RBs will asynchronously differentiate back to EBs. Eventually, lysis of both the inclusion and cell membranes occurs, usually 40–72 h postinfection depending on the species, and infectious particles are released.

The innate immune receptors that recognize *C. pneumoniae* are primarily TLR2 and, to a lesser extent, TLR4. Several groups have shown a clear dependence on expression of TLR2 for cellular responses to *C. pneumoniae* in vitro (4–7), although in vivo, the data are conflicting as to whether TLR2 is important for bacterial clearance, lung inflammation, and the inflammatory cytokine response (6, 8, 9). TLR4 is reportedly involved in recognition of chlamydial heat shock proteins both in vitro (10, 11) and in vivo (12), and the TLR adaptor MyD88 has also been shown to be critical for the resolution of acute inflammation and bacterial clearance in the pneumonia model (9). In addition to the TLR/MyD88 pathway, recent in vivo data also suggest a role for the nucleotide-binding oligomerization domain (Nod)/RIP2 pathway in lung infections (13).
The β form of IL-1 (IL-1β) is a classic proinflammatory cytokine and is an important mediator of inflammation in a variety of clinically stressful conditions, such as sepsis and septic shock (14). It is also postulated to play a role in lung injury during acute respiratory distress syndrome (ARDS) (15). In addition, it has been suggested to play a role in the chronic inflammatory states associated with some rheumatologic diseases, vasculitis, cancer, atherosclerosis, and even aging (reviewed in Refs. 16, 17). IL-1β production is a tightly regulated process that includes regulation at multiple levels and requires two distinct signals for activation and release. A proinflammatory signal first leads to NF-κB activation and synthesis of pro–IL-1β, a 35-kDa precursor protein. The second signal involves activation of the evolutionarily conserved cysteine protease, caspase-1, which cleaves pro–IL-1β into mature and biologically active IL-1β. Caspase-1 itself is made as an inactive protein that also must be activated via a multiprotein complex known as the inflammasome to generate biologically active caspase-1 (reviewed in Ref. 18). The same process of inflammasome activation and caspase-1 cleavage is also responsible for processing pro–IL-18 into its biologically active form.

The inflammasome is a multiprotein complex containing caspase-1, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and a nucleotide-binding oligomerization domain-like receptor (NLR). NLR family members that, with the adaptor ASC (official name PYCARD), have been implicated in caspase-1 activation in response to Salmonella or Legionella infection (19, 20). NLRP3, another cytosolic NLR (also known as NALP3 or cryopyrin), has been implicated with the adaptor ASC for caspase-1 activation and IL-1β production in response to bacterial pore-forming toxins (21, 22), several viruses (23–27), and the fungal pathogen Candida albicans (28, 29). NLRP3 is also involved in the response to endogenous danger signals, such as ATP (21) and uric acid (30). Other particles, such as silica crystals, β-amyloid, asbestos, and alum, have also been reported to activate the NLRP3 inflammasome, possibly via induction of lysosomal damage or the induction of reactive oxygen species (ROS) (31–34). Collectively these studies indicate that the synthesis of IL-1β requires a signal from the extracellular milieu via innate immune receptors, such as TLRs, as well as an additional host danger signal induced intracellularly, that can be pathogenic specific.

In vitro studies indicate that caspase-1 activation and IL-1β release can be derived from the activation of surface-expressed purinergic receptors of the P2X7 subtype, for which ATP is thought to be the main endogenous ligand (35). In vivo, ATP can be considered an intercellular signaling transmitter released from cells during trauma and inflammation. However, the role of P2X7 in response to pathogenic bacteria is not well defined, and recent studies indicate that IL-1β release following infection with intracellular bacteria proceeds normally in the absence of the P2X7 receptor (36). We hypothesized that C. pneumoniae would be a potent inducer of IL-1β, providing an explanation for its association with pneumonia and chronic inflammatory states. Using an in vitro model, we investigated the ability of C. pneumoniae to induce IL-1β secretion and the mechanism behind its processing. We found a requirement for the pattern recognition receptor, TLR-2, in the induction of pro–IL-1β, whereas the NLRP3/ASC inflammasome complex was required for caspase-1 activation and pro–IL-1β cleavage. Caspase-1 cleavage by C. pneumoniae was independent of the P2X7 receptor, and inhibitor data suggested a role for potassium efflux, lysosomal acidification, and cathepsin B release. Furthermore, we also identified an important role for IL-1 signaling in regulating inflammation and the development of fibrosis during severe pneumonia in vivo. We conclude that C. pneumoniae activates the NLRP3/ASC inflammasome via an as yet undefined mechanism, leading to potent induction of biologically active IL-1β, which is essential for regulating the host response that can lead to excessive inflammation and fibrosis in the lung.

Materials and Methods

Reagents

Ultrapure LPS (Escherichia coli serotype O111:B4) was purchased from List Biological Laboratories (Campbell, CA). The synthetic lipopeptide Pam3 Cs,CysSk was from InvivoGen (San Diego, CA). Renograin-60 was purchased from Bracco Diagnostics (Princeton, NJ). RPMI 1640 was from BioWhittaker (Lonza, Walkersville, MD). FBS (low endotoxin) was from Hyclone. Hyclone (Logan, UT). Caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-CHO (Ac- YVAD-CHO, catalog number 60856) was from Anspec (Fremont, CA) and dissolved in H₂O at 2 mM concentration. Glibenclamide (catalog number 0911) was from TOCRIS Bioscience (Ellisville, MO). Bafilomycin A1 (catalog number ALX-380-030) and (2R, 4R)-aminopropyldiolide-2, 4-dicarboxylic acid were from Alexis Biochemicals (Plymouth Meeting, PA). Cathepsin B inhibitor CA-074 Me (catalog number 205531) was from Calbiochem (San Diego, CA). ELISA kits for mouse cytokines and chemokines were as follows: IL-1β, IL-6, IL12p40, IFN-γ, IFN-γ-inducible protein (IP-10), and RANTES from R&D Systems (Minneapolis, MN). TNF-α from eBioscience (San Diego, CA). IL-18 from MBL Medical & Biological Laboratories (Nagoya, Japan). Milliplex custom 4-plex for serum samples was purchased from Millpore (Billerica, MA). Abs against caspase-1 p10 (sc-514), mouse IL-1β (H-153, sc-7884), and GAPDH (sc-25778) were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG conjugated with HRP and ATP disodium salt were from Sigma-Aldrich (St. Louis, MO).

Propagation of Chlamydiae

C. pneumoniae strain A03 was kindly provided by Dr. Charlotte Gaydos (The Johns Hopkins University, Baltimore, MD), and strain AR39 was obtained from Dr. Li Shen (Louisiana State University, New Orleans, LA). Unless otherwise indicated, all experiments were carried out using strain AO3. Chlamydia muridarum (Nigg; formerly MoPn), Chlamydia trachomatis serovar L2/434/As and Chlamyphila philaepia GPI (OPC) were provided by Dr. Catherine O’Connell (Children’s Hospital of Pittsburgh of University of Pittsburgh Medical Center, Pittsburgh, PA). C. trachomatis serovar D was purchased from the American Type Culture Collection (Manassas, VA). Chlamydiae were propagated in L2929 fibroblasts growing in RPMI 1640 medium supplemented with 10% FBS at 37°C, except for C. pneumoniae, which was grown at 35°C in a 5% CO₂ environment. Following infection, cells were harvested and disrupted by glass beads or sonication (Sonicator 4000, Misonix Sonicators, Newtontown, CT), and Chlamydiae were separated from cell debris by ultracentrifugation through 32% Renograin. Chlamydia EBs were further purified by ultracentrifugation through 40% Renograin. After washing twice, the EB pellets were suspended in sucrose-phosphate-glutamate buffer (SPG; pH 7.2) and stored at –80°C. Bacterial titers were calculated as inclusion forming units (IFU) per milliliter. All the Chlamydia stocks used in this study were negative for Mycoplasma contamination by PCR (37). All experimental procedures involving pathogenic bacteria were carried out with approval from the Institutional Biosafety Committee at Boston University Medical Center (Boston, MA).

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All knockout mice were back bred onto the C57BL/6 strain. Tlr2−/− and Tlr4−/− mice, back bred at least six generations, were generated by Dr. Shizuo Akira and used with his permission (38, 39). Nlrp3−/−, Nlrc4−/−, and Asc−/− mice were generated by Millenium Pharmaceuticals (Cambridge, MA), back bred at least seven (Nlrp3−/−) or nine (Nlrc4−/− and Asc−/−) generations before being used. P2X7 receptor-deficient mice, back bred at least 22 generations, were originally generated by Pfizer Global Research and Development, Pfizer (Groton, CT) (40) and provided by Dr. Amy Hise (Case Western Reserve University, Cleveland, OH). IL-1R type 1-deficient mice, strain B6.129S7-IL1r1tm1lmx/J (F2 N1J3), were bred at least 22 generations, were originally generated by Pfizer Global Research and Development, Pfizer (Groton, CT) (40) and provided by Dr. Amy Hise (Case Western Reserve University, Cleveland, OH). IL-1R type 1-deficient mice, strain B6.129S7-IL1r1tm1lmx/J (F2 N1J3), were purchased from The Jackson Laboratory. All animals were housed in a controlled environment, given free access to food and water, and maintained under the supervision of veterinary staff. All experimental procedures were carried out with approval from the Institutional Animal Care and Use Committees at Boston University Medical Center and the University of Massachusetts Medical School (Worcester, MA).

Preparation of bone marrow-derived macrophages and alveolar macrophages

Bone marrow-derived macrophages (BMDMs) were prepared as follows. Briefly, femurs and tibiae were dissected from mice aged 6–8 wk, bone
maraus were flushed, and cells were cultured in RPMI 1640 supplemented with 10% FBS, 20 µg/ml gentamicin, and 20–30% (v/v) L929 condition medium (containing macrophage-CSF). The cells were incubated at 37˚C, 5% CO2 incubator, for 7–9 d to allow macrophage differentiation and removed from gentamicin 1 d prior to infection with Chlamydia. Alveolar macrophages (AMs) were prepared by bronchoalveolar lavage. Briefly, groups of mice aged 6–8 wk were euthanized by CO2 inhalation and the intact lungs lavaged with ~1 ml HBSS. Recovered AMs from the same strain of mice were pooled and washed with PBS, then used for infection studies the same day.

**Infection of macrophages with Chlamydiae and cytokine analysis**

Macrophages were plated in 24-well tissue culture dishes and infected with Chlamydia at the indicated multiplicity of infection (MOI). To initiate infection postinoculation with Chlamydia, plates were centrifuged at 1500 × g, 35 C, for 1 h. When used, the inhibitors were added 150 min postinoculation of Chlamydia, except for the caspase-1 inhibitor, Ac-YVAD-CHO, which was added 30 min prior to addition of Chlamydia. The ATP, when used for Chlamydia-infected cells or LPS- or Pam3CysSK4-treated cells, was added at a concentration of 5 mM for 40–60 min prior to harvesting unless otherwise indicated. Supernatants collected from infected wells were assayed for cytokines using commercially available ELISA kits according to the manufacturer’s instructions. Plates were read in a FLx800 Universal Microplate Reader (BioTek, Winooski, VT) and data analyzed using SoftMax Pro 4.6 software (Molecular Devices, Sunnyvale, CA). The cytokine induction experiments were performed in triplicate wells and the data presented as the mean ± SEM. Statistical analysis was performed by calculating a p value using an unpaired t test.

**Western blot analyses**

Macrophages were plated in six-well tissue culture dishes and infected with Chlamydia as described above. Serum-free supernatants were stored at −80˚C until used in the Western blot assay. Lysates were prepared as follows. Cells were lysed in Triton lysis buffer [20 mM Tris Cl (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, and 25 mM β-glycerophosphate]; protease inhibitor mixture, sodium vanadate (Na3 VO4), and PMSF were added before use. Proteins were precipitated from supernatants using methanol/chloroform as follows. Briefly, an equal volume of methanol and 0.25 volume of chloroform were added to samples, mixed, and centrifuged at 20,000 × g for 10 min. The upper phase was discarded, and another equal volume of methanol was added to the interphase, followed by mixing and centrifugation at 20,000 × g for 5 min. The protein pellets were dried at 55°C. Dried pellets were resuspended in Laemmli’s SDS-Sample Buffer and heated to 100˚C for 5 min prior to loading.

**Murine intranasal infection model**

Male and female C57BL/6 or IL-1R−/− mice aged 6–8 wk were inoculated via the intranasal route under light anesthesia using a ketamine/xylazine mix (60–100/5–10 mg/kg i.p., respectively). All infected mice received 20 µl bacterial suspension in SPG containing 2 × 107 1FU gradient purified C. pneumoniae strain A03; mock-infected mice received 20 µl SPG. Mice were infected in groups of six to nine. At the indicated time points, mice were euthanized by CO2 inhalation. After collection of the blood by cardiac puncture, the lungs and spleens were removed for culture and cytokine measurement. In some mice, the lungs were inflated with 10% neutral formalin, removed en bloc for

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**FIGURE 1. C. pneumoniae potently induces IL-1β production via a TLR2-dependent signaling pathway.** BMDMs (A–C) and AMs (D) were infected with Chlamydia as described at the indicated MOI. Some in cases, LPS (100 ng/ml) or Pam3CysSK4 (100 ng/ml) treatment for 5 h was used as a control. Supernatants collected at 24 h post treatment were assayed for IL-1β and TNF-α by ELISA. A. Comparison of various Chlamydia species in terms of their ability to induce secretion of IL-1β. BMDMs from C57BL/6 mice were infected with C. pneumoniae (Cp), C. caviae (GP4C), or C. muridarum (Nigg), with or without ATP. Supernatants were assayed simultaneously for IL-1β and TNF-α by ELISA. B. Role of TLR2 and TLR4 in IL-1β secretion in Cp-infected BMDMs. BMDMs derived from C57BL/6 mice or mice deficient in TLR2 or TLR4 on the same background were infected with Cp, and supernatant was assayed for IL-1β. C. Requirement for live bacteria in the induction of IL-1β. BMDMs derived from C57BL/6 or TLR2-deficient mice on the same background were infected with either live Cp or the equivalent amount of heat-killed (HK) Cp, and supernatant was assayed for IL-1β. D. Role of TLR2 in Cp-infected AMs. AMs derived from C57BL/6 or TLR2-deficient mice on the same background were infected with Cp or treated with LPS or Pam3CysSK4 in the presence or absence of ATP. These results are representative of three independent experiments. Significance was calculated using a two-tailed t test, and p values calculated as follows: *p < 0.05; **p < 0.01; ***p < 0.001. ND, none detected.
further formalin fixation, and embedded in paraffin for histopathological analysis. All in vivo experiments were carried out with approval from the Institutional Biosafety Committee and the Institutional Animal Care and Use Committees at Boston University Medical Center.

Preparation of lung homogenates for cytokine analysis
Following removal from euthanized mice, lungs were homogenized in 1 ml PBS utilizing a Medimachine System (BDB Biosciences, San Jose, CA). A total of 50 μl lung homogenates were subjected to culture for quantification of bacteria, and remaining homogenates were mixed with equal volume of 2× protease inhibitor mixture and frozen at −80°C for cytokine and chemokine measurement by ELISA and Milliplex custom multiplex assay, according to the manufacturer's instructions. ELISA plates were read in an ELx800 Universal Microplate Reader (BioTek), and multiplex assays were run on the LiquiChip 200 Workstation (Qiagen, Valencia, CA). Each sample was assayed in duplicate. Statistical analysis was performed by calculating a p value using an unpaired t test.

Identification of C. pneumoniae in tissue and blood
Quantitative culture of C. pneumoniae in lung homogenates was carried out as follows. A total of 50 μl homogenates were mixed with 150 μl SPG and then briefly spun to pellet tissue debris. Serially diluted supernatants were inoculated in duplicate onto a monolayer of L929 fibroblasts seeded in a 96-well plate. After 48–56 h incubation, the cells were fixed in ice-cold methanol for 15 min. Chlamydial inclusions were stained using a Chlamydia-specific LPS mAb (gift of Dr. You-Xun Zhang, Boston University Medical Center), followed by FITC-conjugated secondary Ab; cells were counterstained with Evans blue (Sigma-Aldrich). The inclusions were counted under fluorescent microscopy and calculated as the number of IFU per well/per lung. Significance was calculated as a p value using an unpaired t test.

C. pneumoniae DNA was identified in spleen and blood as follows. DNA was extracted from tissue and blood using a commercially available kit (Qiagen). PCR was conducted using primers designed to detect the lipoprotein signal peptidease (LspA) from C. pneumoniae. Primer sequences were: forward: 5'-GATAATATCCCCGTCGTTTCCTAT-3' and reverse: 5'-GAAAGGCGCTGTTCCGTGCCT-3'. PCR conditions were: 94°C for 2 min, 94°C for 30 s, 62°C for 30 s, 72°C for 1 min for one cycle; touchdown −0.5°C for each cycle for 30 cycles; 94°C for 30 s, 52°C for 30 s, 72°C for 1 min for 25 cycles; and 72°C for 7 min. Predicted PCR product size was 180 bp.

Histopathology and immunohistochemical analyses
Embedded lung blocks were cut completely in 7- to 8-μm cryosections and every 10th section stained using routine H&E, chloroacetate esterase, or trichrome protocols. Immunohistochemistry was carried out on deparaffinized tissue using mAb against mouse vimentin (clone V9; Ventana Medical Systems, Tucson, AZ) or its isotype control, followed by HRP for detection. The number of infiltrated lung neutrophils (polymorphonuclear cells [PMNs]) from chloroacetate esterase staining was counted under a 20× objective from 20 random fields. The percentage of fibrosis to total

**FIGURE 2.** Activation of caspase-1 and the release of mature IL-1β require TLR2 signaling. Western blot analyses of the activation of caspase-1 and cleavage of IL-1β in BMDMs infected with C. pneumoniae (Cp). A, BMDMs from C57BL/6 were left untreated, stimulated with LPS (100 ng/ml) for 5 h then with ATP for 20 min, or infected with indicated MOI of Cp for 12 h. Supernatants were harvested and assayed for caspase-1 p10. B, BMDMs from C57BL/6 mice were left untreated or stimulated with LPS (100 ng/ml) for 5 h in the presence or absence of ATP, ATP alone for 1 h, or infected with Cp for 20 h. Samples were assayed for caspase-1 p10 followed mature IL-1β (supernatants), or pro–IL-1β followed procaspase-1 and GAPDH (lysates). C, BMDMs from C57BL/6 mice were left untreated or infected with C. pneumoniae (Cp) at an MOI of 5:1, and supernatants and cell lysates were harvested and assayed for caspase-1 p10 and mature IL-1β (supernatants) or pro–IL-1β and GAPDH (lysates). D, BMDMs were stimulated with live C. pneumoniae (Cp) strain A03 or AR39 at an MOI of 10:1, or the equivalent amount of UV- or heat-killed A03. Supernatants collected at 12 h postinfection were assayed for caspase-1 p10 and IL-1β. E, BMDMs from C57BL/6- or TLR2-deficient mice were left untreated or treated with LPS (100 ng/ml) or Pam3Cys-SK4 (100 ng/ml) for 5 h with ATP or infected with C. pneumoniae (Cp) at an MOI of 5:1. Supernatants and cell lysates were harvested at 20 h postinfection for detecting caspase-1 p10 and mature IL-1β (supernatants) or pro–IL-1β, procaspase-1, and GAPDH (lysates). These results are representative of three independent experiments.
area of lung was quantified using Image J software (National Institutes of Health, Bethesda, MD) from eight images taken with Nikon DXM 1200 digital camera under 5× objective (Nikon, Melville, NY). All slides were reviewed independently by two pathologists who were blinded as to the experimental design.

Results

Chlamydia pneumoniae potently induces IL-1β secretion in BMDMs and AMs via a TLR2-dependent pathway

We first examined the ability of Chlamydia pneumoniae and other Chlamydia species to induce IL-1β secretion in resting BMDMs. As shown in Fig. 1A, we found that C. pneumoniae infection induced the release of IL-1β into tissue culture supernatant in a dose-dependent manner and at a 4–10-fold higher concentration compared with the related Chlamydia species C. caviae and C. muridarum. In contrast, all three species induced similar amounts of the cytokine TNF-α. Although the addition of exogenous ATP was required for LPS-induced IL-1β release and enhanced IL-1β secretion in the C. caviae- and C. muridarum-infected macrophages, it had no effect on IL-1β release by C. pneumoniae. Thus, C. pneumoniae is capable of inducing IL-1β secretion from murine BMDMs without the need for macrophage priming or the addition of exogenous ATP.

A number of innate immune receptors have reported to play a role in host recognition of various Chlamydia species. To determine if TLR2 or TLR4 played a role in C. pneumoniae-induced secretion of IL-1β, we stimulated resting BMDMs from C57BL/6, TLR2-deficient, or TLR4-deficient mice using different MOIs of killed Chlamydia for TLR2 but not TLR4 in IL-1β-dependent experiments. We tested other species of Chlamydia—AO3, isolated from an atheroma in a patient with pharyngitis (43)—in terms of their ability to induce IL-1β secretion in terms of their ability to activate caspase-1 cleavage, albeit much weaker than what we observed with C. pneumoniae (data not shown). Thus, the ability to activate caspase-1 cleavage in cell culture supernatants as early as 6–8 h postinfection, around the same time when pro–IL-1β can first be detected in the cell lysates; mature IL-1β can be detected in the cell culture supernatant by 13 h. This suggests that inflammasome activation might be related to early uptake and trafficking events of the EB or possibly early replicative events of the RB. Again, this required productive infection with live bacteria, as UV- and heat-inactivated C. pneumoniae had no effect on caspase-1 activation (Fig. 2B). As shown in Fig. 2C demonstrates, caspase-1 p10 can be seen in cell culture supernatants as early as 6–8 h postinfection, around the same time when pro–IL-1β can first be detected in the cell lysates; mature IL-1β can be detected in the cell culture supernatant by 13 h. These results demonstrate that C. pneumoniae infection activates caspase-1 cleavage and IL-1β processing.

C. pneumoniae infection activates caspase-1 cleavage and IL-1β processing

Processing of the precursor protein pro–IL-1β to biologically active mature IL-1β requires activated caspase-1. We wanted to know whether C. pneumoniae infection of resting BMDMs could induce caspase-1 activation and whether this was linked to the secretion of mature IL-1β. As shown in Fig. 2A, we found that C. pneumoniae induced activation of procaspase-1 to release the p10 fragment in a dose-dependent manner into cell culture supernatants. In contrast, LPS alone had no effect unless it was combined with ATP (Fig. 2B). As the time course in Fig. 2C demonstrates, caspase-1 p10 can be seen in cell culture supernatants at an early time when pro–IL-1β can first be detected in the cell lysates; mature IL-1β can be detected in the cell culture supernatant by 13 h.

FIGURE 3. Release of mature IL-1β by C. pneumoniae-infected cells requires caspase-1 activation. BMDMs were infected with C. pneumoniae (Cp) in the presence or absence of the caspase-1 inhibitor Ac-YVAD-CHO. A, Supernatants collected at 24 h postinfection were assayed for IL-1β by ELISA. B and C, Supernatant collected at 18 h postinfection was assayed for caspase-1 p10 and mature IL-1β by Western blot. These results are representative of three independent experiments. ***p < 0.001.
To investigate the role of TLR2 signaling in processing of IL-1β and activation of caspase-1, we infected C57BL/6 and Tlr22/2 BMDMs with C. pneumoniae and looked for pro–IL-1β, mature IL-1β, and caspase-1 p10 by Western blot analysis. As shown in Fig. 2E, pro–IL-1β induction by C. pneumoniae requires the expression of TLR2, which is known to activate NF-κB–dependent proinflammatory pathways. In addition, we also found that lack of TLR2 expression had a negative impact on caspase-1 cleavage to release the p10 fragment, although the mechanism behind this is less clear. A recent report by Bauernfeind et al. (44) suggests that TLR-dependent NF-κB signals may also be important for inflammasome activation and might explain our observation.

To link caspase-1 activation to C. pneumoniae-induced IL-1β secretion, we next examined the effect of the cell-permeable, irreversible caspase-1 peptide inhibitor Ac-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) on cytokine release in resting BMDMs. As expected, Ac-YVAD-CHO inhibited the release of mature IL-1β into cell culture supernatant, as detected by ELISA (Fig. 3A) and Western blot analysis (Fig. 3B), as well as caspase-1 cleavage (Fig. 3C), in a dose-dependent manner. Together, these data demonstrate that C. pneumoniae infection of resting macrophages activates caspase-1 cleavage, resulting in processing of pro–IL-1β to mature IL-1β.

C. pneumoniae induces caspase-1 activation and IL-1β processing via the NLRP3/ASC inflammasome

To define the inflammasome components involved in the activation of caspase-1 by C. pneumoniae infection, we infected resting BMDMs from C57BL/6 mice as well as mice deficient in NLRP3, NLRC4, or ASC and examined the cell culture supernatant for IL-1β. As shown in Fig. 4, wild-type macrophages released IL-1β in response to C. pneumoniae infection over time (Fig. 4B) and in a dose-dependent manner (Fig. 4A-C). However, IL-1β secretion by BMDMs derived from mice deficient in NLRP3 or ASC was nearly absent or significantly reduced compared with wild-type C57BL/6 mice and mice deficient in NLRC4 (Fig. 4A–C). In contrast, TNF-α secretion, which is independent of the inflammasome, was comparable regardless of the expression of the inflammasome components, demonstrating that the ability of the bacteria to activate

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** C. pneumoniae infection of BMDM induces IL-1β via NLRP3/ASC-dependent pathway. BMDM from C57BL/6 mice or mice deficient in NLRP3, NLRC4 or ASC on the same background were infected with C. pneumoniae (Cp) at the indicated MOI (A, C, D) or an MOI of 10:1 (B). LPS (100 ng/ml) treatment for 5 h with or without ATP was used as a control. Supernatants were harvested at indicated time points (B) or at 24 h (A, C, D) and then assayed for IL-1β, IL-18, and TNF-α by ELISA. These results are representative of three independent experiments. Significance was calculated using a two-tailed t test, and p value calculated as follows: ***p < 0.001. ND, none detected.
other inflammatory pathways was not compromised (Fig. 4B, 4C). In addition to processing IL-1β, the caspase-1 inflammasome complex is also involved in processing IL-18. We assayed our cell culture supernatants for IL-18 secretion and found a similar dependence on NLRP3 and ASC expression (Fig. 4D).

To further examine the role of the NLRP3/ASC inflammasome in IL-1β secretion, we looked for evidence of caspase-1 activation as well as cleavage of pro–IL-1β in the presence or absence of these proteins. As shown in Fig. 5A, in the absence of NLRP3 or ASC expression, caspase-1 cleavage to release the p10 fragment failed to occur. Consistent with this, we saw upregulation of pro–IL-1β in the cell lysates and supernatant in the absence of NLRP3 or ASC, but failed to observe the release of mature IL-1β into the supernatant (Fig. 5B). Together, these data demonstrate that *C. pneumoniae*-induced activation of caspase-1 cleavage and IL-1β processing in resting macrophages occurs via the NLRP3/ASC inflammasome complex.

**C. pneumoniae-induced activation of caspase-1 is independent of the P2X7 receptor.**

The time point we observed for caspase-1 activation and IL-1β secretion occurred early in the developmental cycle of *C. pneumoniae*, long before cell lysis and release of infectious particles would be expected. Nevertheless, we could not rule out that some cell lysis secondary to toxic effects of the infection on the cell culture monolayer might release small amounts of ATP that could serve as the second signal for inflammasome activation, independent of some effect of the organism. To test this, we infected resting BMDMs derived from C57BL/6- or P2X7-deficient mice with *C. pneumoniae* and looked for IL-1β production by ELISA and caspase-1 activation and pro–IL-1β cleavage by Western blot analysis. As shown in Fig. 6A, BMDMs from P2X7 receptor-deficient mice secreted equivalent amounts of IL-1β into the cell culture supernatant as compared with those from wild-type C57BL/6 mice. In contrast, LPS plus ATP was completely dependent on expression of the P2X7 receptor. Likewise, expression of the caspase-1 p10 fragment and processing of pro–IL-1β to mature IL-1β in *C. pneumoniae*-infected cells was also independent of the P2X7 receptor, as shown by Western blot analysis (Fig. 6B, 6C). In addition, we observed no effect of uricase treatment of *C. pneumoniae*-infected cells on the release of IL-1β by ELISA, suggesting that uric acid release from dead or dying cells was not contributing to inflammasome activation (data not shown). These data suggest that endogenous danger signals, such as released ATP or uric acid from cells, are not responsible for inflammasome activation in resting BMDM by *C. pneumoniae*.

**Potassium efflux, lysosomal acidification, and cathepsin B release may be involved in activation of the NLRP3/ASC inflammasome response to infection of *C. pneumoniae*.**

In addition to endogenous ATP and uric acid release, other mechanisms of NLRP3 inflammasome activation include generation of ROS, potassium efflux into the cytosol, lysosomal acidification, and lysosomal damage with cathepsin B release (34, 45). To determine if any of these mechanisms might be involved in *C. pneumoniae*-induced activation of the NLRP3 inflammasome, we examined the effects of a series of defined inhibitors on the secretion of IL-1β by infected BMDM: (2R, 4R)-4 aminopyrrolidine-2, 4-dicarboxylate (APDC) was used for inhibiting the NADPH oxidase-dependent ROS system; glibenclamide was used as a potassium-channel inhibitor; bafilomycin A1 was used for inhibiting lysosomal acidification; and CA-074-Me was used for inhibiting released cathepsin B. As shown in Fig. 7A and 7B, glibenclamide, bafilomycin A1, and CA-074-Me inhibited IL-1β release from infected BMDMs as measured by ELISA while having no such effect on TNF-α release. In contrast, APDC had no effect on the induction of either cytokine. The blocking effect of the inhibitors could be partially overcome in the case of glibenclamide and bafilomycin A1 by increasing the MOI. As a confirmation of the ELISA data, we did not observe release of the caspase-1 p10 fragment in glibenclamide- and
CA-074-Me–treated cell supernatants by Western blot analysis, although pro–IL-1β was upregulated normally in lysates from the treated cells (Fig. 7C). These data also demonstrate these inhibitors had no effect on the induction of pro–IL-1β.

C. pneumoniae-infected IL-1R−/− mice show some exaggerated cytokine responses at early time points postinfection

To examine the role of IL-1 signaling in vivo during C. pneumoniae-induced pneumonia, we infected C57BL/6 mice, or mice deficient in the IL-1R type 1, intranasally with 2 × 10⁷ IFU C. pneumoniae and looked for evidence of both local and systemic inflammation by measuring cytokine levels in lung homogenates and serum, respectively. We found that concentrations of TNF-α and IL-6 were elevated in lung homogenates on day 3 post-infection in the infected IL-1R–deficient mice compared with the C57BL/6 mice (Fig. 8A, 8B), although levels were reduced and comparable between the two mouse strains by day 5 and remained reduced out to day 12 (data not shown). Serum levels of TNF-α and IL-6 were similarly elevated in infected IL-1R–deficient mice compared with the C57BL/6 mice, but again only at day 3 (Fig. 8G, 8H). In contrast to TNF-α and IL-6, we found no statistically significant change in the levels of the chemokines RANTES or IP-10 between the two mouse strains (Fig. 8C, 8D).

Immunity to Chlamydia is dependent on the induction of IFN-γ, which is required to limit bacterial growth (46), and IFN-γ induction is critically dependent on the production of IL-12 (47). To determine if IFN-γ responses early in the course of infection were intact in the IL-1R–deficient mice, we looked in lung homogenates and found significantly elevated levels of IL-12p40 in the knockout mice compared with the C57BL/6 infected mice at these early time points (Fig. 8E); however, IFN-γ levels were not significantly different between the two mouse strains (Fig. 8F). Although there was a trend toward higher bacterial burden at day 3 in the IL-1R–deficient mice, it did not reach statistical significance, and both strains were able to clear the infection, with the exception of one IL-1R–deficient mouse, by day 12 (Fig. 8I). Neither strain showed evidence of disseminated infection, as evidenced by negative PCR analysis for C. pneumoniae–specific DNA in the spleen and blood.

**FIGURE 7.** Potassium efflux and cathepsin B release are involved in inflammasome activation by *C. pneumoniae*. BMDMs from C57BL/6 were infected with *C. pneumoniae* (Cp) at an MOI of 5:1 or the indicated MOI (B). APDC, glibenclamide (Gliben), bafilomycin A1 (Bafilo), and CA-074-Me (CA-074) were added at the indicated concentration as described in the Materials and Methods. A and B, Supernatants harvested at 24 h postinfection were assayed for IL-1β and TNF-α by ELISA. A shows the dose effect of the inhibitor with a constant MOI of *C. pneumoniae*. B shows the effect of increasing the MOI of *C. pneumoniae* with a constant concentration of inhibitor. C, Supernatants and lysates from infected cells were harvested at 20 h postinfection and assayed for caspase-1 p10 and mature IL-1β (supernatants) or procaspase-1, pro–IL-1β, or GAPDH (lysates). These results are representative of two independent experiments. Significance was calculated using a two-tailed *t* test, and *p* values calculated as follows: *p* < 0.05; **p** < 0.01; ***p*** < 0.001.
These data suggest that IL-1R–deficient mice have a transient local and systemic hyperinflammatory response compared with wild-type mice following *C. pneumoniae* infection that is unrelated to the bacterial burden. IL-1 signaling is important for lung repair during *C. pneumoniae* infection

Although our cytokine data suggested an exaggerated inflammatory response in the absence of IL-1 signaling very early in the course of infection, the question remained as to whether this transient inflammatory response would have any effect on the lung histopathology. To test for this, we again infected C57BL/6- or IL-1R–deficient mice intranasally with 2 × 10^7 IFU *C. pneumoniae* and evaluated the lungs for inflammation, neutrophilic infiltrates, and fibrosis at days 3, 6, and 12 postinfection. Although both strains of mice developed signs of acute inflammation with edema and influx of neutrophils and macrophages in the lungs on H&E staining, they differed significantly in terms of the cellular infiltrate and the extent of repair and fibrosis. Specifically, we found that IL-1R–deficient mice showed extensive proliferation of fibroblasts on H&E staining at the early time point (Fig. 9A, 9B), which remained increased relative to the wild-type mice even at day 12 (Fig. 9E, 9F). To better visualize the extent of tissue repair between the two mouse strains, lung sections were stained using an mAb directed against vimentin, the major intermediate filament protein found in fibroblasts and mesenchymal cells that plays an important role in wound healing (48). We found a marked upregulation of vimentin expression in the IL-1R–deficient mice at day 3 postinfection, whereas the infected C57BL/6 mice had very little similar, in fact, to the uninfected mice (Fig. 9C, 9D); by day 12, the difference between the two strains was less pronounced (Fig. 9G, 9H). In contrast to fibroblasts and mesenchymal cells, the wild-type C57BL/6 mice had a greater predominance of neutrophils infiltrating the lung compared with the IL-1R–deficient mice, and this held true out to day 12 (Table I).

To determine if these changes in cellular infiltrates had a long-term effect on the repair of the lung parenchyma, we carried out trichrome staining of the tissue at days 6 and 12 to look for evidence of lung fibrosis. To our surprise, we observed much more fibrosis in the IL-1R–deficient mice (Fig. 9I, 9J), with more than twice the amount of tissue affected in the IL-1R–deficient mice when compared with the wild-type C57BL/6 mice (Table I). This suggested to us that IL-1 signaling is important for preservation of lung architecture during the tissue repair following acute pneumonia.

**Discussion**

The proinflammatory cytokine IL-1β is a critical mediator of host defense against a variety of infectious states. For example, IL-1β is produced in the lung after intratracheal administration of LPS (49), and its presence is directly associated with lung inflammation in patients with community-acquired pneumonia (50). IL-1 is required for control of a variety of intracellular pathogens, such as *Listeria, Leishmania,* and *Mycobacterium tuberculosis* (51–53). However, little is known about the role of IL-1 in control of *Chlamydia pneumoniae*, an obligate intracellular bacterium that is associated with a variety of acute infectious processes, such as atypical pneumonia, bronchitis, and pharyngitis; chronic infection with *C. pneumoniae* has also been linked to a variety of chronic inflammatory states, such as asthma and atherosclerosis.
A number of receptors have been implicated in the innate immune response to *Chlamydia*, in particular TLR2 and TLR4, which have been shown to activate NF-κB–dependent proinflammatory pathways. Among the NLRs, there have been only a handful of reports suggesting a role for Nod1 and/or Nod2, either in vitro (54) or in vivo (13), in the pathogenesis of *C. pneumoniae* infection, and except for a recent report that the related species *C. trachomatis* can activate caspase-1 in epithelial cells in an NLRP3-dependent manner (55), activation of the inflammasome has been largely ignored. We also observed inflammasome activation by *C. trachomatis* and *C. muridarum*; however, *C. pneumoniae* induced a far higher degree of caspase-1 processing and production of mature IL-1β in resting macrophages. Our data further suggest the activation of two distinct pathways in the *C. pneumoniae*-induced production of mature IL-1β by resting macrophages. First, TLR2 activation leads to the induction of pro–IL-1β (signal 1), followed by activation of the NLRP3/ASC inflammasome, which activates caspase-1 (signal 2) to cleave pro–IL-1β to its mature and biologically active form. Furthermore, *C. pneumoniae*-induced caspase-1 activation does not require macrophage priming or any additional signals, such as ATP activation of the P2X7 receptor. Interestingly, caspase-1 activation occurs relatively early in the developmental cycle of *C. pneumoniae*, before significant RB replication has occurred, suggesting that it does not require a large intracellular bacterial burden. The fact that only live bacteria are capable of inducing caspase-1 cleavage suggests that it requires uptake and trafficking of bacteria to the inclusion and possibly early gene transcription. Although our in vitro data focused only on the processing and activation of IL-1β and IL-18, recent evidence suggests that caspase-1 is also capable of regulating IL-1α and other proteins involved in cytoprotection and cell survival (56). Thus, the potential biological effects of caspase-1 activation by *C. pneumoniae* are quite broad.

The precise mechanism behind activation of the NLRP3/ASC inflammasome is unknown, although it appears to require potassium flux, lysosomal acidification, and cathepsin B release. This is similar to other particulate activators of the NLRP3 inflammasome, which in some models are hypothesized to trigger lysosomal destabilization (34). However, in the case of *C. pneumoniae*, the connection between replicating bacteria secluded within an intracellular inclusion and lysosomal compartments is unclear. *Chlamydia* do possess a type III secretion system (T3SS), and so one possible mechanism could involve injection of a ligand into the cytosol that leads to effects on neighboring lysosomes, indirectly activating NLRP3. This could be related to the also undefined mechanism by which *Chlamydia* actively inhibits lysosomal fusion with the intracellular inclusion. This seems more likely than a cytosolic chlamydial Ag injected via the T3SS acting directly as a NLRP3 ligand. 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Unfortunately, this hypothesis is difficult to test because the T3SS appears to be essential for chlamydial growth and development. In the study by Baily et al. (57), small molecule inhibitors of the *Yersinia* T3SS were found to inhibit *C. pneumoniae*, but not *C. trachomatis*, infections in vitro, blocking the developmental cycle and

![C. pneumoniae infection in IL-1R-deficient mice leads to the development of lung fibrosis. C57BL/6- or IL-1R-deficient mice were treated with PBS (mock) or infected with *C. pneumoniae* intranasally. The mice were euthanized at days 3, 6, and 12 postinfection, and the lungs were inflated and removed for histopathological and immunohistochemical analysis as described in the Materials and Methods. Shown above are the images from the *C. pneumoniae*-infected mice at day 3 (*A–D*) and day 12 (*E–J*). *A, B, E, and F* represent H&E staining; *C, D, G, and H* represent vimentin staining (dark brown); and *I* and *J* represent trichrome staining, in which collagen fibers stain blue. These data are representative of two independent experiments. Original magnification ×100 (*A–H*), ×400 (*I, J*).

In vitro, *C. pneumoniae* has been shown to infect and productively replicate within a number of cell types, including macrophages, leading to the upregulation of a variety of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-18, and IFN-γ, and chemokines like KC and MCP-1. IL-1β and IL-18 are unique among this list in that they are regulated at two levels: first, the induction of transcription of a precursor protein, followed by assembly of the inflammasome complex and activation of cysteine protease caspase-1, which cleaves the precursor protein to the biologically active protein that is secreted. Because of its reported role in host defense, specifically pneumonia and ARDS, and chronic inflammatory states, such as asthma and atherosclerosis, we were interested in examining the regulation and function of IL-1β during *C. pneumoniae* infections.

### Table I. Infiltration of PMN and lung fibrosis

<table>
<thead>
<tr>
<th>Days postinfection</th>
<th>PMN Count per 20 Random Fields</th>
<th>Percent Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL/6</td>
<td>IL-1R KO</td>
</tr>
<tr>
<td>3</td>
<td>1207.7 ± 302</td>
<td>726 ± 219</td>
</tr>
<tr>
<td>6</td>
<td>1163.5 ± 129.4*</td>
<td>342.5 ± 105.3*</td>
</tr>
<tr>
<td>12</td>
<td>41.0 ± 32.5</td>
<td>14.5 ± 5.2</td>
</tr>
</tbody>
</table>

Values shown above are the mean ± SD. *p* < 0.05. Representative of two independent experiments.
the development of inclusions. Our data demonstrate that productive infection is required for caspase-1 activation; thus, it would be impossible to determine if any changes in caspase-1 activation as a result of the inhibitors were a result of their effect on the T3SS or the lack of intracellular bacterial growth.

Finally, our data are the first to examine a role for IL-1 signaling in the pathogenesis of pneumonia. Studies in patients with ARDS have demonstrated the presence of IL-1β in bronchoalveolar lavage fluid (58). However, some data suggest that its presence might not be a poor prognostic factor (59). In fact, several studies have suggested that IL-1β signaling is required for alveolar epithelial repair during acute lung injury (58, 60, 61). Using an established mouse model for 
*Chlamydia pneumoniae* infection, we found a previously unappreciated role for IL-1 signaling in regulating the beneficial host inflammatory response, suggesting that IL-1β, and likely IL-1α, are protective to the host during severe pneumonia. Our data demonstrate that in the absence of IL-1 signaling, lung inflammation is comparable, if not more severe, than that seen in mice with intact IL-1 signaling, and IL-1 does not seem to be required for resolution of the infectious process. However, what we did find was a difference in the cellular infiltrate when IL-1 signaling was absent. We found evidence of increased infiltrating fibroblasts and mesenchymal cells in the lung histology, with an increased vimentin-positive cells in the parenchyma. Interestingly, the absolute number of infiltrating neutrophils was actually reduced in the absence of IL-1 signaling, although the chemokine levels, as best we could determine, were comparable.

The role of neutrophils during infection has always been controversial, as it plays an important role in the control of many infectious processes, but the sequestration of activated neutrophils in the lung has long been known to contribute to acute lung injury (62, 63). However, severe alveolar damage during ARDS can occur even in patients with neutropenia, so there are obviously other mechanisms by which lung damage can occur (64). The combination of both decreased neutrophils and increased lung fibrosis in the IL-1R−/− deficient mice initially seemed counterintuitive. However, Tate and colleagues (65) recently described the outcome of severe influenza pneumonia in neutropenic mice. This group found that depletion of neutrophils in mice infected with influenza virus led to exacerbated pulmonary inflammation, edema, and respiratory dysfunction and that this was not entirely explained by early increased viral replication (65). Like Tate and colleagues’ data (65), ours suggests a novel role for neutrophils in ameliorating inflammation and fibrosis during severe infection. The mechanism behind this might be explained by Zhang and colleagues (66), who recently reported that TLR-stimulated neutrophils are actually poor inducers of proinflammatory signals and, in contrast, produce large amounts of the anti-inflammatory cytokine IL-10. Furthermore, in their model of chronic infection secondary to *Mycobacterium bovis*, they found that neutrophil depletion promoted inflammation. Thus, we conclude that both IL-1 signaling and neutrophils are beneficial to the host, at least in the setting of an acute infectious process.

Questions arising from our studies that remain to be answered are exactly how does *C. pneumoniae* trigger NLRP3/ASC inflammasome activation, and what, if any, role does the T3SS play in the interaction of bacteria with these host cytosolic receptors? Additional studies to determine what cell types are responsible for the beneficial response to IL-1β during lung repair may help us to design better therapies to prevent complications arising from severe pneumonia while preserving the protective immune response that is required for bacterial clearance and the development of lasting immunity. Finally, whereas our data suggest that IL-1 signaling is important during acute *C. pneumoniae* lung infections, its role in chronic inflammatory states may be opposite the given suggestion from a number of sources that IL-1 signaling contributes to the development of atherosclerosis and asthma. Thus, the role of IL-1 during *C. pneumoniae*-induced chronic inflammatory conditions remains to be defined.

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Disclosures

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