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*J Immunol* 2012; 188:2866-2875; Prepublished online 13 February 2012;
doi: 10.4049/jimmunol.1103461
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Significant Role of IL-1 Signaling, but Limited Role of Inflammasome Activation, in Oviduct Pathology during Chlamydia muridarum Genital Infection

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IL-1β has been implicated in the development of oviduct pathology during Chlamydia muridarum genital infection in the mouse model. The goal of this study was to characterize the role of IL-1 signaling and the inflammasome-activation pathways during genital chlamydial infection. Compared with control mice, IL-1R-deficient mice displayed delayed clearance and increased chlamydial colonization. Consistent with the role for IL-1 signaling in infection clearance, mice deficient for the IL-1R antagonist cleared infection at a faster rate. Despite increased infection, IL-1R-deficient mice had significantly reduced oviduct pathology, which was associated with decreased numbers of neutrophils, but more macrophages, in the genital tract. IL-1β secretion is dependent on caspase-1 and apoptosis-associated speck-like protein containing caspase recruitment domain (ASC) inflammasome during in vitro infection of primed macrophages with C. muridarum. To investigate the role of inflammasome components during vivo genital infection, mice lacking NLRP3, NLRC4, and ASC were tested and found to display no reduction in oviduct pathology compared with control mice. Mice deficient for ASC displayed a prolonged course of infection, which was associated with reduced T cell cell recruitment and proliferation. Further, ASC-deficient mice displayed normal levels of IL-1β in genital secretions. However, a significant decrease in caspase-1–dependent IL-18 was observed in both ASC- and NLRP3-deficient mice. These data demonstrate a major role for IL-1 signaling, but a limited role for the inflammasome pathway, in IL-1β secretion and development of oviduct pathology during genital chlamydia infection. The data also suggest an IL-1–independent role for ASC in adaptive immunity during genital chlamydial infection. The Journal of Immunology, 2012, 188: 2866–2875.

Chlamydia trachomatis is the most common bacterial sexually transmitted infection in the world. C. trachomatis infection in women has been identified as an important public health problem because of its negative impact on female reproduction secondary to scarring of the fallopian tubes (reviewed in Ref. 1). The female mouse genital tract infection model using Chlamydia muridarum has been invaluable in dissecting the elements that contribute to upper genital tract pathology following infection. Using this model, it was shown that inflammatory cytokines induced following TLR2 stimulation are major players in development of oviduct pathology during genital C. muridarum infection (2).

We previously showed that IL-1β knockout (KO) mice develop significantly less oviduct pathology during C. muridarum infection (3), suggesting that downstream signaling by IL-1β during chlamydial infection could be the mechanistic basis for this phenotype. IL-1α and IL-1β exert a similar biological function by binding to IL-1R1, a member of a large family of IL-1Rs, and triggering downstream effects, including induction of proinflammatory cytokines (reviewed in Ref. 4). Conversely, IL-1Ra is a natural antagonist of IL-1α and IL-1β, preventing uncontrolled immune activation by IL-1α/β through competitive binding to IL-1R (5). During chlamydial infection, IL-1β is produced in large amounts by infiltrating macrophages and neutrophils (3), suggesting that it is a primary cytokine in downstream IL-1R activation. IL-1β is initially expressed in its proform and is only converted to a biologically active form following proteolytic cleavage by the protease caspase-1 (6, 7). Caspase-1 KO mice infected with C. muridarum exhibit less pathology in the mouse oviducts in comparison with wild-type (WT) controls (8), suggesting that activation of the cysteine protease caspase-1 is important for the development of host pathology.

Because of the integral inflammatory function of IL-1β, caspase-1 activation is controlled in a rigorous manner. Caspase-1 is activated in the cytosol in a multiprotein scaffold termed the inflammasome (9), which forms only in response to different danger signals, including bacterial or viral infections (10–13). Multiple cytosolic Nod-like receptor (NLR) proteins function as the upstream sensor proteins in this pathway. Currently, there are four defined inflammasomes, all of which contain procaspase-1 and the crucial adaptor molecule apoptosis associated speck-like protein containing caspase recruitment domain (ASC) (14), which bridges procaspase-1 to the NLR sensor proteins (15). Inflammasomes are classified by the unique NLR protein present in conjunction with these two common protein components. NLRP3
triggers activation in response to many stimuli, including extracellular ATP, large insoluble structures, and pore-forming toxins (13, 16, 17). NLRC4 is able to recognize a conserved molecular motif present in both bacterial flagellin and many bacterial rod proteins that are found in the bacterial type III secretion (T3S) apparatus (18–20). Nalp1b activates caspase-1 in response to Bacillus anthracis lethal toxin (21). Absent in melanoma 2 (AIM2), which is able to sense cytosolic dsDNA, is the most recently identified inflammasome (18, 22, 23).

The entire development cycle of C. muridarum occurs in an intracellular compartment named the inclusion, so it is intriguing how cytosolic pathways are able to interact with C. muridarum during infection to activate caspase-1. Two studies using RNA interference techniques to knockdown expression of inflammasome components in HeLa and THP-1 cells showed that NLRP3 and ASC are necessary for activation of caspase-1 during chlamydial infection (24, 25). Using macrophages from mice deficient for inflammasome components, a similar dependence on NLRP3 and ASC, but not on NLRC4, for IL-1β secretion was observed during Chlamydia pneumoniae infection (26). However, it is not known whether the same components are involved in inflammasome activation and IL-1β secretion during in vivo genital infection and how they influence infection clearance and disease outcome. In this study, we extend our results from IL-1β KO mice (3) and confirm the important role of IL-1 signaling for infection clearance and its detrimental role for oviduct pathology. Further, we investigated how these results relate to inflammasome activation during genital C. muridarum infection. In vitro infection of prestimulated macrophages results in IL-1β secretion in an ASC-dependent, but NLRP3-independent, manner, suggesting multiple pathways for inflammasome activation. However, during in vivo infection, IL-1β secretion was predominantly ASC independent. As a consequence, mice deficient for inflammasome components develop oviduct pathology similar to WT mice, unlike IL-1R−/− deficient mice. In addition, a significant role for ASC in T cell response and infection clearance was uncovered.

Materials and Methods

Chlamydial stocks

C. muridarum, “Nigg” strain, was propagated in Mycoplasma-free McCoy cells grown in DMEM supplemented with 100 μM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 10% FBS, 50 mg/ml gentamicin sulfate (Gemini Bioproducts), and 0.5 mg/ml cycloheximide. Infectious elementary bodies were isolated at 30 h postinfection by scraping the plates with a cell scraper, followed by sonication for 1 min with a sonic dismembrator with a probe attachment (Fisher Scientific). The resulting cell extracts were spun at 300 g for 5 min, and the pellet containing any remaining cellular debris was discarded. Supernatants were spun down at 30,960 × g for 16,000 rpm for 30 min at 4°C in a Beckman Coulter Avanti J-25 centrifuge fixed with a 25.50 JA rotor. Chlamydial bodies were washed; spun a second time in PBS; resuspended in 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid (SPG buffer) (pH 7.2) in small-volume aliquots (~50 ml); and finally stored at −80°C. The infectious titer of the stock was determined by infecting a fresh McCoy cell monolayer and calculating infection-forming units (IFUs), as described below.

Mouse strains

Mice deficient for the IL-1R1 subunit (IL-1R1 KO; stock no. 003245) and mice deficient for the IL-1R antagonist (IL-1Ra KO; stock no. 004754) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice deficient for inflammasome components NLRP3, NLRC4, and ASC; NLRP3 KO, NLRC4 KO, and ASC KO mice (all on a C57BL/6J background) were kind gifts from Genentech (Dr. Vishva Dixit) (13, 27). All control mice used in this study for in vivo infection or macrophage isolation were C57BL/6 mice (stock no. 000664; The Jackson Laboratory). All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Genital infections of mice

Seven days prior to infection, each mouse received 2.5 mg Depo-Provera s.c. in 100 μl PBS. A week later, mice were anesthetized with Nembutal (200–230 μl 1:100 PBS diluted stock) and infected by administering 1 × 106 infectious-forming units of C. muridarum in 10 μl SPG buffer directly into the vaginal vault.

Specimen collection from in vivo infections

Chlamydial colonization was determined by swabbing the cervix and vaginal vault of infected mice with a calcium alginate swab (Spectrum Medical Instruments, Los Angeles, CA) at various times postinfection. IFUs were calculated as described below. Additionally, genital secretions were collected 1 d prior to infection (d0), as well as throughout the duration of infection, and processed for cytokine analysis. Briefly, mice were anesthetized with Nembutal, and absorbent sponges were placed into the vagina for 30 min. Sponges were removed and stored at −80°C until further processing. At that time, cytokines were eluted from the sponges with PBS containing 0.5% BSA and 0.05% Tween-20 using Spin-X centrifuge tubes (no. 07-200-385; Fisher Scientific). Sponges were placed in the upper chamber of these tubes in 350 μl elution solution and vortexed vigorously for 1 min. Elution was allowed to occur on ice for one additional hour. Finally, after vortexing again for 1 min, samples were spun in a tabletop centrifuge at 9300 × g for 5 min at 4°C.

Histopathology

Genital tract tissues were extracted en bloc from mice sacrificed on day 45 postinfection. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Longitudinal sections (4 μm) were stained with H&E and evaluated by a pathologist blinded to the experimental design. Each anatomic site (exocervix, endocervix, uterine horn, oviduct, and mesosalpinx) was assessed independently for the presence of neutrophils, mononuclear cells (lymphocytes and monocytes), plasma cells, and fibrosis. Right and left uterine horns and right and left oviducts were evaluated individually. A four-tiered, semiquantitative scoring system was used to quantitate the inflammation and fibrosis: 0, normal; 1+, rare foci (minimal presence) of parameter; 2+, scattered (1–4) aggregates or mild diffuse increase in parameter; 3+, numerous aggregates or confluent areas of parameter; and 4+, severe diffuse infiltration or confluence of parameter. Luminal distention of the uterine horns, granulomas, and dilatation of the oviducts were graded from 1 to 4, with grade 4 representing peak severity or frequency of the parameter.

Cytokine quantification

The protein levels of IL-1β in genital secretions and culture supernatants were determined using either an ELISA kit (SMLB00C; R&D Systems) (supernatants) or a MilliplexR Map mouse cytokine/chemokine kit (cat. no. MPXMCYTO-70K-04). For ELISA analysis of IL-1β, eluates were diluted 1:20 prior to the assay. IL-18 and IFN-γ were analyzed directly from the eluates by ELISA using kits from MBL (cat. no. 7625) and R&D Systems (cat. no. MIPF00), respectively. Optical densities taken at 450 nm for quantification were measured using a Biotek plate reader.

IFU determination

IFUs were calculated by reinfecting a fresh McCoy cell monolayer in 96-well black plates. Cells were fixed at 24 h postinfection with methanol. Inclusions were visualized by staining cells using anti-C. muridarum mouse immune sera (1:300 dilution), followed by Alexa Fluor 488-conjugated anti-mouse IgG (1:1000 dilution in PBS) (Southern Biotechnology), with 0.1% Evans blue solution as a counter stain. Inclusions were counted using an Olympus fluorescent microscope to calculate the IFU/ml of each sample. For each sample assayed, the numbers of inclusions were counted in 5–20 fields. The IFU/ml was calculated by multiplying the total number of inclusions by the dilution factor, the field factor (20/no. of fields counted), and the scalar 42.33.

Macrophage isolation and in vitro infection

Peritoneal macrophages were obtained and cultured as described (28) for in vitro infections. All infections were performed in antibiotic-free media on cells that were allowed to rest in culture for ≥48 h following isolation from the mouse. Alternately, some macrophages were frozen (DMEM, 35% FBS, 10% DMSO), stored in liquid nitrogen until needed, and treated upon thawing as above. C. muridarum was added at multiplicity of infection of 1 to cells in tissue culture plates. Next, cells were centrifuged at 1690 × g at 37°C for 1 h. The media were replaced with fresh complete media. To confirm that the cells were infected, macrophages in wells...
containing coverslips were fixed with methanol for 10 min at room temperature at 24 h postinfection and stained with the FITC-conjugated Pathfinder anti-chlamydial mAb (Bio-Rad). Prestimulation of macrophages with *Escherichia coli* LPS at 100 ng/ml was carried out 6 h before infection, as described earlier (3).

**T cell proliferation**

On day 14 postinfection, mice were sacrificed, and iliac lymph nodes were harvested, macerated through a 70-μm mesh filter, and brought into single-cell suspension. The cells were resuspended at 2.5 × 10^6/ml, and 100 μl each suspension was added to 100 μl media or media containing 5 μg/ml Con A or 5 μg/ml chlamydial Ag. Chlamydial Ag was prepared by isolating *C. muridarum* elementary bodies from infected HeLa cells by renografin centrifugation (29). The chlamydiae were UV inactivated and tested for lack of viability prior to use in assays. Each treatment was assayed in at least triplicate. After 96 h of incubation, 20 μl supernatants/well was pulled and replaced with 20 μl alamarBlue (Invitrogen). Fluorescence was read on a 96-well Biotek plate reader.

**FIGURE 1.** IL-1R deficiency results in enhanced chlamydial colonization, whereas the deficiency of its antagonist IL-Ra reduces bacterial colonization and augments infection clearance during genital *C. muridarum* infection. *C. muridarum* infection course in WT (*n* = 5) and IL-1R KO (*n* = 5) mice (A) and in WT (*n* = 5) and IL-1Ra KO (*n* = 5) mice (C). Log_{10} IFU/ml were calculated from genital swabs, as described in Materials and Methods, and graphed as mean ± SEM of animals positive for infection on that day. Significance was determined by two-way ANOVA [WT versus IL-1R KO: *p* = 0.039; WT versus IL-Ra KO: *p* = 0.015 (A)]. (B and D) Percentage of mice that remain infected in each group. *p* = 0.0737 for WT versus IL-1R KO (B) and *p* = 0.0311 for WT versus IL-1Ra KO (D) by Kaplan-Meier survival analysis. A representative of four experiments is shown for (A) and (B), and a representative of two experiments is shown for (C) and (D).

**T cell proliferation**

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**FIGURE 2.** IL-R KO mice display reduced incidence of HS and histopathological dilatation of the oviducts, whereas IL-1Ra KO oviducts have increased inflammation. Percentage of oviducts developing HS in WT and IL-1R KO mice (A) and WT and IL-1Ra KO mice (D). Actual numbers of HS from the total oviduct numbers are represented over the bars. Significance was determined by the Fisher exact test. Mean histopathology scores for WT versus IL-1R KO mice (B) and WT versus IL-1Ra KO mice (E). Acute, PMNs; chronic, mononuclear cells. The grades were classified as 1, very little; 2, mild; 3, moderate; and 4, severe. Distribution of dilatation or acute scores for individual oviducts in (B) and (E) are shown by plotting dilation scores for WT and IL-1R KO mice (C) and acute cell scores for WT versus IL-1Ra KO mice (F). Pathology scores are data combined from four independent experiments for WT (*n* = 25) versus IL-1R KO (*n* = 26) mice and two experiments for WT (*n* = 15) versus IL-1Ra KO (*n* = 10) mice. *p* < 0.05, Mann-Whitney U test.
Flow cytometric analysis of genital tract cells from infected mice

Cervical tissue, uterine horns, and both oviducts were excised from the genital tract of an individual mouse, minced, and incubated separately with 1 ml collagenase I (1 mg/ml, no. C0130; Sigma) for 20 min at 37°C. After neutralizing the enzymes with EDTA (10 μM), 100 μl cell suspension was diluted with an equal volume of PBS, 1% BSA, and 1 mM EDTA and passed through filter-top tubes (no. 352235; BD). Cells (2–5 × 10^6) were passed through a mesh filter (70 μm) using DMEM with 0.5% FBS. Cell suspensions were centrifuged and resuspended in 1 ml FACS buffer containing DAPI (500 ng/ml). The following mAbs (BD Biosciences) were used: CD45 PerCP Cy5.5 (clone 30-11), Ly6G-FITC (clone IA8), F4/80-allophycocyanin (clone BMB), and CD4-PE (clone RM-4). Cells were washed with FACS buffer and resuspended with 200 μl FACS buffer containing DAPI (500 ng/ml). Cells were analyzed immediately after staining using FACSAria (BD Biosciences), and the resulting data were analyzed using FlowJo software (Tree Star).

Statistical analysis

Where indicated, at least three independent experiments were performed to test for significance using SigmaStat (Systat Software). For more than two treatment groups, a one-way ANOVA with pair-wise multiple comparison (Holm–Sidak method) was performed to determine statistically significant differences. A statistical comparison between the two mouse strains for IFU quantification or cytokine analysis over the course of infection was made using two-way ANOVA with the post hoc Tukey test as a multiple comparison procedure. The Fisher exact test was used for determination of significant differences in the frequency of hydrosalpinx (HS) observed in the two groups. For analysis of pathology scores, a Mann–Whitney U test was performed. One-way ANOVA or unpaired Student t test was used for determining the significance between differences in the percentage of positive cells from flow cytometry data.

Results

IL-1R signaling is essential for optimal clearance of genital chlamydial infection but plays a detrimental role in oviduct pathology

We previously showed that IL-1β KO mice displayed an enhanced colonization and delayed clearance of C. muridarum infection in comparison with WT mice (3). To further address the function of IL-1 signaling, the infection course of C. muridarum in the genital tract was studied using IL-1R KO mice. IL-1R KO mice showed significantly enhanced levels of chlamydial colonization and delayed clearance of infection in the genital tract in comparison with WT mice (Fig. 1A, 1B). Consistent with the observed role for IL-1 signaling in controlling infection, IL-1Ra KO mice, which show increased IL-1 response (30), showed reduced bacterial colonization and earlier clearance of infection compared with WT mice (Fig. 1C, 1D).

At day 45 postinfection, mice were sacrificed for assessment of gross pathology and histopathological observations. IL-1R KO mice showed a significantly reduced incidence of oviduct HS (p < 0.0001) (Fig. 2A). Histopathological observation of the oviduct showed significantly reduced dilation in IL-1R KO mice compared with WT mice but no differences in acute polymorphonuclear cells (PMN), chronic (mononuclear cells), and plasma cells between the two groups at this terminal time point (Fig. 2B, 2C). However, the uterine horns showed significantly more dilation and numbers of chronic and plasma cells (data not shown), which could relate to the delayed infection clearance in these mice. Contrary to the IL-1R KO mice, IL-1Ra KO mice showed an increase in the incidence of oviduct pathology in comparison with WT mice (total oviducts with HS: WT = 15/30, IL-1Ra KO = 13/20; mice with HS: WT = 10/15, IL-1Ra KO = 9/10), but this difference was not statistically significant (Fig. 2D). Histopathological observation of IL-1Ra KO oviducts showed significantly increased numbers of acute, chronic, and plasma cells (Fig. 2E, 2F), suggesting increased inflammation, despite early infection clearance (Fig. 1C).

IL-1β secretion from macrophages infected with C. muridarum is dependent on ASC

IL-1β is one of the predominant cytokines in genital secretions during C. muridarum infection and is primarily produced by macrophages and neutrophils (3). Considering its abundance and importance, it is hypothesized to be the major player in IL-1 signaling. To initially determine how IL-1β secretion is regulated by inflammasome components during C. muridarum infection, in vitro infections were performed with macrophages obtained from WT, ASC KO, NLRP3 KO, and NLRC4 KO mice. In LPS-primed macrophages, ATP-induced IL-1β secretion depended on both ASC and NLRP3, but not NLRC4 (Fig. 3A), as was previously shown (13). However, IL-1β secretion in LPS-primed macrophages following infection with C. muridarum was independent of NLRP3 and NLRC4 but dependent on ASC (Fig. 3B). Pro–IL-1β mRNA expression following prestimulation with E. coli LPS was not impaired in ASC KO macrophages (data not shown), implying that the decreased IL-1β secretion is due to lack of caspase-1 activation. Because NLRP3 has been implicated in

FIGURE 3. IL-1β secretion in prestimulated macrophages is NLRP3 independent but ASC dependent. Mouse peritoneal macrophages (8 × 10^5/well) from WT, ASC KO, NLRP3 KO, and NLRC4 KO mice were pre-stimulated with E. coli LPS for 6 h, followed by ATP stimulation for 30 min (A) or infected with C. muridarum at a multiplicity of infection of 1 (B). (C) Unstimulated macrophages were infected with C. muridarum. Culture supernatants were analyzed for IL-1β by ELISA. Data represent IL-1β levels measured by ELISA in supernatant collected 2 h after stimulation (A) or 18 h postinfection (B, C). A representative of four experiments is shown. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.
the activation of caspase-1 following chlamydial infection of unprimed cells (25), these experiments were also performed with unstimulated macrophages. With no prestimulation, macrophages secrete lower levels of IL-1β than do LPS-primed macrophages upon *C. muridarum* infection. Interestingly, unlike prestimulated macrophages, IL-1β secretion from unstimulated macrophages infected with *C. muridarum* was completely dependent on ASC and NLRP3 (Fig. 3C). Pro–IL-1β mRNA expression levels in infected macrophages were similar among WT, ASC KO, and NLRP3 KO macrophages (data not shown). Overall, these data suggest that, in addition to NLRP3, *C. muridarum* activates an inflammasome that contains both ASC and a pyrin domain containing NLR protein, but not NLRC4, during in vitro infection of prestimulated macrophages.

**ASC is required for optimal clearance of *C. muridarum* from the female genital tract**

We have hypothesized that, during in vivo infection, macrophages and neutrophils coming to the site of infection can get prestimulated by various chlamydial TLR ligands (3). To investigate the role of inflammasome components in IL-1β secretion and its relationship to infection clearance and oviduct pathology, ASC KO, NLRP3 KO, NLRC4 KO, and WT mice were infected genitally with *C. muridarum*. ASC KO mice exhibited increased bacterial colonization and prolonged courses of infection in comparison with WT mice (Fig. 4A). NLRP3 KO mice also showed slightly increased chlamydial colonization and a delay in infection clearance, although the differences were not statistically significant in multiple experiments compared with WT mice (Fig. 4B). Conversely, NLRC4 KO mice showed no differences in the rate of chlamydial clearance or colonization (Fig. 4C). To further establish the levels of bacterial colonization, genital tract tissues derived from mice from an independent study were processed for chlamydial IFU at day 10 postinfection. ASC KO mice showed a significant increase in chlamydial IFU, whereas NLRP3 and NLRC4 KO mice showed a marginal increase that was not statistically significant (data not shown).

At day 45 postinfection, the mice were sacrificed, and no significant differences were observed in the incidence of HS in ASC KO, NLRP3 KO, and NLRC4 KO mice in comparison with their corresponding WT controls (Fig. 5). Histopathological observations also showed no significant changes in the oviducts among the groups (data not shown). These data demonstrate that ASC KO mice display increased bacterial colonization just as IL-1R KO mice do, but they do not show any reduction in oviduct pathology as observed for IL-1R KO mice (Fig. 2A) compared with WT mice.

**Inflammasome-independent IL-1β secretion in vivo during *C. muridarum* genital infection**

In vitro infection data support a role for ASC inflammasome in IL-1β secretion (Fig. 3B). However, during in vivo infection, ASC KO mice do not display the reduction in pathology observed in IL-1R KO or IL-1β KO mice (3). To understand this dichotomy, IL-1β levels in genital secretions of inflammasome KO mice were analyzed. IL-1β levels were dependent on ASC and NLRP3 only at day 2 postinfection but were independent of ASC or NLRP3 deficiency from days 3 to 10 postinfection (Fig. 6A). In contrast, the secretion of another caspase-1–dependent protein (IL-18) was...
completely dependent on ASC and was also reduced in NLRP3 KO mice compared with WT mice (Fig. 6B), demonstrating that activation of caspase-1 is indeed compromised in these mice. These data demonstrate that IL-1β production is independent of ASC inflammasome during genital infection and suggest that delayed infection clearance in ASC KO mice is likely to be independent of IL-1β levels and IL-1 signaling.

**Decreased PMN recruitment during infection in IL-1R KO mice is associated with reduced pathology in IL-1R KO mice**

To further understand the mechanism of differential oviduct pathology in IL-1R KO and inflammasome KO mice, recruitment of inflammatory cells to the cervix, horns, and oviducts in IL-1R KO mice was tested at days 10 and 20 postinfection. A fraction of the collagenase-treated tissues was also analyzed for chlamydial burden by measuring IFU. Consistent with the data obtained from genital swabs (Fig. 1A), IL-1R KO horns displayed increased bacterial colonization at both days 10 and 20 postinfection, whereas the cervix showed increased IFU at day 10 postinfection (Fig. 7A, 7B). IL-1R KO oviducts showed equivalent levels of IFU as did WT oviducts (Fig. 7A, 7B), suggesting that the reduced incidence of HS at day 45 was not associated with decreased ascension of bacteria at early time points. Flow cytometric analysis showed a slight, but significant, increase in CD45+ cells in IL-1R KO genital tracts (data not shown). When individual inflammatory cells were analyzed from the total live cell population, a large and significant decrease in the number of Ly6G+ PMNs was observed in IL-1R KO genital tracts in comparison with WT mice (Fig. 7C, 7D). CD4+ T cells were also significantly decreased in the oviducts at day 20 postinfection in the IL-1R KO mice (Fig. 7E, 7F). In contrast, a significant increase in F4/80+ macrophages (Fig. 7G, 7H) was observed, which could account for the increased CD45+ cells in IL-1R KO genital tracts. A similar decrease in Ly6G+ PMNs and CD4+ T cells was observed within the CD45 pop-

**FIGURE 6.** IL-1β levels in genital secretion of ASC and NLRP3 KO mice are comparable to WT mice, but IL-18 levels are significantly reduced. Genital secretions collected using sponges on infected mice were eluted and analyzed for IL-1β (A) and IL-18 (B) by ELISA. *p < 0.001, WT versus NLRP3 KO and WT versus ASC KO IL-18 levels, two-way ANOVA. *p < 0.05, unpaired t test.

**FIGURE 7.** IL-1R KO genital tissues display a reduced number of PMNs, despite increased bacterial burden. WT (*n = 5*) and IL-1R KO (*n = 5*) mice were infected with $1 \times 10^8$ IFU, and cervix, horns, and oviduct tissue were processed at days 10 (A) and 20 (B) postinfection, as described in Materials and Methods, for IFU and flow cytometry. Total live cells were analyzed: CD45+Ly6G+ F4/80- (PMNs) (C, D), CD45+ CD4+ (CD4 T cells) (E, F), and CD45+Ly6G- F4/80+ (macrophages) (G, H). A representative of two experiments is shown. Graph and contour plot of a representative oviduct sample at day 20 postinfection from WT (I) and IL-1R KO (J) mice. Cells were gated for CD45+ cells before analyzing numbers of PMNs and CD4 T cells. Numbers represent an average from five mice/group. *p = 0.003 (CD4+ T cells), p = 0.004 (Ly6G+ PMNs), unpaired t test. *p < 0.05, unpaired t test.
ulation itself, indicating that there was no skewing of data due to changes in CD45 numbers between the two groups during infection. A representative oviduct sample from WT and IL-1R KO mice is shown (Fig. 7I, 7J). The reduction in PMN numbers in IL-1R KO mice was due to lack of IL-1R signaling and not IL-1β levels, because IL-1β levels in the genital secretions of IL-1R KO mice were comparable to those in WT mice (data not shown). Overall, the data indicate that absence of IL-1 signaling is associated with decreased PMN recruitment to the genital tract, despite increased infection. Importantly, this reduction in early PMN recruitment in IL-1R KO mice is associated with the decreased incidence of HS observed at day 45 postinfection in the oviducts (Fig. 2A), consistent with the hypothesized role for neutrophils in ascending infection (31–33).

ASC KO mice display increased PMN recruitment and a significant role for ASC in T cell recruitment and function

Although ASC KO mice have prolonged infection like IL-1R KO mice, the pathological outcome of infection is unlike IL-1R KO mice. To investigate the cellular mechanism of increased infection and oviduct pathology in ASC KO mice, tissues from infected mice at days 10 and 20 postinfection were treated with collagenase, and recruitment of inflammatory cells was analyzed by flow cytometry. No difference in the overall numbers of CD45+ cells was observed in all tissues between WT and ASC KO mice (data not shown). In contrast to IL-1R KO mice (Fig. 7), increased numbers of PMNs were observed at day 20 postinfection in ASC KO mice compared with WT mice (Fig. 8A, 8B, 8E, 8F) in cervix and horn tissues. No significant differences in F4/80+ macrophage populations were observed between the two groups at day 20 postinfection in the oviducts and horns, although some reduction was observed in ASC KO mice cervix at day 10 postinfection (data not shown). However, a significant reduction in T cell recruitment to cervix, horns, and oviducts of ASC KO mice was observed compared with WT mice at day 20 postinfection (Fig. 8B, 8C, 8E, 8F).

To further understand whether the difference in T cell recruitment is reflective of the number of T cells in the iliac lymph nodes, iliac lymph nodes from WT and ASC KO mice were obtained at day 14 postinfection. Surprisingly, there was a small, but significant, increase in the CD3+ cells in the iliac lymph nodes of ASC KO mice compared with WT mice (data not shown). However, ASC KO T cells demonstrated a statistically significant decrease in chlamydial Ag-specific proliferation in comparison with cells isolated from WT mice (Fig. 9A). Conversely, NLRP3 KO and NLRC4 KO T cells showed no decrease in T cell proliferation (data not shown). Culture supernatants from ASC KO T cells from both the mitogen- and Ag-treated groups showed reduced IFN-γ and IL-17 secretion (Fig. 9B, 9C), suggesting a diminished Th1 and Th17 response in ASC KO mice. Overall, our data suggest that ASC KO mice show increased chlamydial colonization as the result of T cell dysfunction and recruitment, despite normal IL-1β levels. Further, increased infection in ASC KO mice results in increased PMN recruitment through an intact IL-1–signaling pathway, a potential explanation for the pathology observed in these mice.

**FIGURE 8.** ASC KO mice have significantly reduced CD4 T cell but increased PMN recruitment to the genital tract. Cervix, horns, and oviducts from days 10 and 20 postinfection (WT, n = 5; ASC KO, n = 5) were analyzed for Ly6G+ PMNs (A, B) or CD4+ T cells (C, D) within the live CD45+ population. Representative contour plot of cervix samples from WT (E) and ASC KO (F) mice at day 20 postinfection. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t test.
Materials and Methods

In mice were infected with C. muridarum directly for IL-17 by ELISA (A). After 4 d, alamarBlue was added, and fluorescence was measured after 24 h. Each sample was set in triplicate, and data show average and SEM for all mice in each group. Supernatants from T cell proliferation were analyzed for IFN-γ at 1:10 dilution (B) or directly for IL-17 by ELISA (C). *p < 0.05, unpaired t test.

Discussion

This study was undertaken to determine how caspase-1 is activated during genital chlamydial infection and its relationship to IL-1 signaling. Previous studies showed that caspase-1 and the caspase-1–dependent cytokine IL-1β are associated with exacerbation of upper genital tract pathology during infection with C. muridarum (3, 8). In this study, we show that the absence of IL-1 signaling significantly protects oviducts from pathology during C. muridarum genital infection. This protection is observed despite increased chlamydial colonization, suggesting that IL-1 signaling is a double-edged sword with a beneficial effect for optimal infection clearance, as well as a deleterious effect by causing oviduct pathology during C. muridarum infection. The downstream effect of IL-1 signaling in host pathology is likely mediated through PMN recruitment to the genital tract, because lack of IL-1 signaling significantly reduced PMN infiltration into the genital tissue. These data are consistent with the important role for neutrophils in ascending infection (31–33). However, one cannot rule out additional cytotoxic roles for IL-1 signaling directly on epithelial cells, as was shown using fallopian tube organ culture (34). IL-1 signaling was also shown to play a role in disrupting epithelial tight junction claudin-4 in a Rho kinase–dependent manner in gastric epithelial cells (35), suggesting additional mechanisms through which it could damage the oviduct epithelial cell junctions.

Inflammasome activation through NLRP3 and ASC is an essential step for IL-1β secretion during in vitro C. trachomatis infection (24, 25) and during C. pneumoniae lung infection (26). We speculate that during in vivo infection, macrophages and neutrophils coming to the site of infection can get prestimulated by various chlamydial TLR ligands. Therefore, it is critical to understand the components of inflammasome activation in prestimulated macrophages. Resting macrophages required ASC and NLRP3 for IL-1β secretion but occurred independent of NLRC4. However, in LPS-prestimulated macrophages, neither NLRP3 nor NLRC4 was required for IL-1β secretion following C. muridarum infection. Only loss of the adaptor protein ASC led to decreased secretion of IL-1β, suggesting that the crucial inflammasome in primed macrophages consists of caspase-1, ASC, and an unidentified pyrin domain containing protein. AIM2, which responds to cytosolic DNA (22, 36, 37), and NALP1 (21) represent two possibilities. AIM2 is essential for inflammasome activation during Streptococcus pneumoniae infection (38) and could be the possible ASC-dependent inflammasome during chlamydial infection. The NALP1 possibility is also intriguing because caspase-1 activation by B. anthracis lethal toxin mediated by NALP1 (21) has the same potassium efflux and proteasome dependence (39–41) as does chlamydial induced IL-1β secretion (3). Above and beyond NALP1 and AIM2, it is also likely that inflammasomes exist that have yet to be discovered. It has been hypothesized that Legionella pneumophila can activate caspase-1 via ASC through an uncharacterized mechanism not involving NLRP3 or NLRC4 (42). Overall, the in vitro results in this study indicate that there are multiple inflammasomes activated by C. muridarum inside the cell, depending on the cell status, as previously shown for Listeria monocytogenes and Yersinia pseudotuberculosis (43, 44). With respect to chlamydial effectors inducing inflammasome activation, it has been speculated that chlamydial T3S apparatus could be an important trigger for caspase-1 activation (3, 45). The fact that NLRC4 played no role might suggest otherwise, but it must be noted that NLRC4 appears to be activated only by flagellin or T3S rod proteins expressing a particular structural motif (18). It would be speculated that the chlamydial T3S rod protein also lacks this motif. Nevertheless, the absence of NLRC4 activation by C. muridarum does not conclude that T3S or T3S effectors (46) are not important for caspase-1 activation.

Despite the critical role of ASC in IL-1β secretion during in vitro infection, IL-1β secretion was dependent on ASC and NLRP3 only at day 2 postinfection during in vivo genital infection. These data are reminiscent of in vitro findings in unstimulated macrophages. However, at all time points after day 2 postinfection, IL-1β secretion was independent of ASC and NLRP3. The caspase-1 activation was indeed ASC dependent, as evidenced by decreased IL-18 levels in ASC KO mice. This dependency could imply that other inflammasome proteins, which do not require the adaptor ASC, could be compensating for the lack of NLRP3 and ASC to result in IL-1β secretion. The more likely interpretation of these results is that a substantial proportion of IL-1β produced during C. muridarum infection in the female genital tract is caspase-1 independent. Along similar lines, IL-1β expression during infection of mice with Mycobacterium tuberculosis was also found to occur in a caspase-1–independent fashion (47). Additionally, in mouse models of rheumatoid arthritis, it was demonstrated that IL-1β from macrophages is caspase-1 dependent, whereas neutrophil–produced IL-1β is caspase-1 independent (48). Neutrophils possess serine proteases, such as elastase and proteinase 3, which can accomplish the catalytic step of cleaving pro–IL-1β near the site where caspase-1 targets, processing the cytokine to its active form (49). We have
shown that neutrophils isolated from genital tissue infected with *C. muridarum* have high expression of IL-1β (3). Consistent with the role of neutrophils for inflammasome-independent IL-1β production, ASC KO mice showed increased PMN recruitment and had significant IL-1β levels in their genital secretions. These data are in contrast to observed findings during *Pneumocystis* infection in lungs, in which a direct correlation of IL-1 signaling, ASC KO, and NLRP3 KO was observed (26, 50). However, it must be noted that *Pneumocystis* seems to be able to activate cytosolic sensors for other cytokine pathways in a manner different from *C. muridarum* (51–53). Therefore, drawing parallels between these two pathogens with respect to caspase-1 activation may not be precise.

Independent of IL-1β levels, ASC KO mice exhibited increased chlamydial colonization of *C. muridarum*. Additionally, infection course and pathology from ASC KO mice during genital chlamydial infection does not mirror the data from caspase-1 KO mice (8), which showed reduced oviduct pathology compared with WT mice. The phenotype in ASC KO mice could be attributed to decreased T cell function and recruitment to the genital tract. ASC is a cytosolic protein, for which an inflammatory role independent of caspase-1 and NLRP3 was demonstrated in an Ag-induced arthritis model (54). Using the same model, it was shown that ASC KO T cells show decreased cellular proliferation (54) and diminished priming from dendritic cells (55). In a mouse model for experimental autoimmune encephalomyelitis, ASC-deficient CD4 T cells exhibited impaired survival (56), illustrating another example where ASC seems to be able to play a crucial caspase-1–independent role. Recently, it was demonstrated that ASC controlled mRNA stability of Dock2, a guanine nucleotide exchange factor that mediates Rac-dependent signaling in immune cells, affecting T cell migration and Ag uptake by dendritic cells, in an inflammasome-independent manner (57). Therefore, during in vivo chlamydial infection, we propose that Ag-specific T cells are likely exhibiting impaired survival and decreased proliferation in the absence of ASC, resulting in delayed infection clearance.

Overall, we extend our studies with IL-1β KO mice (3) and provide a conclusive role for IL-1 signaling in progression of oviduct pathology. The protection from oviduct pathology in IL-1R KO mice is particularly striking, because these mice have increased chlamydial burden. These results suggest that IL-1R signaling is a major host cytokine-signaling pathway contributing directly to oviduct pathology. However, inflammasome activation in vivo is not a major contributor to IL-1β levels during in vivo infection, although ASC has a specific role in T cell function and activation necessary for resolving infection. It remains to be determined how IL-1β is produced during in vivo infection and whether modulating this pathway by antagonists can prevent oviduct pathology during infection.

Acknowledgments

We thank Genentech Inc. (Dr. V. Dixit) for kindly providing NLRP3 KO, NLR4 KO, and ASC KO mice. We also thank Shamnugam Nagarajan (University of Pittsburgh Medical Center, Pittsburgh, PA) and John Gрегan (Arkansas Children’s Hospital Research Institute, Little Rock, AR) for critical and editorial comments on the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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