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Development of an *Ex Vivo* Tissue Platform To Study the Human Lung Response to *Coxiella burnetii*

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***Coxiella burnetii* is an intracellular bacterial pathogen that causes human Q fever, an acute debilitating flu-like illness that can also present as chronic endocarditis. Disease typically occurs following inhalation of contaminated aerosols, resulting in an initial pulmonary infection. In human cells, *C. burnetii* generates a replication niche termed the parasitophorous vacuole (PV) by directing fusion with autophagosomes and lysosomes. *C. burnetii* requires this lysosomal environment for replication and uses a Dot/Icm type IV secretion system to generate the large PV. However, we do not understand how *C. burnetii* evades the intracellular immune surveillance that triggers an inflammatory response. We recently characterized human alveolar macrophage (hAM) infection *in vitro* and found that avirulent *C. burnetii* triggers sustained interleukin-1β (IL-1β) production. Here, we evaluated infection of *ex vivo* human lung tissue, defining a valuable approach for characterizing *C. burnetii* interactions with a human host. Within whole lung tissue, *C. burnetii* preferentially replicated in hAMs. Additionally, IL-1β production correlated with formation of an apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC)-dependent inflammasome in response to infection. We also assessed potential activation of a human-specific noncanonical inflammasome and found that caspase-4 and caspase-5 are processed during infection. Interestingly, although inflammasome activation is closely linked to pyroptosis, lytic cell death did not occur following *C. burnetii*-triggered inflammasome activation, indicating an atypical response after intracellular detection. Together, these studies provide a novel platform for studying the human innate immune response to *C. burnetii*.**

Coxiella burnetii is a Gram-negative, obligate intracellular pathogen that causes Q fever in humans (1). Acute Q fever presents with flu-like symptoms; however, infection can persist and cause life-threatening endocarditis. *C. burnetii* is a category B select agent due to an aerosol mode of transmission, low infectious dose, and environmental stability (2). The pathogen has a unique intracellular lifestyle that requires replication within an acidic lysosome-like parasitophorous vacuole (PV) in macrophages (3). Using a Dot/Icm type IV secretion system (T4SS) to secrete bacterial proteins into the host cytoplasm (4, 5), *C. burnetii* hijacks the host cell to control signaling cascades and heterotypic fusion with endosomes, autophagosomes, and lysosomes to establish the PV (6). Although *C. burnetii* intracellular trafficking has been well characterized, understanding of the initial interactions between the pathogen and its human host is lacking. Although small animal models provide beneficial information about the host response to *C. burnetii*, they do not accurately mimic the human lung response to infection, presenting the need for improved systems. For example, mouse alveolar macrophages degrade *C. burnetii* (7), while the pathogen replicates efficiently in primary human alveolar macrophages (hAMs) (8).

Macrophages are critical for Q fever development and dictate disease outcome by responding to invading pathogens. Macrophages detect bacteria using extracellular Toll-like receptors (TLRs) and intracellular nucleotide-binding oligomerization domain proteins (NODs). TLR signaling ultimately results in nuclear factor-κB (NF-κB) activation and expression of genes encoding proinflammatory cytokines such as interleukin-1β (IL-1β). TLRs recognize bacterial lipopolysaccharide (LPS), composed of lipid A and O antigen. The lipid A moiety of LPS is a conserved invariant portion present in all Gram-negative bacteria and is recognized by TLRs, while the O antigen varies among species and is not typically recognized (9). The LPS structure differs between virulent and avirulent *C. burnetii* strains and is a major determinant of Q fever (10). Virulent organisms, referred to as being in phase I, produce full-length LPS and cause significant disease in guinea pigs and humans. However, continued passage in culture results in truncation of LPS, resulting in attenuation of the pathogen and its characterization as phase II. One clonal phase II isolate of *C. burnetii* (clone 4, RSA439) is exempt from select agent regulations and has been used extensively to study the cell biology of infection. However, we recently demonstrated differing primary hAM cytokine responses to *C. burnetii* pathotypes (8), indicating the need for comparison of virulent and avirulent bacteria.

In addition to TLRs, cytosolic inflammasomes form in response to microbes and result in caspase activation (11, 12). Active caspases process the proinflammatory cytokines IL-1β and IL-18 to mature secreted forms and promote pyroptotic cell death (13, 14). This response typically requires an initial signal that triggers expression of pro-IL-1β and pro-IL-18 and a second signal that initiates complex formation (15). A canonical inflammasome

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contains a NOD-like receptor or PYHIN (pyrin domain and hematopoietic expression, interferon-inducible nature and nuclear localization [HIN] domain-containing), the adapter molecule apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and caspase-1 (16). Although the inflammasome-mediated response has been described for many intracellular pathogen systems, there is a lack of information regarding intracellular sensing of *C. burnetii*.

In this study, we defined a novel approach to study *C. burnetii* infection using *ex vivo* human lung tissue and primary hAMs. Using these infection platforms, we demonstrated that avirulent *C. burnetii* specifically replicates in hAMs within the lung environment and triggers caspase-dependent IL-1β production. We also discovered activation of a potential noncanonical caspase-5-dependent hAM inflammasome that may direct secretion of IL-1β in response to *C. burnetii*. These findings advance understanding of the innate immune response to *C. burnetii* in the human lung.

MATERIALS AND METHODS

Bacteria and eukaryotic cell culture. *C. burnetii* NMII (RSA439, avirulent clone 4) was cultured in acidified citrate cysteine medium (ACCM) for 7 days at 37°C with 5% CO_2 and 2.5% O_2. Cultures were washed three times and resuspended in sucrose phosphate (SP) buffer prior to infection experiments. All experiments using virulent *C. burnetii* were performed in the Centers for Disease Control and Prevention-approved biosafety level 3 facility at the University of Arkansas for Medical Sciences. hAMs were infected with a multiplicity of infection of 10, and precision-cut lung slices (PCLS) were infected with 1.5×10^7 bacteria by addition of organisms to the medium (0 h postinfection [hpi]). At 24 hpi, cells were washed to remove extracellular bacteria.

Primary hAMs were obtained by bronchoalveolar lavage (BAL) from human lungs acquired from the National Disease Research Interchange postmortem. BAL fluid cells were collected by centrifugation and red blood cells lysed by incubation with 0.86% ammonium chloride for 10 min. Ammonium chloride was neutralized by addition of culture medium and removed by centrifugation. Cells were resuspended in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) containing 10% fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin sulfate (50 μg/ml), gentamicin sulfate (50 μg/ml), and amphotericin B (0.25 μg/ml) (Invitrogen). Cells were allowed to adhere to tissue culture dishes for 2 h, and then nonadherent cells were removed by washing, leaving a >95% population of hAMs as confirmed by immunofluorescence microscopy (data not shown).

After BAL, lungs were filled with low-melting-point agarose and allowed to solidify at 4°C. Lung tissue was then processed into two-inch sections. Sections were then cored with an 8-mm-diameter tool, and tissue was sectioned into 750-μm precision-cut lung slices (PCLS) using a vibrating microtome (Compresstome; Precisionary Instruments). PCLS were incubated with shaking in DMEM-F12 containing antibiotics and antimycotics at 37°C and 5% CO_2. PCLS and hAMs were cultured in antibiotic- and antimycotic-containing medium for 3 days, and then antibiotic- and antimycotic-free medium was added and samples incubated for an additional 24 h before infection to ensure absence of contamination.

Confocal fluorescence microscopy. hAMs or PCLS were fixed in 4% paraformaldehyde (PFA) and blocked in solution containing 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100 for 1 h at room temperature. Samples were then incubated with primary antibodies in blocking solution for 1 h at room temperature, followed by Alexa Fluor-conjugated secondary antibodies (Alexa Fluor-488, -594, or -647) for 1 h. Caspase-1 antibody (Cell Signaling) was used at a concentration of 1:100. ASC (Santa Cruz Biotechnologies) and IL-1β (R&D Systems) antibodies were used at a concentration of 1:50. Cells were imaged using a Nikon Ti-Eclipse confocal microscope and images analyzed with Nikon Elements software.

Immunoblot analysis. Following infection, hAMs were lysed in buffer containing 1% SDS by passage through a 26-gauge needle. Total protein was quantified using a DC protein assay (Bio-Rad) and 10 μg of total protein separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a 0.2-μm polyvinylidene fluoride membrane and blocked overnight in Tris-buffered saline (150 mM NaCl, 100 mM Tris-HCl, pH 7.6) containing 0.1% Tween 20 and 5% nonfat milk. After blocking, total protein levels were confirmed by probing with mouse β-tubulin antibody (Sigma). Lysates were probed with caspase-4 or caspase-5 antibodies (Cell Signaling) or an IL-1β antibody (R&D Systems). Appropriate secondary antibodies conjugated to horseradish peroxidase were used with ECL-Plus chemiluminescence reagent to visualize proteins. Positive-control cells were treated with *Escherichia coli* LPS (100 ng/ml; Sigma) for 6 h followed by ATP (5 mM; Sigma) for 30 min.

Cytokine analysis. IL-1β enzyme-linked immunosorbent assays (ELISAs) (BD Biosciences) were performed using supernatants harvested from infected cells or PCLS. Precoated wells were incubated with samples for 2 h at room temperature and then washed, and detection antibody was added and left for 1 h. After removing excess antibody, samples were fixed in 4% paraformaldehyde (PFA) for 30 min to inactivate any remaining virulent bacteria. PFA was removed, and enzyme working reagent was added and left for 30 min, followed by washing and treatment with tetramethylbenzidine (TMB) one-step substrate for 30 min. The reaction was then stopped with stop solution and absorbance measured at 450 nm using a BioTek H2 Synergy plate reader. A standard curve was established using duplicate standards ranging from 0 to 125 pg/ml, and unknown sample concentrations were calculated.

FLICA activity assay. hAMs (1.0×10^5 cells/well) were cultured in black-wall 96-well glass-bottom plates and either left uninfected and untreated or infected for the indicated times. Uninfected hAMs were treated with *E. coli* LPS and ATP as described above (positive control). Cells were then incubated with 6-carboxyfluorescein (FAM)–caspase-1 fluorescein (FLICA) (Immunochemistry Technologies) for 60 min at 37°C. Unbound probe was removed by adding medium to wells and incubating for 60 min to allow unbound probe to diffuse from cells. Medium was then aspirated, cells placed in phosphate-buffered saline (PBS), and fluorescence measured using a BioTek H2 Synergy plate reader (excitation, 490 nm; emission, 525 nm).

LDH assay. hAMs (4×10^5/well) were plated in 24-well plates. hAMs were then either left uninfected and untreated or infected for the indicated times. Positive-control hAMs were treated with *E. coli* LPS and ATP as described above. Supernatants were then harvested and lactate dehydrogenase (LDH) measured using a CytoTox 96 nonradioactive cytotoxicity assay (Promega) according to the manufacturer's protocol. The positive control was set to 100%, and values for experimental samples were calculated as percentages of the positive-control value.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR). Uninfected or infected hAMs were harvested in TRIzol (Invitrogen) by repeated pipetting. Phase separation was performed by addition of 0.2 ml chloroform/ml of TRIzol. Tubes were shaken, and then samples were centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous phase was collected and RNA precipitated with 1 volume of 70% ethanol. RNA was then isolated using an RNeasy kit according to the manufacturer's instructions (Qiagen), performing two on-column DNase digestions (Qiagen). Total RNA (500 ng) was converted to cDNA using a Superscript III first-strand synthesis kit (Life Technologies) and random hexamers. TaqMan-based PCR was performed for each sample using primers and probe (Life Technologies) specific for each gene of interest and TaqMan Fast Advanced master mix (Life Technologies). Samples were normalized to the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene and compared to uninfected samples.

Statistical analysis. All experiments were performed with material from at least three separate donors. Statistical analyses were performed using Prism 6 software and two-way analysis of variance (ANOVA).

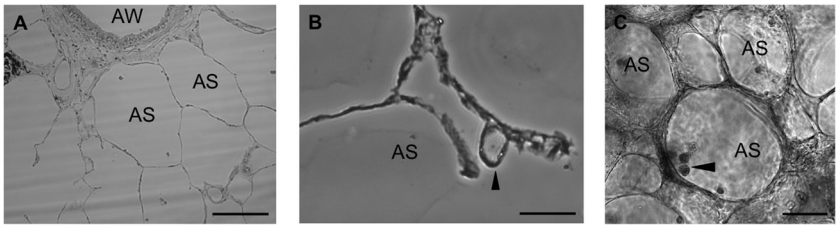

FIG 1 Cells morphologically similar to alveolar macrophages are present in PCLS. PCLS were incubated with avirulent *C. burnetii* for 72 h and then processed for histology and observed by bright-field microscopy. Bars, 50 μm. (A) Four-micrometer-sectioned PCLS showing alveolar spaces (AS) and an airway (AW). (B) AS containing an expanded *C. burnetii* PV (arrowhead). (C) AS containing dense cells morphologically similar to macrophages (arrowhead).

RESULTS

***C. burnetii* preferentially replicates within hAMs in *ex vivo* human lung tissue.** We recently developed a primary hAM approach to study *C. burnetii* host-pathogen interactions in primary human cells (8). This study showed that virulent and avirulent *C. burnetii* strains replicate to high numbers in hAMs and trigger pathotype-specific innate inflammatory responses. However, no previous study has proven that alveolar macrophages are the pathogen's target cell *in vivo*. To determine if hAMs are the true *C. burnetii* target cell, we extended our studies to intact *ex vivo* human lung tissue to study initial interactions between *C. burnetii* and its human host. Lung tissue was sectioned into 750-μm-thick slices termed precision-cut lung slices (PCLS) (17) and incubated in tissue culture plates. We visualized PCLS by bright-field microscopy (Fig. 1A and B) and found cells morphologically indicative of macrophages in alveolar spaces (Fig. 1C, arrowhead). PCLS were then infected with *C. burnetii* for 72 h and visualized by bright-field microscopy, which indicated large bacterium-containing vacuoles in cells on the walls of alveolar spaces (Fig. 1B). It is important to note that the tissue structure was maintained during infection, suggesting that *C. burnetii* does not trigger disruption of the lung structure during intracellular growth, unlike the damage resulting from other bacterial respiratory infections (18, 19).

To determine if cells that support *C. burnetii* replication are macrophages, PCLS were infected for 72 h and then processed for confocal fluorescence microscopy. As shown in Fig. 2A, a subset of alveolar cells contained an expanded CD63-positive PV that supported robust *C. burnetii* replication. Accumulation of bacteria in these PVs was not the result of a large inoculum, as bacterial numbers continually increased throughout the infection time course and type IV secretion system-defective *C. burnetii* did not accumulate in macrophages (data not shown). Although individual *C. burnetii* cells were present in numerous alveolar cells, only macrophages supported bacterial replication, as indicated by the presence of expanded PVs only in cells that were labeled with the macrophage marker CD68 (Fig. 2B). These results demonstrate that *C. burnetii* specifically targets alveolar macrophages for replication in human lung tissue.

Avirulent *C. burnetii* triggers prolonged IL-1β production in hAMs and PCLS. We previously discovered that avirulent, but not virulent, *C. burnetii* triggers robust IL-1β production by primary hAMs (8), a response that is critical for clearing many bacterial pathogens (20). To further characterize the human innate

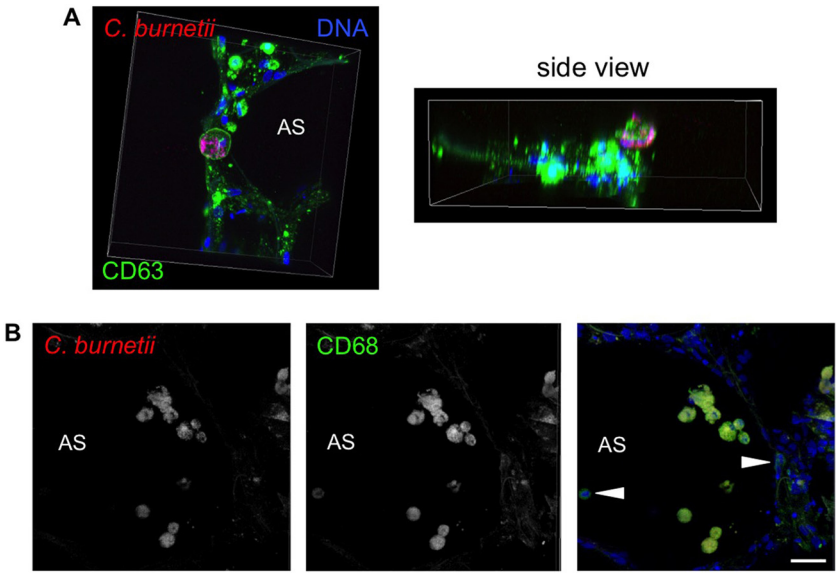

FIG 2 *C. burnetii* replicates specifically within alveolar macrophages in human PCLS. PCLS were incubated with avirulent *C. burnetii* for 72 h and then processed for confocal fluorescence microscopy using antibodies directed against the PV marker CD63 (A) or the macrophage marker CD68 (B) and *C. burnetii* (red). Arrowheads indicate macrophages that do not harbor *C. burnetii*. DAPI (4′,6′-diamidino-2-phenylindole) was used to stain DNA (blue), and colors are shown in the images. Bar, 10 μm. *C. burnetii* replicates in a CD63-positive, lysosome-like PV only in CD68-positive alveolar macrophages.

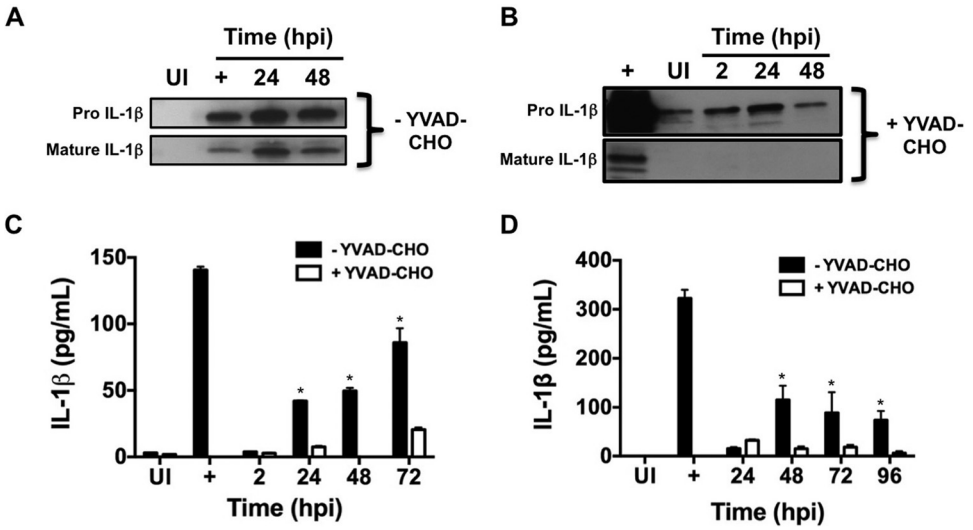

FIG 3 Avirulent *C. burnetii* triggers caspase-dependent IL-1β production in hAMs and PCLS. (A and B) hAMs were infected with avirulent *C. burnetii* for the indicated times in the absence (A) or presence (B) of the caspase-1 inhibitor YVAD-CHO. Samples were subjected to immunoblot analysis using an IL-1β antibody, and the results shown are representative of three individual experiments. UI, uninfected cells; +, cells treated with LPS and ATP. Mature IL-1β production does not occur in the presence of YVAD-CHO, suggesting that caspase-1 activity is required. (C and D) hAMs (C) or PCLS (D) were left uninfected (UI), treated with LPS and ATP (+), or infected for the indicated times, and supernatants were harvested. YVAD-CHO was added where indicated, and IL-1β was detected in supernatants by sandwich ELISA. Avirulent *C. burnetii* triggers secretion of IL-1β by infected hAMs and PCLS, and this response is inhibited by YVAD-CHO. Error bars indicate the standard deviation from the mean; *, $P < 0.05$ according to two-way ANOVA comparing infected cells to uninfected and YVAD-CHO-treated cells. These results indicate that avirulent *C. burnetii* triggers IL-1β production and secretion through a caspase-dependent mechanism in hAMs and PCLS.

response to *C. burnetii*, we monitored IL-1β production and secretion during infection of PCLS or isolated hAMs. Avirulent *C. burnetii* triggered a robust and prolonged IL-1β response, as evidenced by increased mRNA transcripts from 2 to 24 hpi (data not shown), increased production of pro-IL-1β and mature IL-1β in whole-cell lysates from 24 to 48 hpi (Fig. 3A and B) using immunoblot analysis, and increased levels of secreted IL-1β in supernatants of infected hAMs from 24 to 72 hpi (Fig. 3C) using an ELISA. Additionally, IL-18 transcripts increased similarly to those of IL-1β (data not shown). Furthermore, IL-1β production required live *C. burnetii*, as heat-killed bacteria did not trigger cytokine production (data not shown). We next assessed the IL-1β response during infection of PCLS to determine if *C. burnetii* elicits a proinflammatory response during replication in the lung environment. Confirming the hAM results, we observed similar responses to avirulent *C. burnetii* in PCLS from 24 to 96 hpi (Fig. 3D), indicating that this sustained proinflammatory innate response to avirulent organisms occurs in a context relevant to human disease.

Macrophage recognition of intracellular bacteria involves activation of a specific inflammasome in response to bacterial products such as LPS, flagellin, and secreted proteins (12, 21). Inflammasome complexes are composed of a NOD-like protein, a caspase, and, depending on the inflammasome, an adapter molecule (21, 22). Following activation, caspase-1 processes pro-IL-1β to mature IL-1β that is secreted from the cell to trigger an immune response (13). To determine if the mechanism of IL-1β production in response to avirulent *C. burnetii* involves canonical activation of caspase-1 (23), we used the established caspase-1 peptide inhibitor AC-YVAD-CHO during infection (24). When caspase-1 activity was inhibited, mature IL-1β production and secretion

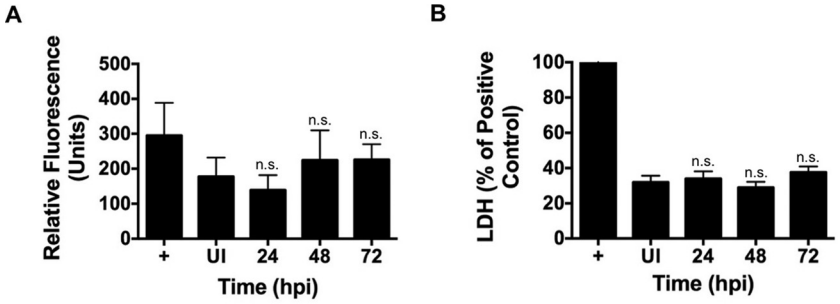

FIG 4 *C. burnetii* infection does not elicit elevated caspase-1 activity or promote cell lysis indicative of pyroptosis. hAMs were left uninfected (UI), treated with LPS and ATP (+), or infected with avirulent *C. burnetii* for the indicated times. (A) Active caspase-1 was detected with a fluorescent FAM-FLICA probe. Avirulent *C. burnetii* does not trigger significant caspase-1 activity. (B) LDH present in the supernatant was measured as an indicator of cell lysis. Values for experimental samples were calculated as percentages of the positive-control value (set to 100%). Data are representative of at least three separate experiments using cells from at least two separate donors, and error bars represent the standard deviation from the mean. n.s., not significantly different from value for uninfected cells. *C. burnetii*-infected cells do not release significantly more LDH than uninfected hAMs.

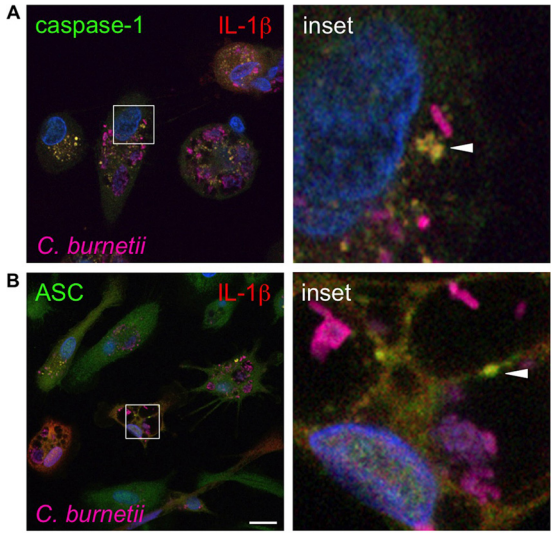

FIG 5 Caspase-1 and ASC colocalize with IL-1β near avirulent *C. burnetii*. hAMs were infected with avirulent *C. burnetii* for 72 h and then processed for confocal fluorescence microscopy using antibodies directed against caspase-1 (A), ASC (B), and IL-1β and *C. burnetii*. DAPI was used to stain DNA (blue), and colors are shown in images. Bar, 10 μm. Caspase-1 and ASC colocalize with IL-1β (yellow; arrowheads) near *C. burnetii*, suggesting that inflammasomes are located near the PV for detection of avirulent bacteria.

were prevented in hAMs and PCLS (Fig. 3B to D). However, using an established FLICA assay, no significant caspase-1 activation was observed (Fig. 4A). Additionally, using a lactate dehydrogenase release assay (Fig. 4B), levels of infected-cell death did not significantly increase compared to those for uninfected cells, similar to the results of our previous apoptosis studies (25–27). This is unexpected if caspase-1 is activated, as this event typically triggers pyroptosis, an inflammatory form of cell death (23). Thus, *C. burnetii* may activate a noncanonical caspase-1-independent inflammasome or may prevent caspase-1-mediated death.

Caspase-1 and ASC colocalize with IL-1β at the PV membrane. Inflammasome formation can be assessed by microscopy to detect formation of characteristic specks containing caspase-1, IL-1β, a NOD-like protein, and the adapter protein ASC (23). To assess inflammasome formation, we used confocal fluorescence microscopy to observe caspase-1, IL-1β, and ASC localization during infection. In avirulent *C. burnetii*-infected hAMs, caspase-1 colocalized with IL-1β in characteristic specks that were in close proximity to intracellular bacteria (Fig. 5A). Specks containing ASC and IL-1β were also evident in *C. burnetii*-infected hAMs (Fig. 5B). Interestingly, a portion of ASC-containing specks were present within the *C. burnetii* PV, suggesting that some inflammasomes are trafficked into the degradative lysosome-like vacuole. Combined with the caspase inhibitor data, these results suggest that avirulent *C. burnetii* triggers ASC-dependent inflammasome activation and caspase-dependent production of IL-1β in primary hAMs and PCLS.

***C. burnetii* triggers increased expression of *nlrp3*, *nod2*, and *casp5*.** To probe the type of inflammasome activated in response to avirulent *C. burnetii*, we used an RT-PCR array to determine inflammasome- and caspase-related gene expression. Inflammasome activation is not typically controlled at the transcriptional level; however, this analysis provides clues about increased expression of products potentially involved in bacterial recognition. As shown in Fig. 6A, expression of *nlrp3* and *nod2* increased significantly during avirulent *C. burnetii* infection compared to that in uninfected hAMs, suggesting that an NLRP3 inflammasome is involved in detecting avirulent organisms.

Caspase-1 peptide inhibitors such as YVAD-CHO target the catalytic site of caspase-1, which is homologous to the catalytic sites of caspase-4 and caspase-5 (24). Due to low levels of caspase-1 activity and cell death during avirulent *C. burnetii* infection, we sought to determine if other caspases are involved in intracellular detection of the pathogen. Indeed, the closely related *Legionella pneumophila* and intracellular *Salmonella* are detected by an inflammasome requiring murine caspase-11, the murine homolog to human caspase-4 and caspase-5 (28, 29). To assess caspase involvement in infection, we monitored expression of caspase-related genes during avirulent *C. burnetii* infection using an RT-PCR array. As shown in Fig. 6B, avirulent *C. burnetii* infection of hAMs elicited increased expression of *casp5*, but not *casp1*, *casp8*, *card6*, or *card18*. Together, these results suggest that a novel caspase-5-containing noncanonical NLRP3 inflammasome is activated in response to avirulent *C. burnetii*.

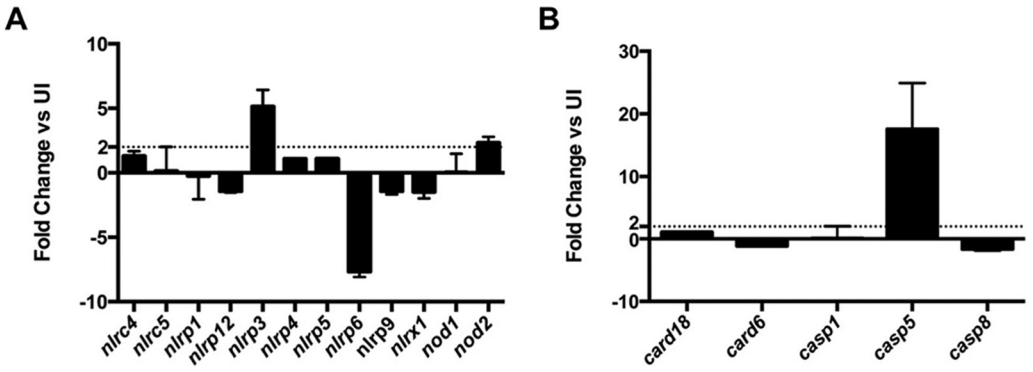

FIG 6 *C. burnetii* infection triggers increased expression of specific inflammasome-related genes. hAMs were infected with avirulent *C. burnetii* for 24 h and total RNA isolated. The resulting cDNA was subjected to analysis using an RT-PCR array specific for relevant inflammasome-related genes (A) or caspase-related genes (B). Transcript levels were compared to uninfected hAMs (UI), and the dashed line in each graph denotes the 2-fold cutoff. Data represent two separate experiments using cells from two separate donors, and error bars represent the standard error. Avirulent *C. burnetii* triggers increased transcription of *nlrp3*, *nod2*, and *casp5*.

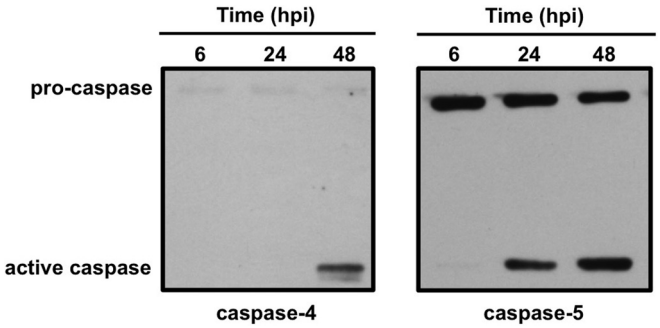

FIG 7 Avirulent *C. burnetii* triggers caspase-4 and caspase-5 processing. hAMs were infected with avirulent *C. burnetii* for 6 to 48 h and samples subjected to immunoblot analysis using antibodies directed against caspase-4 or caspase-5. Caspase-5 processing is evident from 24 to 48 hpi, and active caspase-4 is present at 48 hpi, suggesting noncanonical inflammasome activation during *C. burnetii* infection involving caspase-5.

***C. burnetii* elicits caspase-4 and caspase-5 processing in hAMs.** Similar to other caspases, caspase-4 and caspase-5 are processed from a pro form (~50 kDa) to an active protein (~10 to 20 kDa) to control cellular events. Because *casp5* expression increased during avirulent *C. burnetii* infection, activation was monitored by immunoblotting in hAMs. As shown in Fig. 7, caspase-4 processing was observed at 48 hpi, a time following IL-1β production in avirulent *C. burnetii*-infected hAMs. However, caspase-5 processing showed kinetics similar to those for IL-1β production, with processed caspase apparent by 24 hpi. Unfortunately, attempts to silence expression of *casp5* were unsuccessful due to poor hAM transfection efficiency (data not shown). However, these results indicate that caspase-5 activation corresponds to IL-1β secretion during avirulent *C. burnetii* infection and may be involved in detection of the pathogen.

DISCUSSION

Here, we show that primary hAMs and PCLS mount a proinflammatory IL-1β response following exposure to avirulent *C. burnetii*. We previously showed that this response is absent when hAMs are infected with virulent *C. burnetii* (8), demonstrating a major difference in the cellular response to disease-causing and attenuated bacteria. In over 70 years since its discovery as the causative agent of Q fever (30–32), the human immune response to *C. burnetii* has remained difficult to understand. This difficulty is due, in part, to the fact that ~50% of infected individuals remain asymptomatic, suggesting that an intact immune system can effectively combat the pathogen. Unfortunately, we do not understand how *C. burnetii* avoids the immune response to cause acute or chronic disease in symptomatic cases.

Animal models have previously been used to provide information about the importance of specific cell types and cytokines in the anti-*C. burnetii* response. Indeed, using various species, researchers have shown that tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and T cell activation play a role in the anti-*C. burnetii* response (33). However, most mice are refractory to infection, and few reagents are available to study infection of guinea pigs, the most disease-relevant small animal model of Q fever. To study the human lung response to *C. burnetii*, we developed human PCLS as a novel infection platform. Our findings indicate that PCLS infection is a disease-relevant approach to studying *C. burnetii* innate interactions with humans. Using PCLS, we show, for the first time, that hAMs are the only cell type that supports *C. burnetii* intracellular replication in the lung. Interestingly, the pathogen enters other alveolar cells but does not replicate. In contrast, *C. burnetii* replicates in most cell types *in vitro*, including alveolar epithelial cells. It is possible that infected hAMs alert bystander cells to the presence of *C. burnetii*, allowing other cells to mount effective intracellular defenses against the pathogen, and this possibility is under investigation.

Primary hAMs and PCLS allowed characterization of the IL-1β response to avirulent *C. burnetii*, which is not triggered by virulent isolates. Inflammasomes are intracellular sensors of microbes and control responses that alert other immune cells. Multiple inflammasomes have been described, and intracellular pathogens typically activate a specific type. For example, *L. pneumophila* flagellin is recognized by an NLRC4 inflammasome. NLRC4 coordinates caspase-1 activation and resultant IL-1β production (34, 35). In addition to caspase-1, caspase-11 recognizes *L. pneumophila*-containing vacuoles and restricts growth of the pathogen (29). Furthermore, caspase-4 is activated in cells infected with *Salmonella* spp., leading to clearance of the pathogen from the intestinal epithelium (28, 29). Caspase-1 activation is typically triggered by stimulation of an intracellular NOD-like receptor that promotes assembly of an inflammasome that recruits and activates the caspase (14, 21, 22). Although we do not know the specific inflammasome that responds to *C. burnetii*, TLR ligation is the first step in the IL-1β response, and avirulent *C. burnetii* likely triggers TLR-2 signaling, as the organism does not activate TLR-4 (36, 37).

C. burnetii recognition by hAMs alters inflammasome-related gene expression, including increased expression of *nlrp3*, *nod2*, and *casp5*. These data support NLRP3-dependent detection of *C. burnetii*. Furthermore, caspase-1 and ASC colocalize with IL-1β close to the PV. It is currently not known whether *C. burnetii* actively recruits caspase-1 and ASC to the PV; however, a subset of inflammasome specks appears in the PV lumen, suggesting that they are delivered into the vacuole. The PV is a degradative environment (38) and, *C. burnetii* may recruit inflammasomes to counteract intracellular detection and the proinflammatory response. Indeed, we recently demonstrated that *C. burnetii* uses T4SS effectors to recruit autophagosomes to the PV (39), and inflammasome turnover is controlled by autophagy in other systems (15).

The mechanism of virulent *C. burnetii* suppression of IL-1β production is under investigation and represents a major difference in the hAM response to virulent and avirulent bacteria. The best characterized difference between isolates lies in their LPS, with avirulent bacteria producing severely truncated LPS that stimulates TLR signaling (40, 41). However, LPS differences alone are not entirely responsible for promoting IL-1β production, as infection of hAMs with virulent *C. burnetii* triggers initial production of pro-IL-1β and barely detectable mature IL-1β (8), suggesting that the pathogen combats the response after activation. It is possible that virulent *C. burnetii* produces a subset of proteins not present in avirulent *C. burnetii*. Indeed, a genome study by Beare et al. found a group of genes containing single nucleotide polymorphisms (SNPs) present in NMII (avirulent) compared to NMI (virulent) *C. burnetii* (42, 43). If these SNPs alter protein production, NMI may produce proteins that prevent inflammasome activation. Genes containing SNPs are under investigation to determine if they alter inflammasome-mediated detection of virulent *C. burnetii*.

To our knowledge, this is the first study to implicate caspase-5 in

macrophage detection of intracellular bacteria. Notably, this discovery would not have been possible without our hAM infection platform, because caspase-4 and caspase-5 are human-specific proteins. Additionally, caspase-5 is undetectable in a THP-1 human macrophage-like cell line model (data not shown). *casp5* expression increases substantially during avirulent *C. burnetii* infection. Previous studies proposed that caspase-4 and caspase-5 are homologs of murine caspase-11 that promote noncanonical inflammasome activation (23, 44–46). However, our results suggest that these proteins function independently, as the timing of caspase-5 processing (24 hpi) corresponds to IL-1β production in avirulent *C. burnetii*-infected hAMs, while cleaved caspase-4 is not seen until 48 hpi. These findings open a new avenue of study to define the function of human caspase-5 in the host response to *C. burnetii*.

A recent study using avirulent *C. burnetii* showed that the organism does not trigger IL-1β secretion from mouse macrophages (47). That study presents an intriguing difference between murine and human *C. burnetii* infection and suggests that the two cell models can be used to define unique intracellular pathogen activities. Additionally, that study showed that *C. burnetii* secretes an effector, termed IcaA, that prevents caspase-11 activity. It is possible that avirulent *C. burnetii* uses IcaA to prevent activity of human caspase-4 (as a caspase-11 homolog) yet fails to inhibit caspase-5 activity that controls mature IL-1β secretion. Nonetheless, that study, combined with our results, indicates that *C. burnetii* must confront caspase activity and inflammasome-mediated detection during intracellular growth in macrophages.

Collectively, the current study further defines the primary hAM IL-1β response to *C. burnetii* and establishes human PCLS as a new infection platform. Due to the extensive use of avirulent *C. burnetii* in the field, these results should be considered when planning host response experiments. Numerous studies, including our own, have shown that avirulent and virulent bacteria display nearly identical intracellular behavior, including development of similar PVs and activation of host signaling. The current study supports the existence of important differences in the host cell response to differing pathotypes and promotes enhanced appreciation for using primary hAMs and *ex vivo* human lung tissue to characterize the innate immune response to *C. burnetii*.

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