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Virulent *Coxiella burnetii* Pathotypes Productively Infect Primary Human Alveolar Macrophages

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Summary

The intracellular bacterial pathogen *Coxiella burnetii* is a category B select agent that causes human Q fever. *In vivo*, *C. burnetii* targets alveolar macrophages wherein the pathogen replicates in a lysosome-like parasitophorous vacuole (PV). *In vitro*, *C. burnetii* infects a variety of cultured cell lines that have collectively been used to model the pathogen's infectious cycle. However, differences in the cellular response to infection have been observed, and virulent *C. burnetii* isolate infection of host cells has not been well defined. Because alveolar macrophages are routinely implicated in disease, we established primary human alveolar macrophages (hAMs) as an *in vitro* model of *C. burnetii*-host cell interactions. *C. burnetii* pathotypes, including acute disease and endocarditis isolates, replicated in hAMs, albeit with unique PV properties. Each isolate replicated in large, typical PV and small, non-fused vacuoles, and lipid droplets were present in avirulent *C. burnetii* PV. Interestingly, a subset of small vacuoles harbored single organisms undergoing degradation. Prototypical PV formation and bacterial growth in hAMs required a functional type IV secretion system, indicating *C. burnetii* secretes effector proteins that control macrophage functions. Avirulent *C. burnetii* promoted sustained activation of Akt and Erk1/2 pro-survival kinases and short term phosphorylation of stress-related p38. Avirulent organisms also triggered a robust, early pro-inflammatory response characterized by increased secretion of TNF- α and IL-6, while virulent isolates elicited substantially reduced secretion of these cytokines. A corresponding increase in pro- and mature IL-1 β occurred in hAMs infected with avirulent *C. burnetii*, while little accumulation was observed following infection with virulent isolates. Finally, treatment of hAMs with IFN- γ controlled intracellular replication, supporting a role for this antibacterial insult in the host response to *C. burnetii*. Collectively, the current results demonstrate the hAM model is a human disease-relevant platform for defining novel innate immune responses to *C. burnetii*.

Introduction

Alveolar macrophages are the first line defense that confronts pulmonary bacterial pathogens following inhalation of contaminated aerosols. The ability of these cells to rapidly respond to an invading pathogen is central to mounting an effective antibacterial response during initial stages of infection. Upon confronting a pathogen, alveolar macrophages

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secrete distinct cytokines, including TNF- α , IL-1 β , and IL-6, to recruit additional immune cells to the area, promoting pathogen disposal and development of adaptive immunity (Marriott *et al.*, 2007). Following phagocytosis, bacteria-laden phagosomes mature through the phagolysosomal pathway, acquiring features including acid hydrolases, acidic pH, and reactive oxygen species, all of which are detrimental to the harbored organism (Flannagan *et al.*, 2009). Not surprisingly, intracellular bacterial pathogens have evolved sophisticated mechanisms to avoid lysosomal degradation by alveolar macrophages and subvert the host immune response, allowing propagation of the bacterial population. For example, *Mycobacterium tuberculosis* is a highly prevalent human pathogen that alters macrophage phagolysosomal trafficking to prevent destruction by lysosomal proteases (Deretic *et al.*, 2006).

Coxiella burnetii is a category B select agent that replicates within macrophages and causes human Q fever. *C. burnetii* has a worldwide distribution and diverse animal reservoir, with livestock serving as the primary source of bacterial spread to humans via contaminated aerosols inhaled while working with infected animals (Maurin *et al.*, 1999). Most symptomatic cases present with acute disease characterized by high fever and pneumonia that can be readily treated with antibiotics (Gikas *et al.*, 2010, Raoult *et al.*, 2005). Persistent infections result from ~ 2% of acute cases and have a higher fatality rate (de Valk, 2012, Raoult *et al.*, 1999), typically presenting as endocarditis in immunocompromised individuals and patients with heart valve conditions. A recent Q fever outbreak in the Netherlands resulted in over 4,000 acute Q fever cases, 250 estimated chronic cases, and 14 deaths to date (Forland *et al.*, 2012, Limonard *et al.*, 2010a, Limonard *et al.*, 2010b, van der Hoek *et al.*, 2012), underscoring our lack of understanding basic *C. burnetii* biology and virulence mechanisms.

C. burnetii has been isolated from a variety of geographic regions, hosts, and disease conditions since its discovery in the 1930s. *C. burnetii* isolates are generally placed into one of eight groups with distinct genomic characteristics and disease presentations (Beare *et al.*, 2006). Group I organisms are typified by the Nine Mile I (NMI) reference isolate and typically cause acute disease, while groups IV and V (including the G isolate) are often associated with chronic diseases such as endocarditis. Group VI is composed of organisms collected from rodents in Dugway, UT that are severely attenuated in animal models (Stoenner *et al.*, 1960, Stoenner *et al.*, 1959). Most virulent *C. burnetii* isolates, including NMI, produce a full length lipopolysaccharide (LPS) and are referred to as being in phase I. However, Nine Mile II (NMII) is a clone derived from NMI *C. burnetii* that produces a truncated LPS referred to as being in phase II and does not cause disease. Importantly, NMII can infect cells *in vitro*, providing a model organism for use under biosafety level-2 conditions. Much debate has centered on whether *C. burnetii* genomic composition or host factors play a larger role in disease presentation (Samuel *et al.*, 1985, Thiele *et al.*, 1994). However, *C. burnetii* replication inside host cells is a determinant of disease caused by all isolates.

Following inhalation by a mammalian host, *C. burnetii* is engulfed by macrophages and promotes interactions with autophagosomes, fluid phase endosomes, and lysosomes to establish a host membrane-derived compartment in which to replicate (Voth *et al.*, 2007a). *C. burnetii* metabolism is activated in the harsh, acidic environment of this parasitophorous vacuole (PV) that decorates with the lysosomal markers LAMP-1, -2, -3 and contains active proteases, including cathepsin D. While replicating in the PV, *C. burnetii* continuously directs fusion with other host cell compartments to provide membrane for PV expansion and inhibits apoptotic cell death to allow a prolonged infectious cycle (Howe *et al.*, 2003, Vazquez *et al.*, 2010, Voth *et al.*, 2009b, Luhrmann *et al.*, 2007, Voth *et al.*, 2007b). To regulate infection, the pathogen uses a Dot/Icm type IV secretion system (T4SS) to inject

effector proteins into the host cytosol (Voth *et al.*, 2009a, Voth *et al.*, 2011, Voth *et al.*, 2009c, Pan *et al.*, 2008, Chen *et al.*, 2010, Luhrmann *et al.*, 2010) and organisms deficient in T4SS-mediated secretion do not replicate in eukaryotic cells or prevent intrinsic apoptosis (Beare *et al.*, 2011, Carey *et al.*, 2011, Beare *et al.*, 2012).

Many eukaryotic cell types, including epithelial cells, monocytes, and macrophages have been used to model the *C. burnetii* infectious process using NMI or NMII organisms (Voth *et al.*, 2007a). However, nothing is known about the interaction between virulent *C. burnetii* isolates and their *in vivo* target, the human alveolar macrophage (hAM). Furthermore, cellular infection has not been addressed for other pathotypes, leaving a substantial void in our understanding of *C. burnetii* biology. Here, we assessed the intracellular properties of four *C. burnetii* pathotypes and found that all organisms replicated in primary hAMs with unique PV formation properties. We also determined the cytokine response to infection by each pathotype and assessed the role of IFN- γ in controlling replication. This study is the first to address cellular aspects of endocarditis isolate and naturally attenuated isolate infections and demonstrates the hAM is a human disease-relevant model for studying the unique *C. burnetii* intracellular lifestyle.

Results

Primary human alveolar macrophages support *C. burnetii* growth

No studies have addressed the interaction of *C. burnetii* with its *in vivo* human target cell, the alveolar macrophage. To address this gap in our understanding of this highly infectious pathogen, primary hAMs were harvested by bronchioalveolar lavage and incubated with one of four *C. burnetii* isolates (NMII, NMI, G, or Dugway) for 9 days. Every two days, infected cells were harvested, bacterial DNA isolated, and genome equivalents indicative of intracellular organisms determined. As shown in Fig. 1, all *C. burnetii* isolates productively infected and replicated in hAMs. G and Dugway isolate growth waned slightly through the first 3 days post-infection (dpi), followed by increased growth from 3–9 dpi, while NMII and NMI had similar growth curves indicating avirulent and virulent organisms are not efficiently degraded by hAMs and replicate in these cells similar to studies using primary human dendritic cells (Shannon *et al.*, 2005).

C. burnetii generates a phagolysosome-like PV in hAMs

The hallmark of a *C. burnetii* infection at the cellular level is biogenesis of a spacious, phagolysosome-like PV required for bacterial growth. Following infection with NMII, NMI, G, or Dugway, hAMs were assessed for the presence of prototypical PV containing replicating organisms. As shown in Fig. 2A, all isolates formed a large vacuole that labeled with the late endosome/lysosome PV marker CD63. Additionally, each isolate replicated in a PV containing cathepsin D, which is indicative of an active lysosome (Fig. 2A and data not shown). These observations agree with recent results demonstrating that NMII and NMI replicate in human monocyte-derived macrophages in a functional lysosomal environment that degrades other bacteria (Howe *et al.*, 2010). Electron microscopy analysis confirmed the presence of large PV in many NMII-infected hAMs similar to those observed in THP-1 human macrophage-like cells (Fig. 2B).

C. burnetii's ability to form a replication-permissive PV in cultured cell lines requires a functional Dot/Icm T4SS and accompanying effector proteins (Beare *et al.*, 2011, Carey *et al.*, 2011, Beare *et al.*, 2012). Therefore, we assessed whether the T4SS is needed for PV formation in hAMs. As seen in Fig. 2C, T4SS-deficient *C. burnetii* (*icmD::Tn*) was unable to generate a large PV and did not replicate in tight-fitting phagosomes, confirming the importance of the T4SS in primary hAMs.

***C. burnetii* PV display unique properties in hAMs**

In each infected hAM culture, a subset of cells contained small CD63-positive phagosomes harboring individual organisms when observed by immunofluorescence microscopy (Fig. 3A). In cultured cell lines, *C. burnetii* is only present in a tight-fitting phagosome within the first 12 hpi, at which point the vacuole expands via heterotypic fusion with host compartments (Voth *et al.*, 2007a). In contrast, ~ 50% of NMII-infected hAMs contained at least one large, spacious PV while ~ 50% contained only small phagosomes and no large PV (Fig. 3A). This composition differed substantially from NMII-infected THP-1 cells, where ~ 92% contained large PV and only ~ 8% harbored tight-fitting PV. Additionally, over 90% of hAMs harbored multiple PV of various sizes at 72 hpi (Fig. 3A), while only 20% of THP-1 cells contained multiple PV at this time. Similar phenotypes were observed during infection by NMI, G, and Dugway, with G replicating most efficiently in cells with multiple PV (data not shown). When monitored by electron microscopy, some tight-fitting hAM vacuoles contained a low number of organisms (~ 1–2/phagosome) in various states of degradation as evidenced by disrupted membranes (Fig. 3B).

Electron microscopy revealed an unexpected aspect of *C. burnetii*-host cell interactions, with many PV containing lipid droplets (Fig. 4A). Lipid droplets play a role in cellular infection by intracellular pathogens, including *Salmonella spp.*, *M. tuberculosis*, and *Chlamydia trachomatis* (Peyron *et al.*, 2008, Arena *et al.*, 2011, Cocchiario *et al.*, 2008, Saka *et al.*, 2012, Kumar *et al.*, 2006), but have not previously been reported during *C. burnetii* infection of other eukaryotic cells. Electron microscopy was confirmed by fluorescence microscopy using the lipid-specific probe BODIPY 493/503, which showed lipid droplets present within the PV at 72 hpi (Fig. 4B), a time when the vacuole is expanding to accommodate replicating organisms. Furthermore, fluorescence intensity analysis confirmed that lipid droplets are harbored within intact PV and some associate with *C. burnetii*. Thus, lipid droplets represent a novel area of *C. burnetii* research for future exploration.

***C. burnetii* modulates hAM pro-survival signaling pathways**

Previous studies demonstrated the importance of intracellular signaling cascade activation for *C. burnetii* PV formation and prevention of apoptosis (Hussain *et al.*, 2010, Voth *et al.*, 2009b, Hussain *et al.*, 2012, MacDonald *et al.*, 2012). The organism inhibits apoptosis by activating kinases such as Akt and Erk1/2, promoting an anti-apoptotic transcriptional program, and preventing cytochrome *c* release from mitochondria (Voth *et al.*, 2007b, Luhrmann *et al.*, 2007, Luhrmann *et al.*, 2010). To assess the potential *in vivo* role of host kinase signaling, we probed infected hAMs for phosphorylation (activation) of the cell survival mediators Akt and Erk1/2 and the stress response kinase p38, which have been previously shown in the THP-1 macrophage-like cell line (Voth *et al.*, 2009b). During NMII growth in hAMs, increased phosphorylation of Akt and Erk1/2 occurred from 2–72 hpi (Fig. 5), correlating with phosphorylation profiles in infected THP-1 cells and highlighting the importance of prolonged kinase activation for *C. burnetii* infection. In contrast, p38 phosphorylation increased substantially at early times post-infection (2–24 hpi) with each isolate, then decreased to near uninfected cell levels by 72–96 hpi, suggesting p38 activation is an early hAM response to *C. burnetii*.

hAMs mount a robust inflammatory response to *C. burnetii*

In a pulmonary innate immune response, hAMs must confront an invading pathogen, traffic the organism to a phagolysosome for degradation, and produce appropriate cytokines to recruit other immune cells, promoting development of an adaptive immune response (Marriott *et al.*, 2007). Although hAMs are unable to efficiently degrade *C. burnetii* in a phagolysosome, they are predicted to mount a cytokine response to recruit immune cells to the alveoli. Therefore, we monitored secretion of cytokines from hAMs exposed to *C.*

burnetii pathotypes that are likely critical for clearing infection in the host (Fig. 6A). Each isolate elicited increased secretion of TNF- α at 24 hpi; however, the TNF- α response to avirulent *C. burnetii* was more robust than the response to NMI, G, or Dugway. These results correspond to those reported during *C. burnetii* infection of primary human dendritic cells (Shannon *et al.*, 2005). Similar to TNF- α , levels of secreted IL-6 increased substantially during infection with each isolate at 24 hpi. IL-10 levels also increased in response to each isolate, representing a potential anti-inflammatory event promoted by the pathogen to establish a persistent infection (Meghari *et al.*, 2008, Honstetter *et al.*, 2003). In contrast to TNF- α , IL-6, and IL-10, IL-1 β only increased substantially during NMII infection (Fig. 6B). Using immunoblot analysis, we observed that levels of pro- and mature IL-1 β increased substantially above uninfected levels by 24 hpi with NMII organisms and remained elevated through 72 hpi, indicating hAMs respond to replicating intracellular avirulent *C. burnetii* by activating IL-1 β -dependent signaling. Interestingly, pro- and mature IL-1 β were undetectable after 24 hpi by NMI, G, or Dugway, suggesting organisms in phase I with full length LPS do not provide the triggers necessary to induce an appropriate inflammatory IL-1 β response.

IFN- γ -mediated control of *C. burnetii* replication

In vivo, IFN- γ stimulates macrophage antibacterial responses such as production of reactive oxygen species that promote clearance of intracellular pathogens. IFN- γ is an integral component of the immune response to *C. burnetii* in animal models (Andoh *et al.*, 2007), implicating its importance in humans. Therefore, we monitored *C. burnetii* NMII infection of hAMs in the presence of IFN- γ . hAMs were either pre-treated with IFN- γ for 24 h or treated at the time of infection as previously reported (Ghigo *et al.*, 2002, Dellacasagrande *et al.*, 2002) to determine the impact of the cytokine on *C. burnetii* growth. As seen in Fig. 7A, each treatment condition significantly decreased total infectious *C. burnetii* numbers as determined by a standard foci forming unit assay. Interestingly, many IFN- γ -treated cells allowed large PV formation, but limited *C. burnetii* replication in these vacuoles (data not shown). These results underscore the importance of IFN- γ signaling in controlling *C. burnetii* infection. However, infected hAMs treated with IFN- γ did not undergo high levels of apoptosis to destroy intracellular bacteria (Fig. 7B), as previously reported for *C. burnetii*-infected THP-1 cells (Dellacasagrande *et al.*, 2002, Dellacasagrande *et al.*, 1999).

Discussion

Here, we present the first *in vitro* study demonstrating that primary hAMs are suitable hosts for replication and study of the highly infectious intracellular bacterial pathogen *C. burnetii*. The macrophage has been accepted for many years as the pathogen's *in vivo* target in humans, but no cellular studies of this unique host-pathogen interaction have been reported. Alternatively, a collection of cultured cell lines and primary cells have been used to provide a wealth of relevant information about the infectious process (Voth *et al.*, 2007a), with monkey alveolar macrophages and human monocyte-derived macrophages serving as the models most similar to the *in vivo* human condition studied to date (Voth *et al.*, 2007b, Howe *et al.*, 2010). Animal studies consistently report high numbers of *C. burnetii* in alveolar macrophages (Khavkin *et al.*, 1988, Russell-Lodrigue *et al.*, 2009, Russell-Lodrigue *et al.*, 2006), making the primary hAM a disease-relevant model for probing *C. burnetii* mechanisms of host cell parasitism. While our hAM results correspond in some respects with those derived using cultured cell lines, we present new evidence of non-fused, atypical PV harboring replicating *C. burnetii*, lipid droplet recruitment to the PV, and distinct cytokine responses to infection with four different pathotypes. These results suggest hAMs can be used as a valuable model to uncover novel host-pathogen interactions.

hAMs are an unquestionably critical innate defense against pulmonary pathogens. Arguably the most prevalent pulmonary intracellular pathogen, *M. tuberculosis*, prevents its own destruction in lysosomes, allowing replication in hAMs (Deretic *et al.*, 2006, Clemens *et al.*, 2000, Via *et al.*, 1997, Clemens *et al.*, 1995). To avoid destruction, *M. tuberculosis* prevents phagolysosome formation by stalling phagosome maturation at the stage of an early phagosome that decorates with Rab5 but fails to recruit LAMPs. However, cell surface interactions with *M. tuberculosis* elicit an inflammatory cytokine response characterized by secretion of TNF- α and IL-6 (Yang *et al.*, 2007). *Legionella pneumophila*, the causative agent of Legionnaires' disease that is closely related to *C. burnetii*, infects alveolar macrophages and replicates in an endoplasmic reticulum-derived vacuole that does not fuse with lysosomes (Joshi *et al.*, 2001, Clemens *et al.*, 2000). *L. pneumophila* deploys an arsenal of secreted Dot/Icm T4SS effector proteins to establish its intracellular niche and control host signaling (Hubber *et al.*, 2010). A functional *C. burnetii* Dot/Icm T4SS is also required for generation of large PV that contain lysosomal cathepsin D in hAMs, as organisms with disrupted *icmD* are only found in tight-fitting phagosomes and do not replicate. Intracellular bacterial pathogens must also combat the action of IFN- γ and reactive oxygen species (Katti *et al.*, 2008, Yang *et al.*, 2007, Nathan *et al.*, 2000), which comprise a primary hAM defense mechanism. *C. burnetii* antagonizes production of reactive oxygen species *in vitro* (Hill *et al.*, 2011, Siemsen *et al.*, 2009) and this response is likely necessary for establishing a productive hAM infection. Additionally, IFN- γ efficiently decreased bacterial replication in hAMs, highlighting the importance of this cytokine in controlling *C. burnetii* infection.

An early mouse infection study demonstrated the presence of *C. burnetii* within alveolar macrophages following intranasal challenge (Khavkin *et al.*, 1988). Following this report, other researchers compared differing routes of animal infection and found intranasal or aerosol delivery to be most representative of natural human exposure (Russell-Lodrigue *et al.*, 2009, Russell-Lodrigue *et al.*, 2006, La Scola *et al.*, 1997). Together, these studies support a major role for alveolar macrophages in the initial stages of Q fever. Khavkin and Tabibzadeh also reported that murine alveolar macrophages had the capacity to degrade *C. burnetii* (Khavkin *et al.*, 1988). However, virulent *C. burnetii* replicates efficiently in hAMs in lysosome-like PV similar to cultured cell lines such as THP-1 human macrophage-like cells and primary human monocyte-derived macrophages (Howe *et al.*, 2010), suggesting a large percentage of bacteria survive macrophage antibacterial responses. *C. burnetii* anti-apoptotic activity was originally identified in monkey alveolar macrophages that also support replication, providing evidence of the *in vivo* importance of preventing macrophage death (Voth *et al.*, 2007b). Likewise, our preliminary studies demonstrate that apoptosis prevention is involved in *C. burnetii* infection of hAMs, with virulent organisms preventing staurosporine-induced death (MacDonald *et al.*, unpublished results).

Two novel aspects of infection uncovered in this study are the presence of infected cells with multiple atypical PV and trafficking of lipid droplets into the PV. In most cells, *C. burnetii* forms and maintains a large, spacious PV that fills with hundreds of bacteria (Voth *et al.*, 2007a). It is uncommon to observe cells with multiple PV containing replicating organisms, particularly with the low inocula used in this study. The *C. burnetii* Priscilla isolate was originally thought to replicate in numerous individual PV in murine cells, but further studies confirmed the presence of one, multi-lobed PV (Hechemy *et al.*, 1993). Compared to our established THP-1 infection model, ~ 70% more hAMs contain numerous PV and many of these provide a suitable replication environment. Each isolate replicates in cells with multiple PV, suggesting this phenotype is representative of an *in vivo* scenario. It is currently unknown if all tight-fitting PV in hAMs harbor viable bacteria, as some small vacuoles contain only one or two bacteria. These small phagosomes may possess more efficient lysosomal capacity that aids degradation of *C. burnetii*. Indeed, *C. burnetii* encased in some small vacuoles contain membranes in various states of degradation. Therefore, it

appears that some hAM phagosomes proficiently degrade *C. burnetii*, and the composition of these small vacuoles is under investigation. In support of these findings, a population of *L. pneumophila* is killed by hAMs, with surviving bacteria replicating to high numbers (Jacobs *et al.*, 1984).

An intriguing finding in *C. burnetii*-infected hAMs is the presence of lipid droplets within and adjacent to the PV. Lipid droplets contain sterols and triacylglycerols (Tauchi-Sato *et al.*, 2002) and are involved in eukaryotic cell processes such as vesicular fusion and signaling (Saka *et al.*, 2012), two aspects of cell biology central to intracellular pathogen infections. Lipid droplets associated with *C. trachomatis* replication vacuoles are predicted to influence chlamydial development (Cocchiario *et al.*, 2008, Kumar *et al.*, 2006). Although the mechanistic role of these components in chlamydial infection is unknown, they potentially regulate cholesterol transport, membrane traffic, and signaling, events that are also involved in *C. burnetii* manipulation of host cells. *C. burnetii* exploits host cholesterol metabolism during intracellular growth, triggering increased cholesterol biosynthesis, and the PV decorates with the sterol probe filipin (Howe *et al.*, 2006). Additionally, the pathogen encodes a sterol reductase predicted to process cholesterol precursors (Gilk *et al.*, 2010). *C. trachomatis* produces and secretes at least three proteins that associate with, and possibly recruit, lipid droplets (Kumar *et al.*, 2006). Thus, *C. burnetii* may devote a subset of Dot/Icm substrates to recruitment of lipid bodies during infection.

There is currently a void in *C. burnetii* research regarding host cell interactions with non-NM isolates, including chronic disease pathotypes and severely attenuated Dugway isolates obtained during studies in the 1950s in Utah (Sidwell *et al.*, 1964, Stoenner *et al.*, 1960). Although virulence in humans is unknown, Dugway is highly attenuated in hamsters, mice, and guinea pigs (Stoenner *et al.*, 1960). Russell-Lodrigue *et al.* recently showed that Dugway organisms do not infect wild type mice as efficiently as NMI bacteria and cause no detectable fever in guinea pigs, but are able to infect and replicate in immunodeficient SCID mice, causing severe splenomegaly (Russell-Lodrigue *et al.*, 2009). However, no studies have been performed at the cellular level to determine if Dugway attenuation results from an inability to grow in macrophages. Interestingly, growth of Dugway organisms lags slightly in hAMs between 1 and 3 dpi. However, after 3 dpi, Dugway numbers increase through 9 dpi and organisms replicate in hAMs in a lysosome-like PV that decorates with CD63 and cathepsin D. These results indicate that Dugway can replicate in macrophages and attenuation *in vivo* likely requires an intact immune system.

Alveolar macrophages mount an inflammatory cytokine response upon confrontation with a pathogen to recruit other immune cells. The cytokine response to NMI *C. burnetii* is typified by production of inflammatory TNF- α and IL-6 in host serum and at the cellular level (Shannon *et al.*, 2009, Russell-Lodrigue *et al.*, 2009), while production of IL-10 may regulate persistent infection (Meghari *et al.*, 2008, Honstetter *et al.*, 2003). Indeed, alveolar macrophages can produce high levels of these cytokines in response to bacteria (Marriott *et al.*, 2007). hAMs demonstrate a robust inflammatory response to NMII organisms at 24 hpi as evidenced by dramatic increases in secreted TNF- α and IL-6. hAMs respond similarly to NMI, G, and Dugway, but overall cytokine levels are substantially reduced compared to NMII, suggesting organisms with phase I LPS activate a less intense inflammatory immune response. This hypothesis is supported by the observation that NMI LPS shields immune mediators on the bacterial cell surface and prevents activation of toll-like receptor 4 signaling (Shannon *et al.*, 2005). Surprisingly, hAMs do not produce high levels of IL-10 in response to virulent *C. burnetii* as expected based on previous reports supporting an *in vivo* role for the cytokine (Meghari *et al.*, 2008, Meghari *et al.*, 2006, Honstetter *et al.*, 2003). However, the current study only assessed cytokine secretion at 24 hpi, representing an early stage in the *C. burnetii* infectious cycle. Additionally, this study only monitored hAM

supernatants and not whole host serum levels. Thus, non-macrophage cells may serve as the major source of IL-10 during active disease.

This is the first study to report production and secretion of mature IL-1 β during *C. burnetii* infection of macrophages. IL-1 β is an inflammatory mediator produced by alveolar macrophages in response to pathogenic situations (Descamps *et al.*, 2012). Following stimulation by a pathogen-associated molecular pattern such as LPS, pro-IL-1 β is produced and subsequently processed to mature IL-1 β by caspase-1 after receiving a second stimulus that activates the inflammasome. Mature IL-1 β is then secreted from the cell. hAMs respond to NMII with prolonged production of pro- and mature IL-1 β from 24–72 hpi, supporting a role for the cytokine in mediating the innate immune response to avirulent organisms. Interestingly, much lower levels of pro- and mature IL-1 β are observed at 24 hpi with phase I *C. burnetii* isolates and are undetectable from 48–72 hpi, suggesting virulent organisms either combat or fail to fully trigger this inflammatory response. The inflammasome is activated in response to a variety of pathogen products in the cytoplasm and is inhibited by several secreted bacterial proteins (Barker *et al.*, 2011). For example, *L. pneumophila* flagellin triggers activation of the inflammasome, leading to clearance of the organism from wild type mice (Case *et al.*, 2009), while *M. tuberculosis* Zmp1 prevents caspase-1 activation and subsequent IL-1 β secretion (Master *et al.*, 2008). Although *C. burnetii* does not produce flagellin, NMII LPS may trigger inflammasome activity during phagocytic uptake by hAMs. Once inside the cell, secreted bacterial proteins are candidates for cytosolic detection, and hAMs may detect *C. burnetii* T4SS effectors, resulting in inflammasome activation and mature IL-1 β production. However, if phase I isolate LPS provides a weak trigger of pro-IL-1 β production, only low levels of mature IL-1 β would be produced in response to T4SS effectors, allowing virulent organisms to persist in the absence of this critical inflammatory response. This intriguing scenario is supported by our hAM results and is currently under further investigation.

Collectively, the current results indicate the hAM is a disease-relevant model of *C. burnetii*-host interactions that can be used to test hypotheses about *in vivo* infection events at the cellular level, particularly those investigating the innate immune response to infection. The hAM model is particularly relevant in regard to virulent *C. burnetii* isolates associated with differing forms of Q fever. Furthermore, hAMs support replication of the Dugway isolate that is severely attenuated in animal models. Numerous studies support a major role for LPS in the immune response to *C. burnetii*, with NMII truncated LPS stimulating an immune response that clears the organism from infected animals. However, Dugway produces phase I LPS, suggesting a more complex host immune response to attenuated organisms. Finally, hAMs can be used to probe shared and distinct parasitic mechanisms employed by *C. burnetii* and related pulmonary pathogens. For example, little is known about growth of the macrophage pathogens *L. pneumophila* and *Chlamydia pneumoniae* in their *in vivo* target cell. Interestingly, an early report showed that > 97% of *L. pneumophila* is destroyed by hAMs within 30 minutes of uptake, with remaining bacteria surviving, replicating, and eventually killing hAMs (Jacobs *et al.*, 1984). Similarly, *C. pneumoniae* growth is restricted in primary murine alveolar macrophages compared to cell lines (Redecke *et al.*, 1998, Haranaga *et al.*, 2003). Together with our *C. burnetii* results, these studies indicate alveolar macrophages elicit distinct responses to related intracellular pathogens and should not be neglected for validating cellular studies of the innate immune response.

Experimental Procedures

C. burnetii and mammalian cell culture

C. burnetii Nine Mile II (RSA439), Nine Mile I (RSA493), G (Q212), and Dugway (7E65-68) were cultured in acidified citrate cysteine medium (ACCM) for 7 days at 37°C in

5% CO₂ and 2.5% O₂ (Omsland *et al.*, 2009). Isolates were collected by centrifugation and washed with sucrose phosphate buffer prior to use. Work with virulent *C. burnetii* isolates was performed in the UAMS biosafety level-3 laboratory that is approved by the Centers for Disease Control and Prevention.

Vero (Green monkey kidney) cells and human monocytic THP-1 cells (American Type Culture Collection) were cultured in RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37°C in 5% CO₂. THP-1 cells were incubated with 200 nM phorbol 12-myristate 13-acetate (PMA; EMD Biosciences) overnight to promote differentiation into macrophage-like cells (Voth *et al.*, 2007b). Prior to infection, medium containing PMA was replaced with PMA-free medium. Primary human alveolar macrophages were isolated by bronchioalveolar lavage with phosphate buffered saline (PBS) from lung tissue obtained postmortem from the National Disease Research Interchange. No donors were excluded unless obvious contamination, indicative of infection, was observed after culturing macrophages. All experiments were performed with at least two different donor macrophage populations. Cells were collected from lavage fluid by centrifugation at 1,000 rpm for 5 min. Red blood cells were lysed by resuspending cells in 0.0084% ammonium chloride for 10 min. Ammonium chloride was then neutralized by addition of medium and removed from cells by centrifugation. Cells were then resuspended in Dulbecco's modified Eagle medium/F-12 medium containing 10% FBS, penicillin (50 U/mL), streptomycin sulfate (50 µg/mL), gentamicin sulfate (50 µg/mL), and amphotericin B (0.25 µg/mL) (Invitrogen) and plated at 4 × 10⁵ cells/well in 24-well plates, or 1.5 × 10⁶ cells/well in 6-well plates. Macrophages were allowed to adhere to plates for 2 h at 37°C in 5% CO₂, then non-adherent cells were removed and remaining cells washed with fresh medium. hAMs were routinely assessed for homogeneity by immunofluorescence and immunoblot analysis (Fig. S1) with antibodies directed against the macrophage-specific markers CD11/18b, CD14, and CD68 (Abcam). Lavage cultures typically contained > 90% macrophages. hAMs and THP-1 cells were infected with *C. burnetii* isolates at a multiplicity of infection (MOI) of ~ 10 in the absence of gentamicin, penicillin, streptomycin, and amphotericin B.

Growth curve analysis

hAMs were infected and samples collected for DNA analysis at 1, 3, 5, 7, and 9 dpi. Cells were harvested by scraping in 1 mL of medium, and DNA was isolated using a bacterial DNA extraction kit according to the manufacturer's instructions (MO BIO Laboratories, Inc.). Genomes were quantified using quantitative real time PCR and primers (DotA-F 5'-GCG CAA TAC GCT CAA TCA CA-3' and DotA-R 5'-CCA TGG CCC CAA TTC TCT T-3'; Integrated DNA Technologies) that recognize *C. burnetii dotA*, and calculated as previously described (Samuel *et al.*, 2009). Platinum Taq and SYBR green (Invitrogen) were used to amplify and detect DNA with an Applied Biosystems 7300 Real Time PCR cycler.

Fluorescence microscopy

Following infection of hAMs or THP-1 cells, cells were fixed and permeabilized with 100% ice-cold methanol for 3 min, then blocked for 1 h in PBS containing 0.5% bovine serum albumin (BSA; Cell Signaling) at room temperature. Cells were then incubated with either mouse anti-CD63 (BD Biosciences), mouse anti-cathepsin D (Enzo Life Sciences), rabbit anti-*C. burnetii*, or guinea pig anti-*C. burnetii* primary antibodies for 1 h at room temperature. Cells were then washed 3 times with ice cold PBS and incubated in 0.5% BSA-PBS containing AlexaFluor-488, -594, or 647-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Indicated samples were incubated with BODIPY 493/503 (Invitrogen) at a concentration of 1:500 for 30 min to detect lipid droplets. Cells were then incubated with DAPI (4',6'-diamidino-2-phenylindole dilactate; Invitrogen) for 5 min at

room temperature to detect host and bacterial DNA. Fluorescence microscopy was performed using a Ti-U microscope with a 60X oil immersion objective (Nikon). Images were obtained with a D5-QiIMc digital camera and analyzed using NIS-Elements software (Nikon). PV and apoptotic nuclei were quantified from 5–10 fields containing ~ 25 cells/field.

Electron microscopy

hAMs or THP-1 cells were infected for 72 h, then washed with PBS. Cells were fixed on ice for 15 min in PBS containing 4% paraformaldehyde and 2.5% glutaraldehyde (Invitrogen). Cells were then gently scraped from plates and incubated in fixative for an additional 45 min at room temperature. Cells were then collected by centrifugation at 1000 rpm for 5 min and resuspended in PBS. Samples were subsequently processed and analyzed by Dr. Wendy Beatty at the Washington University School of Medicine Molecular Imaging Facility.

Immunoblot analysis

C. burnetii-infected hAMs were lysed in buffer containing 1% sodium dodecyl sulfate (SDS) by 10 passages through a 26-gauge needle and boiled for 5 minutes. Total protein concentration was determined using a DC assay (Bio-Rad). Ten μ g of total protein was separated by SDS-polyacrylamide gel electrophoresis, transferred to a 0.2- μ m-pore-size polyvinylidene fluoride membrane (Bio-Rad), and blocked in Tris-buffered saline (150 mM NaCl, 100 mM Tris-HCl [pH 7.6]) containing 0.1% Tween 20 and 5% nonfat milk for 1 h at room temperature. After blocking, membranes were probed for equal protein loading using a mouse anti- β -tubulin primary antibody (Sigma-Aldrich). After confirming equal loading, lysates were probed for total and phosphorylated p38, Erk1/2 (Thr202/Tyr204) or Akt (Ser473) (Cell Signaling), IL-1 β (R & D Systems), or CD14, CD11/18b, or CD68 (Abcam). A positive control for mature IL-1 β production was established by treating hAMs with 100 ng/mL of *E. coli* LPS for 6 h and 5 mM ATP (Adenosine 5'-triphosphate disodium salt hydrate; Sigma-Aldrich) for 30 minutes. In all immunoblots, reacting proteins were detected using anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Cell Signaling).

Multiplex cytokine analysis

TNF- α , IL-6, and IL-10 concentrations were determined using a multiplex Bio-Plex cytokine assay (Bio-Rad). Supernatants were harvested from *E. coli* LPS-treated or infected hAMs at 24 h and centrifuged at 14,000 rpm for 15 min to clear any precipitate. Standards and buffers were prepared according to the manufacturer's instructions and equilibrated to room temperature for 20 min before use. A 96-well filter bottom plate was incubated with assay buffer prior to use and removed using a vacuum manifold. Magnetic coupled beads were prepared and added to all wells followed by addition of standards or samples to individual wells. Plates were shaken at 1,100 rpm for 30 sec, then at 300 rpm for 30 min at room temperature. Wells were then washed three times with wash buffer and detection antibodies added for 30 min. Plates were then washed and Streptavidin PE was added for 10 min. After washing, samples were analyzed using a Bio-Plex 200 system and cytokine concentrations calculated using Bio-Plex Manager 6.0 software.

IFN- γ treatment and foci forming unit assays

hAMs were left untreated, pretreated for 24 h, or treated at the time of infection with 10, 100, or 1000 U/mL IFN- γ (Invitrogen). Cells were then either mock-infected or infected with *C. burnetii* isolates for 72 h in the presence of IFN- γ . At 24 hpi, hAMs were washed to remove excess organisms and fresh IFN- γ added. At 72 hpi, cells were harvested and sonicated to release intracellular organisms and samples applied to Vero cell monolayers.

Infected Vero cells were incubated for 96 h, then processed for fluorescence microscopy using an anti-*C. burnetii* primary antibody and an AlexaFluor-488-conjugated anti-rabbit secondary antibody (Invitrogen) to detect infectious foci. Foci-forming units (FFUs) were visualized using a 40X objective lens and quantified as FFU/mL as previously described (Cockrell *et al.*, 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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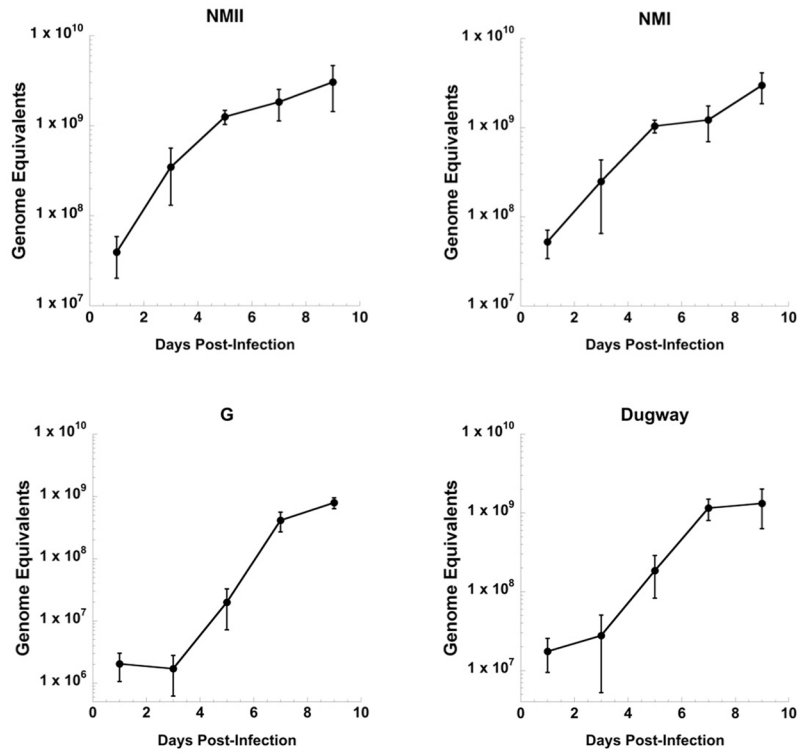


Fig. 1. hAMs support growth of virulent *C. burnetii* pathotypes

hAMs were infected with NMII, NMI, G, or Dugway for nine days. Cells were harvested and bacterial genomic DNA isolated every two days in triplicate. Bacterial genomes were quantified using quantitative PCR based on *dotA* using a *DotA*-encoding plasmid to generate a standard curve. Samples were analyzed in triplicate and error bars represent the standard deviation from the mean. *C. burnetii* isolates replicated efficiently in hAMs across a nine-day time course, with G and Dugway growth lagging until after three dpi.

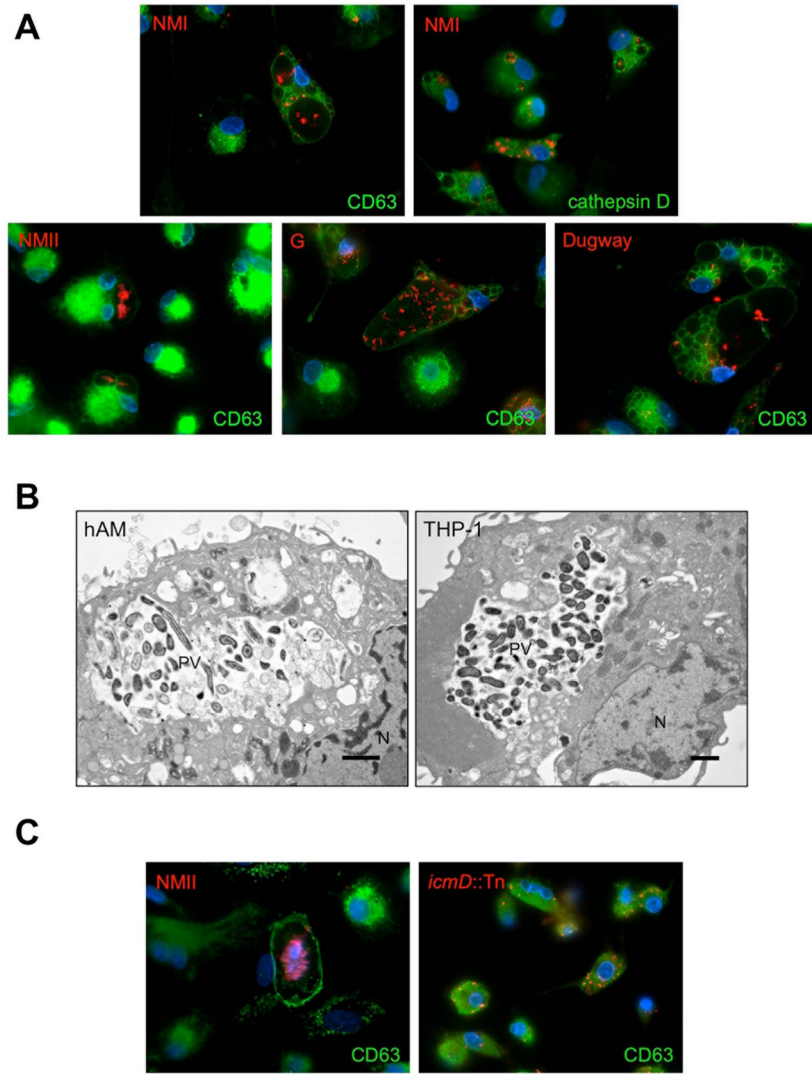


Fig. 2. Biogenesis of a large, spacious PV in hAMs is T4SS-dependent

hAMs or THP-1 cells were infected for 72 h with the indicated isolate, then processed for fluorescence (A and C) or electron (B) microscopy. The PV markers CD63 or cathepsin D are indicated in the figure and shown in green, *C. burnetii* in red, and DAPI-stained DNA in blue. Isolates are indicated in panel A and PV and nuclei (N) are denoted in panel B. Each wild type *C. burnetii* isolate generates a large, phagolysosome-like PV in hAMs while T4SS-deficient organisms (*icmD::Tn*) are housed in tight-fitting phagolysosomes and fail to replicate (C). Furthermore, electron microscopy analysis (B) shows that large vacuoles in hAMs (left) contain many replicating NMII *C. burnetii* similar to that seen in THP-1 cells (right).

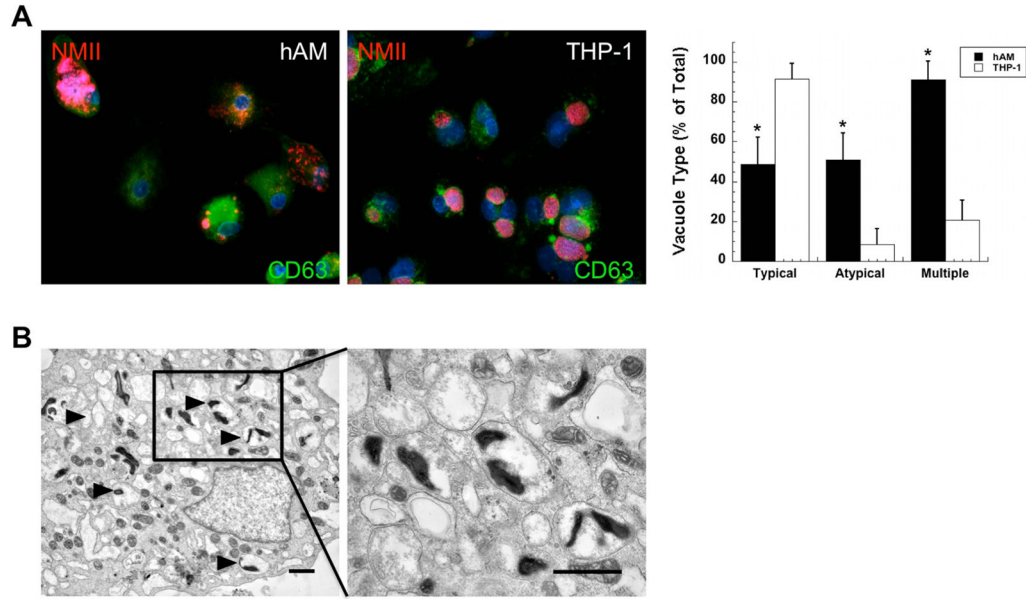


Fig. 3. *C. burnetii* generates atypical PV in hAMs

hAMs were infected for 72 h with NMI *C. burnetii*, then processed for fluorescence (A) or electron (B) microscopy. The PV marker CD63 is shown in green, *C. burnetii* in red, and DAPI-stained DNA in blue. Cells containing at least one typical, only atypical, or multiple PV were quantified from at least 250 NMI-infected cells and expressed as a percent of the total population. Samples were analyzed in triplicate and error bars represent the standard deviation from the mean. * indicates a *P* value < 0.0001 compared to infected THP-1 cells using a Student's *t* test. Typical = > 15 μ M, atypical = < 15 μ M, and multiple = many PV of various sizes in each cell. Approximately 90% of hAMs harbor *C. burnetii* in multiple phagolysosome-like PV, many that support replication and some of which contain degrading organisms (arrowheads in panel B).

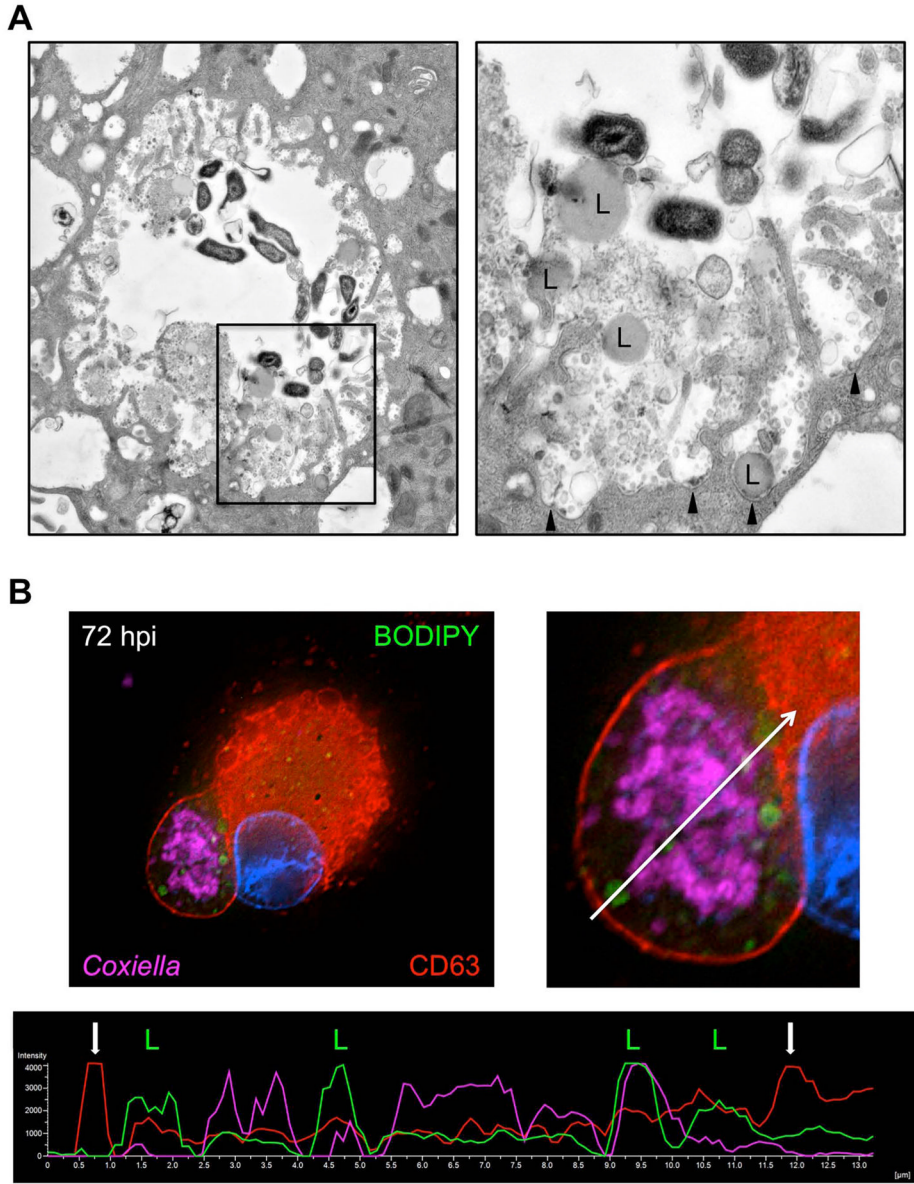


Fig. 4. Lipid droplets associate with the PV
 hAMs were infected for 72 h with NMII *C. burnetii*, then processed for electron (A) or fluorescence microscopy (B). The neutral lipid stain BODIPY 493/503 (green) was used to label lipid droplets, *C. burnetii* is shown in violet, CD63 is shown in red, and DAPI-stained DNA in blue. L = lipid droplet and arrowheads denote the intact PV membrane. The intensity profile (bottom panel) depicts fluorescence along the white arrow drawn across the PV. In the intensity profile, white arrows indicate the CD63-positive limiting membrane of the PV on each side of a typical vacuole. Lipid droplets are found in and near the PV at 72 hpi and associate with *C. burnetii*.

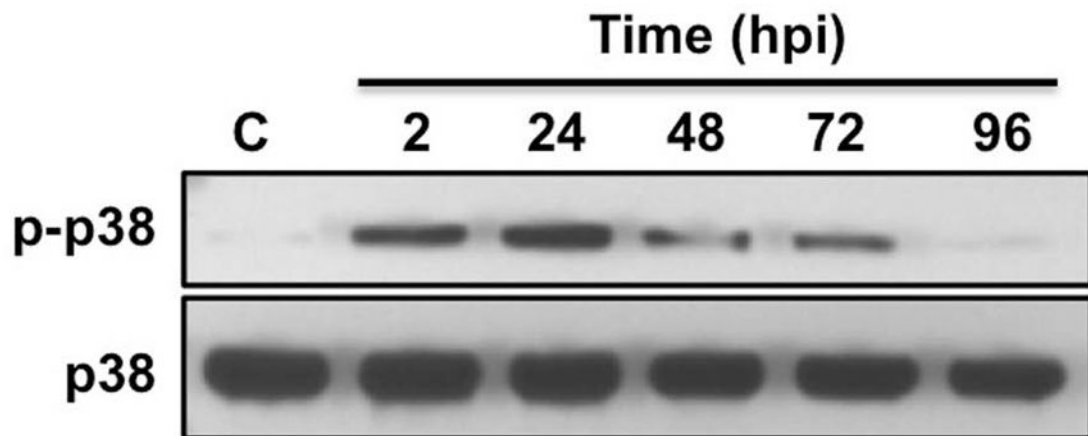
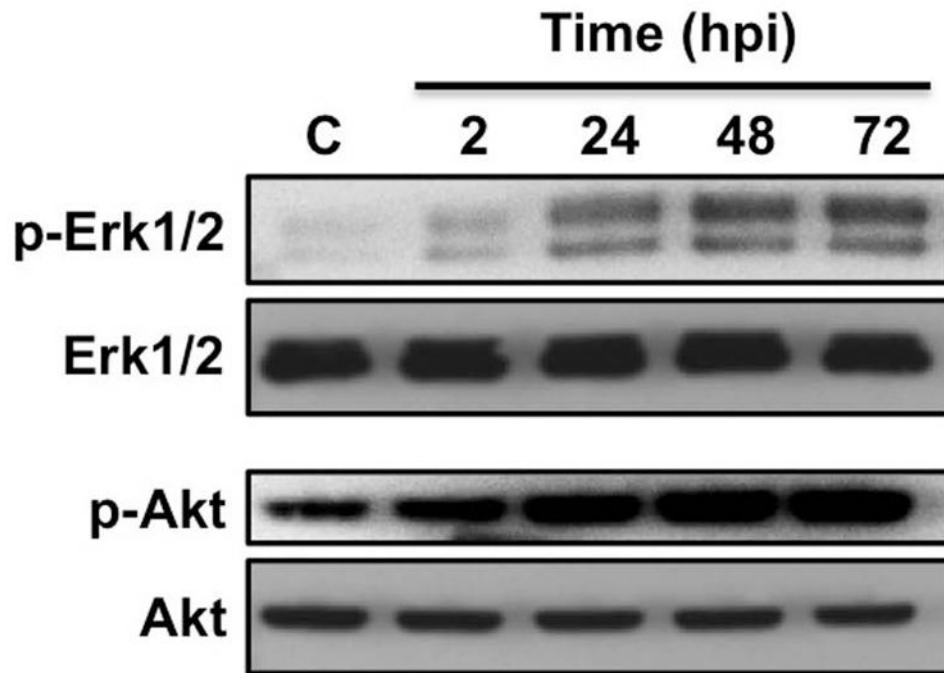


Fig. 5. *C. burnetii* modulates intracellular hAM signaling

hAMs were infected with NMII *C. burnetii* for the indicated times and whole cell lysates analyzed by immunoblot using antibodies specific for the indicated proteins. C = control, uninfected cells and p = phosphorylated. *C. burnetii* triggers sustained phosphorylation (indicative of activation) of the pro-survival kinases Akt and Erk1/2 and short term phosphorylation of the stress response protein p38.

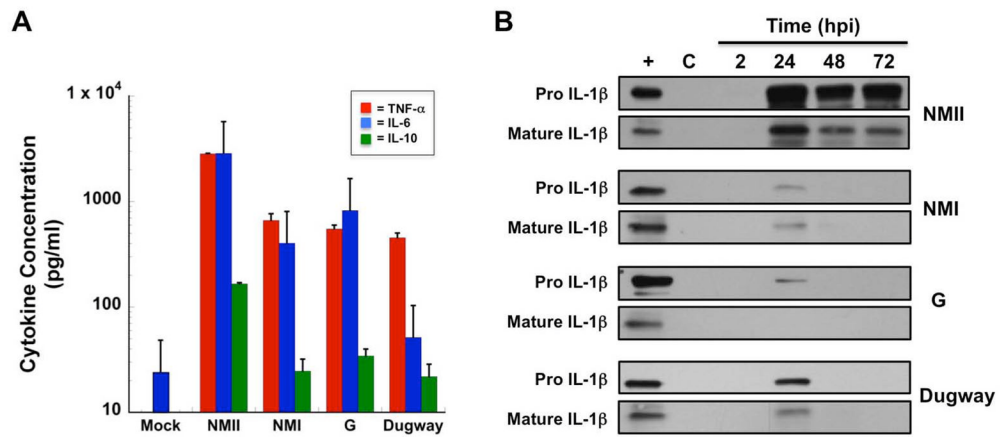


Fig. 6. hAMs mount a robust cytokine response to *C. burnetii*

(A) hAMs were infected for 24 h with NMII, NMI, G, or Dugway and supernatants analyzed with a multiplex cytokine assay. Results are from one experiment with infections performed in duplicate and are representative of three independent experiments using hAMs from three different donors. Error bars represent the standard error of the mean and Mock = uninfected cells. hAMs respond to each isolate with increased production of TNF- α , IL-6, and IL-10 at 24 hpi, with levels consistently higher in NMII-infected hAMs. (B) Cells were infected for the indicated times with the isolates shown, then harvested for immunoblot analysis. C = control, uninfected cells and + = uninfected cells treated with *E. coli* LPS and ATP. Pro and mature IL-1 β production was greatest in NMII-infected hAMs and was sustained from 24–72 hpi. Phase I isolates triggered production of low pro- and mature IL1 β levels at 24 hpi and levels were undetectable from 48–72 hpi.

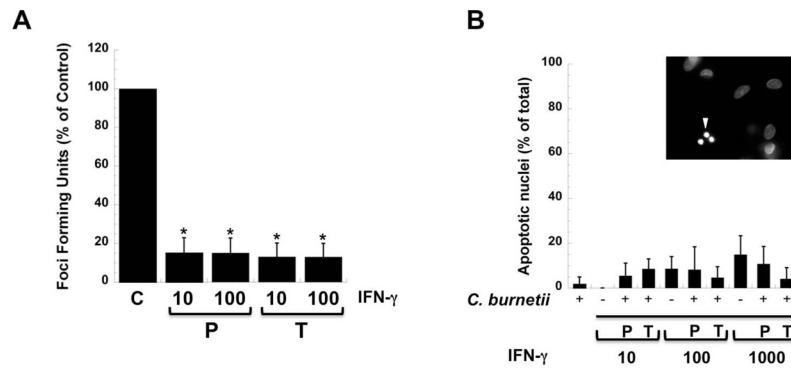


Fig. 7. IFN- γ treatment controls *C. burnetii* growth in hAMs

hAMs were either pre-treated for 24 h or treated at the time of infection with NMII *C. burnetii* with the indicated concentrations of IFN- γ (U/ml) and incubated for 72 h. (A) Infectious bacteria were assessed by plating hAM lysates on Vero cell monolayers and quantifying FFU/ml. Experiments were performed in triplicate and results are expressed as % of control (C) untreated infected cell FFUs. Error bars represent the standard deviation from the mean. P = 24 h pre-treatment with IFN- γ and T = treatment at time of infection. * indicates a P value < 0.0001 compared to untreated cells using a Student's t test. IFN- γ treatment before or at the time of infection significantly lowers the number of infectious *C. burnetii*. (B) hAM viability was assessed by quantifying apoptotic nuclei using DAPI staining after treatment with IFN- γ at the indicated concentrations. Inset shows infected cells treated with 1000 U/ml IFN- γ and arrowhead denotes a fragmented nucleus indicative of an apoptotic cell. Samples were analyzed in triplicate and error bars represent the standard deviation from the mean. P values were > 0.05 for all conditions compared to uninfected treated cells using a Student's t test. IFN- γ controls *C. burnetii* replication without significantly affecting hAM viability.