Chapter 8
The *Coxiella burnetii* Parasitophorous Vacuole

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Contents

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Abstract *Coxiella burnetii* is a bacterial intracellular parasite of eucaryotic cells that replicates within a membrane-bound compartment, or "parasitophorous vacuole" (PV). With the exception of human macrophages/monocytes, the consensus model of PV trafficking in host cells invokes endolysosomal maturation culminating in lysosome fusion. *C. burnetii* resists the degradative functions of the vacuole while at the same time exploiting the acidic pH for metabolic activation. While at first glance the mature PV resembles a large phagolysosome, an increasing body of evidence indicates the vacuole is in fact a specialized compartment that is actively modified by the pathogen. Adding to the complexity of PV biogenesis is new data showing vacuole engagement with autophagic and early secretory pathways. In this chapter, we review current knowledge of PV nature and development, and discuss disparate data related to the ultimate maturation state of PV harboring virulent or avirulent *C. burnetii* lipopolysaccharide phase variants in human mononuclear phagocytes.

Keywords Macrophage • Parasitophorous vacuole • Endosome • Lysosome • Autophagy • Integrin • Lipopolysaccharide • Monocyte • Phase variation • Secretory pathway

8.1 Introduction

Soon after identification of *Coxiella burnetii* as the cause of human Q fever, the organism was classified as a rickettsial agent due to its obligate intracellular nature and staining properties (Cox 1939). The original assignment of *C. burnetii* to the α-proteobacterial order *Rickettsiales* (Weiss and Moulder 1984) was changed during the molecular era with genetic information indicating appropriate placement within the γ-proteobacteria order *Legionellales*, which includes the facultative intracellular pathogen *Legionella pneumophila* (Weisburg et al. 1989). Indeed, *C. burnetii* and *L. pneumophila* share several traits including aerosol transmission, a tropism for alveolar macrophages during natural infection, developmental forms adapted to intracellular survival and environmental transmission, and subversion of host cell functions by effector proteins secreted by a specialized Dot/Icm type IV secretion system (T4SS) (Voth and Heinzen 2007; Newton et al. 2010). Although macrophage invasion and survival are central to pathogenesis by both *C. burnetii* and *L. pneumophila*, the organisms occupy unique intracellular niches and utilize different subversion strategies once internalized by their macrophage hosts.

Natural isolates of *C. burnetii* undergo a virulent to avirulent transition upon serial passage in embryonated eggs or tissue culture. This transition can be serologically defined using post-vaccination antisera and is referred to as "phase variation". Lipopolysaccharide (LPS) *O*-antigen of virulent organisms is the primary surface antigen recognized by phase I antiserum. Avirulent *C. burnetii,* recognized by phase II, but not phase I antiserum, produce a severely truncated LPS lacking *O*-antigen and some core sugars (Hackstadt et al. 1985). Some, but not all, *C. burnetii* in phase

II contain a large chromosomal deletion enriched in genes involved in *O*-antigen biosynthesis (Denison et al. 2007; Beare et al. 2006). The virulence function of *C. burnetii* LPS is unrelated to lipid A, as both phase I and phase II organisms of the Nine Mile reference strain have the same tetra-acylated structure that weakly interacts with Toll-like receptor (TLR) 4 (Zamboni et al. 2004). Instead, virulence appears related to *O*-antigen masking of the organism's outer surface that inhibits complement deposition (Vishwanath and Hackstadt 1988) and antibody access to surface proteins (Hackstadt 1988). Masking is also proposed to restrict TLR receptor interactions of immune cells with non-LPS ligands (Shannon et al. 2005). Interestingly, despite weak lipid A-TLR4 interactions, full-length phase I LPS stimulates dramatic F (filamentous)-actin rearrangement in human macrophages in a TLR4-dependent manner (Capo et al. 1999, 2003; Honstettre et al. 2004). Consequently, *C. burnetii* protein interactions with complement receptor 3 (CR3) are inhibited, an effect speculated to result in different trafficking of phase I and phase II organisms in human mononuclear phagocytes (discussed in more detail below) (Barry et al. 2011).

LPS *O*-antigen does not appear to act directly as a ligand in *C. burnetii* uptake as phase II organisms are internalized 10–100 times more efficiently then phase I organisms by cultured professional phagocytes, epithelial cells and fibroblasts (Moos and Hackstadt 1987). The lower carbohydrate content of phase II LPS is thought to make the organism hydrophobic, thereby facilitating non-specific host plasma membrane interactions (Williams et al. 1981). The more accessible surface protein ligands of phase II *C. burneii* may further enhance uptake (Hackstadt 1988).

By default, the phagocytic process results in material being sequestered within a nascent phagosome that traffics through the endocytic pathway (Haas 2007). The first step of phagosome maturation is the intermingled fusion/fission events with early endosomes. Phagosomes acquire several markers, such as early endosome antigen-1 (EEA 1) and the small GTPase Rab5. Early phagosomes progressively transform into compartments that present features of late endosomes. Markers of late endosomes, such as the small GTPase Rab7 and lysosomal membrane-associated protein-1 (LAMP-1), gradually replace early endosomal markers (Scott et al. 2003; Henry et al. 2004). The pH of early phagosomes is around 6.0, with acquisition of the vacuolar proton pump ATPase (V-H^+-ATPase) by late phagosomes leading to an intraphagosomal pH of 4.5–5.5 (Scott et al. 2003). Finally, late phagosomes fuse with lysosomes that contain hydrolytic enzymes such as cathepsin D, thus leading to the formation of phagolysosomes in which bacteria can be destroyed (Scott et al. 2003). Consequently, numerous bacterial pathogens have developed specific strategies to avoid this intracellular fate, thereby enhancing their survival within host cells (Scott et al. 2003). Bacteria such as *Listeria*, *Shigella* and *Rickettsia* escape from nascent phagosomes to the cytosol to avoid destruction in phagolysosomes (Cossart and Sansonetti 2004). A different strategy used by several pathogens involves interference with normal phagolysosome biogenesis that leads to formation of vacuoles supporting replication (Alonso and Garcia-del Portillo 2004). For example, *B. abortus* vacuoles interact with the endoplasmic reticulum but not with the classical endocytic network (Meresse et al. 1999b). Furthermore,

Mycobacterium phagosomes exchange material such as transferrin with early endosomes but are unable to fuse with late endosomes (Scott et al. 2003; Rohde et al. 2007). Finally, *Salmonella* resides in atypical phagosomes that are neither early nor late phagosomes. Membranes of the trans-Golgi network surround *Salmonella*-containing phagosomes, suggesting interactions with endocytic and biosynthetic pathways (Holden 2002).

In contrast with other intracellular bacteria, the *C. burnetii* parasitophorous vacuole (PV) has extensive interactions with the endolysosomal pathway. With the exception of human mononuclear phagocytes, studies using several cell types have consistently demonstrated PV containing virulent phase I strains of *C. burnetii*, or the avirulent Nine Mile phase II (NMII) RSA439 strain, fully mature through the endolysosomal cascade to resemble a large phagolysosome (Voth and Heinzen 2007). However, disparate models have been published on trafficking of vacuoles harboring virulent Nine Mile phase I (NMI) RSA493 organisms or NMII in human mononuclear phagocytes (Ghigo et al. 2002; Howe et al. 2010). NMI is considered a reference strain with NMII being a high passage isogenic variant of NMI. NMII has been extensively characterized and harbors a large chromosomal (~26 kb) deletion of *O*-antigen biosynthesis genes (Moos and Hackstadt 1987; Hoover et al. 2002). The clonality and large chromosomal deletion of NMII render it non-revertable to full virulence. For the purpose of discussion, we will refer to the opposing models of NMI and NMII trafficking as the differential trafficking (DT) and similar trafficking (ST) models. In the DT model, maturation of PV containing NMI stalls at a late endosomal stage, resulting in a vacuole permissive for pathogen survival and growth (Ghigo et al. 2002). Conversely, PV sheltering NMII fully mature into a phagolysosomal-like compartment that contains active lysosomal hydrolases and is bacteriocidal (Ghigo et al. 2002). Thus, in the DT model, virulence of phase variants is attributed to the ultimate maturation state of their respective PVs. In the ST model, NMI and NMII both replicate in phenotypically-indistinguishable PV that fully mature through the endolysosomal pathway to fuse with lysosomes. In this phagolysosome-like vacuole, phase variants resist degradation and replicate at equal rates (Howe et al. 2010).

Here, we review early and recent work on *C. burnetii* PV nature and development. Furthermore, we discuss disparate data related to the ultimate maturation state of PV harboring *C. burnetii* NMI or NMII phase variants in human mononuclear phagocytes.

8.2 Early Studies of the *Coxiella* Vacuole

In 1937, Edward Derrick of Brisbane, Australia, published his careful and extensive description of a new clinical entity in humans called Q (query) fever (Derrick 1937). He successfully isolated the infectious agent – now known as *Coxiella burnetii* – in guinea pigs but was unable to visualize the pathogen in infected tissues. Using inocula provided by Derrick, Macfarlane Burnet and Mavis Freeman

(Burnet and Freeman 1937) infected mice and successfully stained organisms in spleen sections that they stated "occur as intracellular microcolonies of close-packed individuals, nearly always sharply circumscribed within an oval or circular outline." Although not specifically mentioned, it is clear from their elegant sketches that organisms were restrained in an intracellular compartment. Interestingly, unpublished notes of Derrick showed strikingly similar drawings (Cooke 2008). A year later, Harold Cox at the Rocky Mountain Laboratories in Hamilton, Montana, USA, successfully cultivated the newly isolated NMI strain of *C. burnetii* in tissue cultures of minced chick embryos and alluded to their vacuolar nature (Cox 1938). Subsequent electron microscopy clearly demonstrated intracellular NMI surrounded by a limiting membrane (Handley et al. 1967).

The biological nature of the *C. burnetii* PV began to unravel with the seminal findings of Burton and co-workers (Burton et al. 1971, 1978). Based on cytochemical localization of the lysosomal enzymes acid phosphatase and 5´-nucleotidase in NMI-infected mouse L929 cells, they suggested *C. burnetii* resides in a secondary lysosome, i. e., the product of primary lysosome fusion with a phagosome. They proposed both significant replication and degradation of *C. burnetii* in the vacuole (Burton et al. 1971). A similar fusion event in spleen reticular cells from mice infected with the Luga strain of *C. burnetii* was described by Ariel et al. (1973) who used the term "phagolysosome" to describe the *C. burnetii* vacuole. Burton et al. (1978) went on to show acid phosphatase activity in NMI and NMII vacuoles of persistently infected (6–10 months) mouse L929 cells and African green monkey kidney (Vero) cells, with Vero cell vacuoles also containing abundant membrane whorls, or "myelin configurations" (Fig. 8.1).

By definition, a phagolysosome is a degradative organelle containing acid activated hydrolases. The proton pumping V-H^+-ATPase is responsible for lowering the vacuole's lumenal pH to approximately 5.0 to achieve optimal hydrolytic enzyme activity (Luzio et al. 2007). Consistent with the presence of two active lysosomal enzymes (Burton et al. 1971), Akporiaye et al. (1983) subsequently demonstrated that NMI PV in persistently-infected J774 murine macrophages acquire thorium dioxide from primary lysosomes and acidify to pH 5.2, thus strengthening the emerging model of PV-lysosome fusion.

8.2.1 Acid Activation of Metabolism

Shortly after the original clinical isolation of *C. burnetii,* robust growth of the pathogen was achieved in embryonated hen's eggs and cultured cell lines (Ormsbee 1952; Cox 1938). Thus, it was perplexing in early metabolic studies why intact host cell-free bacteria, unlike bacterial lysates, displayed little metabolic activity in different physiologic buffers (Weiss 1973). This puzzle was solved by Hackstadt and Williams (1981) who astutely recognized the implications Burton et al.'s (1975, 1978) findings. In a landmark study published in 1981, and before the actual determination of PV pH, they showed that transport, catabolism and incorporation

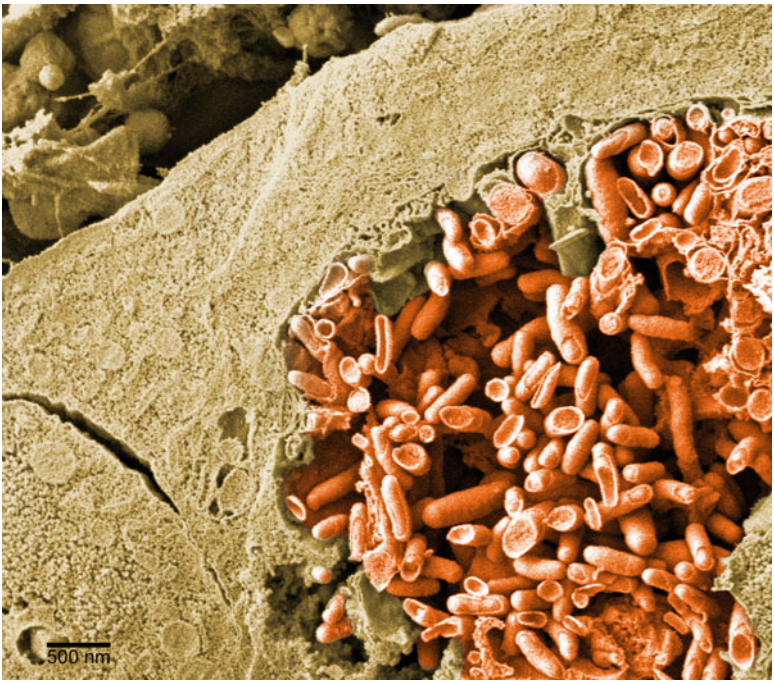

Fig. 8.1 The *C. burnetii* parasitophorous vacuole (PV). Scanning electron micrograph showing a cryo-prepared Vero cell infected with *C. burnetii* Nine Mile (phase II) for 4 days. The host cell cytoplasm (tan) contains a large PV filled with *C. burnetii* (*orange*) (Image courtesy of Beth Fischer, Rocky Mountain Laboratories)

of both glucose and glutamate by host cell-free *C. burnetii* NMI are highly stimulated under acidic conditions mimicking that of a phagolysosome (Hackstadt and Williams 1981). Importantly, they also demonstrated an intracellular growth requirement for vacuolar acidity by showing cessation of NMI growth following neutralization of the PV with lysosomotropic amines (Hackstadt and Williams 1981). The discovery of "acid activated" *C. burnetii* metabolism led to a series of studies that identified optimal conditions for transport and utilization of nutrients, maintenance of the ATP pool, and membrane energization (Hackstadt and Williams 1984).

8.3 The *Coxiella* Parasitophorous (PV) Vacuole

A resurgence in *C. burnetii* PV characterization occurred in the mid-1990s. Enabled by new reagents, such as vital stains and organelle-specific antibodies, and enhanced imaging capabilities, such as laser scanning confocal microscopy, researchers could now more precisely monitor phagosome maturation. These tools, coupled with a variety of cultured cell model systems, allowed several groups to dramatically

expand our knowledge of *C. burnetii* PV biogenesis. With the exception of NMI organisms in human mononuclear phagocytes (discussed in detail below), cumulative evidence supports a model whereby *C. burnetii* PV fully mature through the endolysosomal cascade to acquire characteristics of a phagolysosome. However, this is an overly simplified model as *C. burnetii* modifies the vacuole to create a specialized compartment with unusual fusogenicity with other endolysosomal compartments and interactions with autophagy/secretory pathways.

8.3.1 PV Maturation/Biology in Animal Cells and Human Epithelial Cells/Trophoblasts

Although the primary targets of *C. burnetii* during natural infection are monocytes/macrophages (Stein et al. 2005; Khavkin and Tabibzadeh 1988), the organism has an impressive ability to infect a wide variety of cultured epithelial cells, fibroblasts, and macrophage-like cells (Voth and Heinzen 2007). In this section, we discuss *C. burnetii* interactions with animal cells (both professional and non-professional phagocytes) and human epithelial cells/trophoblasts.

In mouse L929 cells and P388D1 macrophages, live or dead NMI or NMII *C. burnetii* are internalized by phagocytosis at equal rates, indicating *C. burnetii* plays a passive role in cellular uptake (Baca et al. 1993a; Tujulin et al. 1998). Rearrangement of host cell F (filamentous)- actin is required for pathogen uptake as internalization is inhibited by treating cells with actin depolymerizing agents such as cytochalasin D (Baca et al. 1993a). The bacterial ligand(s) mediating attachment are unknown; however, it is likely proteinaceous as treatment of *C. burnetii* with proteases hinders uptake (Baca et al. 1993a). Pretreatment of NMI with the cationic peptide CAP37 dramatically enhances infection of L929 cells without a deleterious effect on the pathogen (Aragon et al. 1995). An opsonon-like activity of the peptide is speculated to promote invasiveness (Aragon et al. 1995).

In addition to a role in pathogen uptake, F-actin rearrangements regulate the size and formation of the *C. burnetii* PV (Aguilera et al. 2009). In HeLa cells, a web of F-actin surrounds mature PV harboring NMII. Treatment with latrunculin B or cytochalasin D following uptake results in smaller PV with reduced fusogenicity. PV decorate with both wild type and constitutively active forms of the Rho family GTPases Cdc42 and RhoA, noted regulators of actin dynamics. Actin accumulation may benefit PV expansion by providing tracks for vesicular fusion and/or enhancing structural integrity. A similar actin web surrounds the vacuole (inclusion) occupied by the intracellular bacterium *Chlamydia trachomatis,* and is thought to provide a structural scaffold (Kumar and Valdivia 2008).

In animal cells and human epithelial cells/trophoblasts, studies assessing pH, enzymatic activities, and lysosomal protein markers agree that nascent NMI or NMII-containing phagosomes fully mature through the default endocytic pathway to acquire characteristics of a phagolysosome (Heinzen et al. 1996; Howe and Mallavia 2000;

Burton et al. 1971, 1978; Akporiaye et al. 1983; Howe et al. 2003; Romano et al. 2007; Campoy et al. 2011; Aguilera et al. 2009; Grieshaber et al. 2002). Ratiometric pH-sensitive probes consistently show PV with a phagolysosomal-like pH (~5) in J774 mouse macrophages infected with NMI (Akporiaye et al. 1983), Vero cells infected with NMII (Grieshaber et al. 2002), and P388D1 and L929 cells infected with NMII or the virulent Priscilla and Q212 strains of *C. burnetii* (Maurin et al. 1992a, b). A cursory examination of the kinetics of PV maturation has been conducted in several cell lines. In CHO cells, the early endosomal marker Rab5 is found on PV containing NMII as early as 5 min after bacterial uptake, with maximum association at 20 min (Romano et al. 2007). The late endosomal/lysosomal marker Rab7 has maximal PV association between 40 and 60 min post-infection, when Rab5 levels are decreasing (Romano et al. 2007). Phagosome recruitment of lysosomal hydrolases is a defining marker of lysosome fusion; however, trafficking of the lysosomal enzymes acid phosphatase and cathepsin D to the *C. burnetii* PV appears delayed in NMI-infected J774 macrophages and NMII-infected CHO cells respectively, taking approximately 1–2 h to reach high levels (Howe and Mallavia 2000; Romano et al. 2007). These maturation kinetics are substantially slower than those of phagosomes containing inert particles such as latex beads, which acquire lysosomal markers by 15 min after uptake (Oh and Swanson 1996). Cathepsin D localizes to mature PV harboring replicating NMII in both HeLa and CHO cells (Heinzen et al. 1996; Aguilera et al. 2009; Romano et al. 2007) and PV containing replicating NMI in human BeWo trophoblasts (Ben Amara et al. 2010). General protease activity has also been demonstrated for mature NMII PV in HeLa cells (Campoy et al. 2011). Other late endosome/lysosomal markers that decorate mature PV in animal cells and human epithelial cells/ trophoblasts include the V-H^+-ATPase (Heinzen et al. 1996), the lysosomal glycoproteins LAMP-1, LAMP-2 and LAMP-3 (CD63) (Ghigo et al. 2002; Heinzen et al. 1996; Shannon et al. 2005; Sauer et al. 2005; Beare et al. 2009a), and syntaxin 8 (Fig. 8.2), a t-SNARE involved in late endosome-lysosome fusion (Luzio et al. 2009).

8.3.1.1 Autophagy/Secretory Pathway Interactions

A potential mechanism for delayed *C. burnetii* phagosome maturation involves interactions with autophagosomes (Gutierrez et al. 2005; Romano et al. 2007). As early as 5 min after NMII uptake by CHO cells, the nascent phagosome acquires the autophagosomal marker microtubule-associated protein light-chain 3 (LC3) and the marker remains associated through at least 3 days post infection (Romano et al. 2007; Beron et al. 2002; Campoy et al. 2011; Gutierrez et al. 2005). Rab24, a small GTPase involved in autophagy, also decorates mature NMII PV (Gutierrez et al. 2005). Overexpression of LC3 or starvation-induced autophagy both result in reduced recruitment of cathepsin D by the *C. burnetii* phagosome at 1 h post-infection (Romano et al. 2007). Thus, engagement of the autophagy pathway promotes delayed PV-lysosome fusion. In CHO and HeLa cells, NMII infection itself induces autophagy as evidenced by increased conversion of LC3-1 to LC3-II

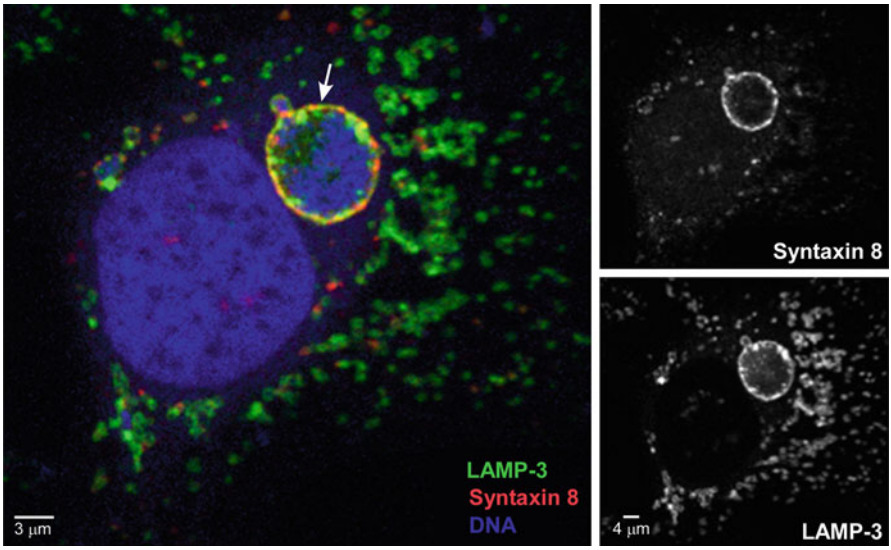

Fig. 8.2 The *C. burnetii* PV decorates with the t-SNARE syntaxin 8. HeLa cells infected with *C. burnetii* Nine Mile (phase II) for 2 days were stained by immunofluorescence for syntaxin 8 (*red*) and LAMP-3 (CD63) (*green*). Host and *C. burnetii* DNA were stained with DRAQ5 (*blue*). syntaxin 8 and LAMP-3 decorate the PV membrane (*arrow*) (Image courtesy of Dale Howe, Rocky Mountain Laboratories)

(Vazquez and Colombo 2010; Romano et al. 2007). An increasing body of evidence indicates that autophagy mediates innate immunity by delivering both intravacuolar and cytoplasmic bacterial pathogens to lysosomes for degradation (Deretic and Levine 2009). However, *C. burnetii* clearly benefits from interactions with the autophagic pathway as induction enhances PV formation and bacterial replication (Gutierrez et al. 2005).

An interplay between autophagy and apopotosis pathways in *C. burnetii* PV development has recently been described (Vazquez and Colombo 2010). In NMII-infected HeLa cells, Beclin 1, an important regulator of autophagy, is recruited to the PV membrane. Beclin 1 overexpression favors development of PV while siRNA knockdown reduces PV size. Beclin 1 has a BH2 domain that binds Bcl-2 family proteins involved in apoptosis, including anti-apoptotic Bcl-2. Interestingly, the beneficial effect of PV-localized Beclin-1 is optimized upon binding Bcl-2. Thus, *C. burnetii* infection modulates both autophagy and apoptotic pathways through Beclin 1/Bcl-2 interactions to promote successful infection.

New data indicates that *C. burnetii* benefits from PV interactions with the early secretory pathway (Campoy et al. 2011). In CHO and RAW murine macrophages cells, NMII PV recruit Rab1b, a small GTPase responsible for anterograde transport between the endoplasmic reticulum (ER) and Golgi apparatus. Overexpression of a dominant-negative form of Rab1b results in smaller PV and less *C. burnetii* replication. Disruption of the Golgi apparatus, and consequently the secretory pathway,

by treating infected cells with brefeldin A or expression of a dominant-negative form of the small GTPase Sar1, also results in significantly smaller PV. Rab1b decoration is most noticeable on mature PV (>24 h post-infection), suggesting secretory pathway engagement is important during the expansion phase of PV development. Interestingly, autophagosome formation also requires Rab1 and functional ER exist sites (Zoppino et al. 2010), thereby implicating interactions between ER transport processes and autophagy in PV biogenesis.

8.3.1.2 *Coxiella* Modulation of PV Biogenesis

Collectively, the current model of *C. burnetii* PV biogenesis now includes endocytic, autophagy and secretory pathway interactions. As mentioned above, the *C. burnetii* phagosome matures with delayed kinetics. This behavior requires active *C. burnetii* protein synthesis as PV containing organisms inactivated with formalin or chloramphenicol mature with normal phagosome kinetics (Romano et al. 2007; Howe and Mallavia 2000) and fail to recruit LC3 (Romano et al. 2007). How *C. burnetii* benefits from stalled phagosome maturation is unclear. Two possibilities are (1) stalling affords the organism extra time to metabolically activate and express proteins ultimately required for survival within a phagolysosomal environment, and (2) stalling is necessary for differentiation of the non-replicating small cell variant (SCV) developmental form into the replicating large cell variant (LCV) developmental form (Voth and Heinzen 2007; Coleman et al. 2004). However, contrary data demonstrate that NMII inactivated with chloramphenicol persist in a viable state for days in lysosome-like vacuoles of Vero cells (Howe et al. 2003), and that purified NMII LCV and SCV are equally infectious (Omsland et al. 2009; Coleman et al. 2004).

In addition to PV biogenesis, *C. burnetii* protein synthesis is required for maintenance of the mature PV structure and its recruitment of lysosomes (Howe et al. 2003). Coincident with *C. burneti* entry into log phase (~2 days post-infection), mature PV containing NMII in Vero cells undergo a dramatic expansion, resulting in a large and spacious vacuole that is easily visible by light microscopy (Coleman et al. 2004). However, following a 12 h treatment with chloramphenicol, these vacuoles collapse and lose their spaciousness and phase translucent appearance (Howe et al. 2003). Moreover, fusion between mature PV and the lysosomal compartment, as evidenced by recruitment of latex bead-containing phagolysosomes, is substantially reduced in infected J774 macrophages treated with chloramphenicol (Howe et al. 2003). Although the *C. burnetii* protein effectors of PV maturation and maintenance are unknown, some are presumably delivered directly to the host cytosol via the pathogen's T4SS (Voth and Heinzen 2009a).

The PV filled with stationary phase *C. burnetii* (~6 days post-infection) can encompass nearly the entire host cell cytoplasm (Coleman et al. 2004). Interestingly, this parasitic burden does not impose obvious cytopathic effects and there is no concerted lytic event associated with *C. burnetii* host cell egress. Indeed, L929 and J774 infected with NMI or NMII continue to divide normally with the PV segregating

to one of two daughter cells (Roman et al. 1986; Baca et al. 1985). Segregation of NMII PV does not apparently lead to defects in host cell cytokinesis as shown for vacuoles harboring *C. trachomatis* (Grieshaber et al. 2006).

8.3.2 PV Maturation/Biology in Human Mononuclear Phagocytes

As mentioned above, there are conflicting models regarding the ultimate maturation state of PV harboring phase I and phase II *C. burnetii* in human mononuclear phagocytes and the outcome of infection (Ghigo et al. 2002; Howe et al. 2010). These studies have exclusively used the NMI and NMII strains of *C. burnetii*.

8.3.2.1 NMI and NMII: Differential Trafficking (DT) Model

In the following sections, data supporting the DT model are presented. Selective receptor engagement by NMI and NMII that induces different cytoskeletal rearrangements and signaling is discussed, as is the involvement of TLR4 in host cell interactions. Moreover, the maturation process of PV containing NMI or NMII is described along with the modulation of trafficking by cytokines. The DT model asserts that the avirulence of NMII is associated with residence in a vacuole that fuses with lysosomes. Conversely, virulence of NMI is associated with residence in a vacuole that stalls at a late endosomal stage, thereby allowing pathogen growth (Ghigo et al. 2002).

Integrin-Dependent Phagocytosis

A critical element of the DT model is the engagement of different phagocyte receptors by NMI and NMII that ultimately affects PV outcome. Phagocytosis is an ancestral defense mechanism directed against microbial invasion. Professional mononuclear phagocytes, including circulating monocytes and tissue macrophages, ingest and degrade a wide variety of microorganisms such as bacteria, virus, fungi and protozoa. To discriminate between infectious agents, mononuclear phagocytes possess a restricted number of phagocytic receptors including Fc receptors and complement receptor (CR3, a heterodimer consisting of αM and β2 integrin proteins CD18 and CD11b) that recognize opsonized microorganisms, the mannose receptor that recognizes conserved motifs on pathogens, scavenger receptors that recognize diacyl lipids from the bacteria surface, and TLRs that recognize microbial structures such as LPS, peptidoglycan or flagellin (Taylor et al. 2005).

Numerous pathogens exploit the phagocytic process to infect host cells. In human mononuclear phagocytes, *C. burnetii* has developed a survival strategy based on subversion of receptor-mediated phagocytosis (Capo et al. 1999, 2003). In contrast

to NMII, NMI organisms are poorly internalized by monocytes. Whereas the uptake of NMII is mediated by both αvβ3 integrin and CR3, the internalization of NMI involves only the engagement of αvβ3 integrin. The phagocytic efficiency of CR3 depends on its activation through αvβ3 integrin and an integrin-associated protein (CD47), a molecule physically and functionally associated with β3 integrins. Indeed, macrophages from CD47-deficient mice are unable to ingest NMII bacteria through CR3. It has been demonstrated that the inhibitory mechanism mediated by NMI does not target CD47 since they do not down-regulate the expression of CD47 (Capo et al. 1999).

The functional consequence of inhibition of αvβ3 integrin/CR3 crosstalk is survival of NMI in monocytes (Capo et al. 1999). Conversely, monocytes eliminate NMII, suggesting CR3 engagement is deleterious to *C. burnetii*. The involvement of CR3 in the microbicidal activity of myeloid cells such as monocytes and macrophages seems to depend on the nature and opsonization of pathogens (Stuart and Ezekowitz 2005). *Escherichia coli*, *Salmonella* sp. and *Pseudomonas aeruginosa* are eliminated after CR3-mediated internalization. In contrast, *Bordetella* sp. avoid killing by taking advantage of CR3 uptake (Agramonte-Hevia et al. 2002). CR3 engagement does not affect the intracellular survival of *Mycobacterium tuberculosis* (Hirsch et al. 1994) and CR3 is not involved in the development of *M. tuberculosis* infection (Hu et al. 2000).

Cytoskeleton Remodelling

C. burnetii virulence is associated in monocytes with cytoskeletal rearrangement (Meconi et al. 1998). NMI stimulates membrane protrusions accompanied with increased content in F-actin and transient and intense reorganization of F-actin. In contrast, NMII does not induce any change in cell morphology, actin polymerization and F-actin reorganization. The mechanism used by NMI to induce cytoskeletal rearrangement likely requires actin polymerization and the tension of actin-myosin filaments since F-actin colocalizes with myosin in cell protrusions. It has also been demonstrated that contact between *C. burnetii* and monocytes is necessary to induce cytoskeleton reorganization: bacterial supernatants do not stimulate F-actin reorganization and bacteria are in close apposition with F-actin protrusions.

The cytoskeletal reorganization induced by NMI is related to its inefficient uptake by human monocytes (Capo et al. 2003). Indeed, CD11b and CD18 molecules, the two components of CR3, are excluded from the protrusions induced by these bacteria, but not αvβ3 integrin, suggesting that a physical cross-talk between αvβ3 integrin and CR3 is needed to activate CR3. When CR3 is localized within protrusions induced by unrelated inducers of cytoskeletal rearrangement, including a chemoattractant such as RANTES (Regulated on Activation Normal T cell Expressed and Secreted), or THP-1 monocytes expressing Nef protein, the uptake of NMI is increased, demonstrating that the localization of CR3 in the vicinity of αvβ3 integrin facilitates *C. burnetii* uptake. In RANTES-stimulated monocytes and Nef-expressing monocytes, in which CR3 is distributed in the proximity of αvβ3 integrin, the replication of NMI is inhibited. Again, the mode of entry of *C. burnetii* into monocytes

seems to govern the intracellular fate of organisms. Hence, the localization of CR3 is critical for *C. burnetii* uptake and also for the control of bacterial replication.

The cytoskeleton reorganization induced in monocytes by NMI is related to activation of protein tyrosine kinases (PTK). NMI induces early PTK activation and the tyrosine phosphorylation of several endogenous proteins, including Hck and Lyn, two Src-related kinases (Meconi et al. 2001). PTK activation reflects *C. burnetii* virulence since NMII bacteria do not stimulate PTK. Tyrosine-phosphorylated molecules colocalize with F-actin inside cell protrusions induced by NMI. PTK and Src kinase inhibitors block the formation of cell protrusions and F-actin rearrangement induced by NMI, demonstrating that tyrosine kinases are involved in the cytoskeletal reorganization induced by these bacteria. These inhibitors also increase the uptake of NMI but have no effect on the uptake of NMII, demonstrating functional links between PTK activation, cytoskeletal reorganization and *C. burnetii* uptake. It has been demonstrated that the activation of PTK also provides an uptake signal for several invasive pathogens such as *Listeria monocytogenes*, enteropathogenic *E. coli*, *Helicobacter pylori* and *Campylobacter* species (Cossart and Sansonetti 2004). Concerning the interaction of *C. burnetii* and monocytes, PTK activation may result in the formation of membrane ruffles that limit the redistribution of CR3 in contact areas between αvβ3 integrin and *C. burnetii*. Alternatively, PTK may target αvβ3 integrin, thus interfering with the cross-talk between αvβ3 integrin, CR3, and the actin cytoskeleton (Meconi et al. 1998; Patil et al. 1999). In contrast, some bacterial pathogens inhibit PTK and PTK-mediated microbicidal responses. For example, a tyrosine phosphatase of *Yersinia* spp. and *Salmonella enterica* serovar Typhimurium induces the disruption of the actin cytoskeleton and regulates bacterial uptake (Cossart and Sansonetti 2004).

Involvement of Toll-Like Receptors

Innate and adaptive immune responses are initiated by the recognition of microbial molecules through TLRs. Among TLRs, TLR4 and TLR2 are involved in the recognition of Gram-negative bacteria and Gram-positive bacteria, respectively. TLR4 specifically recognizes prototypic LPSs whereas TLR2 recognizes bacterial lipoproteins, proteoglycans, lipopeptides or LPS from *Porphyromonas gingivalis* (Underhill 2004).

TLR4 is involved in uptake of NMI by macrophages (Honstettre et al. 2004). Polymyxin B, which interferes with LPS binding, inhibits uptake of NMI, but not NMII. The uptake of NMI is also reduced in murine macrophages that do not express TLR4 whereas the uptake of NMII organisms remains unaffected. It has been also demonstrated that the uptake of NMI is independent of TLR2. Besides its role in the uptake of NMI, TLR4 is also involved in *C. burnetii*-induced F-actin reorganization, highlighting again the close relationship between *C. burnetii* uptake and cytoskeleton organization. The mechanism that connects TLR4 and the actin cytoskeleton remains hypothetical, even if the LPS-TLR complex is known to induce a transient F-actin remodeling in a p38- and ERK-dependent pathway (West et al. 2004).

TLR4 controls the immune response against *C. burnetii* through granuloma formation and cytokine production (Honstettre et al. 2004). NMI infection of wild type mice results in sustained formation of granulomas, indicative of a protective immune response, but granuloma formation is only transient in TLR4-deficient mice. The altered granuloma formation is associated with decreased levels of interferon (IFN)-γ. The levels of interleukin (IL)-10, known to impair the production of inflammatory type 1 cytokines, are also reduced in TLR4-deficient mice, suggesting that cytokines are involved in the defective formation of granulomas.

In contrast, TLR4 is dispensable for the survival of NMI in macrophages as growth occurs in TLR4-deficient macrophages (Honstettre et al. 2004). TLR4 is also dispensable for NMI infection of mice. Indeed, NMI infection of tissues and bacterial clearance are similar in wild type and TLR4-deficient mice (Honstettre et al. 2004). Similarly to TLR4, TLR2 is involved in inflammatory and immune responses to NMI but is not necessary for bacterial clearance (Meghari et al. 2005). These results may be explained by the results of Zamboni et al. (2004) who demonstrated that purified lipid A from NMI or NMII fails to activate TLR2 and TLR4. We can suppose that some functions induced by *C. burnetii* need TLR4 (and/or TLR2) whereas other functions are independent of TLR4 (and/or TLR2). It has been suggested that TLRs may regulate phagosome maturation in macrophages (Blander and Medzhitov 2004) even if this hypothesis has been debated (Yates and Russell 2005). TLR4 does not control the maturation of *C. burnetii* phagosomes since NMI colocalize with LAMP-1 but not with cathepsin D in wild type and TLR4-deficient macrophages (Honstettre et al. 2004).

Intracellular Trafficking

In human monocytes and macrophages, NMI survive and replicate whereas NMII is eliminated (Fig. 8.3a, b) (Ghigo et al. 2002, 2006). During the first hours after uptake, nascent NMI and NMII phagosomes interact with intracellular compartments related to the early endosomal network, as revealed by the presence of a marker such as EEA1. This association of *C. burnetii* with the early endocytic network is transient, demonstrating that the interaction between the early endosomal compartment and *C. burnetii* phagosomes is normal. Later, *C. burnetii* phagosomes interact with late endosomes, as demonstrated by their colocalization with LAMP-1, CD63, and mannose-6-phosphate receptor. Mature PV containing NMI and NMII also accumulate V-H^+-ATPase and acquire a pH around 4.5–5, demonstrating that *C. burnetii* virulence is not related to the acidic pH of PV. In contrast, *C. burnetii* virulence seems associated with a defective acquisition of Rab7, a small GTPase involved in phagosome maturation (Desjardins et al. 1994). Indeed, Rab7 is acquired by PV containing NMII but only in part by those containing NMI (Ghigo et al. 2002). The different levels of Rab7 acquisition may explain the defective fusion of PV containing NMI with lysosomes because the amount of Rab proteins on endocytic organelles is critical for the fusion process (Henry et al. 2004; Rink et al. 2005). The partial acquisition of Rab7 by PV containing NMI also suggests that

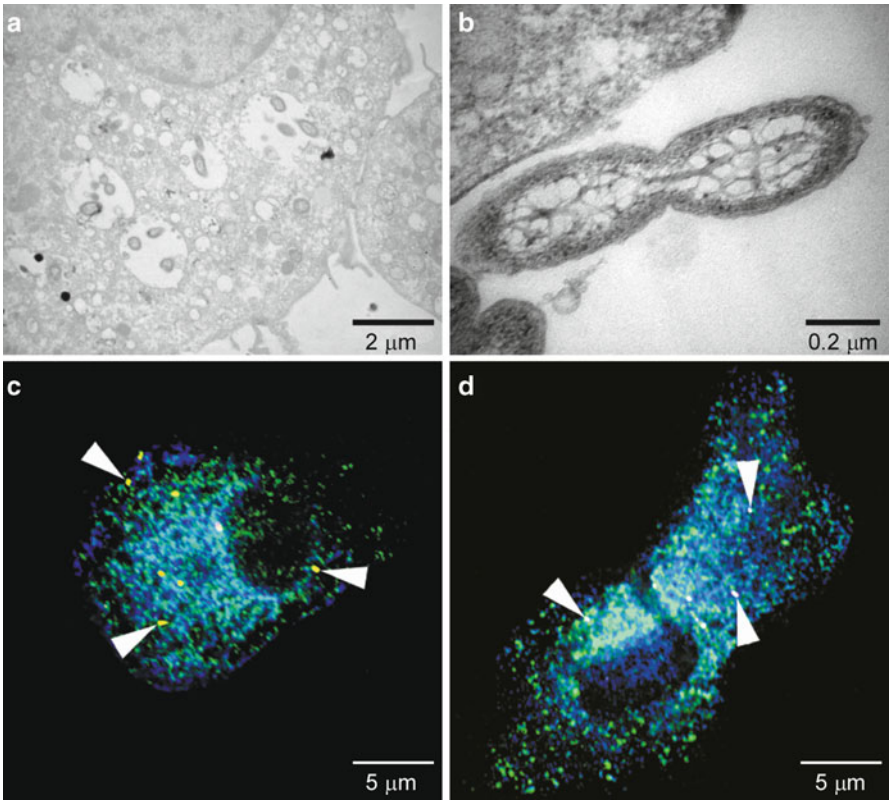

Fig. 8.3 Differential trafficking of *C. burnetii* Nine Mile phase I (NMI) and Nine Mile phase II (NMII) in human mononuclear phagocytes. (**a** and **b**) Electron micrographs of vacuoles containing NMI in human monocyte-derived macrophages. A dividing organism is observed in (**b**). Confocal microscopy of human monocyte-derived macrophages infected with NMI (**c**) or NMII (**d**) for 96 h. Cells were stained by immunofluorescence using antibodies directed against *C. burnetii* (*red*), LAMP-1 (*blue*), and cathepsin D (*green*). Cathepsin D localizes with NMII, but not NMI (*arrowheads*) (Images courtesy of Eric Ghigo, Université de la Méditerranée)

downstream maturation events are impaired. Cathepsin D, a lysosomal protease, does not accumulate in PV containing NMI. The lack of cathepsin D colocalization with NMI is due to defective fusion of PV with lysosomes, as evidenced with a lysosomotropic probe. The impaired acquisition of cathepsin D by PV containing NMI is related to bacterial virulence since NMII colocalize with cathepsin D (Fig. 8.3c, d). Taken together, these data indicate that the survival of NMI in mononuclear phagocytes is associated with an altered maturation of PV.

This hijacking strategy is reminiscent of the escape mechanism used by *S. typhimurium* (Holden 2002). However, the involved molecular mechanisms are different: while NMI PV acquire Rab7 only in part, *Salmonella*-containing vacuoles recruit LAMP-1 in a Rab7-dependent manner (Meresse et al. 1999a). It is hypothesized that Rab7 regulates vesicle traffic in late endocytosis (Bucci et al. 2000;

Zerial and McBride 2001). The fact that NMI does not modify the early acquisition of EEA1 but alters the acquisition of Rab7 by its PV strengthens this hypothesis. It is likely that the survival strategy of *C. burnetii* in mononuclear phagocytes is based on the interference of Rab7 recruitment at the surface of *C. burnetii*-containing vacuoles, thus leading to the formation of PV unable to interact with lysosomes.

The discrepancies in the literature regarding NMI and NMII trafficking may reflect the nature of engaged receptors. As discussed earlier, the route of *C. burnetii* internalization may influence the intracellular fate of the bacteria (Capo et al. 1999, 2003). The importance of pathogen routes of internalization has been clearly demonstrated for mycobacteria. Indeed, IgG-opsonised mycobacteria internalized via Fc receptors are delivered to phagolysosomes and killed whereas unopsonized mycobacteria replicate in vacuoles having characteristics of an early endosome (Scott et al. 2003). These observations suggest that the nature of the receptor engaged during bacterial entry may govern the molecular mechanisms involved in phagosome maturation (Scott et al. 2003).

Modulation of Trafficking by Cytokines

Cytokines are well known to modulate the microbicidal activity of macrophages. IFN-γ, an inflammatory Th1 cytokine, activates macrophages, leading to the control or elimination of several intracellular pathogens (Santic et al. 2005). Intracellularly, IFN-γ induces the killing of NMI by naive monocytes (Dellacasagrande et al. 1999) and the maturation of PV containing NMI as demonstrated by their acquisition of cathepsin D (Ghigo et al. 2002). IFN-γ acts through two distinct mechanisms. First, the addition of IFN-γ to monocytes infected with NMI stimulates PV-lysosome fusion without affecting PV pH. In contrast, IFN-γ decreases the pH of *Mycobacterium*-containing phagosomes (Schaible et al. 1999) and rescues phagosome maturation (Tsang et al. 2000). Second, the treatment of monocytes by IFN-γ prior to infection with NMI induces the alkalinization of *C. burnetii* PV independently of V-H^+-ATPase exclusion. IFN-β also induces the alkalinization of the trans-Golgi network by inhibiting V-H^+-ATPase activity (Sidhu et al. 1999). The mechanism of phagosome alkalinization mediated by IFN-γ is still unknown. It has been shown that IFN-γ inhibits the remodeling of *Legionella*-containing phagosomes into endoplasmic reticulum-derived vesicles (Santic et al. 2005). It is noteworthy that acute Q fever is characterized by the production of IFN-γ whereas in patients with chronic Q fever, the production of IFN-γ is defective (Koster et al. 1985; Izzo and Marmion 1993). Monocytes from patients with chronic Q fever are unable to kill NMI and exhibit a defective maturation of bacterial phagosomes (Ghigo et al. 2004). The production of IFN-γ by patients with acute Q fever may control *C. burnetii* infection through PV-lysosome fusion (Ghigo et al. 2002, 2004).

IL-10, an immunoregulatory cytokine, also modulates trafficking of NMI. IL-10 is known to support the intracellular replication of numerous bacterial pathogens (Blauer et al. 1995; Park and Skerrett 1996). Q fever is characterized by the overproduction of IL-10 (Capo et al. 1996a, b; Honstettre et al. 2003) and IL-10 stimulates

NMI replication (Ghigo et al. 2004). Monocytes from convalescent patients recovering from acute Q fever exhibit fusion of NMI PV with lysosomes and, subsequently, efficient bacterial killing. It is related to the activity of the disease since NMI killing and PV-lysosome fusion are restored in patients who have recovered from chronic Q fever. Interestingly, NMI killing by monocytes and PV maturation are modulated by IL-10. The neutralization of endogenous IL-10 overproduced by patients with ongoing chronic Q fever enhances NMI killing by monocytes to the level of cured patients and restores PV-lysosome fusion. Similarly, adding recombinant IL-10 to monocytes from patients with cured chronic Q fever prevents NMI killing and induces PV maturation blockage (Ghigo et al. 2004). These results concerning the role of IL-10 in the inhibition of phagolysosome fusion are consistent with those of Via et al. (1998) who found that maturation of mycobacterial phagosomes is improved in macrophages from IL-10 deficient mice.

8.3.2.2 NMI and NMII: Similar Trafficking (ST) Model

An opposing model by Howe et al. (2010) proposes similar trafficking (ST) of NMI and NMII in human mononuclear phagocytes, and that the virulence of *C. burnetii* is unrelated to PV maturation status. Data for the ST model is primarily derived using human THP-1 cells, differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate, and primary human peripheral blood monocyte-derived macrophages (HMDM).

In both HMDM and THP-1 cells, coinfection experiments show a common PV can support growth of both NMI and NMII (Howe et al. 2010). In THP-1 cells, there is no difference in the percentage of NMI and NMII PV decorated with Rab5, Rab7, CD63, and cathepsin D at early (8 h) and late (72 h) time points post-infection. At 72 h post-infection, greater than 80% of NMI and NMII PV colocalize with the late endosomal/lysosomal markers Rab7, CD63, and cathepsin D. Mature PV containing NMI or NMII readily degrade bovine serum albumin in a cathepsin D-dependent manner, and contain proteolytically-active cathepsins B, K and L as assessed by the cleavage of cathepsin-specific fluorogenic peptides. Protease activity correlates with the ability of NMI and NMII PV to completely degrade *E. coli* within 15 min. Collectively, these data suggest NMI and NMII reside in phenotypcially-indistinguishable PV that fully mature through the endolysosomal pathway. In both HMDM and THP-1 cells, phase variants replicate with similar kinetics, achieving roughly 2–3 logs of growth before reaching stationary phase (Howe et al. 2010). Thus, in the ST model, NMI and NMII phase variants replicate in PV that retain phagolysosome degradative activities, and *C. burnetii* resistance to this environment represents a pathogenic strategy.

Using microscopy and/or genome equivalent PCR assays, several independent studies have also revealed growth of NMII in human monuclear phagocytes. NMII grows robustly in CD63-positive PV of human monocyte-derived dendritic cells (Shannon et al. 2005; Omsland et al. 2010; Sauer et al. 2005). Primary alveolar macrophages from humans (Fig. 8.4a, b) and cynomolgous monkeys (*Macaca fascicularis*)

also support growth of NMII, with an approximately 3 log increase in genome equivalents at 6 days post-infection documented for the latter cell type (J. Hill and R. A. Heinzen, unpublished data). With respect to human monocyte/macrophage-like cell lines, NMII infection of THP-1 cells was used to decipher *C. burnetii* anti-apoptotic signaling (Voth et al. 2007; Voth and Heinzen 2009b), probe the roles of kinases/phosphatases in PV formation (Hussain et al. 2010), identify pathogen proteins secreted into the host cell cytoplasm (Chen et al. 2010; Voth et al. 2011), and to define host cell transcriptional responses to infection (Mahapatra et al. 2010; Ren et al. 2003). Furthermore, NMII-infected MonoMac-1 cells were employed to characterize pathogen clearance activities of TLR agonists (Lubick et al. 2007). Electron micrographs of PV harboring NMII in THP-1 cells are depicted in Fig. 8.4c, d.

8.3.2.3 Reconciliation of DT and ST Trafficking Models

Subtleties between laboratories in the microscopic scoring of PV cellular markers might partially explain the different phenotypes of vacuoles harboring NMI in the DT and ST models. The complicated post-translational processing of cathepsin D makes this marker particularly problematic (Zaidi et al. 2008). What is more difficult to reconcile is why NMII grows robustly in the ST model while showing no growth in the DT model. Studies related to both models used non-opsonized bacteria; thus, the routes of NMI and NMII entry should be similar. In fact, Fc receptor-mediated uptake of NMI by HMDM, J774 cells, or primary guinea pig peritoneal macrophages does not inhibit pathogen replication (Hinrichs and Jerrells 1976; Baca et al. 1984; Shannon et al. 2009). An intriguing possibility is that the NMII strain used in DT studies has a novel genetic mutation(s) that restricts growth in human mononuclear phagocytes, but not in continuous lines such as L929 cells (Ghigo et al. 2002). NMII is a high passage derivative of low passage NMI that was plaque-cloned after 90 egg passages (Amano and Williams 1984). The strain has subsequently undergone extensive (and undocumented) laboratory passages in various labs. Thus, it is possible that the NMII strains used in ST and DT studies are genetically different. Minor genetic differences can confer permissive or non-permissive inter-strain growth phenotypes in intracellular bacteria. For example, mutations in *C. trachomatis trpA*, encoding tryptophane synthase, are associated with growth in the presence of IFNγ and implicated in tissue tropisms (Caldwell et al. 2003). A *C. burnetii* strain that cannot adequately metabolize and grow inside a given host cell should, by default, traffic to a phagolysosomal compartment, a behavior observed with chloramphenicol-treated organisms (Howe et al. 2003). The NMII used in studies related to the ST model was derived from a single source (Rocky Mountain Laboratories, Hamilton, Montana). Recent derivation of this strain's genome sequence shows that, in addition to the 25,992 bp deletion of LPS biosynthesis genes, it has 18 single nucleotide polymorphisms relative to NMI, none of which

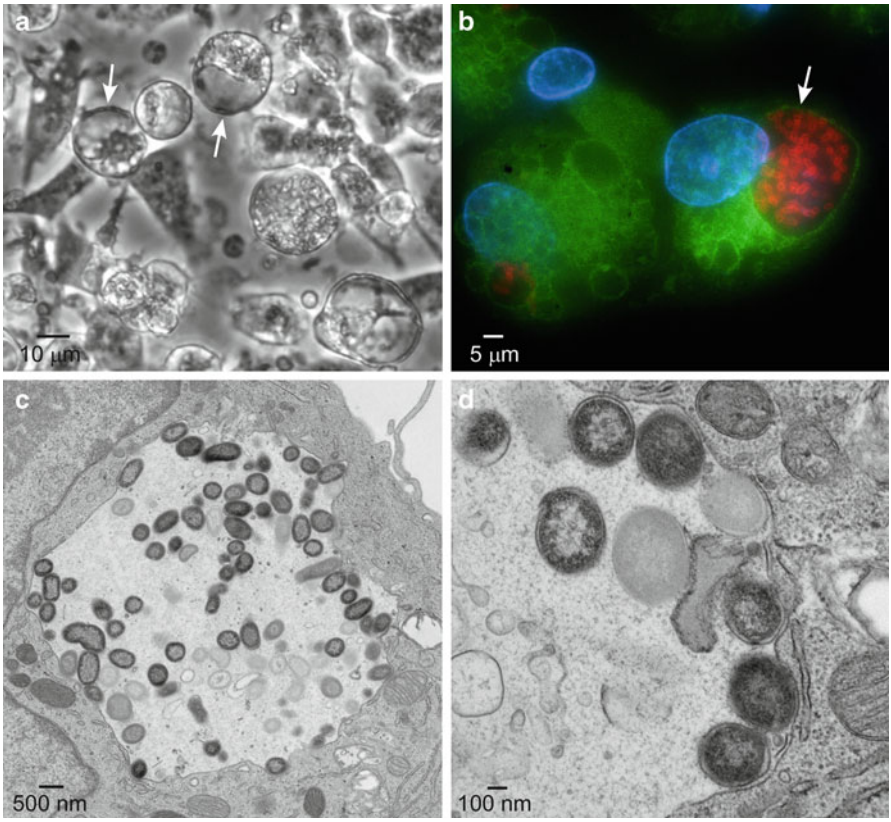

Fig. 8.4 Growth of *C. burnetii* Nine Mile (phase II) in mononuclear phagocytes. Phase contrast (**a**) and immunofluorescence (**b**) images of primary human alveolar macrophages infected with *C. burnetii* Nine Mile (phase II) for 3 days. Cells in (**b**) were stained by immunofluorescence using antibodies directed against *C. burnetii* (*red*) and the LAMP-3 (CD-63) (*green*). Host and *C. burnetii* DNA were stained with DRAQ5 (*blue*). Large PV (*arrows*) containing *C. burnetii* are seen in both micrographs. (**c**) and (**d**) Electron micrographs of THP-1 human monocyte-like cells infected with *C. burnetii* Nine Mile (phase II) for 4 days. Prior to infection, cells were differentiated into adherent, macrophage-like cells by treatment with PMA. Micrographs show large and spacious PV harboring multiple *C. burnetii*. Many organisms have intimate contact with the PV membrane that may facilitate cytosolic delivery of effector proteins required for PV biogenesis (Images in panel **a** and **b** are courtesy of Dan Voth, University of Arkansas for Medical Science. Images in panels **c** and **d** are courtesy of Dale Howe and Beth Fischer, Rocky Mountain Laboratories)

are predicted to disrupt proteins required for intracellular growth (P. A. Beare and R. A. Heinzen, unpublished data). Comparison of the genome sequence of NMII organisms associated with DT and ST models might be illuminating. Perhaps more informative would be testing of additional non-revertable phase II clones of different *C. burnetii* strains. Unfortunately, such clones are currently unavailable.

8.3.3 The Murine Macrophage Anomaly

While debate continues on the outcome of NMII infection of human mononuclear phagocytes, the strain clearly has severe growth defects relative to NMI in primary mouse macrophages (Honstettre et al. 2004; Sauer et al. 2005; Zamboni et al. 2002, 2004; Zamboni 2004; Zamboni and Rabinovitch 2003, 2004). This behaviour contrasts markedly with the proficient growth observed in multiple mouse macrophage-like cells (Baca et al. 1981; Briggs et al. 2008; Roman et al. 1986; Maurin et al. 1992b; Zamboni et al. 2002) and L929 fibroblasts (Howe et al. 2002; Baca et al. 1985; Burton et al. 1978; Roman et al. 1986; Maurin et al. 1992b; Zamboni et al. 2002). As mentioned above, surface TLR ligands of NMII, such as lipoproteins, are thought to be more accessible due the strain's lack of LPS *O*-antigen (Shannon et al. 2005). Thus, potent activation of the primary mouse macrophage pathogen recognition system by NMII may induce production of a cellular effector that limits replication. Consistent with this idea is the observation that primary mouse macrophages from TLR2 knockout mice are markedly more permissive for growth of NMII than wild type macrophages, and produce less pro-inflammatory IL-12 and TNF-α (Zamboni et al. 2004). Moreover, the corresponding knockout mouse is more susceptible to NMII infection (Ochoa-Reparaz et al. 2007). Nitric oxide (NO) induced by TNF-α may be the critical effector limiting NMII growth in primary mouse macrophages (Brennan et al. 2004; Zamboni and Rabinovitch 2003, 2004; Howe et al. 2002). NMII infection induces significant amounts of NO and treatment of macrophages with inducible NO synthase (iNOS) inhibitors, such as aminoguanidine, enhances NMII replication (Zamboni and Rabinovitch 2003). Furthermore, NMII growth is improved in macrophages from iNOS knockout mice (Zamboni and Rabinovitch 2004). TNF-α also controls mycobacteria infection of primary mouse macrophages in an iNOS-dependent manner (Bekker et al. 2001).

C. burnetii appears exquisitely sensitive to oxidative and nitrosative stress (Brennan et al. 2004) and the organism has evolved active mechanisms of avoidance. For example, infection of J774 macrophages or human neutrophils by *C. burnetii* results in little superoxide anion production (Baca et al. 1984; Akporiaye et al. 1990; Hill and Samuel 2011; Siemsen et al. 2009). In human neutrophils, this behavior involves pathogen inhibition of the host cell NADPH oxidase complex via the activity of a secreted acid phosphatase (Hill and Samuel 2011; Siemsen et al. 2009; Baca et al. 1993b, 1994; Li et al. 1996). The heightened sensitivity of *C. burnetii* to oxidants may partially explain the requirement for a microaerobic (2.5% O_2) environment to support pathogen growth in a recently established host cell-free growth system (Omsland et al. 2009).

Interestingly, relative to NMI, NMII also stimulates potent activation and maturation of human monocyte-derived dendritic cells and their subsequent release of pro-inflammatory cytokines (i. e., IL-12 and TNF-α) (Shannon et al. 2005). Despite activation, NMI grows with the same kinetics as NMII in these cells (Shannon et al. 2005). However, this behavior is predicted to result in potentiated innate and adaptive immune responses that prevents productive NMII infection of animals (Shannon et al. 2005).

8.4 Summary

The *C. burnetii* PV is a unique intracellular niche and a fascinating example of pathogen-host adaptation. The acid activated metabolism of the organism represents a biochemical strategy that ensures replication only within an acidified compartment while also conferring metabolic quiescence and stability outside the host cell (Hackstadt and Williams 1981). Lysosomal fusion with the PV is documented in many cells types (Voth and Heinzen 2007). How does *C. burnetii* resist degradation by lysosomal hydrolases and other toxic elements such as antimicrobial peptides? Resistance appears to be an inherent property of *C. burnetii*, as non-metabolizing organisms remain viable for several days in lysosome-like vacuoles of Vero cells (Howe et al. 2003). Full-length LPS is not required for resistance as NMI and NMII grow equally well in multiple cells types. An unusual cell wall containing protease-resistant peptidoglycan-associated proteins may be a resistance mechanism (Amano et al. 1984).

Early elucidation of *C. burnetii*'s metabolic requirements set the stage for the recent development of a hypoxic (2.5% O_2) host cell-free growth method using Acidified Citrate Cysteine Medium (ACCM) (Omsland et al. 2009). The precise biochemical and physiochemical character of the PV luminal environment remains an interesting question. However, one would surmise that it likely resembles ACCM in being rich in amino acids/peptides, critical carbon and energy sources of *C. burnetii*, with low oxygen content. Amino acids are likely delivered to the PV via fusion with autophagosomes and/or late endosomes/lysosomes carrying cargo expected to be rich in peptides (Gutierrez et al. 2005; Heinzen et al. 1996). A similar mechanism for nutrient delivery is proposed for *Leishmania mexicana*, a protozoan pathogen with a PV phenotypically similar to *C. burnetii*'s (Schaible et al. 1999). Furthermore, oxygen gradients across biological membranes can be associated with oxygen concentrations considerably lower than atmospheric oxygen levels (i.e., ~20%) (Hu et al. 1992).

Biogenesis of the PV begins with receptor-mediated phagocytosis. The *C. burnetii* protein adhesin(s) mediating internalization is unknown. The organism encodes multiple proteins with integrin binding RGD motifs that could potentially interact with cellular integrin receptors (Ruoslahti 1996; Seshadri et al. 2003; Beare et al. 2009b). The nascent phagosome quickly becomes fusogenic with other cellular vesicles, including autophagosomes, that contribute membrane to the enlarging PV. Bacterial pathogens utilize several strategies to manipulate the host cell membrane machinery including subversion of phosphoinosotide metabolism (Weber et al. 2009), and the functions of SNAREs and Rab family GTPases (Wesolowski and Paumet 2010; Cossart and Roy 2010). *C. burnetii* proteins predicted to modulate these and other host factors reside within a repertoire of proteins secreted into the host cytosol by the pathogen's Dot/Icm T4SS. To date, 49 *C. burnetii* Dot/Icm secretion substrates have been identified (Voth et al. 2009, 2011; Pan et al. 2008; Luhrmann et al. 2010). However, only AnkG currently has a defined function in inhibiting apopotosis via a mechanism involving binding of the pro-apoptotic protein gC1qR (p32) (Luhrmann et al. 2010). An F-actin mesh surrounds the mature

PV, but what functional role(s) does this structure play and are other cytoskeletal elements, such as microtubules, involved in PV development?

Another lingering question regards the immune function of *C. burnetii* PV. In HeLa cells, the vacuole functions as the loading compartment of the class II antigen presentation pathway (Lem et al. 1999). The class II MHC molecules HLA-DM and HLA-DR accumulate in the vacuole, which seems to affect the dynamics and repertoire of peptide loading. Consequently, defects in immune recognition of *C. burnetii* may occur. The distribution of MHC class I is unaffected by *C. burnetii* infection (Lem et al. 1999); however, whether the PV participates in antigen cross-presentation (Houde et al. 2003) is unknown.

The prior obligate intracellular nature of *C. burnetii* imposed significant experimental obstacles to unraveling the molecular details of PV biogenesis and host cell manipulation. However, novel genetic tools and host cell-free growth (Beare et al. 2011; Omsland et al. 2009) will enable novel lines of investigation that should entice new investigators into the field. Studies from additional research groups will eventually resolve discrepant data on NMI and NMII trafficking in human mononuclear phagocytes. An improved understanding of *C. burnetii* cellular microbiology will aid our ability to model mechanisms used by the pathogen to cause animal and human disease.

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