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Review

Leading a Sheltered Life: Intracellular Pathogens and Maintenance of Vacuolar Compartments

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Many intracellular pathogens survive in vacuolar niches composed of host-derived membranes modified extensively by pathogen proteins and lipids. Although intracellular lifestyles offer protection from humoral immune responses, vacuole-bound pathogens nevertheless face powerful intracellular innate immune surveillance pathways that can trigger fusion with lysosomes, autophagy, and host cell death. Strategies used by vacuole-bound pathogens to invade and establish a replicative vacuole are well described, but how the integrity and stability of these parasitic vacuoles are maintained is poorly understood. Here, we identify potential mechanisms of pathogenic vacuole maintenance and the consequences of vacuole disruption by highlighting select bacterial and protozoan parasites.

Introduction

Adaptation to an intracellular lifestyle offers most pathogens the ability to escape recognition by humoral immune responses such as circulating antibodies and complement. However, within an infected cell, a pathogen is further challenged by intracellular defense mechanisms. Prominent among these is the fusion of pathogen-containing vacuoles with lysosomal compartments (Ramachandra et al., 2009). The ability of infected cells to dispose of microbial invaders depends on the cell type and cytokine-dependent activation. Activated macrophages and dendritic cells, for example, provide the least hospitable environment, while nonimmune cells are more permissive.

To avoid lysosomal fusion, a pathogen could potentially escape the membrane-bound vacuole. However, Nod-like receptors (NLRs) and Rig-like receptors (RLRs) recognize pathogen-associated molecular patterns (PAMPs) in the cytoplasm and induce the production of proinflammatory cytokines and chemokines. These molecules influence adaptive immune response and can trigger host cell death via activation of the inflammasome (Franchi et al., 2008; Yu and Finlay, 2008). Additional antimicrobial responses include autophagosome formation on the surface of cytoplasm-exposed bacteria and their eventual fusion with lysosomes. Some pathogens, like *Listeria* and *Shigella*, have specifically adapted to life in the host cell cytoplasm by engaging in actin-based motility (Perrin et al., 2004) and by suppressing induction of autophagy (Ogawa et al., 2005).

Most intracellular pathogens studied to date replicate within membrane-bound compartments (Casadevall, 2008). It can be argued that perhaps in the context of an intact immune system and inflammatory responses, sequestration within membrane-bound vacuoles is a more desirable outcome for survival. Although the molecular mechanisms underlying the establishment of replicative niches by a number of membrane-bound intracellular pathogens are fairly well understood, how these pathogens maintain the stability and integrity of their vacuoles and the consequences of vacuole disruption on pathogenesis are not. Here, we review how pathogenic vacuoles are formed and their stability is maintained, and we describe pathways that specifically aid in the avoidance of innate immune responses.

Pathogen-Containing Vacuoles Are Customized Host Organelles

Intracellular pathogens invade their hosts via a cell entry process that culminates in the formation of a plasma membrane-derived pathogen-containing vacuole. Shortly after entry, many intracellular pathogens hijack the endomembrane system of the host cell to prevent or delay their fusion with lysosomes. Initially, the pathogen-containing vacuole is composed of host-derived membranes and shares molecular features of early endosomes. Soon thereafter, these compartments are modified by pathogen-derived proteins, and lipids and their fusogenicity with vesicular carriers and other organelles is altered (Figure 1). As a result, pathogen-containing vacuoles become unique organelles with features of late endosomes/lysosomes (*Salmonella*), early endosomes (*Mycobacteria*), or endoplasmic reticulum (*Legionella*) or appear devoid of any salient features (*Chlamydia* and *Toxoplasma*) (reviewed in Méresse et al., 1999).

The Salmonella*-Containing Vacuole*

Salmonella species are facultative intracellular bacteria that cause gastroenteritis and enteric fever (Grassl and Finlay, 2008). Soon after entry, the *Salmonella*-containing vacuole (SCV) displays markers of early endosomes such as early endosomal antigen 1 and transferrin receptor. The SCV recruits Rab7 and a subset of lysosomal markers (LAMP1 and vATPase) by selective fusion with late endosomes (LEs) and/or lysosomes. Some markers of LEs/lysosomes, such as the mannose-6-phosphate receptor (M6PR), are removed from the SCV via a Rab11-dependent recycling pathway (reviewed in Bakowski et al., 2008). The SCV then traffics along microtubules to the microtubule-organizing center (MTOC) in a process that is independent of *Salmonella* pathogenicity island 2 (SPI-2)-secreted effectors (Ramsden et al., 2007). The SCV is retained at this location via recruitment of the dynein complex by Rab7-interacting lysosomal protein (RILP)-dynein tethers. The bacterial effectors SseF and SseG are also required for SCV positioning at the MTOC (Abrahams et al., 2006; Deiwick et al., 2006). The SCV is a pleomorphic organelle characterized by filamentous projections termed *Salmonella*-induced filaments (Sifs) (Garcia-del Portillo et al., 1993). Both Sif formation and juxtanuclear positioning

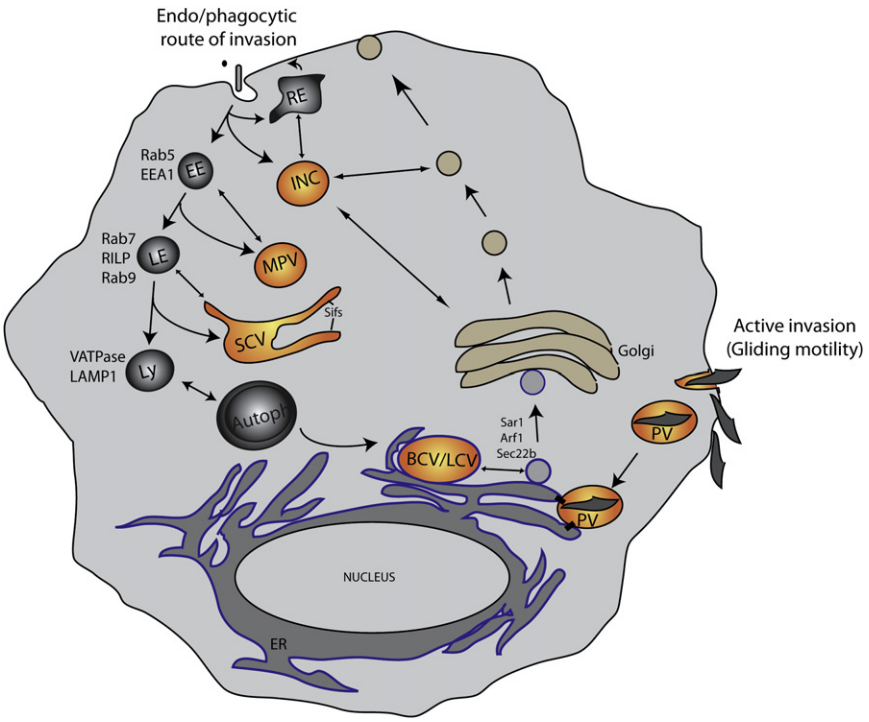

Figure 1. Interaction of Vacuolar Niches of Intracellular Pathogens with Host Endomembrane System

Bacterial pathogens invade their hosts via the endophagocytic pathway and establish vacuolar niches that share features with one or more host organelles. These niches include the *Mycobacteria* pathogen vacuole (MPV), *Salmonella*-containing vacuole (SCV), *Brucella*-containing vacuole (BCV), and *Legionella*-containing vacuole (LCV). Others, such as the *Chlamydia trachomatis* inclusion (INC) and *Toxoplasma* parasitophorous vacuole (PV), appear devoid of any markers of endocytic traffic. While avoiding fusion with degradative compartments, these organelles retain the ability to selectively intercept host vesicular traffic for nutrient acquisition. EE, early endosomes; RE, recycling endosomes; LE, late endosomes; Ly, lysosomes; Autophag, autophagosomes.

of the SCV are dependent on interactions with dynein and kinesin and are required for efficient bacterial replication (Beuzon et al., 2002; Salcedo and Holden, 2003). The secreted *Salmonella* effector proteins SifA and SseJ modulate Sif formation, with mutations in *sifA* and *sseJ* leading to lower or higher Sif formation, respectively (Brumel et al., 2002a; Ruíz-Albert et al., 2002). Recent studies indicate that SseJ is a cholesterol acyltransferase that may regulate SCV membrane dynamics by removing free cholesterol (Lossi et al., 2008).

A small portion of intracellular *Salmonella* exits the SCV and resides in the host cytoplasm. These bacteria are targeted for ubiquitination (Perrin et al., 2004) and subsequently cleared by autophagy (Birmingham et al., 2006). How these bacteria escape the vacuole is unclear, although type III secretion (T3S)-mediated damage of the SCV membrane has been proposed as one mechanism (Birmingham et al., 2006). Although the small proportion of cytosolic bacteria makes its significance questionable, *sifA* mutants readily spill into the cytoplasm (Beuzon et al., 2000), indicating a role for SifA in maintaining vacuole integrity. However, these mutants are not ubiquitinated and do not activate autophagy (Birmingham et al., 2006). While the basis for differential recognition of two forms of cytoplasmic *Salmonella* is unknown, it is possible that only bacteria associated with or enclosed in damaged SCV membranes are recognized. Indeed, ubiquitinated forms of cytoplasmic wild-type bacteria, unlike *sifA* mutants, colocalize with SCV markers (Birmingham et al., 2006). In epithelial cells, cytoplasmic *Salmonella* populations replicate more efficiently than the SCV-enclosed ones (Beuzon et al., 2002). This is in contrast to murine macrophages and fibroblasts where *sifA* mutants grow poorly (Beuzon et al., 2002; Brumell et al., 2001). This likely reflects cell-type-specific differences in the microbicidal capacity of cytoplasmic innate immune responses. Maintenance of SCV integrity is likely important for systemic disease, since *sifA* mutants are attenuated in their ability to establish infection in mice (Stein et al., 1996).

The Mycobacterial Phagosome

Mycobacterium tuberculosis, the causative agent of tuberculosis, replicates in macrophages (Cosma et al., 2003). The *Mycobacteria* pathogen vacuole (MPV) arrests at the early endosomal (EEA1, Rab5-positive) stage in a process partially mediated by bacterial mimics of phosphatidylinositol that inhibit phosphoinositide 3-kinase (PI3K) activity (Philips, 2008). The MPV retains the ability to interact with early and recycling endosomes through the action of another mycobacterial lipid, phosphatidylinositol mannoside (PIM) (de Chastellier, 2009), and Rab proteins 11 and 14, presumably to acquire nutrients delivered by endosomal recycling pathways (Kyei et al., 2006). In addition, proteins translocated by the ESX-1 type VII secretion machinery are likely involved in mediating the arrest of MPV maturation (MacGurn and Cox, 2007).

Despite the arrest in MPV maturation, *Mycobacteria* can be delivered to phagolysosomes in macrophages after induction of autophagy with rapamycin treatment or in response to IFNγ treatment (Gutierrez et al., 2004; Hope et al., 2004). As with the SCV, ubiquitination of bacterial products and autophagy are potent host defenses against establishment of the MPV (Alonso et al., 2007). Not surprisingly, *Mycobacteria* have acquired mechanisms to minimize the induction of autophagy. One such mechanism proposed is via mycobacterial inhibition of PI3K, a central regulator of autophagy and phagosome maturation (Deretic, 2008).

Whether *Mycobacteria* reside exclusively within membrane compartments has been the subject of controversy. *M. marinum*, a close relative of *M. tuberculosis*, escapes from the phagosome by secreting ESAT-6, a pore-forming substrate of the ESX-1 secretion system (Smith et al., 2008). Recent studies have revealed that during the lag between phagosome escape and initiation of actin-based motility, cytoplasmic *M. marinum* are targeted for ubiquitination by the host and subsequently engulfed in LAMP1-positive autophagosome-like compartments (Collins et al., 2009). Interestingly, many bacteria escape this form of degradation by shedding cell wall material as "decoys" (Collins

et al., 2009). Although *M. tuberculosis* and *M. leprae* have also been reported to exit the MPV in dendritic cells and macrophages in an ESX-1 dependent manner (van der Wel et al., 2007), their fate in the cytoplasm has not been reported.

Brucella- *and* Legionella-*Containing Vacuoles*

For pathogens like *Brucella abortus* and *Legionella pneumophila*, avoidance of lysosomal compartments takes a circuitous route through the endoplasmic reticulum (ER). The early *Brucella*-containing vacuole (BCV) bears all the markers of early endosomes (Rab5, EEA1, and transferrin receptor) (Gorvel and Moreno, 2002). The BCV rapidly sheds these markers and embarks on a unique maturation pathway (Celli et al., 2003; Starr et al., 2008). After transient acidification, BCVs mature into organelles with features of both autophagosomes (e.g., LAMP1- and monodansylcadaverine-positive) and ER (e.g., calreticulin and Sec61b). These organelles likely represent ER-derived autophagosomes (Pizarro-Cerdá et al., 1998a). This branch of the autophagic pathway may be important for the pathogen, since inhibition of PI3K increased *Brucella* killing while stimulation of autophagy by amino acid starvation enhanced replication (Pizarro-Cerdá et al., 1998b). Eventually, the BCV becomes enriched in ER markers (Gorvel and Moreno, 2002). The sustained acquisition of ER markers by the BCV requires the host GTPase Sar1 and interaction with ER exit sites (Celli et al., 2005). In addition, acquisition of ER markers may involve the proliferation of ER-associated autophagosomes by *Brucella*-mediated activation of the IRE1α kinase, an inducer of the unfolded protein response (Qin et al., 2008).

The *Legionella*-containing vacuole (LCV) interacts with ER-derived vesicles to mature into a vacuole primarily consisting of rough ER-derived membranes (reviewed extensively in Isberg et al., 2009). The LCV resembles autophagosomes with double membranes and associates with autophagy markers Atg7 and Atg8 (Am and Swanson, 2005; Swanson and Isberg, 1995), although the role of autophagy in *Legionella* replication is unclear, since bacterial growth is not impaired in autophagy-deficient *Dictyostelium* (Otto et al., 2004).

Small GTPases Sar1, Rab1, and Arf1 are required for the LCV to acquire Sec22b-containing, ER-derived vesicles (Kagan and Roy, 2002). Rab1 recruitment and function at the LCV surface is modulated by *Legionella* effectors DrrA/SidM and LepB via their guanine nucleotide exchange factor (GEF) and guanine-activating protein (GAP) activities, respectively (Ingmundson et al., 2007; Machner and Isberg, 2007). The association of secreted *Legionella* effectors such as DrrA and SidC with the LCV surface is mediated by their affinity for phosphatidylinositol 4-phosphate (PI4P), a lipid that is abundant on the LCV surface (Brombacher et al., 2009; Ragaz et al., 2008).

Both *Legionella* and *Brucella* use a type IV secretion (T4S) apparatus to deliver effectors into host cells. It is likely that, similar to T3S (Coombes and Finlay, 2005), T4S may also cause membrane damage that could compromise the integrity of the pathogenic vacuole. In macrophages and amoebae, *L. pneumophila* has been proposed to escape into the host cytoplasm late in infection (Molmeret et al., 2004). The significance of this is unclear.

The Chlamydia *Inclusion*

Chlamydia species are obligate intracellular bacterial pathogens that infect genital, ocular, and pulmonary epithelial surfaces. In contrast to other bacterial vacuoles described above, the *Chlamydia* pathogenic vacuole ("inclusion") is rapidly segregated from stereotypical endomembrane trafficking pathways. Like the SCV, the nascent inclusion travels on microtubules to the MTOC (Grieshaber et al., 2006), where it intimately interacts with the Golgi apparatus. At this stage, *Chlamydia* induces Golgi fragmentation by cleaving Golgin84, but the Golgi fragments remain in close association with the inclusion (Heuer et al., 2009), presumably to allow the efficient acquisition of sphingolipids and cholesterol.

Despite its segregation from classical endocytic traffic, a number of Rab GTPases (e.g., Rab1, 4, 6, 10, and 11) associate with the inclusion (Rzomp et al., 2003), which suggests that the inclusion may selectively interact with ER and Golgi-derived vesicles. While no Rab7 or Rab9 are present on the inclusion, the vacuole can acquire markers of multivesicular body endosomes (Beatty, 2006). The inclusion may also acquire lipids and nutrients by scavenging organelles from the host cytoplasm. For example, lipid droplets, neutral lipid storage organelles, are translocated across the inclusion membrane into the inclusion lumen (Cocchiaro et al., 2008).

The inclusion membrane is extensively modified by a set of poorly characterized integral membrane proteins (Incs) (Rockey et al., 2002). Inc proteins likely play central roles in inclusion biogenesis and maintenance by recruiting Rab and Rab effector proteins (Rzomp et al., 2006) and maintaining a fusogenic state (Hackstadt et al., 1999). IncA, for example, may act as a SNARE mimic that permits homotypic fusion of inclusions (Delevoye et al., 2008).

Toxoplasma *Parasitophorous Vacuole*

Toxoplasma gondii is a widely disseminated protozoan parasite of human and animal cells. Attachment and invasion of host cells results in the formation of a plasma membrane-derived vacuole, the parasitophorous vacuole (PV) (Plattner and Soldati-Favre, 2008), which is disconnected from classical host vesicular trafficking pathways (Plattner and Soldati-Favre, 2008). Nevertheless, the PV acquires extracellular LDL-cholesterol by intercepting postlysosomal cholesterol-loaded vesicles destined for the ER via a noncanonical pathway independent of host fusion proteins (Sehgal et al., 2005). This acquisition likely occurs by direct translocation of cholesterol-loaded lysosomes into the PV lumen in a process mediated by Gra7, a parasite protein secreted from dense granules (Coppens et al., 2006b). The close apposition of PV membranes with mitochondria and ER, the latter mediated by parasite protein Rop2 (Sinai and Joiner, 2001; Sinai et al., 1997), may also allow lipid acquisition by a direct membrane transfer. Several dense granule proteins (Gra2, 4, 6, 9, and 12) localize to a membrane tubular network (MTN), connecting the PV membrane with the parasite, presumably to increase membrane surface area for nutrient acquisition. Adding further complexity, however, Gra2 deletion mutants that fail to form MTNs are attenuated for acute infection in mice, but not in cultured fibroblasts (Mercier et al., 1998).

In HeLa cells, autophagy is required for parasite replication, and *atg5*$-/-$ mouse embryo fibroblast cells are less permissive for growth (Wang et al., 2009). In contrast, activated macrophages use autophagosome formation and fusion with lysosomes to clear *T. gondii* infections (Andrade et al., 2006; Ling et al., 2006).

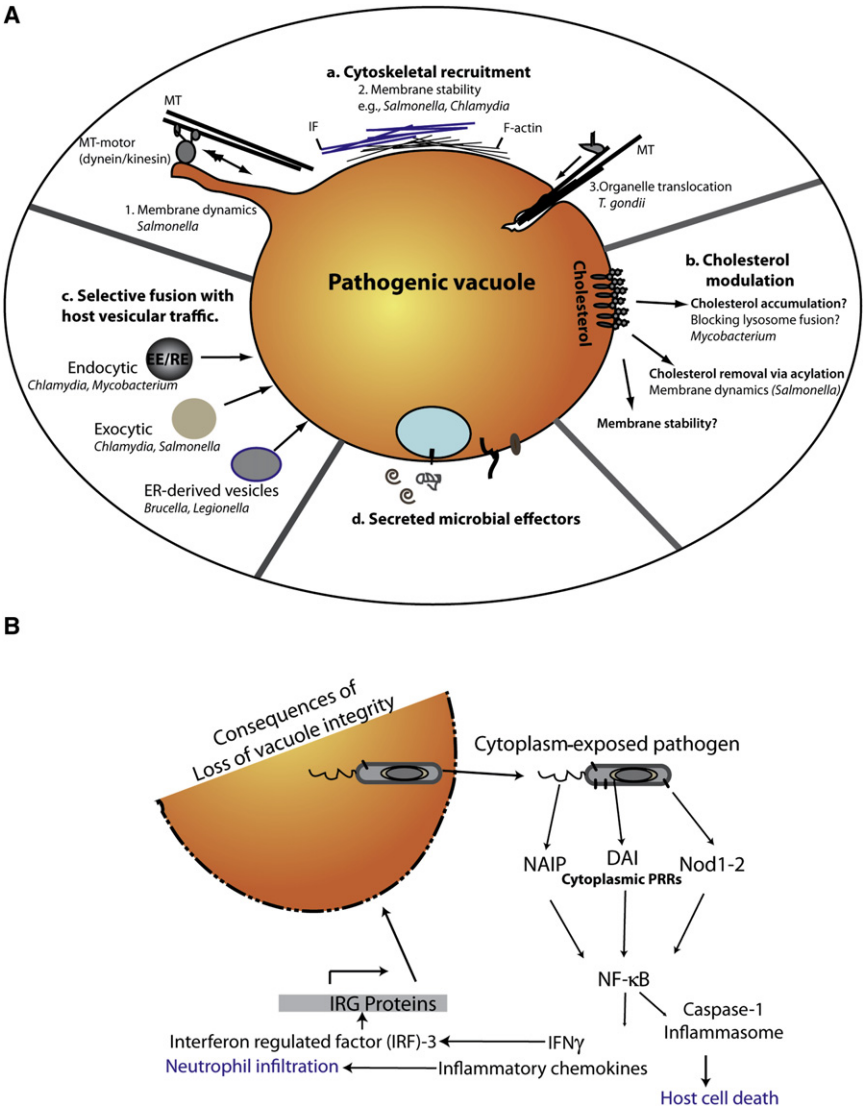

Figure 2. Integrity of Pathogenic Vacuoles Is Essential for Avoidance of Host Immune Surveillance

(A) Pathogens such as *Chlamydia* and *Salmonella* modulate interactions with host cytoskeleton and cytoskeletal motors (a), which may directly influence structural stability as well as membrane dynamics of their vacuoles. In *T. gondii*, interactions with host microtubules (MTs) mediate acquisition of host membranes via organelle scavenging. Interception of host membrane traffic is also mediated by differential interactions with host fusion machinery (b). Modulation of vacuolar membrane lipids, especially cholesterol (c), may further influence vacuolar stability. Pathogens likely regulate the unique properties and interactions of their vacuoles via secretion of effector proteins (d).

(B) Maintenance of vacuolar integrity is essential to avoid cytoplasmic immune defense pathways that include detection of microbial ligands by pathogen recognition receptors (PRRs). Such recognition is followed by activation of diverse pathways that can trigger not only host cell death and adaptive immune responses, but also direct disruption of pathogenic vacuoles by IRG proteins.

In astrocytes, although parasite clearance is dependent on *atg5*, it is not inhibited by autophagy inhibitors, and the PV is seen to fuse with the ER prior to its disruption (Halonen, 2009). Mice with macrophage-specific *atg5* deficiencies are more susceptible to *T. gondii* infection (Zhao et al., 2008), implying that in the context of a systemic infection, the host autophagic pathways in immune effector cells are critical in pathogen control. However, autophagy proteins such as Atg5 may mediate parasite clearance independent of their role in autophagy via recruitment of IFN-regulated GTPases (IGTPs) (Zhao et al., 2008).

Molecular Determinants of Pathogenic Vacuole Integrity

While pathogens can escape from membrane-bound compartments, it is apparent that residence in the cytoplasm is not necessarily an advantage and can lead to the engagement of powerful antimicrobial responses in professional phagocytic cells and the onset of robust inflammatory responses. Potentially, many pathways may be critical for maintenance of pathogenic vacuoles.

Interactions with the Cytoskeleton and Cytoskeletal Motors

The cytoskeleton and cytoskeletal motors play a critical role in organelle positioning and membrane traffic. Not surprisingly, many pathogens co-opt cytoskeletal functions to maintain and stabilize their intracellular niches (Figure 2A). In the case of *Salmonella*, the mature SCV is surrounded by an F-actin network that requires the secreted bacterial effector SteC, a Raf-like kinase (Poh et al., 2008). SifA may also contribute to actin assembly at the SCV via its interaction with RhoA (Ohlson et al., 2008). Prolonged treatment with F-actin depolymerizing agents or inhibition of the actin motor myosin causes loss of SCV integrity and cytoplasmic exposure of bacteria (Méresse et al., 2001; Wasylnka et al., 2008).

In the case of *Mycobacterium*, the role of the actin cytoskeleton in the integrity of the MPV is less clear. In contrast to the SCV, assembly of F-actin is inhibited at the MPV (Anes et al., 2003). Because treatment of infected cells with lipids that stimulate actin assembly (ceramide and sphingosine) promote phagosomal maturation and bacterial killing, it has been proposed that *Mycobacteria* inhibits actin assembly at the MPV as a defense mechanism (Anes et al., 2003).

Unlike the SCV and the MPV, the chlamydial inclusion is a large organelle that exhibits structural rigidity within intact infected cells. This is achieved by forming a structural scaffold consisting of F-actin and intermediate filaments (vimentin and cytokeratins) (Kumar and Valdivia, 2008). These structures are dynamic and require the GTPase RhoA and a bacterial protease (CPAF) to increase the flexibility of the intermediate filament network (Kumar and Valdivia, 2008). Disruption of this dynamic scaffold

leads to a loss of vacuole integrity and the leakage of inclusion contents to the host cytoplasm. Other actin-binding proteins, like α-adducin, also localize to the inclusion and may play a role in F-actin ring assembly and maintenance (Chu et al., 2008).

The *T. gondii* PV recruits γ-tubulin and nucleates microtubule growth in vivo, leading to a major reorganization of the microtubule network (Walker et al., 2008). Microtubule-dependent deformations of the PV membrane facilitate the internalization of host organelles such as lysosomes and recycling endosomes (Coppens et al., 2006a). In addition, as in *Chlamydia* vacuoles, vimentin is reorganized around the PV (Halonen and Weidner, 1994). Although *T. gondii* replication is unaffected in vimentin-deficient fibroblasts (Sehgal et al., 2005), it is unclear whether the intermediate filament reorganization influences PV stability, which would be predicted by analogy to *Chlamydia*.

Host Membrane Trafficking Pathways

Microtubules and actin filaments are required for the transport of vesicles between membrane-bound organelles. The specificity of membrane fusion events is controlled by SNAREs, Rab proteins, and tethering factors (Pfeffer, 2007). Not surprisingly, many intracellular pathogens modulate Rab recruitment for the establishment of replicative vacuoles (Brumell and Scidmore, 2007). Expression of dominant-negative forms of Rab7 or constitutively active forms of Rab5 disrupts integrity of the SCV and increases the frequency of cytoplasmic *Salmonella* (Brumel et al., 2002b), indicating that membrane trafficking events mediated by these Rabs contribute to vacuolar integrity. The mycobacterial phagosome requires Rab14 to maintain its early endosome-like characteristics (Kyei et al., 2006), while inhibition of Rab5 reduces growth by limiting access to iron-rich early endosomes (Kelley and Schorey, 2003). Similarly, dominant-negative Rab1 prevents delivery of ER markers to the LCV and impairs *Legionella* survival (Kagan et al., 2004). Whether interfering with Rab function disrupts MPV or LCV integrity has not been explored.

Cholesterol Modulation at the Pathogenic Vacuole

Cholesterol is an important structural component of membranes and an essential organizer of membrane subdomains (Edidin, 2003). Many membrane-bound pathogens accumulate cholesterol on their PVs, and inhibition of cholesterol biosynthesis and transport pathways negatively impacts pathogen replication. Given its structural role, does modulation of cholesterol levels in PV membranes contribute to vacuole stability? Depletion of cholesterol in macrophages infected with *M. avium* triggers phagolysosomal fusion and bacterial degradation (de Chastellier and Thilo, 2006). In reconstituted liposomes, mycobacterial lipid lipoarabinomannan disrupts cholesterol-rich membrane microdomains, suggesting that this may be a mechanism by which it influences phagosome maturation (Hayakawa et al., 2007). The *Salmonella* effector SseJ, a glycerolipid-cholesterol acyltransferase, similarly depletes cholesterol from the SCV membrane via acylation of free cholesterol (Lossi et al., 2008). SseJ and SifA regulate membrane tubulation and Sif formation (Ohlson et al., 2008), indicating a potential role for cholesterol levels in SCV membrane dynamics. Cholesterol depletion from the BCV membrane by cyclic β-glucans presumably shed from the *Brucella* periplasm facilitates lysosomal evasion and interactions with ER (Arellano-Reynoso et al., 2005). Cholesterol is also an abundant component of *Chlamydia* inclusion membranes, and free cholesterol is incorporated into bacterial membranes (Carabeo et al., 2003). The role of cholesterol in inclusion stability is not known.

Cholesterol levels also regulate lysosomal function. Accumulation of cholesterol in late endosomes/phagosomes inhibits fusion with lysosomes (Huynh et al., 2008), while cholesterol depletion disrupts lysosome membrane permeability (Deng et al., 2009). Therefore, cholesterol accumulation in pathogenic vacuoles could represent a strategy to limit lysosomal recognition. Alternatively, given that association of T3S translocons with the target cell plasma membrane has been shown for several bacterial pathogens to be cholesterol dependent (Hayward et al., 2005), secretion of effectors necessary for vacuole maintenance may itself require high levels of cholesterol. Although many studies have focused on the role of cholesterol-containing raft domains in pathogen entry, there are limited data on impact of cholesterol depletion on mature pathogenic vacuoles. Whether pathogenic strategies to modulate cholesterol directly or indirectly influence the stability of pathogenic vacuoles remains to be determined.

Consequences of Disruption of Pathogenic Vacuoles: Perils and Advantages of Living in a Vacuole

The evolution of complex strategies for intravacuolar survival hints at a significant selective advantage. Paradoxically, several lines of evidence indicate that life in a vacuole may not be optimal for pathogen replication. In epithelial cells, cytoplasmic *Salmonella* has a shorter doubling time than membrane-enclosed bacteria (Beuzon et al., 2002). During the exponential growth phase in macrophages, *M. tuberculosis* is predicted to reside in the cytoplasm (van der Wel et al., 2007), while at late stages of infection, *Legionella* can replicate in the macrophage cytoplasm (Molmeret et al., 2004). We speculate that the survival advantage gained by life in a membrane-bound organelle is derived from avoidance of cytosolic surveillance pathways (Figure 2B) and the potent inflammatory signaling cascades that they activate.

Cytosolic Surveillance Pathways

The existence of a cytosolic immune surveillance pathway was first identified in studies of cytosolic pathogens *Listeria*, *Francisella*, and *Shigella*. In these pathogens, mutants that cannot escape their vacuoles fail to activate NF-κB- and IFN-regulated factor-3 (IRF3)-dependent immune-related functions (Henry et al., 2007b; O'Riordan et al., 2002; Philpott et al., 2000). Cytoplasmic pathogen recognition receptors of the NLR family such as Nod1 and Nod2, NAIP, and DNA-dependent activator of IFN-regulatory factors (DAI) have been implicated in recognition of bacterial ligands like peptidoglycan, DNA, and flagellin in the cytoplasm (Martinon et al., 2009). Activation of this pathway leads to proinflammatory cytokine production, including type I IFNs, and activation of the inflammasome complex. Inflammasome-mediated cell death has emerged as a central immune defense mechanism against intracellular pathogens. Whether bacterial components of vacuole-bound pathogens can escape vacuoles and trigger similar signaling pathways is unclear, although pathogens such as *Chlamydia* (Nagarajan et al., 2008) and *Legionella* (Opitz et al., 2006) activate type I IFN-regulated pathways during infection. Additionally, type I IFN-regulated genes such as nitric oxide synthase and immunity-related GTPases (IRGs) have

been implicated in defense against a variety of vacuole-bound pathogens (Decker et al., 2005).

IRG P47 GTPases

In murine cells, a family of IGTPs determines IFN-mediated resistance to a variety of membrane-bound intracellular pathogens (Taylor, 2007), including *Toxoplasma* (Taylor et al., 2000), *Mycobacteria* (MacMicking, 2005), *Salmonella* (Henry et al., 2007a), and *Chlamydia* (Bernstein-Hanley et al., 2006). In IFNγ-activated mouse embryonic fibroblasts, IRG proteins accumulate at the surface of the PVs formed by avirulent *T. gondii* strains and may be involved in the subsequent rupture of PV membrane and parasite release into the host cytoplasm (Zhao et al., 2009). This release is followed by host cell necrosis and parasite death. In contrast, more virulent strains prevent the accumulation of IRG proteins at the PV surface. In macrophages, IRG proteins promote MPV maturation (MacMicking et al., 2003) and trigger autophagy-mediated destruction of the MPV (Singh et al., 2006), suggesting that the IRG proteins may vary in their mechanism of action against vacuole-bound pathogens.

Context-Dependent Advantages of a Vacuole-Sequestered Lifestyle

The microbicidal capacity of the mammalian cell cytoplasm varies significantly in different cell types. While epithelial cells are relatively permissive for bacterial replication, macrophages are not, as exemplified in the differential ability of *Salmonella* to replicate in epithelial versus macrophage cytoplasm. Indeed, several nonpathogenic bacteria will replicate efficiently in the cytoplasm of the host. For instance, *Bacillus subtilis* expressing listeriolysin O (LLO) (Bielecki et al., 1990) and *E. coli* expressing *Yersinia* invasin and coated with LLO (Monack and Theriot, 2001) can escape intracellular vacuoles and replicate in the macrophage and epithelial cell cytoplasm, respectively. The macrophage cytoplasm is rich in antimicrobial molecules, such as ubiquicidin (Hiemstra et al., 1993; Hiemstra et al., 1999), requiring additional strategies by cytoplasmic pathogens to counter them. As a direct consequence of this challenge, a pathogen's choice of a vacuole-sequestered lifestyle may reflect their host cell tropism.

Pathogens such as *Mycobacterium*, *Salmonella*, *Brucella*, and *Legionella* target macrophages where vacuole sequestration may be essential for survival. In contrast, "opportunistic" vacuolar lifestyles have been observed in some cytoplasmic pathogens. In immunocompromised mice and less frequently in macrophages in culture, *Listeria monocytogenes* inhabits and replicates in LAMP1-positive spacious *Listeria*-containing phagosomes (Birmingham et al., 2008). In murine macrophages, *Francisella*, after initial escape from phagosomes, re-enters and inhabits LAMP1-positive autophagosome-like vacuoles (Checroun et al., 2006). These findings suggest that a vacuolar lifestyle may be the preferred option in situations where the host cytoplasmic environment is most potent in its microbicidal capacity, with cytoplasmic replication being the exception rather than the rule. All in all, vacuolar lifestyles may be an evolutionary response to antimicrobial defense strategies of the host cytoplasm.

Concluding Remarks

For many pathogens, the evolutionary choice of a sequestered lifestyle within specialized vacuoles over the nutrient-rich cytoplasm appears to be based on compromising optimal growth in favor of avoidance of immune surveillance pathways. This may in turn govern both host cell tropism and ability of the pathogen to cause systemic and persistent infections. Although mechanisms of long-term maintenance of pathogenic vacuoles are poorly understood, the integrity and stability of their compartments may be central to pathogenicity. We predict that identification of critical determinants (e.g., microbial effectors and co-opted host pathways) of pathogenic vacuole stability will not only enhance our understanding of parasitic strategies but also offer novel therapeutic avenues.

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