Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*

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**Summary**

*Legionella pneumophila* is an intracellular pathogen that replicates in a unique vacuole that avoids endocytic maturation. Previous studies have shown host vesicles attached to the *L. pneumophila*-containing vacuole (LCV) minutes after uptake. Here we examine the origin and content of these vesicles by electron microscopy (EM). Our data demonstrate that the attached vesicles are derived from endoplasmic reticulum (ER) based on the presence of the resident ER proteins glucose-6-phosphatase, protein disulphide isomerase (PDI) and proteins having the ER-retention signal lysine-aspartic acid-glutamic acid-leucine (KDEL). After tethering occurred, ER markers inside of attached vesicles were delivered into the lumen of the LCV, indicating ER fusion. Treatment of cells with brefeldin A did not interfere with the attachment of ER vesicles with the LCV, suggesting that tethering of these vesicles does not require activities mediated by ADP-ribosylation factor (ARF). ER vesicles were not tethered to the LCV in cells producing the Sar1H79G protein, indicating that vesicles produced by the Sar1/CopII system are necessary for vesicle attachment. From these data we conclude that formation of the organelle that supports *L. pneumophila* replication is a two-stage process that involves remodelling of the LCV by early secretory vesicles produced by the Sar1/CopII system, followed by attachment and fusion of ER.

**Introduction**

*Legionella pneumophila* grows naturally within freshwater protozoan hosts in most aquatic ecosystems (Fields, 1996) but are also capable of growth within mammalian host cells, such as alveolar macrophages, if inhaled into the human lung (Horwitz, 1983; Jacobs et al., 1984). Inhalation of *L. pneumophila* can result in Pontiac fever or Legionnaires' disease, which manifest as flu-like symptoms or a severe pneumonia respectively (Fraser et al., 1977; McDade et al., 1977; Kaufmann et al., 1981). In the eukaryotic host, *L. pneumophila* replicates within a compartment surrounded by endoplasmic reticulum (ER) (Horwitz and Silverstein, 1980; Horwitz, 1983; Swanson and Isberg, 1995; Tilney et al., 2001). The generation of a compartment that supports *L. pneumophila* growth requires the Dot/Icm transporter, a bacterial type IV secretion system (Segal et al., 1998; Vogel et al., 1998). The Dot/Icm system is used by *L. pneumophila* to translocate proteins into the host cell cytosol to promote biogenesis of the *L. pneumophila* replicative organelle (Segal et al., 1998; Vogel et al., 1998; Nagai et al., 2002; Luo and Isberg, 2004). Although many proteins translocated into host cells by *L. pneumophila* have been identified (Nagai et al., 2002; Conover et al., 2003; Chen et al., 2004; Luo and Isberg, 2004; Campodonico et al., 2005; Ninio et al., 2005), their functions in vacuole remodelling are generally not well understood.

Several studies by electron microscopy (EM) have shown that the vacuole in which *L. pneumophila* resides recruits host vesicles that remain associated with the *L. pneumophila*-containing vacuole (LCV) for several hours after infection (Horwitz and Silverstein, 1980; Swanson and Isberg, 1995; Tilney et al., 2001). In the late stages of infection, these vesicles dissipate and ribosomes-studded membranes surround the LCV (Horwitz and Silverstein, 1980; Tilney et al., 2001). Analysis of the LCV by fluorescence microscopy has shown that within 30 min of uptake in macrophages, the proteins ADP-ribosylation factor (ARF) and Rab1, cytosolic host GTPases that regulate transport of vesicles between the ER and Golgi, associate with the LCV (Kagan and Roy, 2002; Derre and Isberg, 2004; Kagan et al., 2004). A YFP protein with a C-terminal KDEL or HDEL motif that is targeted to the lumen of the ER has also been shown to colocalize with the early LCV (Kagan and Roy, 2002; Lu and Clarke, 2005). From these studies, it has been hypothesized that the vesicles observed tethered to the LCV by EM are ER-derived compartments involved in remodelling of the LCV, however, because of the limiting resolution of light micros-
copy the precise localization of fluorescent markers in relation to attached vesicles and the LCV could not be determined from these studies.

In this report, we utilize the resolution of the electron microscope in conjunction with immunohistochemistry and cytochemical procedures to determine the content of the vesicles attached to the LCV as well as explore the fate of those vesicles and host processes required for remodelling of the LCV into a replicative vacuole.

Results

**Vesicle recruitment to the LCV is conserved in murine bone marrow-derived macrophages (BMMs)**

The macrophage-like cell line U937 has been used in previous studies describing vesicles attached to the LCV (Tilney et al., 2001). Because U937 cells are highly permissive for *L. pneumophila* replication, supporting the growth of several *dot* and *icm* mutants that are unable to grow in primary macrophages and protozoan hosts (Coers et al., 2000), we investigated whether the LCV in murine BMMs from A/J mice was surrounded by attached vesicles. Using the same fixation and staining procedures as Tilney et al. (2001), it was determined that the process of vesicle recruitment and tethering to the LCV observed previously in U937 cells occurs similarly in murine BMMs.

In the BMMs, LCVs formed by wild-type *L. pneumophila* were surrounded by smooth vesicles that were tethered to and match the contours of the vacuole within 1 h after uptake (Fig. 1A and B). In BMMs infected with *L. pneumophila dotA* mutants defective in type IV secretion, the LCV was barren (Fig. 1C and D), the vesicles seen tethered to LCVs containing wild-type bacteria were completely absent. Thus, the Dot/Icm system is required for vesicle recruitment and tethering to the LCV in BMMs.

We conclude from these observations that recruitment of vesicles to the LCV occurs by subversion of host cellular processes that are conserved in both primary macrophages and macrophage-like cells.

**Vesicles tethered to the LCV contain ER markers**

The origin and content of the vesicles tethered to the LCV remain unknown. Because previous studies are consistent with these vesicles being derived from the ER, we investigated whether these vesicles contained markers found in the ER and vesicles cycling between the ER and Golgi. Towards this end we used antibodies against the ER retention and retrieval epitope KDEL and protein disulphide isomerase (PDI) for immunogold labelling of BMMs infected with *L. pneumophila*. Immunogold labelling of infected BMMs (Fig. 2) at 1 h post infection revealed that the vesicles associated with the LCV were positive for the presence of both the KDEL epitope and PDI. The presence of the KDEL epitope and PDI in vesicles tethered to the LCVs indicate that vacuoles containing *L. pneumophila* are interacting either with the ER directly or with a vesicular compartment of the early secretory pathway.

To assess the presence of ER content in vesicles tethered to the LCV, infected BMMs were stained to detect glucose-6-phosphatase (G6Pase) activity, which will specifically label the lumen of the ER (Griffiths et al., 1983; Gagnon et al., 2002; Celli and Gorvel, 2004). Vesicles tethered to vacuoles containing wild-type *L. pneumophila* stained positive for G6Pase activity (Fig. 3A and B). LCVs were mostly surrounded by tethered, G6Pase-positive compartments at 1 h post infection, and by 3 h post infection, LCVs were almost completely surrounded by G6Pase-positive compartments. Interestingly, by 3 h post infection, LCVs began to exhibit out-pocketings of membrane indicating the possibility that these tethered vesicles were interacting with the LCVs in a manner beyond simply being attached to the exterior of the LCV. This could indicate either fusion of a compartment with the LCV or a pinching off of membrane from the LCV, possibly a recycling of membrane involved in the initial formation of the vacuole as *L. pneumophila* continues the remodelling of its compartment. In the case of cells infected with *L. pneumophila dotA* mutants, we observed that the LCV showed no appreciable association with G6Pase-positive compartments (Fig. 3C and D), which is in concurrence with earlier observations that LCVs containing *dotA* mutants do not associate with host vesicles in a manner similar to wild-type LCVs. Finding that *dotA* LCVs did not interact with G6Pase-positive vesicles further indicates that the *L. pneumophila Dot/Icm* system is required for intimate interactions with host ER.

**Endoplasmic reticulum vesicles fuse with the LCV**

Because tethered vesicles stained positive for G6Pase activity, and the pocketing of membranes observed at 3 h post infection indicated the possibility of fusion of these vesicles with the LCV, we examined the localization of G6Pase activity at later time points after infection to see if ER fusion with the LCV had occurred. Lumenal G6Pase staining of the LCV was used as an indicator of ER fusion. These data show that G6Pase activity was detected in the lumen of the LCV with increasing frequency over time, demonstrating that tethered G6Pase-positive compartments had fused with the LCV (Fig. 4). Although G6Pase activity was not present within all LCVs at 3 h post infection, the increase in lumenal staining from 1 to 3 h post infection was significant (*P* = 0.03) with 6% of vacuoles at 1 h post infection showing lumenal G6Pase activity and 31% being positive at 3 h post infection (Table 1). By 8 h...
and 10 h after infection, G6Pase staining was clearly evident within the lumen of vacuoles containing replicating \textit{L. pneumophila}. At 8 h post infection, 55\% of the LCVs examined exhibited lumenal G6Pase staining, a significant ($P = 0.04$) increase from the 31\% of LCVs with lumenal staining at 3 h post infection. At 10 h post infection, 40\% of LCVs show lumenal G6Pase activity. This decrease in the number of LCVs exhibiting G6Pase activity was not significant ($P = 0.17$). These data indicate that at 8–10 h post infection, the majority of mature vacuoles containing replicating \textit{L. pneumophila} demonstrate G6Pase staining, consistent with ER fusion being involved in the formation of this organelle.

\textit{ARF function is required for efficient ER fusion with the LCV, but not for vesicle tethering}

After determining that ER vesicles were tethered to and fused with the LCV, we examined whether ARF function was required for either process by examining LCV mor-

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\textbf{Fig. 1.} Host vesicles attach to the LCV in primary macrophages. BMMs infected with \textit{L. pneumophila} and fixed at 1 h post infection were stained by the method of Tilney to visualize attached vesicles.

A and B. Vacuoles containing wild-type \textit{L. pneumophila}.

C and D. Vacuoles containing dotA \textit{L. pneumophila}. Arrowheads indicate compartments tethered to the LCV. Lp, \textit{L. pneumophila}. Bar = 100 nm.
phology in brefeldin A (BFA)-treated cells. BFA has been shown to disrupt the secretory pathway and redistribute elements of the Golgi apparatus back to the ER by interfering with guanine nucleotide exchange on ARF (Donaldson et al., 1992; Helms and Rothman, 1992). It has been shown that pretreatment of BMMs with BFA inhibits the ability of L. pneumophila to replicate intracellularly and markedly reduces the association of YFP-KDEL and GFP-

Fig. 2. Vesicles attached to the LCV contain ER proteins. Cryosections of BMMs infected with L. pneumophila were fixed at 1 h post infection, and stained with an antibody against the KDEL (A, B) epitope or protein disulphide isomerase (C, D). Antibody staining was visualized with protein A labelled with 10 nm gold particles. Arrowheads indicate staining associated with the LCV. Lp, L. pneumophila. Bar = 100 nm.
p58 with the LCV, as assayed by intracellular growth curves and fluorescence microscopy respectively (Kagan and Roy, 2002).

Most LCVs had attached host vesicles in BMMs pre-treated with BFA (Fig. 5A and B). Although these data suggest that production and association of host vesicles with the LCV does not require the activity of ARF, the vesicles tethered to the LCV in BFA-treated cells were larger and more spacious than the vesicles attached to the LCV in untreated cells, suggesting that they differed from the ER vesicles associated with the LCV in untreated cells. Cells were stained for G6Pase activity to assess whether the compartments tethered to the LCV in BFA-treated BMMs were similar in content to those seen in untreated cells (Fig. 5C–F). At 1 h post infection, 56% of the LCVs examined had attached G6Pase-positive vesi-

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Fig. 4. Endoplasmic reticulum (ER) fusion with the LCV. G6Pase staining is detectable at later time points in the lumen of vacuoles containing wild-type \textit{L. pneumophila}. Vacuoles containing wild-type \textit{L. pneumophila} at 1 h (A, B), 3 h (C, D), 8 h (E, F, G) and 10 h (H) post infection. Closed arrowheads indicate G6Pase staining at the periphery of LCV. Closed arrows indicate G6Pase staining in the lumen of the LCV. Open arrowheads indicate G6Pase staining in the ER and nuclear envelope. Open arrows indicate staining in the bacterial periplasm. Lp, \textit{L. pneumophila}. Bar = 100 nm.
cles, and at 3 h post infection, 72% of the LCVs examined were associated with G6Pase-positive vesicles. These data indicate the vesicles attached to the LCV in BFA-treated BMMs contain resident ER proteins.

To determine whether ER fusion with the LCV occurs in BFA-treated cells, vacuoles were analysed for luminal precipitate generated by G6Pase activity. Although some BFA-treated cells did exhibit luminal staining of the LCV (18% at 1 h post infection and 21% at 3 h post infection, Table 1), this population appeared to be stable and did not show a significant increase between 1 h and 3 h post infection ($P=0.14$), rising just 3% over time as compared with the 26% rise seen in untreated cells. Because a defect in ARF-dependent retrograde transport functions required for retrieval of ER proteins that have escaped in early secretory vesicles would result in higher levels of G6Pase in early secretory vesicles, the higher baseline of luminal LCV G6Pase staining in BFA-treated cells at 1 h likely reflects the fusion of early secretory vesicles with the LCV. We conclude from these results that the endogenous activity of ARF is required for efficient fusion of ER with the LCV, but is not required ER tethering.

**Sar1 function is important for ER tethering to the LCV**

To further investigate host requirements for LCV biogenesis, we examined the morphology of LCVs in Chinese hamster ovary (CHO) cells producing the GTP-restricted Sar1H79G protein. These cells were cotransfected with a plasmid encoding FcγRII, which mediates the uptake of IgG-opsonized *L. pneumophila* into transfected cells. Sar1 is a GTPase that promotes vesicle budding from the ER in eukaryotic cells through the recruitment of the CopII coat complex to ER membranes (Barlowe et al., 1994). Although ER exit functions are retained in cells producing Sar1H79G, this GTP-restricted protein inhibits the release of CopII from vesicles that have budded from the ER. As a result, the expression of Sar1H79G leads to a defect in transport and fusion of these vesicles with acceptor membranes (Aridor et al., 1995). The limitations in introducing Sar1H79G into BMMs made this experiment infeasible in these cells, however, both Sar1 function and vesicle tethering to the LCV is conserved in CHO cells (Kagan and Roy, 2002; Kagan et al., 2004), so the effects of Sar1H79G should be the same in both cell types. In untransfected CHO cells, host vesicle attachment was observed to wild-type LCVs (Fig. 6A) but not dotA LCVs (Fig. 6B). In CHO cells producing Sar1H79G, attached vesicles were not observed tethered to the wild-type LCV (Fig. 6C). LCVs in the Sar1H79G-producing cells were morphologically similar to vacuoles containing dotA *L. pneumophila* in untransfected cells (Fig. 6B). These data indicate that Sar1 function is important for the recruitment and tethering of ER vesicles with the LCV.

### Discussion

In this study we have used immunogold and histochemical staining to investigate LCV biogenesis and maturation at high resolution by EM. These studies demonstrate an intimate association between the LCV and host ER. Recently, it was suggested that ER fusion during *L. pneumophila* phagocytosis may provide membrane during the initial stages of LCV formation (Gagnon et al., 2002). Although the ER may be an important source of membrane for some phagocytosis events, particularly when large hydrophobic objects are internalized (Becker et al., 2005), our data do not support a role for ER fusion during uptake of *L. pneumophila*. This conclusion is based on the observation that ER content markers were absent from the lumen of most LCVs during the first hour of infection. Additionally, vacuoles harbouring the dotA mutant strain of *L. pneumophila* did not exhibit any interactions with compartments that were identified as ER, indicating that the interactions of vacuoles containing wild-type *L. pneumophila* with ER are not due to an endogenous host process, but are bacterially driven.

There are some interesting similarities and differences between results presented in this study using EM to characterize LCV interactions with host vesicles and previous studies using fluorescence microscopy. Both approaches have shown that proteins cycling between the ER and
Fig. 5. Inhibition of ARF function prevents fusion of ER vesicles with the LCV, but not tethering.
A and B. BFA-treated BMMs were infected with wild-type *L. pneumophila* and fixed by the method of Tilney to visualize attached vesicles at 1 h (A) and 3 h (B) post infection.
C and D. Untreated BMMs infected with wild-type *L. pneumophila* for 1 h (C) and 3 h (D) were stained for G6Pase activity.
E and F. BFA-treated BMMs infected with wild-type *L. pneumophila* for 1 h (E) and 3 h (F) were stained for G6Pase activity. Asterisks indicate enlarged tethered compartments. Closed arrowheads indicate G6Pase staining at the periphery of LCV. Closed arrows indicate G6Pase staining in the lumen of the LCV. Open arrows indicate staining in the bacterial periplasm. Lp, *L. pneumophila*. Bar = 100 nm.
Fig. 6. Attachment of host vesicles to the LCV is blocked by Sar1H79G.

A and B. Untransfected CHO cells were fixed 1 h after infection with wild-type (A) and dotA (B) L. pneumophila by the method of Tilney to visualize vesicle attachment.

C and D. CHO cells producing Sar1H79G were fixed 1 h after infection with wild-type L. pneumophila to visualize vesicle attachment. The low magnification image (C) shows L. pneumophila vacuoles in a cell with no visible Golgi apparatus, confirming the effects of the Sar1H79G protein. The boxed region was enlarged (D) to show that host vesicles are not attached to the LCV in cells producing Sar1H79G. Closed arrowheads indicate attached vesicles at the periphery of LCV. Lp, L. pneumophila. Bar = 100 nm.
Golgi, such as proteins having the KDEL motif, are recruited to the LCV within the first hour of infection (Kagan and Roy, 2002; Lu and Clarke, 2005). The kinetics of recruitment of resident ER proteins, such as calnexin, occurs more slowly when examined by immunofluorescence microscopy, with optimal staining being observed only after 4 h of infection (Kagan and Roy, 2002; Lu and Clarke, 2005). By contrast, in this study we found vesicles attached to the LCV within the first hour of infection that contained the resident ER protein G6Pase. This result suggests that EM is a more sensitive method for detecting early ER interactions with the LCV in situ.

G6Pase staining revealed fusion of ER with the LCV. At the 1 h time point, the proportion of LCVs staining positive for G6Pase was relatively low, but gradually increased over the next 3 h, and reached a maximum of 55% at 8 h post infection. The kinetics of luminal G6Pase staining of the LCV were similar to the kinetics of calnexin staining of the LCV in situ by immunofluorescence microscopy, and indicate that over the first hour of infection, fusion of ER with the LCV is limited. By contrast, the host protein Sec22b, which is a transmembrane protein present on the ER membrane and on the membrane of vesicles exiting the ER in transit to the Golgi, is detected on the limiting membrane of the LCV within the first hour of infection (Kagan et al., 2004), suggesting LCV fuses with secretory vesicles exiting the ER before fusion with ER occurs.

Further evidence that the LCV interacts with secretory vesicles prior to fusion with ER comes from studies showing that Rab1 and ARF localize to the LCV within the first hour of infection (Kagan and Roy, 2002; Derre and Isberg, 2004; Kagan et al., 2004). In uninfected cells, ARF and Rab1 localize to Golgi membranes, and secretory vesicles transported between the ER and Golgi, but do not show extensive colocalization with the ER. ARF and Rab1 staining of the LCV is observed within the first hour of infection, and begins to diminish 2–4 h after infection. Importantly, 2–4 h is the time at which significant luminal G6Pase staining of the LCV was detected. Thus, data obtained using fluorescence microscopy and EM indicate that the LCV initially interacts and fuses with secretory vesicles transiting between the ER and Golgi. Association and fusion of ER membranes with the LCV, resulting in the formation of a vacuole that supports L. pneumophila replication, follow these events.

Cells treated with BFA and infected with L. pneumophila were analysed by EM to elucidate events in LCV maturation that require ARF. Vesicles having ER markers were observed attached to LCVs in cells treated with BFA. Although treating cells with BFA disrupts retrograde membrane transport from the Golgi to the ER, recent studies have shown that ARF function is not required for the formation of vesicles that exit the ER and for fusion of these vesicles into pre-Golgi compartments (Barlowe et al., 1994; Barlowe, 1997; Xu and Hay, 2004). However, in the absence of ARF function, proteins and membrane in these ER-derived compartments are not recycled as needed, which blocks further maturation of these secretory organelles and prevents the assembly of the Golgi apparatus (Aridor et al., 1995; Scales et al., 1997; Ward et al., 2001). Our data showing that ER compartments attach to the LCV in BFA-treated cells are consistent with in vitro data from both yeast and mammalian systems showing that ER-derived secretory vesicles do not require ARF function for tethering and fusion with Golgi membranes, and further support the hypothesis that the LCV is acting as an acceptor compartment for vesicles in transit from the ER to the Golgi.

Although ER vesicles were tethered to the LCV in BFA-treated cells, these vesicles were much larger. Additionally, delivery of G6Pase to the lumen of the LCV was impaired in the BFA-treated cells, indicating fusion of the ER with the LCV requires ARF function. These data suggest that the attached ER vesicles in BFA-treated cells are different from the vesicles attached to the LCV in untreated cells, and are unable to confer upon the LCV the capacity to efficiently fuse with ER and remodel the LCV into an organelle that can maintain L. pneumophila replication.

We hypothesize that in untreated cells, the recruitment and fusion of secretory vesicles exiting the ER delivers proteins that enable the LCV to fuse with ER and acquire luminal components of the ER. In BFA-treated cells, this remodelling process is inhibited. Although secretory vesicles in BFA-treated cells may be functional for fusion, in the absence of ARF activation, production of these vesicles will be limited, preventing multiple rounds of fusion with the LCV from occurring. Multiple rounds of fusion of these early secretory vesicles with the LCV would result in accumulation of early secretory proteins on the LCV, and a block in this process mediated by BFA could explain why these secretory markers are difficult to detect on the LCV by fluorescence microscopy, but are still detected by EM. BFA is also a potent inhibitor of retrograde membrane transport to the ER, which may prevent fusion of the LCV with the ER and explain the defect in delivery of ER proteins into the lumen of the LCV.

Further evidence that secretory vesicles are necessary for remodelling of the LCV comes from data using the Sar1H79G protein, which inhibits fusion of vesicles exiting the ER by preventing the release of the CopII coat from these vesicles. Importantly, cells producing the Sar1H79G protein have functional ER exit sites, and it has been shown that vacuoles containing Brucella abortus, another bacterial pathogen that replicates in an ER-derived vacuole, are still able to fuse with ER in cells producing Sar1H79G (Celi et al., 2005). By contrast, we were unable to detect the association of vesicles with the LCV in cells producing Sar1H79G, which indicates that the fusion of ER-derived
section vesicles is essential for a stage in LCV maturation that leads to host vesicle association.

These data fit a biphasic model of LCV maturation where, in the first phase, vesicles exiting the ER fuse with the LCV shortly after uptake. This process is dependent upon the *L. pneumophila* Dot/Icm system and vesicle production by the host Sar1/CopII system. This first stage is essential for association of the LCV with ER membranes. In the second phase of remodelling, LCVs fuse with ER membranes resulting in ER content being delivered to the lumen of the LCV. Inhibiting ARF function disrupts this second stage of maturation, possibly by preventing multiple rounds of fusion of secretory vesicles with the LCV, which could be essential for delivering host proteins to the LCV that mediate ER fusion. Additionally, ARF function could be important for a retrograde event that drives LCV fusion with the ER. Fusion with the ER may be important for the delivery of peptides and other nutrients into the lumen of the LCV that are used for bacterial multiplication.

This would explain why inhibitors of ARF function interfere with intracellular replication of *L. pneumophila*.

**Experimental procedures**

**Plasmids**

The plasmid containing the dominant interfering Sar1H79G has been previously described (Kagan and Roy, 2002). Briefly, cDNA encoding the Sar1H79G protein was ligated into an EcoRI and BgIII digested derivative of the pSG5 vector (Stratagene).

**Cell and bacterial culture**

Bone marrow-derived macrophages were prepared as previously described (Celada *et al*., 1984) with some modifications. A/J mice were sacrificed by euthanasia and femurs were dissected. Bone-ends were removed and the bone marrow flushed from the bone with RPMI (Gibco-BRL). Cells were pelleted by centrifugation and the media replace with RPMI supplemented with 20% fetal bovine serum (Gibco-BRL) and 30% L-cell conditioned media. Cells were plated in 20 ml of the above media at a density of 4 × 10^6 in 10 cm non-tissue culture-treated Petri dishes and grown for 6–8 days. Ten milliliters of media were added on the fourth day after plating. Cells were harvested with cold PBS and replated to six-well tissue culture dishes at a density of 10^6 per well 1 day prior to use.

Chinese hamster ovary cells cultured as previously described. Briefly, cells were cultured in MEM plus 10% fetal bovine serum. Cells were plated to six-well tissue culture dishes 1 day prior to transfection at a density of 2.5–5 × 10^5. Cells were cotransfected with a plasmid encoding Sar1H79G and a plasmid encoding FcRII using FuGene6 (Roche) as described previously.

Strains of *L. pneumophila* derived from serogroup 1 strain LP01 were passaged on CYE agar plates as previously described (Roy *et al*., 1998). Cells were infected with *L. pneumophila* as previously described (Roy *et al*., 1998). Briefly, for infections lasting less then 6 h, cells were infected at a multiplicity of infection (moi) of 10 followed by a brief (10 min × 1000 r.p.m.) centrifugation to settle the bacteria onto the monolayer, incubated at 37°C for 15 min, washed three times with cold PBS, the media replaced and the remainder of the infection incubated at 37°C. For infections lasting longer than 6 h, cells were infected at an moi of 1, briefly centrifuged as above, and incubated for 1 h prior to washing three times with cold PBS. Cells were incubated at 37°C for the remainder of the infection. The duration of the infection is taken from the time at which *L. pneumophila* are added to the cells.

**Sample preparation for EM**

Culture media was removed and bone marrow-derived macrophages were fixed in situ with a freshly made solution of 1% glutaraldehyde [from an 8% stock from Electron Microscopy Sciences (EMS), Fort Washington, PA], 1% OsO₄ (EMS) in 0.05 M phosphate buffer at pH 6.2 for 60 min on ice. After fixation, cells in Petri plates were rinsed three times with cold distilled water and en bloc stained with 1% uranyl acetate (EMS) overnight. Cells were rinsed 3 × 5 min with water, dehydrated in a graded series of ethanol washes, stripped from the culture dish with propylene oxide, rinsed in 100% propylene oxide, infiltrated in a 50:50 mix of propylene oxide and Embed812 (EMS), and embedded in Embed812.

**Glucose-6-phosphatase staining**

Cytochemical staining for G6Pase activity performed as previously described (Griffiths *et al*., 1983; Celli *et al*., 2003) with the following modifications. Cells were fixed in 1.25% glutaraldehyde (EMS) in 0.1 M PIPES (Sigma-Aldrich, St Louis, MO), pH 7.0, containing 5% sucrose (Fisher Scientific, Hampton, NH) on ice for 30 min. Following fixation, cells were washed 3 × 3 min at room temperature (RT) with 0.1 M PIPES, pH 7.0, containing 10% sucrose, then 1 × 30 s, 0.08 M Tris-maleate (Sigma-Aldrich) buffer, pH 6.5. Cells were incubated in 1 mL of reaction media (0.95% glucose-6-phosphate (Sigma-Aldrich), 0.096% lead nitrate (Sigma-Aldrich) in 0.08 M Tris-maleate, pH 6.8) for 2 h at 37°C. Following cytochemical reaction, cells were washed 3 × 3 min at RT with 0.08 M Tris-maleate buffer then 3 × 2 min at RT with 0.1 M cacodylate (Sigma-Aldrich) buffer, pH 7.2, containing 0.1 M sucrose, 5 mM CaCl₂ and 5 mM MgCl₂. Cells were then placed in 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 0.1 M sucrose, 5 mM CaCl₂ and 5 mM MgCl₂, for 1 h at 4°C. Following fixation, cell were washed 2 × 15 min with the above cacodylate buffer at RT followed by osmication in 1% OsO₄ (EMS) in the same cacodylate buffer but without sucrose for 1 h at RT and then rinsed three to four times rapidly in cacodylate buffer without sucrose to remove the excess of osmium. Following osmication, cells were stained with 1% Uranyl Acetate (EMS) in water overnight at 4°C. Cells were dehydrated and embedded as described above. G6Pase staining is generally specific for the ER and nuclear envelope as seen in Figs 3B and 4E. However, *L. pneumophila* does appear to produce a phosphatase that can generate a reaction product that is restricted to the bacterial periplasm.

**Electron microscopy**

For Epon812 embedded samples, thin sections (∼90Å) were collected on naked 100 mesh copper grids (EMS) and stained.
with 1% uranyl acetate and 1% lead citrate. Grids were viewed in a Phillips Technai BioTwin B12 electron microscope at 80 kV.

**Immunoelectron microscopy**

Cells were fixed in 4% paraformaldehyde (EMS) in 0.25 M Hepes, pH 7.4, for 1 h at RT, followed by 8% paraformaldehyde in the same buffer overnight at 4°C. Cells were scraped, pelleted, and embedded in 10% bovine skin gelatin in PBS. Pieces of the pellet were infiltrated overnight with 2.3 M sucrose in PBS at 4°C, mounted on aluminum studs, and frozen in liquid nitrogen. Blocks were sectioned at −108°C in an Ultracut cryo-ultramicrotome (Leica, Wetzlar, Germany). The 60 nm thick sections were collected using a 1:1 mixture of 2.3 M sucrose and 2% methyl acetate for 10 min on ice. Sections were rinsed in distilled water and incubated with 1.8% uranyl acetate and 1% lead citrate. Grids were viewed in a Philips Tecnai BioTwin B12 electron microscope at 80 kV.

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