

Article Addendum

Endosomal and secretory markers of the Legionella-containing vacuole

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The Gram-negative opportunistic pathogen *Legionella pneumophila* replicates in phagocytes within a specific compartment, the Legionella-containing vacuole (LCV). Formation of LCVs is a complex process requiring the bacterial Icm/Dot type IV secretion system and more than 100 translocated effector proteins, which putatively subvert cellular signaling and vesicle trafficking pathways. Phosphoinositide (PI) glycerolipids are pivotal regulators of signal transduction and membrane dynamics in eukaryotes. Recently, a number of Icm/Dot substrates were found to anchor to the LCV membrane by binding to PIs. One of these effectors, SidC, specifically interacts with phosphatidylinositol-4 phosphate [PtdIns(4)P]. Using an antibody against SidC and magnetic beads coupled to a secondary antibody, intact LCVs were purified by immuno-magnetic separation, followed by density centrifugation. This purification strategy is in principle applicable to any pathogen vacuole that carries specific markers. The LCV proteome determined by LC-MS/MS revealed 566 host proteins, including novel components of the endosomal pathway, as well as the early and late secretory trafficking pathways. Thus, LCV formation is a robust process that involves many (functionally redundant) Icm/Dot substrates, as well as the interaction with different host cell vesicle trafficking pathways.

The Vacuolar Pathogen *Legionella pneumophila*

The Gram-negative bacterium *Legionella pneumophila* parasitizes environmental protozoa and grows in alveolar macrophages of the human lung, thus possibly causing the severe pneumonia Legionnaires' disease.¹ *L. pneumophila* replicates in amoebae and macrophages within a unique compartment, the Legionella-containing vacuole (LCV),² which in either phagocyte is formed by a seemingly conserved mechanism. Therefore, amoebae and in particular the genetically tractable social amoeba *Dictyostelium discoideum*,

are valuable model systems to dissect *L. pneumophila*-phagocyte interactions on a molecular and cellular level.³

L. pneumophila promotes the uptake by phagocytes^{4,5} and LCV formation by means of the Icm/Dot type IV secretion system.⁶ To date, more than 100 Icm/Dot substrates have been identified, many of which modulate host cell vesicle trafficking.⁷ While some of these effector proteins subvert host cell GTP turnover or PI metabolism,⁸⁻¹⁰ most have not been characterized mechanistically. LCVs avoid fusion with lysosomes, interact with early secretory vesicles at endoplasmic reticulum (ER) exit sites and eventually fuse with the ER.^{2,11} Yet, multiple trafficking pathways converge to form a replicative LCV, several of which need to be inactivated to impair intracellular replication of *L. pneumophila*.¹²

A comprehensive description of LCV formation requires a catalogue of the factors involved. To determine the LCV proteome, we purified LCVs by a simple two-step protocol, using *D. discoideum* amoebae producing the ER marker calnexin-GFP and *L. pneumophila* labeled with the red fluorescent protein DsRed.¹³ Intact LCVs were enriched by immuno-magnetic separation with an antibody against the Icm/Dot substrate SidC (selectively binding to LCVs) and magnetic beads coupled to a secondary antibody, followed by density centrifugation. The proteome determined by LC-MS/MS revealed 566 host proteins, including factors associated with mitochondria, the endosomal pathway and the secretory pathway.

Endosomal Markers of LCVs

LCVs avoid fusion with lysosomes, but still interact with the endosomal pathway, as indicated by the presence of the late endosomal small GTPase Rab7,^{13,14} (Fig. 1). LCVs also acquire the *D. discoideum* inositol polyphosphate 5-phosphatase (IP5P) Dd5P4 and its mammalian homologue OCRL1 (oculocerebrorenal syndrome of Lowe).¹⁵ Dd5P4 plays a role in phagocytosis,¹⁶ and in its absence, *L. pneumophila* replicates much more efficiently in the amoebae. OCRL1 localizes to endosomes and the trans Golgi network (TGN), where it promotes (retrograde) trafficking between the two compartments.¹⁷

Additional endosomal factors were identified in the LCV proteome, including the small GTPases Rab8 and Rab14, the putative copper transporter p80 and the coat protein clathrin.^{13,18} The GTPases and p80 are markers of LCVs containing wild-type but not *icm/dot* mutant *L. pneumophila*. While Rab14 accumulates on lysosomes,^{19,20} Rab8 localizes to endosomes, where the GTPase promotes fusion with vesicles from TGN exit sites in the late secretory pathway.²¹ The

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endosomal enzymes Rab8 and OCRL1 might be recruited to LCVs by (i) direct fusion of the two compartments, (ii) interaction of LCVs with transport vesicles at late secretory entry sites (Rab8) and early retrograde exit sites (OCRL1), or (iii) acquisition from the cytoplasm (Fig. 1).

In pull-down experiments using GST-Rab8 coupled to glutathione beads and *L. pneumophila* lysate, we identified LidA as a Rab8-interacting protein (Fig. 2). The Icm/Dot substrate LidA localizes to the LCV membrane, promotes intracellular replication and avoidance of the endosomal pathway,²² interferes with the early secretory pathway²³ and causes secretion defects upon production in the yeast *Saccharomyces cerevisiae*.⁷ Furthermore, LidA enhances the Rab1 guanine nucleotide exchange factor (GEF) activity of SidM/DrrA, and GST-LidA was found to bind Rab1, Rab6 and Rab8, which promote early secretory, Golgi to ER, or Golgi to plasma membrane transport, respectively.²⁴ The promiscuous binding of LidA to different small GTPases suggests a role in subverting multiple host trafficking pathways.

Secretory Markers of LCVs

LCVs acquire ER markers, including calnexin, calreticulin, protein disulfide isomerase, and peptides containing the KDEL/HDEL retrieval motif.^{2,11,13} The formation of LCVs involves interactions with the early secretory pathway at ER exit sites and depends on the activity of the small GTPases Arf1, Sar1 and Rab1.^{12,25-27} Arf1 and Rab1, as well as the v-SNARE Sec22b also localize to LCVs.

Arf1 and Rab1 are recruited to LCVs and activated by two Icm/Dot substrates, which function as GEFs: RalF is a GEF for Arf family GTPases,²⁸ and SidM is a Rab1 GEF^{24,29} that also functions as a GDP dissociation inhibitor (GDI) displacement factor (GDF).^{30,31} Other Icm/Dot substrates promoting interactions of the LCV with the secretory pathway include SidJ, which is involved in the recruitment of ER to LCVs³² and SidC. The 106 kDa protein SidC and its paralogue SdcA anchor to LCVs by binding to phosphatidylinositol-4 phosphate [PtdIns(4)P] via a 20 kDa "P4C" [PtdIns(4)P-binding of SidC] domain near the C-terminus, which is unrelated to eukaryotic PI-binding folds.^{5,18} A 70 kDa N-terminal fragment of SidC is sufficient to bind ER vesicles, and correspondingly, *L. pneumophila sidC-sdcA* deletion mutants do no longer recruit ER vesicles to LCVs, wherein the bacteria replicate at wild-type rate. Thus, communication with the ER is dispensable for the formation of replication-permissive LCVs.¹⁸

PtdIns(4)P is a lipid component of LCVs⁵ and mediates exit of early secretory vesicles from the ER,³³ but preferentially localizes to the TGN, where it is produced by an Arf1-dependent recruitment of PtdIns 4-kinase III β (PI4K III β) to promote trafficking along the secretory pathway.³⁴ Depletion by RNA interference of Rab8, Arf1, PI4K III β but not other small GTPases or PI4Ks reduces the amount of the PtdIns(4)P-binding effector SidC on LCV membranes.^{13,35} PtdIns(4)P might either accumulate on LCVs by direct fusion with

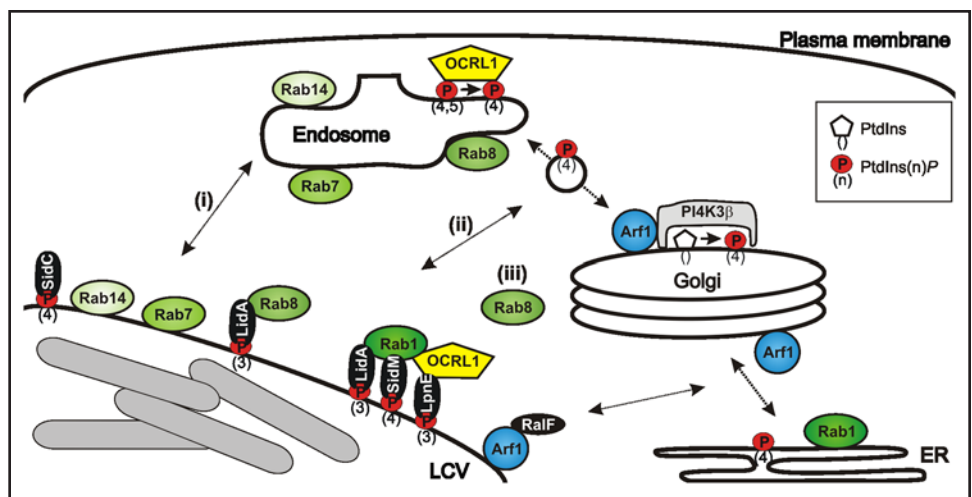


Figure 1. Communication of LCVs with endosomal and secretory trafficking pathways. LCVs are decorated with endosomal and secretory markers, which might be acquired by (i) direct fusion with cell organelles, (ii) interaction with transport vesicles shuttling between compartments, or (iii) recruitment from the cytoplasm. *L. pneumophila* produces two distinct classes of Icm/Dot-translocated effector proteins, which interact with PtdIns(4)P (SidC, SidM)^{5,18,35} or PtdIns(3)P (LpnE, LidA),^{15,35} respectively.

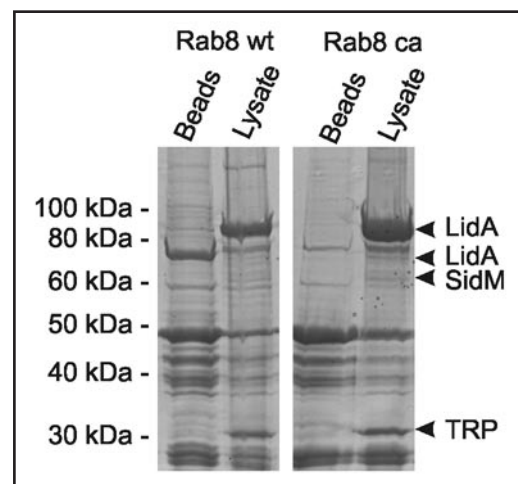


Figure 2. GST-Rab8 interacts with the Icm/Dot substrate LidA in *L. pneumophila* lysates. GST fusion proteins of Rab8 wild-type (wt) or constitutive active (ca; Rab8_{Q74L})³⁶ were used to precipitate binding partners in *L. pneumophila* lysates. Protein bands eluting from washed Rab8-coated beads but not from control beads were identified by LC-MS/MS as the Icm/Dot substrate LidA, the Rab1 GEF SidM and a putative tellurite resistance protein (TRP).

(a) compartment(s) harboring this PI, or be synthesized on LCVs by PI4K III β recruited by Arf1 from the cytoplasm (Fig. 1). Since an *L. pneumophila* Δ ralF mutant strain is defective for Arf1 but not SidC acquisition,³⁵ and since we failed to localize PI4K III β on LCVs in *D. discoideum* and macrophages (unpublished observation), a mechanism involving the direct fusion of LCVs with a compartment decorated with PtdIns(4)P seems more likely.

Conclusions and Outlook

Formation of LCVs is a robust yet complex process involving more than 100 (functionally redundant) Icm/Dot-translocated effector proteins and multiple host cell vesicle trafficking pathways.

While it is firmly established that LCVs interact with the early secretory pathway and ER, it is becoming apparent that this interaction is not required to form a replication-permissive vacuole. Moreover, LCVs also communicate with vesicle trafficking pathways between the TGN and endosomes, suggesting that the late secretory pathway, as well as retrograde endosome to TGN trafficking plays a role in LCV formation. A current challenge in the field is to define the minimal set of *L. pneumophila* effector proteins and host trafficking pathway required to form a replication-permissive vacuole.

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