**Legionella pneumophila** infection is enhanced in a RacH-null mutant of Dictyostelium

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Recently we reported that Dictyostelium cells ingest *Legionella pneumophila* by macropinocytosis, whereas other bacteria, such as *Escherichia coli*, *Mycobacterium avium*, *Neisseria meningitidis* or *Salmonella typhimurium*, are taken up by phagocytosis. In contrast to phagocytosis, macropinocytosis is partially inhibited by PI3K or PTEN inactivation, whereas both processes are sensitive to PLC inhibition. Independently from reduced uptake, *L. pneumophila* proliferates more efficiently in PI3K-null than in wild-type cells. PI3K inactivation also neutralizes resistance to infection conferred by constitutively expressing the endo-lysosomal iron transporter Nramp1. We have shown this to be due to altered recruitment of the endo-lysosomal iron transporter Nramp1. We have shown this to be due to altered recruitment of the V-H⁺ ATPase, but not Nramp1, in the Legionella-containing vacuole (LCV) early during infection. As further evidence for impaired LCV acidification we examine here the effects of disrupting the small G protein RacH on Legionella infection.

Phagocytosis and macropinocytosis can be discriminated in Dictyostelium by several criteria, including requirement for tight particle binding for phagocytosis under shaking, differential involvement of membrane phosphoinositides in both processes, absence of macropinocytosis in wild type natural isolates. By exploiting these features, we have provided evidence that Legionella is taken up by macropinocytosis. In particular we have shown both that Legionella uptake is negligible in wild type natural isolates, which are strictly dependent on phagocytosis for bacterial ingestion, and that uptake by axenic strains, which are capable of macropinocytosis, is strongly reduced under shaking but stimulated upon sodium meta-periodate pre-treatment of the bacteria, favoring the idea that Dictyostelium cells do not possess membrane receptors for Legionella. Finally, we showed that Legionella uptake is reduced upon genetic or pharmacological inactivation of PI3K or the antagonistic PTEN phosphatase.

In Dictyostelium as well as macrophages, PI3K inactivation partially inhibits fluid-phase macropinocytosis as well as phagocytosis of large particles, such as yeast, whereas fails to inhibit phagocytosis of ~1 μm bacteria, such as *E. coli*.

As additional evidence in favor of macropinocytosis, we have now tested a RacH-null mutant, that was generated and characterized by Somesh et al. RacH belong to the Rho family of small GTPases that are ubiquitously distributed across the eukaryotes. By interacting with various effectors, these GTPases regulate the actin cytoskeleton and other cellular responses. In Dictyostelium, the Rho family includes 18 members mostly belonging to the Rac subfamily. Some members of this family have been shown to regulate cell shape, spontaneous and chemotactic motility and phagocytosis. RacH gene disruption resulted in inhibition of macropinocytosis, whereas phagocytosis as well as cell motility and chemotaxis were unaffected. We found that Legionella uptake was strongly reduced in the mutant (Fig. 1A), thus supporting the notion that Legionella is taken up by macropinocytosis. We further investigated whether RacH inactivation affects intracellular proliferation of...
L. pneumophila and found that Legionella multiplied better in RacH-null cells than in the parental AX2 cells (Fig. 2B), thus resembling PI3K-null cells.

The major defect in the RacH-null mutant has been reported to be defective acidification of early endosomes.9 Impaired acidification can explain the stimulatory effect on Legionella intracellular proliferation. LCV fusion with vesicles carrying V-H⁺ ATPase is inhibited by wild type Legionella, a process further enhanced by PI3K inactivation, resulting in increased Legionella proliferation.1,12 If LCV acidification in the RacH null mutant is impaired, Legionella proliferation is expected to be stimulated in comparison to the parental strain, similarly to what found for PI3K-null mutants. Macropinosome or phagosome acidification in Dictyostelium is mainly due to recruitment of the V-H⁺ ATPase from late endosomes.13,14 RacH could regulate traffic of acidic vesicles along actin filaments or microtubules. Following ingestion, the Legionella-containing vacuole is rapidly transported about the cell along

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Figure 1. Legionella pneumophila uptake and intracellular proliferation in the RacH-null mutant. (A) Bacterial uptake was measured by plating parental AX2 or RacH-null cells on 24-well tissue culture plates and adding L. pneumophila at a MOI of 10. To promote uptake, the bacteria were sedimented onto the cell layer by centrifugation. At the time indicated on the abscissa, extracellular bacteria were killed by short incubation with gentamycin and the number of L. pneumophila per cell was calculated by determining the colony forming units (CFU) per ml and normalizing for the cell number. (B) L. pneumophila intracellular growth in parental AX2 cells or RacH-null mutant was measured as above by using a MOI of 1 and by omitting gentamycin treatment. The CFU was determined at the time indicated in the abscissa. Under the conditions used, extracellular bacteria failed to grow.

Figure 2. Legionella pneumophila uptake (A) and intracellular growth (B) in cells treated with the microtubule inhibitor colchicine. AX2 cells were preincubated on ice for 15 min, colchicine at 0.05 mM was added and the cells were warmed up to 25°C before addition of the bacteria. L. pneumophila uptake (A) and growth (B) were assessed as described in the legend to Figure 1.
microtubules, a process closely resembling phagosome or macropinosome transport. We tested whether cell treatment with colchicine would affect Legionella infection. To favor the depolymerizing effect of the drug, wild type AX2 cells were first incubated on ice for 15 min, the drug was then added and the cells were warmed up, before incubation with the bacteria. As shown in Figure 2, neither uptake nor intracellular proliferation of Legionella were altered by the drug in comparison to control cells. Actin inhibitors, such as cytochalasin A or latrunculin A, on the other hand, inhibited uptake but not intracellular replication of Legionella. In addition, Somes et al. reported that cell motility was unaffected in the mutant, ruling out major potential effects of RacH disruption on the actin cytoskeleton. Thus, how RacH inactivation alters vesicle acidification remains unclear.

Acidification regulates the activity of the iron transporter Nramp1. Both in Dictyostelium and macrophages, Nramp1 is located on endo-lysosomal membrane. Nramp1 is essential for iron transport across the phagosome or macropinosome membrane, and we have shown that Nramp1 pumps iron outside the vesicles by a proton gradient-driven mechanism, as originally proposed by Gros and coworkers for macropages. Iron promotes Legionella infection, therefore recruitment of Nramp1, but not V-H+ ATPase, to the LCV generates a friendly environment for replication of Legionella. Accordingly, the bacteria proliferate better in Nramp1-null mutants, whereas Nramp1 constitutive overexpression inhibits Legionella proliferation; this inhibition is reversed by the PI3K inhibitor LY294002. This latter effect can be explained by the additional inhibitory effect of LY294002 on vacuolar ATPase recruitment to the LCV.

In addition to Legionella, intracellular multiplication of Mycobacteria (M. avium and M. marinum) was previously shown to also be enhanced in Dictyostelium RacH-null cells. This suggests that neutralization of Nramp1, and possibly other divalent metal ion transporters, in the replication vacuole via impairment of acidification is a common feature of both pathogens. There are similarities and differences between Legionella and Mycobacterium infection. Hagedorn and Soldati reported transient acidification in a majority of M. marinum phagosomes during the first 90 min of infection, though afterwards the signal became undetectable. Acidification was strongly reduced in the RacH-null mutant. For L. pneumophila, we found that only a minority (10–15%) of the LCV’s co-localized with V-H+ ATPase during the first 2 h post-infection, consistent with previous data. We did not quantify V-H+ ATPase recruitment in the RacH-null mutant during Legionella infection, though we can bona fide assume a value near zero, similarly to what found upon PI3K inhibition.

Recruitment of the post-lysosomal marker vacuolin, a member of the flotillin family, was strongly delayed both in L. pneumophila and M. marinum infection, an indication that both pathogens interfere not only with acidification of the replication vacuole but also with its post-lysosomal traffic. Nevertheless, M. marinum proliferates and escapes the cell, probably via cell-to-cell transmission, after rupturing the replication vacuole and without extensive cell lysis. In the case of Legionella we observed, instead, extensive cell rounding up and lysis after 24–48 h post-infection. Thus the results suggest that both pathogens avoid acidification, albeit with different modalities, and by so doing impair divalent metal ion depletion in the replication vacuole. Following proliferation M. marinum exploits an exocytic pathway to leave the cell, whereas L. pneumophila apparently induces cell lysis. Whether the acquisition in the LCV of post-lysosomal markers involved in exocytosis plays any functional role in the case of Legionella remains open.

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