Phosphoinositides differentially regulate bacterial uptake and Nramp1-induced resistance to *Legionella* infection in *Dictyostelium*

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Summary

Membrane phosphatidylinositides recruit cytosolic proteins to regulate phagocytosis, macropinocytosis and endolysosomal vesicle maturation. Here, we describe effects of inactivation of PI3K, PTEN or PLC on *Escherichia coli* and *Legionella pneumophila* uptake by the professional phagocyte *Dictyostelium discoideum*. We show that *L. pneumophila* is engulfed by macropinocytosis, a process that is partially sensitive to PI3K inactivation, unlike phagocytosis of *E. coli*. Both processes are blocked by PLC inhibition. Whereas *E. coli* is rapidly digested, *Legionella* proliferates intracellularly. Proliferation is blocked by constitutively expressing Nramp1, an endolysosomal iron transporter that confers resistance against invasive bacteria. Inactivation of PI3K, but not PTEN or PLC, enhances *Legionella* infection and suppresses the protective effect of Nramp1 overexpression. PI3K activity is restricted to early infection and is not mediated by effects on the actin cytoskeleton; rather *L. pneumophila*, in contrast to *E. coli*, subverts phosphoinositide-sensitive fusion of *Legionella*-containing macropinosomes with acidic vesicles, without affecting Nramp1 recruitment. A model is presented to explain how *Legionella* escapes fusion with acidic vesicles and Nramp1-induced resistance to pathogens.

Key words: Phagocytosis, Macropinocytosis, Host–pathogen interaction, Nramp1, PI3K, PTEN, PLC, V-H$^+$ ATPase, *D. discoideum*, *L. pneumophila*, *E. coli*, Macrophages

Introduction

Phagocytosis is a property of 'professional' phagocytes capable of ingesting and killing a large variety of microorganisms. Professional phagocytes include specialized metazoan cells of the immune system as well as unicellular amoebae, such as *Dictyostelium* or *Acanthamoeba*, which feed on bacteria by phagocytosis (Bozzaro et al., 2008; Haas, 2007). Phagocytosis transition from a process for ingesting food to an immune defense mechanism requires multicellularity and cell specialization. *Dictyostelium* cells are at the edge of this evolutionary change, because they are both unicellular and multicellular. During the growth phase of their life cycle, *Dictyostelium* cells grow by grazing on bacteria and multiply as solitary amoebae. When the bacterial lawn is depleted, cells stop growing and enter the social phase of the life cycle; they assemble into multicellular aggregates and undergo cell differentiation and morphogenesis (Kessin, 2001). The phagocytic totipotentiality typical of growth-phase cells is gradually reduced after aggregation and restricted at the slug stage of development to a small subpopulation of immune-like cells, named 'sentinel cells'. These cells circulate within the slug body, ingest invading bacteria and toxic substances and are left behind the slug during its migration (Chen et al., 2007). The notion of a primitive immune system in *Dictyostelium* is supported by the occurrence in the *Dictyostelium* genome of homologues of innate immunity signaling proteins found in animal or plants, some of which are upregulated during phagocytosis (Chen et al., 2007; Cosson and Soldati, 2008; Sillo et al., 2008).

Several features of phagocytosis are conserved from *Dictyostelium* to macrophages, such as the crucial role of the actin cytoskeleton for phagosome formation, the intracellular traffic machinery for phagosome maturation, its acidification and fusion with lysosomal vesicles (Bozzaro et al., 2008; Stuart and Ezekowitz, 2005). A regulatory role in these processes is played by membrane phosphoinositides (Di Paolo and De Camilli, 2006; Flannagan et al., 2009; Lemmon, 2007; Vicinanza et al., 2008). The inositol ring of these phospholipids is phosphorylated and dephosphorylated by different phosphoinositide kinases (PIKs) and phosphatases, giving rise to different molecules that generate docking sites for small GTPases and other proteins with specific recognition domains, such as PH, PX, FYVE or FERM domains (Lemmon, 2007; Vicinanza et al., 2008). The major phosphoinositide in the plasma membrane, phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2], is also hydrolyzed by phosphatidylinositol phospholipase C (PI-PLC) to diacylglycerol (DAG) and inositol 3-phosphate (IP3). DAG recruits proteins with a C1 domain, such as protein kinase C (PKC), Rac GAP or Rap1 (Botelho et al., 2000).

PtdIns(4,5)P_2 is a major regulator of actin assembly beneath the plasma membrane and the phagocytic cup. Actin-nucleation factors, nucleation regulatory proteins and several actin-binding proteins possess PH domains that bind to PtdIns(4,5)P_2 (Heck et al., 2007; Ling et al., 2006; Takenawa and Itoh, 2001). Disappearance of PtdIns(4,5)P_2 from the membrane of the closing phagosome, concomitantly with transient increase of DAG, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 sites, correlates with disassembly of the actin coat and further maturation of the phagosome (Botelho et al., 2000; Bozzaro et al., 2008; Dormann et al., 2004; Loovers et al., 2006; Scott et al., 2005; Vieira et al., 2001). In *Dictyostelium* PLC, but not PI3K, inhibitors inhibit *E. coli* phagocytosis (Peracino et al., 1998; Seastone et al., 1999). By contrast, PI3K inactivation inhibits macropinocytosis or phagocytosis of yeast particles

(Dormann et al., 2004; Zhou et al., 1998). Similarly, in macrophages, PI3K inhibitors affect uptake of larger, but not smaller (<3 μm) particles (Botelho et al., 2000; Scott et al., 2005; Vieira et al., 2001).

As a result of their regulatory role in phagosome maturation, membrane phosphoinositides are targeted by many pathogens in their strategy to exploit phagocytosis or macropinocytosis for entering the cell, but escaping the degradative machinery of the host to proliferate intracellularly (Flannagan et al., 2009; Weber et al., 2006). *Dictyostelium* shares with macrophages the susceptibility to several invasive pathogens, such as *Legionella*, *Mycobacterium* and *Neisseria* (Bozzaro et al., 2008; Clarke, 2010). *Legionella pneumophila* infection in particular has been most studied in *Dictyostelium* and has already led to the identification of several host resistance genes (Bozzaro et al., 2008; Clarke et al., 2010).

L. pneumophila is a Gram-negative bacterium that grows in association with amoebae in the aquatic environment. If inhaled by humans and ingested in alveolar macrophages it might lead to severe pneumonia. Infection depends on the type IV Icm/Dot translocation system, a protein complex that secretes hundreds of proteins into the host cell (Isberg et al., 2009; Shin and Roy, 2008). A role for phosphoinositides in *L. pneumophila* infection in *Dictyostelium* has been proposed by Hilby and co-workers (Weber et al., 2009a). These authors showed that both PI3K and the inositol polyphosphate 5-phosphatase Dd5P4 attenuate *Legionella* intracellular growth (Weber et al., 2009b; Weber et al., 2006). They also showed enrichment of the phosphoinositide PtdIns(4)*P* in the membrane of the *Legionella*-containing vacuole and recruitment to PtdIns(4)*P* sites of proteins released by the bacteria (Brombacher et al., 2009; Weber et al., 2006).

How *Legionella* enters *Dictyostelium* cells is unclear. Centrifugation of the bacteria onto a cell layer is required for efficient uptake and infection, whereas it is not required for other bacteria, such as *E. coli*. This raises the possibility that *Legionella* enters *Dictyostelium* cells by means other than phagocytosis. In this paper, we provide evidence that *L. pneumophila*, in contrast to *E. coli*, is taken up by macropinocytosis, a process that is sensitive to PI-PLC, PI3K or PTEN inactivation, albeit with different degrees. By using mutants, in which the single *pten* gene or all class I *pi3k* genes were disrupted, we show that PI3K and PTEN are dispensable for *E. coli* phagocytosis but partially required for *L. pneumophila* uptake, and that PI3K is a resistance host gene against *Legionella* infection. In addition, we present evidence that PLC activity is required for uptake of the pathogen, but not for establishment of a replicative niche. We finally show that pharmacological PI3K inactivation also interferes with Nramp1-induced resistance to infection. Nramp1 (also named Slc11a1) is a divalent metal transporter with preference for iron and manganese, which is found on the membrane of phagosomes and endolysosomes (Cellier et al., 2007; Courville et al., 2006; Nevo and Nelson, 2006; Peracino et al., 2006). Nramp1-deficient mice are more sensitive to infection by invasive bacteria, and *NRAMP1* polymorphisms in humans have been linked to mycobacterial diseases, such as leprosy and tuberculosis (Courville et al., 2006; Nevo and Nelson, 2006). In Dictyostelium *nramp1*-null mutants display increased susceptibility to infection by *Legionella* and *Mycobacterium.* Conversely, overexpressing the Nramp1 protein protects *Dictyostelium* cells against *Legionella* (Peracino et al., 2006). Here, we show that the latter effect is reversed by inhibiting PI3K. Sensitivity to PI3K inactivation is restricted to the initial hours of infection and appears to be linked to inhibition of acidification of the *Legionella*-containing vacuole. A model is presented to explain how inhibition of acidification might affect Nramp1 activity.

Results

L. pneumophila, in contrast to *E. coli*, is taken up by macropinocytosis, a process differentially sensitive to PI-PLC, PI3K and PTEN

Legionella uptake and infection are usually assayed by centrifuging the bacteria onto a cell layer to favor bacterial attachment to the cell surface. Under these conditions, less than 10% of the added bacteria were ingested by *Dictyostelium* cells (Fig. 1A). The uptake rate increased only 1.35-fold during the 2 hours of incubation (Fig. 1B). Uptake is nevertheless actin dependent, and is inhibited by cytochalasin A or latrunculin A (Fig. 1A,B). Negligible uptake of *L. pneumophila* was detected under shaking, whereas other bacteria, such as *E. coli*, *Salmonella thyphimurium* or *Neisseria meningitidis*, were phagocytosed, albeit at different rates (Fig. 1C) (Bozzaro et al., 1987; Colucci et al., 2008; Sillo et al., 2008).

Phagocytosis under shaking requires tight particle binding to the cell surface because shear forces are generated in the liquid suspension because of the speed of rotation. The washing procedure at the end of the incubation removes more than 90% of tightly bound bacteria, such as *E. coli*; thus an increase in the number of bacteria per cell over time is a measure of the uptake rate of the cells, with a modest contribution by bacteria sticking to the membrane (Bozzaro et al., 1987; Peracino et al., 1998). The minimal engulfment of *Legionella*, both in the centrifugation assay and under shaking suggests that it binds very weakly or not at all to *Dictyostelium* cell surface. To test this hypothesis, we pre-treated the bacteria with sodium meta-periodate and assayed phagocytosis under shaking. The fluorescence intensity of the cells as result of bacterial uptake was determined by flow cytometry. Sodium periodate treatment strongly reduced the viability of the bacteria as assessed by plating them on agar (data not shown).

In flow cytometry, the cell and bacterial populations can be neatly separated by particle size, therefore the washing procedure in this assay was omitted. To distinguish binding from uptake under these conditions, cytochalasin-A-treated cells were also tested in parallel. The percentage of fluorescent cells increased sharply upon sodium periodate treatment in the control and to a lower extent in cytochalasin-treated cells (Fig. 1D), indicating that periodate-treated *Legionella* bind much better than untreated ones to the cell surface. Visual examination of the cells confirmed binding and uptake of sodium-periodate-treated *Legionella* in the absence of cytochalasin A, and binding only to the cell surface for cytochalasin-A-treated cells (Fig. 1E).

Thus periodate oxidation of carbohydrate moieties from the lipopolysaccharide (LPS) coat enhances binding and uptake, indicating that *Dictyostelium* cells probably do not possess receptors for the distal *Legionella* LPS sugars. These results also suggest that living *Legionella* are not taken up by phagocytosis by *Dictyostelium* cells, raising the possibility that the low uptake detected in the centrifugation assay might be due to macropinocytosis, a process that does not require particle binding to the cell surface.

To test this hypothesis, we took advantage of previous studies showing that macropinocytosis, in contrast to phagocytosis, is partially sensitive to PI3K inactivation, is strongly defective in *racH*-null cells (Somesh et al., 2006) and is quite negligible in wild-type strains (Kessin, 2001). Fig. 2A,B shows the differential

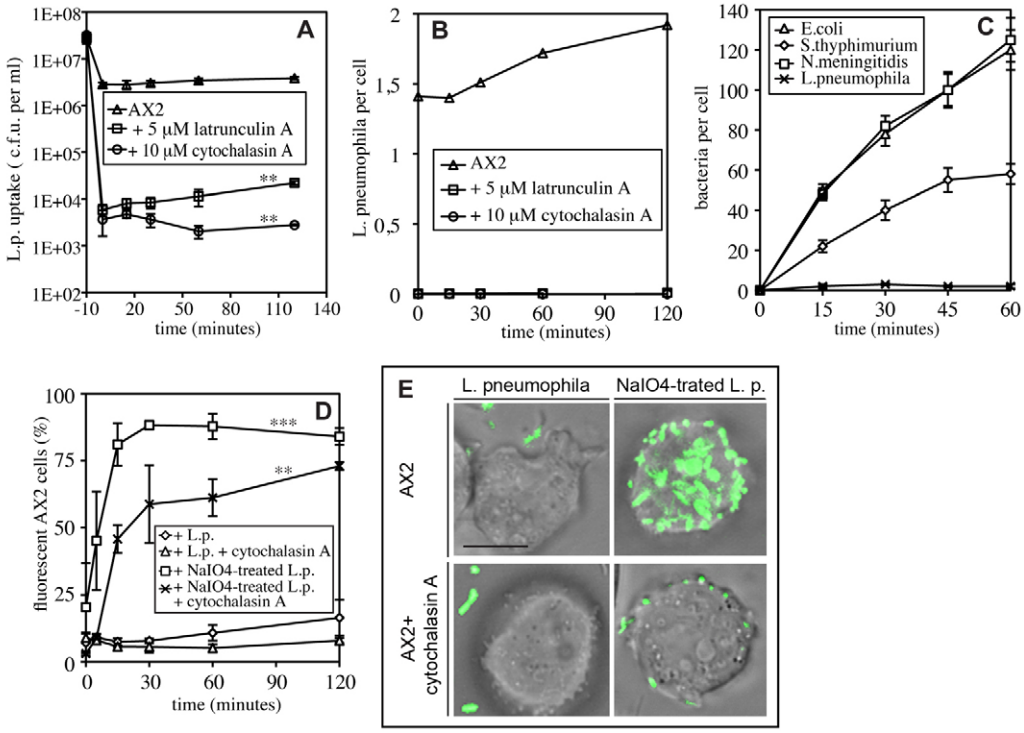

Fig. 1. Characterization of *L. pneumophila* uptake by AX2 cells. (**A**) AX2 cells were plated on 24-well tissue culture plates for 10 minutes and incubated with latrunculin A or cytochalasin A for additional 10 minutes, where indicated. *L. pneumophila* at a MOI of 10 was added to each well and uptake assessed. The CFU at time –10 minutes represents the inoculum. (**B**) The data from A were expressed as number of *L. pneumophila* per cell to allow comparison with C. (**C**) Phagocytosis of the indicated bacterial strains was assayed under shaking at 150 r.p.m. for the time indicated in the abscissa. A 1000-fold excess of bacteria was used in all cases. (**D**,**E**) Phagocytosis assay under shaking of FITC-labeled *L. pneumophila* pre-treated or not with sodium periodate (MOI 100). Where indicated, cells were pre-treated with cytochalasin A for 10 minutes. At the time indicated in the abscissa, samples were withdrawn from the suspension, and the percentage of fluorescent cells in the sample calculated by flow cytometry (D). In E, representative cells are displayed to show that periodate treatment of the bacteria leads to both increased binding and uptake, or to increased binding only in the presence of cytochalasin A. Scale bar: 5 µm. Except for the representative experiments in C, all other measurements are mean values of two or more independent experiments in duplicate with s.e.m. The asterisks in A and D denote statistically significant differences in cell treated with drugs or sodium periodate compared with their controls (two-sample two-tailed *t*-test assuming unequal variance and non parametric Kruskal–Wallis test: $^{**}P<0.01$, $^{***}P<0.0001$).

effects of pharmacologically inhibiting PI3K or PLC on *L. pneumophila* or *E. coli* uptake. *Legionella* engulfment, tested by the centrifugation assay, was partially inhibited by the PI3K inhibitor LY294002, whereas *E. coli* phagocytosis, assayed under shaking, was not significantly inhibited. The PI-PLC inhibitor U73122 completely inhibited engulfment of both *L. pneumophila* and *E. coli* (Fig. 2A,B).

LY294002 is a widely used PI3K inhibitor, but the drug has also been shown to interfere with other targets, affecting pathways potentially unrelated to PI3K (Gharbi et al., 2007). *Dictyostelium* has five genes encoding class I PI3Ks and a single gene for the PTEN phosphatase. Quintuple and sextuple knockout mutants were generated (Hoeller and Kay, 2007), in which either all five class I PI3K genes (mutant HM1284), or in addition the *pten* gene (mutant HM1295) were disrupted. To confirm the results obtained with LY294002, we tested these two mutants as well as a *pten*-null mutant (Iijima and Devreotes, 2002) for *E. coli* or *L. pneumophila* uptake. The results paralleled those obtained with the drug: *E. coli* phagocytosis was not significantly inhibited in the mutants (Fig. 2D), whereas *Legionella* engulfment was inhibited by about 40% and 60% in HM1284 and HM1295, respectively (Fig. 2C). In the *racH*-null mutant, which was previously shown to be normal for *E. coli* phagocytosis (Somesh et al., 2006), *Legionella* ingestion dropped to about 30% of that in the control. Inhibition in the *pten*-null mutant was not statistically significant.

In all these experiments, axenic strains were tested. Axenic strains are widely and routinely used in *Dictyostelium* labs because of several advantages: (1) they can be grown in defined axenic media in the absence of any host bacteria; (2) development can be induced by washing the cells free of medium; (3) for host–pathogen interaction studies, infection can be followed in a half-starving axenic medium, avoiding possible contamination by bacteria used for foraging the cells. Fluid-phase endocytosis, mostly macropinocytosis (Hacker et al., 1997), is a gain-of-function of the axenic mutation (Williams et al., 1974). Therefore, if *Legionella* in the AX2 strain is engulfed by macropinocytosis, its uptake in the natural wild-type isolates should be negligible. Indeed, as shown in Fig. 3, *Legionella* uptake is strongly reduced in the AX2 parental strain NC4, in a second wild-type strain, V12M2, and even in AX2 cells when grown on *E. coli* for several generations.

Taken together, these results indicate that *Legionella* ingestion in *Dictyostelium* axenic strains occurs mostly by macropinocytosis, which also explains its low efficiency. In addition, the process is completely blocked by PI-PLC inactivation and, in contrast to *E. coli* phagocytosis, is partially sensitive to genetic or pharmacological inactivation of PI3K or PTEN.

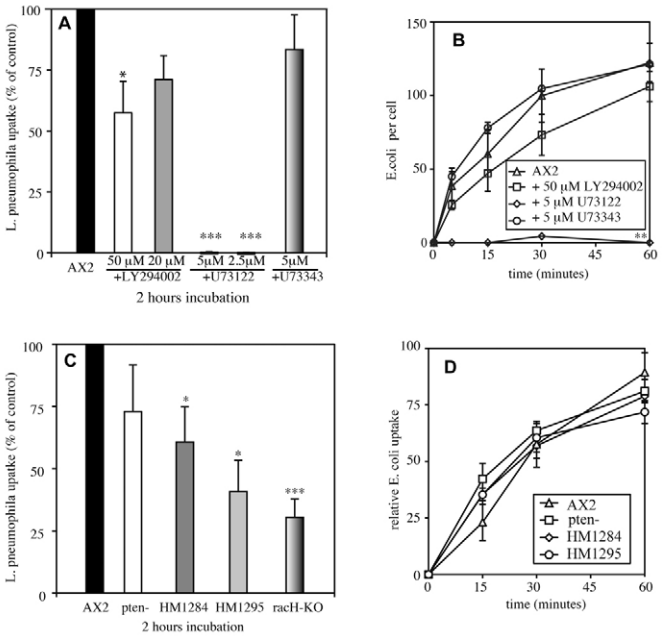

Fig. 2. Differential effects of PI3K, PTEN or PLC inactivation on *L. pneumophila* or *E. coli* uptake. (**A**,**B**) *L. pneumophila* or *E. coli* uptake was tested in the centrifugation assay or under shaking, respectively, in the presence or absence of the indicated inhibitors. U73343 is an inactive analog of U73122. (**C**,**D**) *L. pneumophila* or *E. coli* uptake in *pten* mutant, in class I *pi3k* quintuple null HM1284, sextuple *pi3k*- and *pten*-null HM1295 mutants and *racH*-null mutant were tested in the centrifugation assay or under shaking. HM1284 and HM1295 cells are half the volume of AX2 cells, determined by a microhematocrit tube test. In D, 'Relative *E. coli* uptake' corrects for this difference in volume, and represents the number of bacteria corrected for cell volume. A similar correction has been made for C. Mean values of two representative experiments in duplicate with s.e.m. The asterisks denote significant effects in AX2 cells treated with drugs compared with untreated cells (A,B) or the null mutants compared with AX2 (C) [two-sample two-tailed *t*-test assuming unequal variance or non parametric Kruskal–Wallis test: **P*<0.02; ***P*<0.001; (C); ****P*<0.0001]. No statistically significant differences were found in the other cases.

Inactivation of PI3K, but not PTEN or PLC, stimulates *Legionella* intracellular growth and abolishes protective effect of Nramp1

Despite the very low uptake, ingested *Legionella* survive and proliferate efficiently in *Dictyostelium* cells, as already shown by different labs (Farbrother et al., 2006; Hagele et al., 2000; Peracino et al., 1998; Solomon and Isberg, 2000; Weber et al., 2006). To investigate the involvement of phosphoinositides in *Legionella* infection, we first assayed *Legionella* growth in *pi3k*-null and/or *pten*-null mutants. Compared with the parental AX2 cells, *Legionella* replication was stimulated on average 15-fold and 4-fold, at 72–96 hours after infection in the HM1295 and HM1284 mutant, respectively (Fig. 4A).

We have previously shown that disruption of the gene encoding the iron membrane transporter Nramp1 results in increased susceptibility to *L. pneumophila* as well as *Mycobacterium avium* infection, whereas constitutive overexpression of Nramp1 protects effectively against *Legionella* infection (Peracino et al., 2006). As confirmed in Fig. 4B, the ingested bacteria failed to grow in cells expressing constitutively Nramp1–GFP, whereas in AX2 cells,

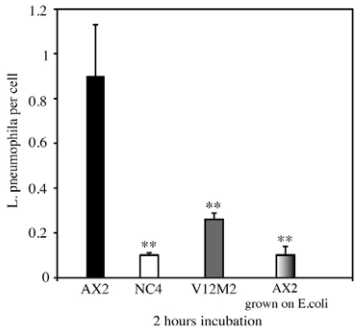

Fig. 3. *L. pneumophila* uptake in wild-type cells growing on bacteria. Phagocytosis was assayed with the centrifugation assay. The AX2 parental strain NC4 and V12M2 are both wild-type isolates growing strictly on bacteria (*E. coli* B/r). For comparison, AX2 grown overnight on *E. coli* B/r was also tested. Mean values of two experiments in duplicate with s.e.m. (two-sample two-tailed *t*-test assuming unequal variance: ***P*<0.006).

Legionella growth was observed. In contrast to extracellular bacteria that rapidly die in the culture medium used during infection, the engulfed *Legionella* in cells overexpressing Nramp1 remained viable but their growth is blocked, as suggested by the flat curve. Addition of the PI3K inhibitor LY294002 further stimulated bacterial growth in AX2 cells and reverted the inhibitory effect of Nramp1 constitutive overexpression (Fig. 4B). Thus, LY294002 has opposing effects on *Legionella* uptake or intracellular replication, which are inhibited or stimulated, respectively, both in AX2 and in cells overexpressing Nramp1. The latter result also suggests that PI3K directly or indirectly controls Nramp1 activity.

To dissect potential pathways regulated by PI3K, we tested the effect of LY294002 at different times during infection, by treating the cells continuously or adding the drug at 2 hours after infection. If the drug was present from the beginning, *Legionella* growth was stimulated; addition at 2 hours after infection was ineffective (Fig. 4C).

In parallel, we tested the effects of the PI-PLC inhibitor U73122. If added at the beginning of infection, U73122 inhibited *Legionella* intracellular growth, whereas addition 2 hours later did not affect the outcome of infection (Fig. 4D). As already shown in Fig. 1A, however, U73122 strongly inhibited bacterial uptake, thus the inhibitory effect on *Legionella* growth when the drug was present from beginning can be fully explained with inhibition of uptake. Indeed, the growth curve in the presence of U73122 paralleled that of the bacteria incubated without cells, which are known to die under these conditions (Fig. 4D).

Taken together, these results delimit a 2 hour time interval at the onset of infection that is sensitive to PI-PLC and PI3K inhibitors, albeit with opposite effects: inhibition of PI-PLC impairs *Legionella* uptake with no additional effects on growth, whereas inhibition of PI3K partially reduces uptake but strongly stimulates intracellular growth of the bacteria.

Actin assembly is required for *Legionella* uptake, but not for intracellular growth

Phosphoinositides regulate actin assembly to phagosomes and macropinosomes. An actin coat is required for phagosome and macropinosome formation, and is immediately disassembled upon internalization of the vesicle (Insall et al., 2001; Maniak et al., 1995; Peracino et al., 1998). Actin depolymerization correlates

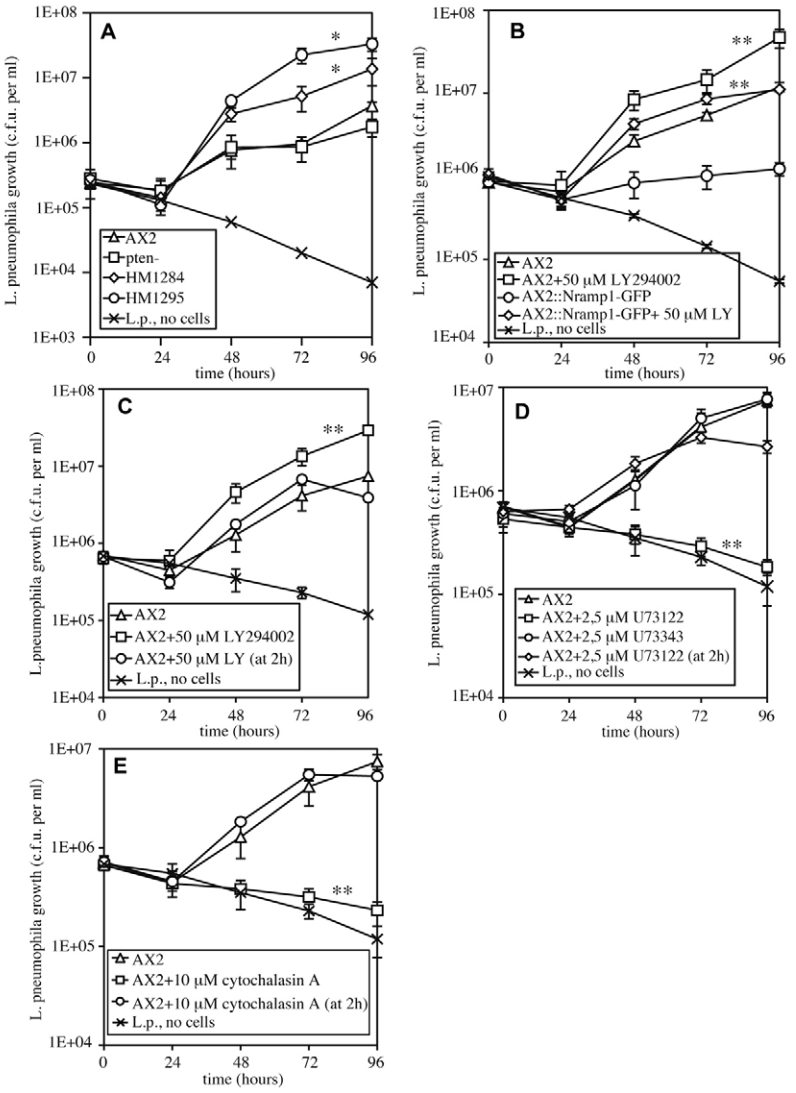

Fig. 4. Effects of inactivation of PI3K, PTEN, PLC or actin polymerization inhibitor on *L. pneumophila* intracellular growth in the wild type and in cells constitutively expressing Nramp1. AX2 parental strain cells and mutants were infected by *L. pneumophila* in the presence of drugs where indicated, and intracellular growth of the bacteria assessed by the CFU assay at the time indicated in the abscissa. In the absence of cells (L.p., no cells), *Legionella* slowly die. (**A**) *Legionella* growth in AX2 cells, in class I *pi3k* quintuple null HM1284, sextuple *pi3k*- and *pten*-null HM1295 mutants and in the single *pten*-null mutant. (**B**) *Legionella* growth in AX2 cells and AX2 cells constitutively overexpressing Nramp1–GFP in the presence or absence of LY294002. (**C–E**) Effects on *Legionella* intracellular growth upon treatment with the indicated drug, added either from beginning of infection or at 2 hours after infection. Mean values of two representative experiments in duplicate with s.e.m. The asterisks denote significant effects in mutant vs AX2 cells (A) or drug-treated vs untreated cells (B–E). In A, the two-sample two-tailed *t*-test assuming unequal variance or the non parametric Kruskal–Wallis test gave a value of $*P<0.05$ for HM1295, whereas for HM1284 a one-tailed *t*-test of the null hypothesis (which stated that the infection level was not significantly higher in the null mutant) was $*P=0.04$. The two-sample two-tailed *t*-test assuming unequal variance or the non-parametric Kruskal-Wallis gave $**P<0.001$ in the indicated comparisons in B–E.

with the disappearance of PtdIns(4,5)P_2 from the closing phagosome or macropinosome, which might be due to the activity of enzymes such as PLC, PI3K or the OCRL1 homolog Dd5P4 phosphatase (Blanc et al., 2005; Bozzaro et al., 2008; Dormann et al., 2004; Loovers et al., 2007). The impaired uptake of *Legionella* upon inactivation of PLC or PI3K can be fully explained with the regulatory activity of these enzymes on actin depolymerization.

Whether the actin cytoskeleton has a role in the establishment of a proliferative niche and intracellular replication of *Legionella* is unknown. The finding that LY294002 treatment stimulated *Legionella* intracellular growth prompted us to test whether effects of the drug on actin remodeling could have such a role. To this purpose, we tested the effects of cytochalasin A on *Legionella* infection. Cytochalasin A inhibited intracellular replication of the bacteria if added at the beginning of infection; addition at 2 hours after infection had no effect on intracellular growth (Fig. 4E). The inhibitory effect at the onset of infection can, however, be fully explained by the strong inhibition of uptake (Fig. 1A,B), similarly to what was observed by inhibition of PI-PLC. Thus, we conclude that post-ingestion events leading to intracellular replication of *Legionella* are insensitive to PI-PLC and to changes in the actin polymerization state of the host. In addition, enhancement of intracellular growth by inactivation of PI3K is not mediated by regulatory effects on the actin cytoskeleton.

Effects of PI3K inhibition on fusion of the *Legionella*-containing vacuole or *E. coli* phagosome with vesicles of the endolysosomal pathway

The intracellular traffic of the *Legionella*-containing vacuole (LCV) differs in many respects from phagosomes containing non-pathogenic bacteria. Both in *Dictyostelium* and macrophages *Legionella* forms a replicative vacuole that avoids fusion with lysosomes, recruits mitochondria and acquires proteins of the endoplasmic reticulum and other trafficking routes (Derre and Isberg, 2004; Fajardo et al., 2004; Francione et al., 2009; Kagan and Roy, 2002; Lu and Clarke, 2005; Robinson and Roy, 2006; Swanson and Isberg, 1995). To investigate whether the stimulatory effect of PI3K inactivation on *Legionella* intracellular growth could be mediated by alterations in LCV fusion with vesicles of the endolysosomal pathway, we assayed the effects of LY294002 on recruitment to the LCV of calnexin, V-H^+-ATPase, Nramp1 and vacuolin A. The chaperonin calnexin is a resident marker of the endoplasmic reticulum (Müller-Taubenberger et al., 2001). V-H^+-ATPase decorates both the contractile vacuole network and acidic

vesicles and is the major agent of phagosomal and endosomal acidification. Vesicles decorated with the vatA, vatB or vatM subunits of the V-H^+-ATPase, with the exception of only the contractile vacuole network, have been shown to colocalize strictly with acidic markers, such as neutral red or Lysotracker Red (Clarke et al., 2002).

Nramp1 is found in the *trans*-Golgi network (TGN) and in vesicles trafficking from the TGN to endosomes and phagosomes (Peracino et al., 2006). Vacuolin A (vacA), together with vacuolin B, is expressed in post-lysosomal vesicles and is required for exocytosis (Rauchenberger et al., 1997).

To investigate recruitment of these proteins in the LCV, and potential alterations by the PI3K inhibitor, AX2 cells expressing Nramp1–GFP or calnexin–GFP were incubated with Ds-red express-labeled *Legionella* in the presence or absence of the drug, and the number of *Legionella* residing in vesicles decorated with the GFP-fused proteins was determined by confocal microscopy. Similarly, GFP-expressing *Legionella* were incubated with AX2 or with mutant HM1284 cells, fixed and labeled with antibodies against the vatA subunit of the vacuolar ATPase or against vacA. As control, the *Legionella* ΔicmT mutant, which fails to grow intracellularly (Weber et al., 2006), heat-treated *Legionella* or *E. coli* were used. Fig. 5 shows representative examples of treated cells at different times during infection. The bacteria were found in vesicles either decorated or not with the markers. The average LCV diameter was 2.5±0.5 μm and did not change significantly during the first 4 hours of infection in the absence or presence of LY294002. For any given marker, vesicles contained only one *Legionella* or were empty, and no relevant changes were detected in vesicle shape and distribution during the first 12–24 hours after infection. Only 48 hours after infection in the case of virulent *Legionella* was a significant increase in the number of bacteria per cell evident, with bacterial clumps enclosed in very large vesicles decorated with the marker, and single non-decorated bacteria dispersed in the cytosol (see Fig. 5, vacA). At this stage, only vatA or vacA could be used as markers, because GFP–Nramp1 expression was strongly reduced.

The extent of and changes in colocalization over time are displayed in Fig. 6. About 50% or 40% of total wild-type *Legionella* in the cells were found after 2 hours of chase in vesicles labeled with calnexin or Nramp1, respectively, with no significant changes upon treatment with LY294002 (Fig. 6A,B). In sharp contrast, about 15% of *Legionella* were found in vatA-decorated vesicles immediately after centrifugation (incubation time: t_0), both in AX2 and in the HM1284 mutant, and the number slowly decreased during the next 2 hours. In the presence of LY294002, colocalization dropped to about 5% and 3% at t=0 and t=15–30 minutes, respectively (Fig. 6C).

In contrast to wild-type *Legionella*, 75% of engulfed ΔicmT, *E. coli* or heat-treated *Legionella* were located in vatA-decorated vesicles after 15 minutes of chase, with a decline to 40% in the subsequent 120 minutes (Fig. 6D and data not shown). In all three cases, LY294002 did not significantly affect the extent of colocalization.

In conclusion, from beginning of infection and during the subsequent 2 hours, 85% of wild-type *Legionella*, in contrast to the avirulent ΔicmT mutant, to heat-killed *Legionella* or to *E. coli*, reside outside vatA-positive, acidic vesicles, whereas a significant proportion was found in calnexin- or Nramp1-decorated vesicles. LY294002 further inhibited LCV fusion with vatA vesicles, with no effects on LCV fusion with calnexin- or Nramp1-decorated vesicles. Remarkably, the inhibitory effect of the drug on fusion with vat-A-decorated vesicles was observed only with living *Legionella*, not with the non-virulent ΔicmT mutant, *E. coli* or with heat-treated *Legionella*. The finding that HM1284 cells did not differ from the parental AX2 cells for LCV and vatA colocalization seems to exclude class I PI3Ks as target of LY294002 inhibition.

No wild-type *Legionella* were found in vacA-decorated vesicles during the first 2 hours after infection (data not shown). Colocalization with vacA, as well as vatA, increased from 4–8 hours up to 24 hours after infection, whereas the values remained constant for GFP–Nramp1 (Fig. 6E). At 48 hours after infection, GFP–Nramp1 expression declined sharply and no data could be collected. It must be also pointed out that at this stage many cells failed to adhere or underwent lysis upon manipulation. Therefore, the 48 hour values for vatA and vacA (Fig. 6E) refer only to the small number of cells that could be observed. In these cells, several bacteria were dispersed in the cytoplasm or clustered in very large vesicles coated with vatA or vacA (see Fig. 5, vacA), but it was unclear whether this was representative of the culture.

Effects of PI3K inhibition on *Legionella* uptake and intracellular growth in macrophages

To study whether *Legionella* uptake by macrophages is also sensitive to PI3K inhibition, we used the macrophage cell line J774. In contrast to *Dictyostelium*, centrifugation was not required for efficient infection with macrophages, thus *Legionella* uptake was assayed by incubating *L. pneumophila* on a macrophage monolayer for different periods of time, followed by addition of gentamycin to kill the extracellular bacteria, and measuring the number of ingested bacteria by the c.f.u. method. As depicted in Fig. 7A, the number of engulfed *Legionella* by macrophages, in contrast to *Dictyostelium*, increased significantly during the 2 hours of incubation, but the process was similarly sensitive to LY294002, with an inhibition of about 40% after 2 hours (Fig. 7B).

When tested in the infection assay, *Legionella* growth in macrophages was delayed during the first 48 hours in the presence of LY294002 (Fig. 7C). It must be pointed out, however, that the c.f.u. score during the first 24 hours reflects the viability of extracellular bacteria rather than intracellular growth, as evident from the declining curve of *Legionella* in the absence of cells (Fig. 7C). In addition, the bacteria continue to be taken up by the cells at a different rate in the presence or absence of LY294002, as already shown in Fig. 7A,B. Therefore, the starting number of intracellular bacteria was expected to be much lower in LY294002-treated cells compared with untreated cells, but the actual difference during the first 24 hours cannot be estimated, because of the higher number of colonies formed by the extracellular viable bacteria (Fig. 7A). A more precise measurement of growth is thus the ratio of *Legionella* intracellular replication at 96 versus 48 hours of incubation. When calculated in this way, *Legionella* intracellular growth increased only 8.5-fold in the control, but 15- and 24-fold in the presence of 20 μM and 50 μM LY294002, respectively (Fig. 7D). Thus, in macrophages, similarly to *Dictyostelium*, the PI3K inhibitor LY294002 partially inhibits uptake, but stimulates intracellular growth.

Discussion

Dictyostelium infection by *Legionella* occurs only when the bacteria are centrifuged onto a cell layer or are in close contact with the cells, e.g. by incubating bacteria in a thin liquid layer with cells. It

is assumed that these procedures simply enhance bacterial attachment to the cell surface, thus favoring phagocytosis. However, similar precautions are not necessary, though they are often used, for infection by *Mycobacterium* (Hagedorn and Soldati, 2007; Peracino et al., 2006; Skriwan et al., 2002), *Neisseria* (Colucci et al., 2008) or *Salmonella* (A. Sillo, University of Turin, Italy, unpublished results). Moreover, even in the centrifugation assay, the number of ingested *Legionella* is consistently very low, amounting to 5–10% of the added bacteria, with negligible increase over the 2 hour co-incubation following centrifugation. This is in contrast with the avidity displayed by *Dictyostelium* cells when phagocytosing other bacterial strains, such as *E. coli*.

The very low rate of uptake suggested to us that *Legionella* might be taken up by macropinocytosis, rather than phagocytosis. In contrast to phagocytosis, macropinocytosis does not require particle binding to the cell surface and, for the same reason, is less

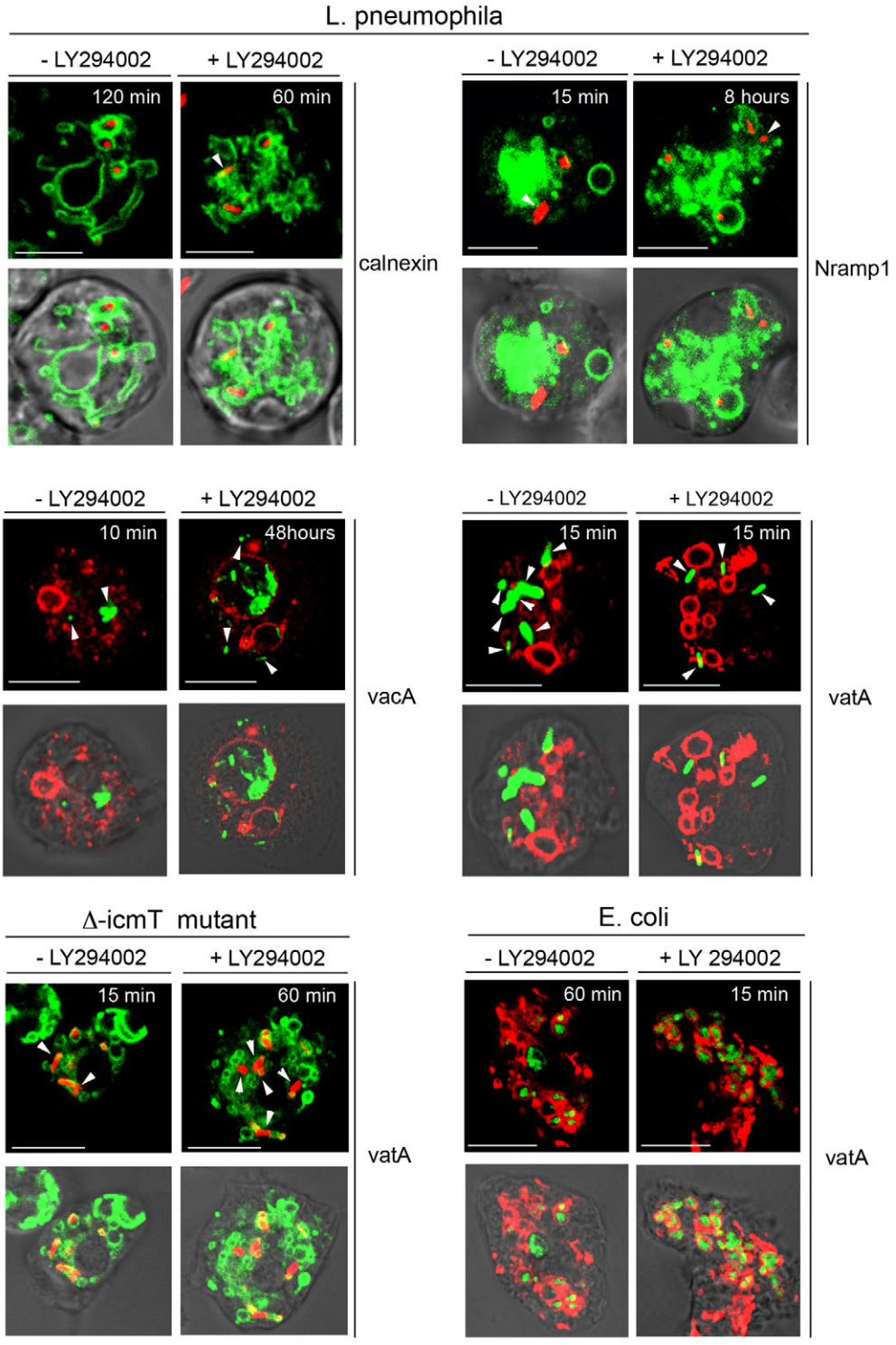

Fig. 5. Confocal microscopy imaging of cells expressing different markers and incubated with wild-type *L. pneumophila*, *ΔicmT* mutant or *E. coli*. Representative samples of fluorescently labelled bacteria in living cells expressing calnexin–GFP or Nramp1–GFP or in fixed cells stained with fluorescent antibodies against the vatA subunit of the V-H^+-ATPase or vacuolin-A. Cells at different time points of infection in the presence or absence of LY294002 are shown to give an idea of changes in cell shape, vacuole size and number of bacteria. Arrowheads pinpoint bacteria outside decorated vesicles. In all cases, merge of the green and red channels (top) or of green, red and phase channels (bottom) are shown. In contrast to the living cells expressing calnexin–GFP or Nramp1–GFP, the cell contour is less evident for vatA- and vacA-labeled cells, because cells were permeabilized with ice-cold methanol for labeling with antibodies. Note that for both wild-type and mutant *Legionella*, the vesicles were either empty or filled with only one bacterium and no relevant differences in cell shape and vesicle size were detected during the first 12 hours of infection. Large vesicles and increased number of bacteria, as evident in the vacA panel are visible only 24–48 hours after infection. Experimental details and quantitative data on the colocalization of bacteria with a given marker are shown in Fig. 6. Scale bars: 5 μm.

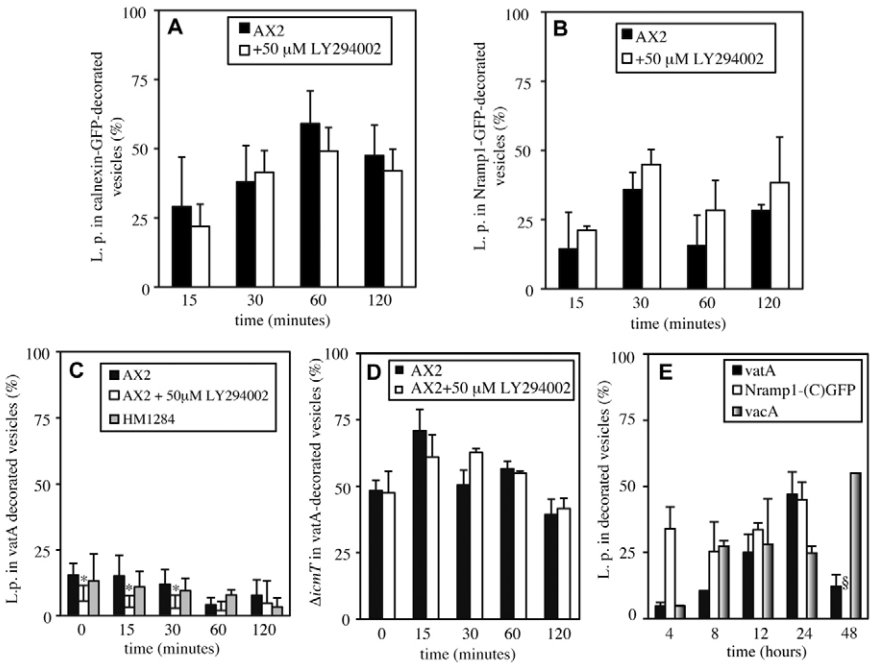

Fig. 6. Effects of LY294002 on the dynamics of bacterial recruitment in endolysosomal vesicles and on bacterial degradation. (**A**–**C**) Percentage of wild-type *Legionella* or (**D**) ΔicmT mutant colocalizing with vesicles decorated with the indicated marker during the first 2 hours after infection. (**E**) Colocalization of wild-type *Legionella* with the indicated marker in AX2 cells at later time points of infection. Wild-type *Legionella* (MOI, 10) and ΔicmT mutant (MOI, 30) were centrifuged onto plated AX2 cells, AX2 cells expressing GFP-fused proteins or HM1284 cells for 10 minutes, washed and chased for the time indicated in the abscissa. Where indicated, LY294002 was present all the time. The number of bacteria colocalizing with the indicated marker was assessed by examining confocal microscopy images of the type shown in Fig. 5. On average, 100 cells containing about 100 *L. pneumophila* were counted per time point per experiment. The data in each panel are from two to three experiments, except for AX2 and vat-A labelling, for which five experiments were done. Asterisks in C denote statistically significant differences between AX2 cells treated or not with LY294002 (two-sample two-tailed *t*-test assuming unequal variance: **P*=0.04). No significant differences were found between AX2 and HM1284 nor between treated and untreated cells in panels A, B and D. In E, § indicates that no data could be collected at this time point for Nramp1–GFP, because its expression declined sharply.

efficient. In this paper, we have provided several lines of evidence in favor of this hypothesis: (1) engulfment by wild-type isolates, which are unable to macropinocytose, is ten-times lower than with axenic AX2 cells; (2) uptake by axenic strains under shaking is negligible; (3) the partial PI3K dependence of uptake; and (4) the defective uptake in *racH*-null cells. This also evidence that ingestion occurs by macropinocytosis, because bacterial phagocytosis is independent of PI3K (Peracino et al., 1998; Rupper et al., 2001b) and macropinocytosis, but not phagocytosis, is defective in the *racH*-null mutant (Somesh et al., 2006); (5) *Legionella* binding to *Dictyostelium* cells and ingestion under shaking are dramatically improved by treating the bacteria with sodium periodate. Periodate treatment leads to oxidative removal of distal carbohydrate residues from the lipopolysaccharide (LPS) coat, exposing internal core sugar residues linked to the lipid A backbone. *Legionella* are Gram-negative bacteria that possess a non-classical LPS structure, characterized by the distal O-polysaccharide chains, which are homopolymers of legionaminic acid, a carbohydrate similar to sialic acid (Kooistra et al., 2002). *Dictyostelium* cells do not possess receptors for sialic-acid-like residues (Bozzaro, 1985; West et al., 1978) and this could be the reason for the very low binding of *Legionella*.

The differential requirement for PI3K for *Legionella* macropinocytosis compared with *E. coli* phagocytosis can be related to basic differences between phagocytosis and macropinocytosis. In contrast to phagocytosis, which leads to local membrane extrusions induced by particle binding, macropinocytosis implies constitutive and extensive ruffling everywhere on the plasma membrane independently of particle binding, and thus requires more intense re-shaping of the actin cortex (Hacker et al., 1997). Disappearance of PtdIns(4,5)P_2 sites from the cytosolic leaflet of the membrane is essential for actin disassembly from the closing phagosome or macropinosome. PLC activity is sufficient to rapidly hydrolyze PtdIns(4,5)P_2 sites in ~1- to 1.5-μm-diameter bacterial phagosomes (Botelho et al., 2000; Stauffer et al., 1998), whereas for macropinosomes, or larger phagosomes, the additional activity of PI3K, or other enzymes such as OCRL1 (Loovers et al., 2007), might contribute to a more rapid disappearance of PtdIns(4,5)P_2 sites, facilitating actin disassembly and vesicle sealing. This explains why phagocytosis of *E. coli* or 1 μm latex beads is dependent on PLC, but independent of PI3K (Botelho et al., 2000; Peracino et al., 1998; Rupper et al., 2001b; Zhou et al., 1998), whereas phagocytosis of yeast particles and large (>3 μm) latex beads and macropinocytosis are sensitive to PI3K in addition to PLC inhibitors, both in *Dictyostelium* and macrophages (Botelho et al., 2000; Bozzaro et al., 2008; Dormann et al., 2004; Scott et al., 2005; Vieira et al., 2001).

Legionella entry into cells by macropinocytosis might not be restricted to *Dictyostelium* cells. *Legionella* uptake by the macrophage cell line J774 is also partially inhibited by LY294002, similarly to results found with *Dictyostelium*. An inhibitory effect of LY294002 on *Legionella* uptake by macrophages has been

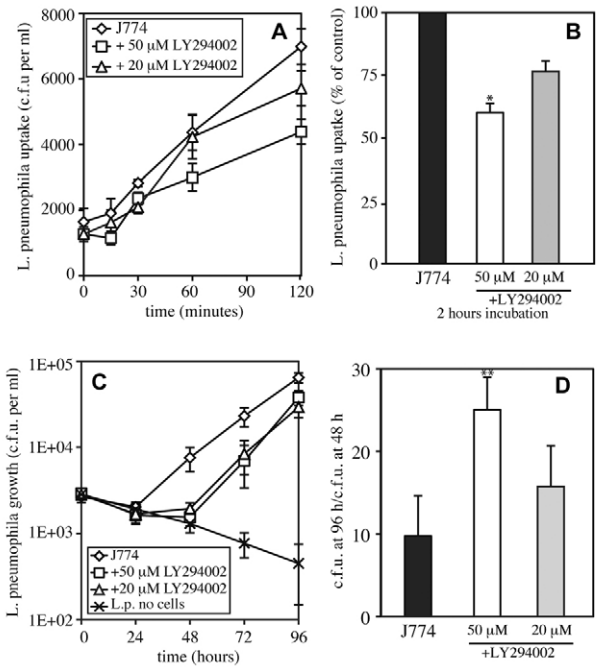

Fig. 7. Effects of LY294002 on *Legionella* uptake and intracellular growth in macrophages. (**A–D**) *Legionella* MOIs were 10 and 0.01 for uptake and infection assay, respectively. Data are means of assays done in duplicate ± s.e.m. Similar results were obtained in at least two independent experiments. In B and D, asterisks denote significant effects of LY294002-treated vs untreated cells (two-sample two-tailed *t*-test assuming unequal variance: **P*=0.004; ***P*=0.0001).

reported by Tachado and colleagues (Tachado et al., 2008). In contrast to *Dictyostelium*, however, *Legionella* uptake by macrophages does not require centrifugation, and the number of ingested bacteria increases steadily over the 2 hours of incubation. This suggests that *Legionella* binds well to the cell surface of macrophages and might be internalized by phagocytosis, although macropinocytosis events cannot be excluded. Insofar as LY294002 affects macropinocytosis rather than phagocytosis also in macrophages, as shown by Grinstein and co-workers (Botelho et al., 2000; Scott et al., 2005; Vieira et al., 2001), the uptake sensitivity of the drug might be considered evidence for *Legionella* uptake by macropinocytosis, in parallel to phagocytosis. Dot/icm-dependent macropinocytosis has been confirmed as an uptake mechanism in mouse macrophages harboring a permissive *Lgn1* allele (Watarai et al., 2001). These authors found that macropinocytosis in the *Lgn1*-restrictive cells was inhibited and the *Legionella* were internalized by phagocytosis and targeted to lysosomes.

Thus one possibility to be considered is that *Legionella* might resemble *S. thyphimurium*, where the degree of virulence has been correlated with macropinosome rather than phagosome formation (Alpuche-Aranda et al., 1994; Hernandez et al., 2004). It can be speculated that macropinosomes, owing to their larger volume, are better suited than tight-fitting phagosomes for favoring bacterial survival, by slowing down the acidification process and diluting potential antimicrobial substances of the host (Watarai et al., 2001).

Despite the reduced uptake, pharmacologically or genetically inhibiting PI3K enhances *Legionella* intracellular proliferation in *Dictyostelium* and, based on the preliminary results reported above, also in macrophages. We have tested two *Dictyostelium* knockout mutants, in which either all five class I PI3K genes or, in addition, the single PTEN gene were disrupted. Stimulation of growth was evident in the sextuple mutant whereas results in the quintuple mutant were statistically less significant. No effects were found in the *pten*-null mutant, compared with the wild type. Weber and co-workers (Weber et al., 2006) have reported a stimulatory effect by LY294002 in a double mutant, where only two of the five PI3K genes were disrupted.

LY294002 treatment also overcomes the protective effect of overexpressing the iron transporter Nramp1. Nramp1 has emerged as a key resistance gene against infection by invasive bacteria in different systems (Courville et al., 2006; Nevo and Nelson, 2006). *Dictyostelium Nramp1*-null mutants are more sensitive to *Legionella* infection, whereas overexpression of Nramp1 from a constitutive promoter effectively inhibits intracellular growth of the pathogen (Peracino et al., 2006). We have shown that this last effect is reversed by LY294002.

Sensitivity to PI3K or PI-PLC inhibitor is restricted to a 2 hour window at the beginning of infection. However enhancement of *Legionella* intracellular growth by LY294002 is not mediated by increased bacterial uptake or by effects of the drug on the actin cytoskeleton. PI-PLC and actin changes are instead exclusively involved in the uptake, confirming the crucial role of modulation of PtdIns(4,5)P_2 in regulating actin assembly and formation of phagosomes and macropinosomes.

Since the stimulatory effect of PI3K inactivation on *Legionella* intracellular growth is a post-uptake event, we investigated whether PI3K affects LCV fusion with endolysosomal vesicles. Our results confirm that the LCV undergoes rapid association and fusion with vesicles originating from the ER (Fajardo et al., 2004; Kagan and Roy, 2002; Lu and Clarke, 2005; Weber et al., 2006), and with Nramp1-decorated vesicles (Peracino et al., 2006), whereas fusion with acidic vesicles (Lu and Clarke, 2005; Sturgill-Koszycki and Swanson, 2000) and with post-lysosomal markers (vacA) was delayed for several hours. In addition, we have shown that LY294002 treatment further inhibits recruitment of vatA, but not calnexin or Nramp-1, to the LCV. However, vatA recruitment to LCV was comparable in AX2 and in the quintuple class I PI3K mutant HM1284, raising the possibility that the additional inhibitory effect of LY294002 is due to inhibition of class III rather than class I PI3K, or to inhibition of both classes of PI3K.

The findings that *Legionella* is able to selectively avoid acidification and that LY294002 treatment further favors this process can explain not only the enhanced growth of *Legionella* upon PI3K inactivation but offers a mechanistic explanation for the reversal of the protective effect of Nramp1 against *Legionella* infection by LY294002, as depicted in the model in Fig. 8. Nramp1 is essential for iron transport in *Dictyostelium* phagosomes and its activity is regulated by a proton gradient providing the force for iron depletion from the phagosome via Nramp1 (Peracino et al., 2006). *Legionella* is known to assimilate significant amounts of iron, and iron has been shown to promote *Legionella* infection (Byrd and Horwitz, 1989; Pope et al., 1996; Robey and Cianciotto, 2002). The culture medium used for the infection assay contains approximately 0.05 mM iron (Franke and Kessin, 1977). If the *Legionella* recruits Nramp1 but not V-H^+-ATPase in the LCV membrane, iron will not be pumped out via Nramp1; in fact, iron might accumulate inside the LCV and be assimilated by the *Legionella*, thus favoring the establishment of a replicative niche.

In striking contrast to virulent wild-type *Legionella*, the vast majority of non-virulent ΔicmT mutant, heat-treated *Legionella* or

E. coli were found soon after engulfment in V-H$^+$-ATPase-decorated vesicles, and this process was insensitive to LY294002. This result is in agreement with previous data, showing that LY294002 treatment did not affect delivery of V-H$^+$-ATPase subunits to *E. coli* phagosomes (Rupper et al., 2001a). The strongly reduced recruitment of V-H$^+$-ATPase in the macropinosomes containing living *Legionella*, and the sensitivity of this process to LY294002 indicate that during the first minutes after infection, *Legionella*, in contrast to non pathogenic bacteria, is able to divert the macropinosome from its normal route, inhibiting fusion with acidic vesicles, in a process that in some way involves inhibition of PI3K, confirming previous results (Weber et al., 2006).

Evidence that pharmacological PI3K inhibition impairs acidification and stimulates intracellular replication of living pathogens is not limited to *Legionella*, but it has been reported for *Neisseria* (Booth et al., 2003), *Salmonella* (Steele-Mortimer et al., 2002) and *Mycobacterium* (Fratti et al., 2001; Fratti et al., 2003; Gillooly et al., 2001), although different mechanisms are involved. In both *Neisseria* and *Mycobacterium* inhibition of PtdIns(3)*P* formation interferes with recruitment of the Rab5 effector EEA1 in the mycobacterial phagosome, blocking delivery of V-H$^+$-ATPase and inhibiting phagosome maturation (Fratti et al., 2001; Fratti et al., 2003). PtdIns(3)*P* formation is also inhibited by toxins that affect binding of class III PI3Ks to the phagosome (Deretic et al., 2006).

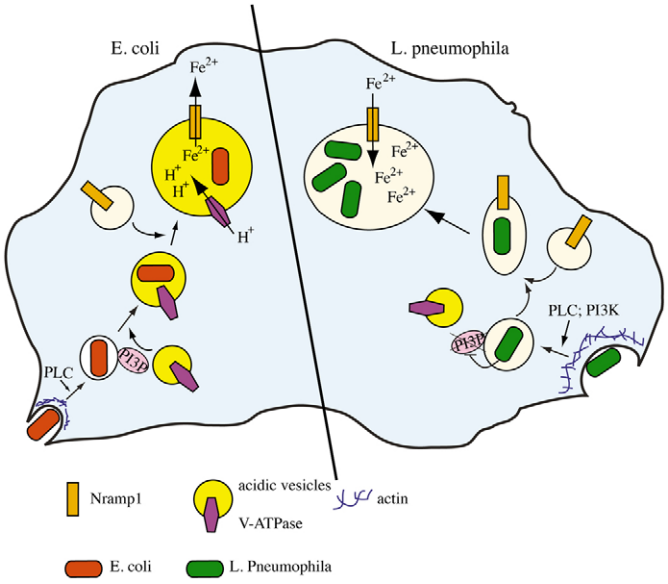

Fig. 8. Model for the differential involvement of PI3K and PLC in the uptake of *L. pneumophila* or *E. coli* and for regulation of Nramp1 activity by PI3K in *Legionella* infection. (Left) Non-pathogenic bacteria, such as *E. coli*, are taken up by phagocytosis, a PLC-dependent, but PI3K-independent process. Upon disassembly of the actin coat from the phagosomal membrane, owing to PtdIns(4,5)P_2 depletion by PLC, the phagosome fuses with acidic vesicles bearing the V-H$^+$-ATPase and shortly thereafter the iron transporter Nramp1 is recruited to phagosomes. V-H$^+$-ATPase delivery requires generation of PtdIns(3)*P* (PI3P) sites on the phagosome. The proton gradient generated by the V-H$^+$-ATPase furnishes the electrogenic force necessary for Nramp1 to pump divalent metal cations outside the phagosome, depleting the phagosome of essential cations, such as iron. (Right) *Legionella* is taken up by macropinocytosis, a PLC- and PI3K-dependent process. Both enzymes act synergistically to deplete PtdIns(4,5)P_2 from the phagosomal membrane, favouring actin disassembly. Soon after engulfment, *Legionella* inhibits V-H$^+$-ATPase recruitment to the *Legionella*-containing macropinosome or vacuole (LCV), with little effect on Nramp1 recruitment. Inhibition of V-H$^+$-ATPase delivery is proposed to depend on inhibition of PtdIns(3)*P* formation on LCV, due to either a 3-phosphatase secreted by *Legionella* or a 3-phosphatase of the host that is anchored to LCV by an effector protein secreted by *Legionella*. PI3K inhibition by LY294002 might favor disappearance of PtdIns(3)*P*, but is not essential. The absence of a proton gradient in the LCV results in neutralization of Nramp1 activity, and thus in storage of iron, a divalent metal cation essential for the pathogen virulence.

A correlation between transient accumulation of PtdIns(3)*P* and delivery of V-H$^+$-ATPase to phagosomes and macropinosomes, with dynamics similar to that of macrophages, has been recently shown in *Dictyostelium* (Clarke et al., 2010). PtdIns(3)*P* accumulates shortly after phago- or macropinosomal vesicle sealing and persists for 5–8 minutes, concomitantly with V-H$^+$-ATPase recruitment to the vesicle membrane. These observations raise the possibility that *Legionella* blocks V-H$^+$-ATPase recruitment by inhibiting PtdIns(3)*P* formation in the LCV, and would be consistent with our data suggesting that LY294002 establishes a replicative niche if present from beginning of infection and not if added later. There is, however, a prima facie discrepancy between the finding that LY294002 affects *Legionella*-induced inhibition of V-H$^+$-ATPase recruitment to the LCV but has no effects on phagosome fusion with acidic vesicles in the case of non-pathogenic bacteria. This discrepancy can be solved by assuming that *Legionella* inhibits PtdIns(3)*P* formation in the LCV not by acting on PI3K, but by either secreting a 3-phosphatase or anchoring a 3-phosphatase of the host to the LCV (Fig. 8). If this hypothesis is correct, inhibition of class I PI3Ks genetically, or inhibiting both classes of PI3K with LY294002, would indirectly favor a more rapid disappearance of PtdIns(3)*P*.

Legionella virulence depends on a functional Dot/icm type-IV secretion system, a conjugation apparatus that is encoded by several genes and that delivers more than 100 effector proteins in the host cell (Ninio and Roy, 2007). Although most of these proteins contribute to the biogenesis of a replicative niche for *Legionella*, their biochemical function and specific effector activities are unknown. In a proteomic analysis of purified LCV, a putative inositol or phosphatidylinositol monophosphatase of the host has been recently found to be transiently enriched in the LCV (Urwyler et al., 2009). Further studies with *Legionella* mutants will help to identify factors that circumvent LCV fusion with acidic vesicles and their potential relationship with phosphoinositides.

Materials and Methods

Chemicals

PI3K inhibitor LY294002, PLC inhibitor U73122 and the inactive analog U73343, actin polymerization inhibitors cytochalasin A and latrunculin A were from Sigma (Milan, Italy), DMEM, fetal calf serum and penicillin-streptomycin were from from Cambrex (Milan, Italy). All other chemicals were analysis-grade.

Cell and bacterial strains and culture methods

D. discoideum axenic strain AX2 and wild-type strains NC4 and V12M2 were used. Mutants HM1294 (class I 1–5 *pi3k*-null) and HM1295 (class I 1–5 *pi3K* and *pten* null) are derived from AX2 and were generated by homologous recombination (Hoeller and Kay, 2007). The single *pten*-null disruptant mutant is derived from AX3 (Iijima and Devreotes, 2002). The mutants were furnished by the dicty stock center (www.dictybase.org). AX2 cells expressing GFP–Nramp1 were generated previously (Peracino et al., 2006), whereas AX2 cells expressing calnexin-GFP were kindly provided by Annette Müller-Taubenberger (Müller-Taubenberger et al., 2001).

Cells of the NC4 and V12M2 strains were grown in a suspension of 1×10^{10} per ml *E. coli B/r* in 0.017 M Soerensen phosphate buffer pH 6.1 while shaking at 150 r.p.m. and 23±1°C, and harvested in exponential phase, when the concentration of bacteria was reduced to $1–2\times10^9$ per ml (Bozzaro et al., 1987). AX2 and mutant strains were grown axenically in AX2 medium either under shaking (Watts and Ashworth, 1970), or, in the case of HM1294 and HM1295, in tissue culture plates

at 23°C. For strains expressing GFP, 20 mg/ml G418 was added to the cultures. For starvation, cells of all strains and mutants were washed twice in 0.017 M Sorensen phosphate buffer, pH 6.1, and shaken in the same buffer at a concentration of 10^7 per ml (Bozzaro et al., 1987). *L. pneumophila* Corby, the icmT mutant GSR011 (Segal and Shuman, 1998; Weber et al., 2006), and strains expressing GFP or Ds-red Express were grown on buffered charcoal yeast extract agar (BYCE), containing 5 mg/ml chloramphenicol, and incubated for 72 hours at 37°C and 5% CO_2 (Hagele et al., 2000).

Dictyostelium uptake assays with *E. coli* and *L. pneumophila*

Phagocytosis of FITC-labeled *E. coli* B/r was assayed as described (Peracino et al., 1998; Peracino et al., 2006). Briefly, cells were mixed with a 1000-fold excess of bacteria in Sorensen phosphate buffer and shaken at 150 r.p.m. and 23°C in Erlenmeyer flasks. Phagocytosis was stopped by withdrawing samples and diluting in 10-fold excess of ice-cold Sorensen buffer, followed by two washes in buffer at 120 ***g*** for 3 minutes. After cell lysis, the fluorescence intensity was read in a spectrofluorimeter and the number of bacteria per cell calculated by reference to a standard curve of FITC-labeled bacteria obtained by serial dilution. All assays were done in duplicate.

For uptake assay with *Legionella*, fresh GFP- or Ds-red Express-labeled bacteria were used. In some experiments the bacteria were labeled with FITC (Peracino et al., 2006). AX2 cells in axenic medium without maltose were plated in 24-well tissue culture plates. Bacteria were added at a MOI of 10:1, immediately centrifuged at 600 ***g*** for 10 minutes and further incubated for 120 minutes at 25°C. Uptake was blocked by adding 0.08 mM gentamycin for 1 hour to kill extracellular bacteria. After cell lysis, the number of viable *L. pneumophila* was determined by measuring CFU (colony forming units) per ml (Solomon and Isberg, 2000). In some experiments, uptake was assayed under shaking, as described for *E. coli*.

Sodium periodate treatment of *L. pneumophila* and analysis of phagocytosis by flow cytometry

L. pneumophila were incubated in the dark under gentle shaking with 20 mM $NaIO_4$ for 2 hours. The reaction was stopped by adding 20 mM glucose followed by two washes. The bacteria were labeled with FITC and immediately used for phagocytosis assay under shaking at a MOI of 100:1. Phagocytosis rate was assessed by measuring cell-bound fluorescence in a FACS CyAn ADP flow cytometer (Beckman Coulter) using the Summit 4.3 software.

Infection assay and intracellular growth of *L. pneumophila* in *Dictyostelium*

For *L. pneumophila* infection, 10^5 AX2 and mutant cells in modified AX2 medium were plated in 96-well plates and left to adhere for 15 minutes. Freshly collected *L. pneumophila* were added at a MOI of 1:1 and immediately centrifuged at 600 ***g*** for 10 minutes at room temperature. The number of viable *L. pneumophila* was calculated by counting the CFU per ml immediately after the centrifugation and during the subsequent 96 hours.

Phagocytosis and infection assay with macrophages

The macrophage cell line J774 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 4 mM L-glutamine. Cells were activated with 1 μg per ml of LPS 1 day before the experiment.

For phagocytosis assay, cells at a concentration of approximately 2×10^5 per ml were plated in 24-well culture plates overnight at 37°C in humidified atmosphere containing 5% CO_2, washed twice in PBS and incubated with 2×10^6 *L. pneumophila* per ml in DMEM without antibiotics. At the end of incubation, gentamycin was added for 1 hour to kill extracellular bacteria, then washed well with PBS, and cells were lysed by pipetting with sterile cold water. The number of intracellular bacteria was measured by plating appropriate dilutions in BYCE agar and counting the number of colonies per ml.

For infection assay, macrophages were activated with LPS as above and incubated with 2×10^3 bacteria per ml (MOI 1:0.01) in DMEM. At intervals of 24 hours, cells were lysed in cold water and the number of viable *L. pneumophila* was calculated after appropriate dilution on agar and colony formation by counting the CFU per ml immediately at t_0 and during the subsequent 96 hours.

In vivo microscopy and fluorescence imaging

AX2 Cells expressing Nramp1(C)-GFP or GFP-(N)Calnexin were incubated with *L. pneumophila* as described for the infection assay. At the desired time point, cells were harvested and plated on a 6×6 cm glass coverslip. Confocal serial images were taken on an inverted Zeiss LSM510 microscope equipped with a 100 Neofluar 1.3 oil-immersion objective, by using a multi-track configuration as follows. Track 1: excitation with a 543 nm band from a Helio-Neon laser line, emission collected with a 620 nm long pass filter for TRITC. Track 2: excitation with a 488 nm band from an argon-ion laser line and emission collected with a 510–525 nm bandpass filter for FITC. The scan time of each track was 3.9 seconds.

Antibodies and immunofluorescence labeling

For immunofluorescence studies, cells were fixed with cold methanol, incubated with antibodies against vatA or vacA, stained with the second antibody and mounted with Gelvatol as described (Peracino et al., 1998). TRITC-labeled rabbit anti-mouse (Jackson ImmunoResearch, West Grove, PA) at a final concentration of 0.03 mg/ml was used as a secondary antibody.

Statistical techniques

To compare *Legionella* uptake or proliferation in control and drug-treated or mutant cells, the two-sample *t*-test assuming unequal variances and the one way and non parametric Kruskal–Wallis test were employed. The two-sample *t*-test was also used for comparing colocalization of bacteria-containing vesicles with endosomal markers.

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