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Amphiphysin IIm Is Required for Survival of *Chlamydia pneumoniae* in Macrophages

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Abstract

Macrophages play a critical role in both innate and acquired immunity because of their unique ability to internalize, kill, and degrade bacterial pathogens through the process of phagocytosis. The adaptor protein, amphiphysin IIm, participates in phagocytosis and is transiently associated with early phagosomes. Certain pathogens, including *Chlamydia pneumoniae*, have evolved mechanisms to subvert macrophage phagosome maturation and, thus, are able to survive within these cells. We report here that, although amphiphysin IIm is usually only transiently associated with the phagosome, it is indefinitely retained on vacuoles containing *C. pneumoniae*. Under these wild-type conditions, *C. pneumoniae* do not elicit significant nitric oxide (NO) production and are not killed. Abrogation of amphiphysin IIm function results in *C. pneumoniae*–induced NO production and in the sterilization of the vacuole. The data suggest that *C. pneumoniae* retains amphiphysin IIm on the vacuole to survive within the macrophage.

Key words: innate immunity • bacterial infection • pathogen • phagocytosis • nitric oxide

Introduction

Chlamydia pneumoniae are obligate intracellular pathogens that cause multiple types of respiratory tract infections and are responsible for 10% of all cases of community-acquired pneumonia. Exposure to this pathogen is quite common; by age 20 yr, 50% of the population has evidence of past infection (1). *C. pneumoniae* causes persistent infection in multiple organs and may be associated with several chronic inflammatory conditions, including atherosclerosis (2–4), asthma (5, 6), reactive arthritis (7, 8), and Alzheimer's disease (9). Interestingly, macrophages are the primary host cell where *C. pneumoniae* are found in all of these chronic conditions.

Macrophages play a critical role in both innate and acquired immunity because of their unique ability to internalize and degrade bacterial pathogens through the process of phagocytosis. Once internalized, the bacterium is contained in a specialized vacuole known as a phagosome. Phagosomes are dynamic organelles that mature over time through a series of fusion and fission events with vesicles of the endosome/lysosome system. Usually the terminal step in macrophage phagocytosis is the formation of a mature phagolysosome in which the bacterium is killed and processed for eventual presentation to $CD4^+$ T cells. Although it is understood that several bacterial pathogens have evolved strategies to subvert macrophage phagosome maturation and thus evade host defense systems (10), little is known about the mechanism by which this is accomplished.

We have shown previously that a novel adaptor protein, amphiphysin IIm, is required for particle internalization during phagocytosis (11). Amphiphysin IIm binds the GTPase dynamin and recruits it to the nascent phagosome (11). Deletion of the SH3 domain of amphiphysin IIm generates a mutant protein that no longer binds dynamin, and functions as a dominant negative inhibitor of amphiphysin IIm activity (11). Thus, expression of $AmphIIm^{SH3-}$ in macrophages prevents the internalization of large particles (>1 μm) by inhibiting membrane extension around the particle.

We report here that, although amphiphysin IIm usually trafficks off phagosomes early in their maturation (∼3–5 min after particle binding), it is retained on the *C. pneumoniae* vacuole for >72 h and its function appears to be a critical factor in the survival of the bacterium.

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Abbreviations used in this paper: MOI, multiplicity of infection; NO, nitric oxide.

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Materials and Methods

DNA Expression Vectors, Cell Lines, and Transfections. Details of the construction of all vectors used in this work have been described previously (11, 12). pTIGZ2 is a bicistronic vector containing a tetracycline-regulated promoter followed by a multiple cloning site, followed by a cap-independent translational enhancer region and the coding region for enhanced GFP. pNeo/Tak was constructed to direct the expression of the tetracycline transactivator under neomycin selection. pTIGZ2-AmphIIm^{SH3-} allows for the coexpression of GFP and the dominant negative form of amphiphysin IIm, in the absence of tetracycline.

The cell line RAW-TT10 is a stable line of RAW 264.7 cells that expresses the tetracycline transactivator. In all experiments in this paper, RAW-TT10 cells were transiently transfected by electroporation. All experiments were performed in the absence of tetracycline to allow for high-level expression of the transfected vectors.

Primary Cells. Murine resident peritoneal macrophages were isolated from CD1 mice (Charles River Laboratories) and cultured as described previously (13). No antibiotics were added to any of the media used in the experiments described in this paper. Bone marrow macrophages were also obtained from CD1 mice. Bone marrow cells were obtained from femurs, plated in Petri dishes in RPMI 1640 with 10% FCS and 20% L-cell media and cultured for 5 d. The macrophages were transferred to glass coverslips and used the next day for experiments.

Bacterial Culture and Infection. *C. pneumoniae* (strain AR-39) was cultured in HL cells and purified by density gradient centrifugation (Hypaque-76; Winthrop-Breon Laboratories; reference 14). The purified organisms were resuspended in sucrose phosphate glutamic acid and frozen at −70°C until use. Infectivity was determined by direct fluorescent staining of chlamydial inclusions using the FITC-conjugated Chlamydia genus-specific Mab, CF-2 (15). To determine the viability and growth of *C. pneumoniae* in macrophages, the macrophages were infected at a multiplicity of infection (MOI) of 10:1, the cells were harvested 3 d after the infection and sonicated, and the infectivity titers were assayed in HL cells. Cells were analyzed with confocal microscopy. Inclusions were counted in 25 high-power from three coverslips for each experiment. Data shown represent the average from at least three separate experiments.

The viability of the macrophages before infection was determined by sorting a known number of cells expressing either p-TIGZ2 or pTIGZ2-AmphIIm^{SH3-} into 96-well plates. These cells were lifted, and viable cell number was determined by counting cells in the presence of trypan blue. The values expressed are corrected for input cell number.

Immunofluorescence, FACS, and Nitric Oxide (NO) Measurement. The antiamphiphysin IIm antibody, M8D10, was generated and characterized as described previously (13). M8D10 was detected with either FITC or Texas red anti–rat IgG secondary antibody (Cappel and ICN Biomedicals). Immunofluorescence and FACS staining was performed as described previously (11, 12). Lysine fixable dextran, which has a molecular weight of 10,000, conjugated to Alexa-Fluor 568 (Molecular Probes) was added to macrophages at a concentration of 500 μg/ml for 2 h, the cells were washed, and the dextran was chased into the macrophage lysosomes for 24 h before particle loading or infections. Zymosan, *Staphylococcus aureus*, and *Escherichia coli* were all labeled with FITC before infections.

Nitrite concentration was measured in supernatant from macrophage cultures using the Griess reagent (Molecular Probes).

Phagocytosis Assays. Full details of the FACS phagocytosis assay and the immunofluorescence procedure have been described previously (12). In this work, 18 h after transfection, RAW-TT10 cells were incubated with the indicated particles (polystyrene "pink" particles; Spherotech, Inc.) for 10 min. The particles were washed off with PBS, and the cells were fixed and analyzed by flow cytometry.

Microscopy was used to confirm that the uninternalized particles were indeed being removed before analysis. RAW-TT10 macrophages were transfected, plated on glass coverslips and, after 18 h, were incubated with biotin-coated polysterene "pink" fluorescent beads (Spherotech, Inc.). The cells were washed and fixed with formalin. The uninternalized beads were stained with streptavidin conjugated to Cy5 (The Jackson Laboratory). Cells were analyzed on a confocal microscope (Leica).

For the *C. pneumoniae* internalization assay, RAW-TT10 cells were transfected with the indicated construct. 18 h later, high-expressing cells were sorted onto coverslips using a FACStar^{PLUS}. The cells were allowed to recover for 24 h, were infected with *C. pneumoniae* at an MOI of 10:1 for 1 h at 37°. Uninternalized bacteria were removed by incubating the cells in trypsin/EDTA for 10 min at 37°. The cells were fixed with formalin, permeabilized with 0.25% Triton X-100 in PBS, incubated with primary antibody (CF2 antibody) and with the secondary antibody (anti–mouse TxR; Cappel and ICN Biomedicals), and analyzed by FACS.

Transmission Electron Microscopy. 18 h after transfection, cells expressing high levels of the indicated vectors were sorted into

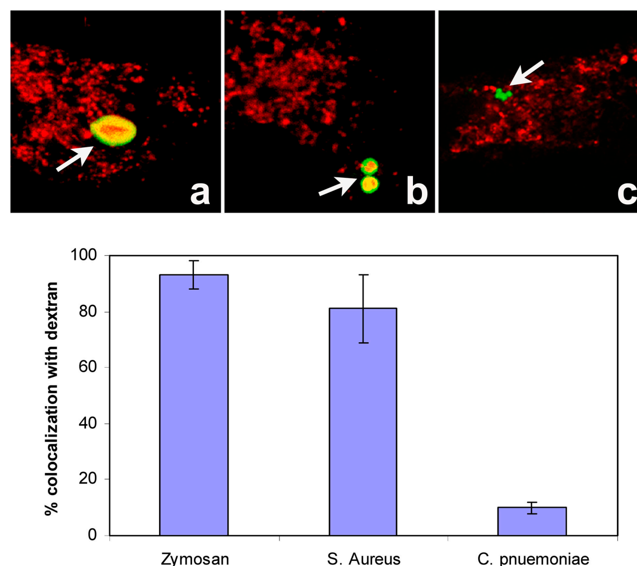

Figure 1. *C. pneumoniae* vacuoles do not develop into mature phagolysosomes. Bone marrow–derived macrophages were pulsed with Alexa-Fluor 568 (red)–labeled dextran, which has a molecular weight of 10,000, and the dextran was chased into lysosomes for 24 h. The cells were allowed to ingest either (a) FITC-Zymosan, (b) FITC–*S. aureus*, for 2 h or (c) *C. pneumoniae* (stained with FITC-CF2 antibody) for 24 h. The pathogens are shown in green and indicated with arrows, dextran is shown in red, and colocalization is indicated by yellow in these merged images. Colocalization of the internalized pathogen with dextran was determined for 100 cells per coverslip. The data shown represent three separate experiments, and the error bars reflect the standard error of the mean.

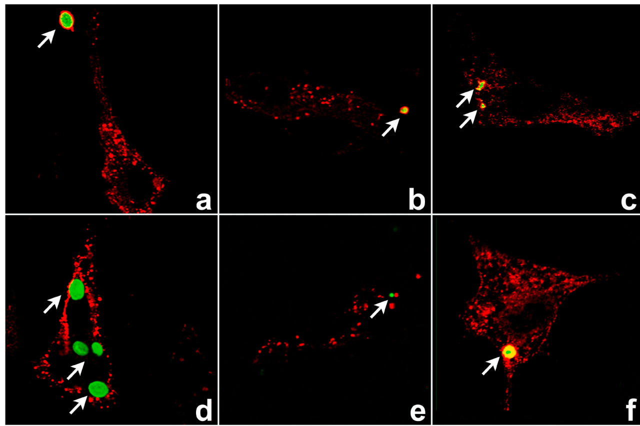

Figure 2. *C. pneumoniae* retain amphiphysin on their vacuole indefinitely. Bone marrow–derived macrophages internalized FITC-Zymosan for 5 min (a) or 15 min (d), FITC–*S. aureus* for 5 min (b) or 15 min (e), or *C. pneumoniae* for 24 h (c) or 72 h (f). *C. pneumoniae* was visualized with FITC-CF2. Amphiphysin was visualized with the M8D10 antibody and an Alexa-Fluor 568 anti–rat secondary. The pathogens are shown in green and are indicated with arrows, amphiphysin is shown in red.

tissue culture dishes using a FACStar^{PLUS}. The cells were infected for 72 h, fixed with 3% glutaraldehyde (in a 0.1-M cacodylate and 0.1-M sucrose buffer) for 1 h, and rinsed with the cacodylate buffer. Cells were post-fixed for 1 h in 1% OsO_4 and dehydrated through a graded ethanol series. They were infiltrated with epoxy resin, polymerized, and cut into 80-nM sections. These were post-stained with 3% urnanyl acetate and lead citrate and examined on a Jeol 1200 ExII transmission electron microscope.

Results

Amphiphysin IIm Is Retained on the C. pneumoniae Vacuole. Certain bacteria, including *C. pneumoniae*, are able to survive inside the normally hostile environment of a macrophage vacuole (16), and by doing so are able to set up a chronic infection. Therefore, we hypothesized that *C. pneumoniae* must be able to interfere with normal macrophage phagosome maturation. In fact, although other pathogens localized to dextran positive late phagolysosomes in murine peritoneal and bone marrow macrophages, within hours of internalization, *C. pneumoniae* were almost never found in this compartment, even 72 h after internalization (Fig. 1 and not depicted).

To identify the mechanism by which *C. pneumoniae* evades killing by macrophages, we characterized the phenotype of vacuoles containing *C. pneumoniae* using 150 novel antiphagosomal monoclonal antibodies (13). Using this screen, we found that amphiphysin IIm transiently associated with early phagosomes containing Zymosan, *S. aureus*, or *E. coli* was shed from these vacuoles before phagosome–lysosome fusion, and remained absent from these vacuoles indefinitely (Fig. 2, a, b, d, and e and not depicted). In contrast, amphiphysin IIm was retained on the *C. pneumoniae* vacuole for at least 72 h (Fig. 2, c and f). Because *C. pneumoniae* survives within its vacuole, the data raised the intriguing possibility that retention of amphiphysin IIm on the *C. pneumoniae* vacuole kept this vacuole in an immature state.

Macrophages Expressing AmphIIm^{SH3-} Internalize C. pneumoniae Normally but Do Not Allow Their Growth. To test this hypothesis, we infected macrophages that expressed the

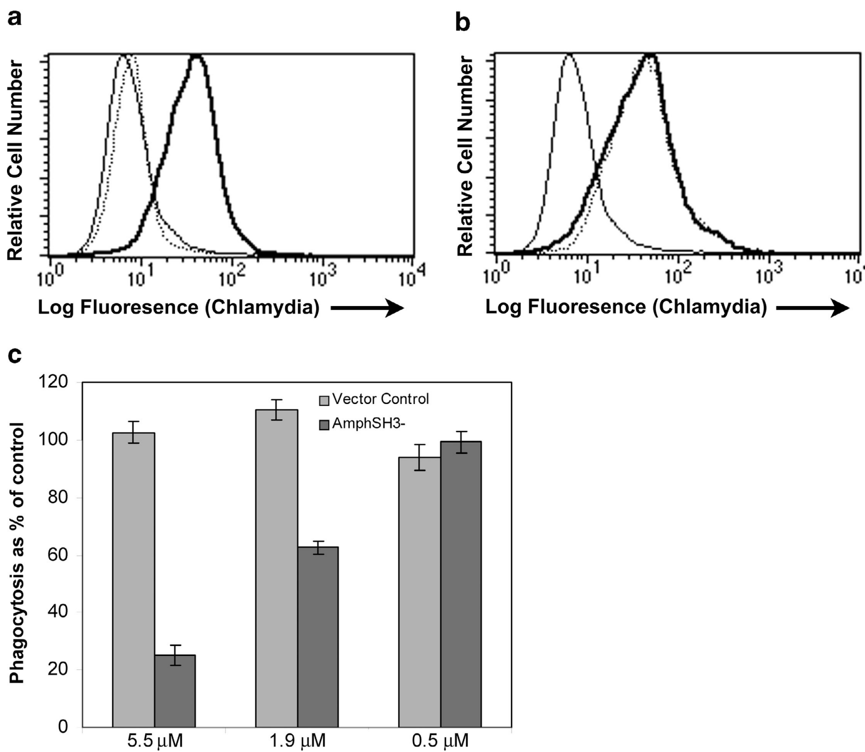

Figure 3. The dependence of particle internalization on amphiphysin function varies with particle size. (a) To ensure that uninternalized bacteria were being removed from the surface of the macrophages, nonpermeabilized RAW-TT10 macrophages that had not been incubated with *C. pneumoniae* (thin line), that had been incubated with bacteria but not washed (thick line), or that had been incubated with bacteria and treated with trypsin-EDTA (dotted line) were stained with the antichlamydial antibody CF-2. (b) RAW-TT10 macrophages were transfected with the control pTIGZ2 vector or the AmphIIm^{SH3-} pTIGZ2 construct. 24 h after transfection, cells expressing high levels of the dominant negative were identified by green fluorescence and sorted by flow cytometry. The cells were allowed to interact with *C. pneumoniae* (MOI: 10:1) for 1 h. Macrophages that had either not been exposed to bacteria (thin line), or had been transfected with control vector and infected (dotted line), or that had been transfected with dominant negative amphiphysin IIm and infected (thick line) were trypsinized, permeabilized, and stained with CF-2 antibody. (c) Transiently transfected RAW-TT10 macrophages internalized particles of the indicated size for 10 min. Uninternalized particles were removed, and the cells were analyzed by flow cytometry. Phagocytosis is expressed as the percent of highly expressing transfected cells internalizing particles relative to the percent of untransfected cells internalizing particles. Control and AmphIIm^{SH3-} cells expressing the same level of GFP were compared. The data shown represent a minimum of three independent experiments, and error bars reflect standard error of the mean.

amphiphysin dominant negative mutant, AmphIIm^{SH3-}, in a bicistronic vector with GFP (pTIGZ2 vector). This allowed transiently transfected cells to be identified by their green fluorescence (12, 17). Interestingly, RAW-TT10 macrophages expressing AmphIIm^{SH3-} internalized *C. pneumoniae* normally (Fig. 3, a and b), suggesting that these particles are internalized in a different way to opsonized sheep red blood cells and Zymosan, whose internalization required functional amphiphysin (11). This difference is due to the small size of the bacterium because latex particles of similar size ($<$0.5 μm) are also internalized in the presence of AmphIIm^{SH3-}, whereas larger latex particles are not (Fig. 3 c). Cox et al. have also demonstrated that particle size governs the route of entry into macrophages (18); large particles require the recruitment of new membrane to the forming phagosome to provide the necessary capacity within the vacuole.

We systematically assayed the role of amphiphysin IIm in *C. pneumoniae* survival in macrophages. RAW-TT10 macrophages expressing the dominant negative form of amphiphysin IIm or empty vector were infected, the cells were lysed, and the lysate was used to infect HL cells; this reinfection step allowed the detection of viable *C. pneumoniae* recovered from the macrophages. We found that AmphIIm^{SH3-} inhibited *C. pneumoniae* growth in macrophages by 87% (Fig. 4 a). This confirmed our hypothesis that retention of amphiphysin IIm on the *C. pneumoniae* vacuole is critical for the survival of the bacteria. Transmission electron microscopy demonstrated large numbers of *C. pneumoniae* contained within vacuoles in control macrophages, whereas cells expressing AmphIIm^{SH3-} had vacu-

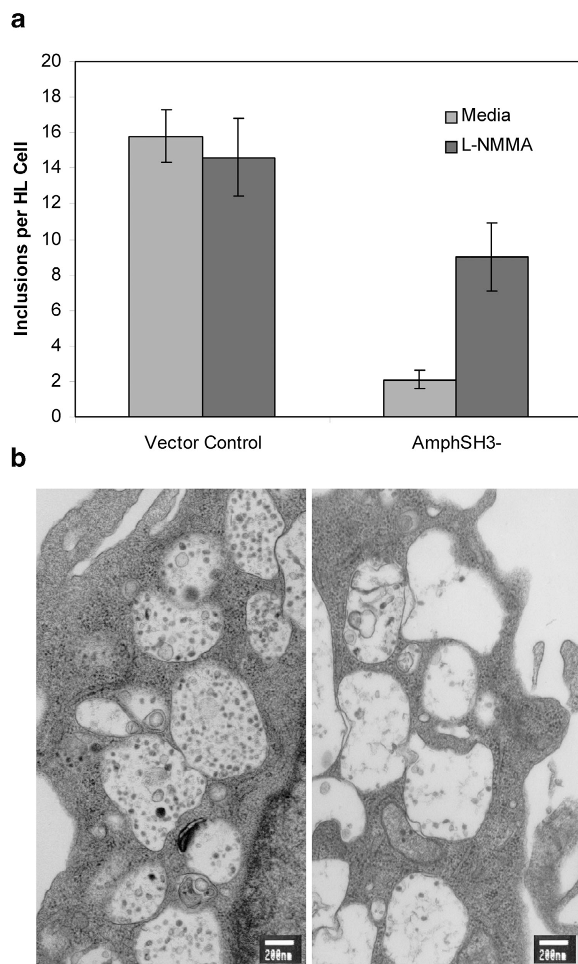

Figure 4. Amphiphysin function is critical for *C. pneumoniae* survival in macrophages. (a) RAW-TT10 macrophages were transiently transfected with either control vector or vector expressing AmphIIm^{SH3-}, and the highest expressers were sorted using FACS®. The cells were treated with media alone (light gray bars) or L-NMMA (dark gray bars) overnight, infected with *C. pneumoniae* for 72 h, and lysed; the lysate was used to infect HL cells. 72 h after the second infection, the HL cells were stained with the CF2 antibody. Inclusions were counted on 25 high-power fields from three coverslips for each experiment. The data represent the average from at least three separate experiments, and the error bars represent standard error of the mean. (b) Transiently transfected RAW-TT10 macrophages were sorted, infected, and analyzed by transmission electron microscopy. The left panel shows a typical cell expressing the vector control and the right panel shows a typical cell expressing AmphIIm^{SH3-}.

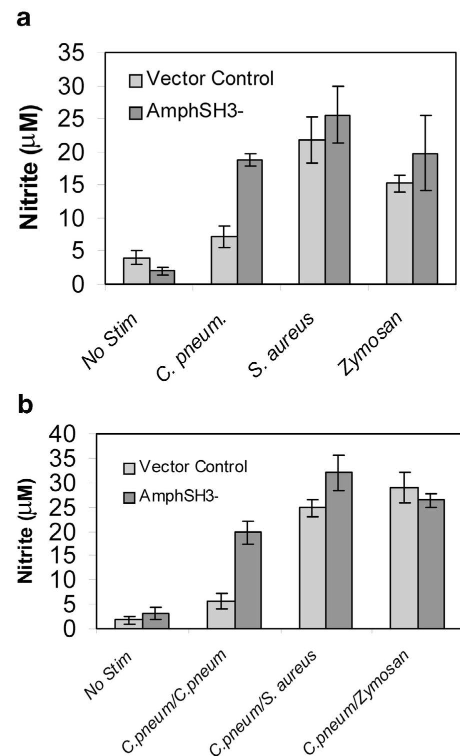

Figure 5. NO production correlates with vacuolar sterilization. (a) RAW-TT10 macrophages were transfected with vector alone (light gray bars) or plasmid-expressing AmphIIm^{SH3-} (dark gray bars), and highly expressing cells were sorted using FACS. The cells were allowed to internalize the indicated particles. 72 h later, NO production was assayed using the Griess reagent. The experiment was repeated four times, and the data are from a typical experiment and represent the average of triplicate samples. The error bars are standard error of the mean. (b) Transfection and sorting was performed as detailed before. The cells were infected with *C. pneumoniae* for 72 h and exposed to the indicated particles for an additional 72 h. NO production was assayed using the Griess reagent. The experiment was repeated three times, and the data are from a typical experiment and represent the average of triplicate samples. The error bars are standard error of the mean.

oles containing remnants of the bacteria with few *C. pneumoniae* visible (Fig. 4 b). Pretreatment of the cells with L-NMMA, an inhibitor of NO, partially rescued *C. pneumoniae* survival in AmphIIm^{SH3-}-expressing cells (Fig. 4 a), suggesting that at least some of the killing is due to induction of NO in these cells. Interestingly, *C. pneumoniae* did not elicit significant NO production from control macrophages, whereas *S. aureus* and Zymosan did (Fig. 5 a). However, when amphiphysin function was inhibited, the cells produced high levels of NO after infection with *C. pneumoniae*, and this correlated with sterilization of the vacuole (Fig. 5 a). There was no difference in Zymosan and *S. aureus* elicited NO production in control cells and cells expressing dominant negative amphiphysin IIm (Fig. 5 a). Although control macrophages did not produce NO in response to *C. pneumoniae* infection, this was not due to a general inhibition of NO production because these cells produced high levels of NO upon exposure to *S. aureus* or Zymosan (Fig. 5 b).

Discussion

C. pneumoniae are able to establish persistent infections in multiple organ systems and this appears to contribute to several chronic diseases, including atherosclerosis, chronic pulmonary disease, and arthritis (2–8). In all of these conditions, the bacteria are found surviving and replicating in the normally hostile environment of macrophage vacuoles. Therefore, we hypothesized that *C. pneumoniae* are able to subvert macrophage phagosome maturation, and we show here a role for amphiphysin in this process. We demonstrated previously that amphiphysin IIm is recruited to nascent phagosomes, where it regulates the insertion of membrane (11). Usually, the molecule trafficks off phagosomes soon after particle internalization, but in the case of *C. pneumoniae* it remains on the vacuole indefinitely, and this is critical for the bacteria's survival.

Amphiphysin has numerous roles in membrane trafficking. Initially, it was shown to be critical in receptor mediated endocytosis; the NH_2-terminal domain of amphiphysin interacts with clathrin, whereas its COOH-terminal SH3 domain interacts with the proline-rich domain of dynamin (19, 20). This results in the targeting of dynamin to forming endosomes, which is necessary for scission of the endosome from the plasma membrane (21). More recent work has demonstrated that amphiphysin also has roles in exocytosis (22, 23) and intracellular vesicular trafficking (24). Amphiphysin also appears to have a direct effect on membrane deformation and curvature (25, 26). In addition to its general role in regulating membrane traffic, we and others have also demonstrated that amphiphysin has a role in phagosome maturation by controlling membrane insertion into the forming phagosome (11, 27). This function of the molecule appears to be related to its capacity to activate dynamin and recruit PI-3 kinase (11). In turn, PI-3 kinase has a central role in regulating membrane traffic (28). Thus the role for amphiphysin in membrane traffic is unequivocal, and it is reasonable to postulate that its capture on the immature phagosome is the underlying reason why the chlamydial vacuole does not progress to a fully fledged phagolysosome where effective killing of the bacterium can occur.

It has been demonstrated recently that a related Chlamydia species, *Chlamydia trachomatis,* is able to survive in macrophages and that this was reversed by the activation of phospholipase D (29). Amphiphysin is known to bind directly to phospholipase D and inhibit its activity by doing so (30); it is possible that by retaining amphiphysin on its vacuole, *C. pneumoniae* may inhibit phospholipase D activity and promote its own survival.

The precise mechanism by which amphiphysin prevents chlamydial killing is not fully elucidated, but it does appear to involve the induction of inducible NO synthase. Macrophage production of NO is known to play a key role in host defense against a wide variety of pathogens including *Mycobacterium tuberculosis*, *Leishmania major*, *Listeria monoctogenes*, and *Toxoplasma gondii* (31). NO also has an important role in control of *C. pneumoniae* infection as evidenced by the observation that inducible NO synthase$^{-/-}$ mice have increased susceptibility to respiratory infection with these bacteria (32), and are more susceptible to *C. pneumoniae* induced exacerbation of atherosclerotic lesions (33).

We demonstrate here that macrophages produce very little NO when they internalize *C. pneumoniae*. However, when amphiphysin function is abrogated, *C. pneumoniae* provokes robust NO production and this correlates with sterilization of the vacuole. *C. pneumoniae* does not globally disable the machinery necessary for NO production, as infected macrophages are still able to produce NO when exposed to a second stimulus.

Together, our data suggest that by retaining functional amphiphysin IIm on the vacuole, *C. pneumoniae* are able to prevent vacuole maturation, thereby avoiding the detection mechanisms that activate NO production.

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