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Analyzing Association of the Endoplasmic Reticulum with the *Legionella pneumophila*–Containing Vacuoles by Fluorescence Microscopy

Alyssa Ingmundson and Craig R. Roy

Summary

A unique feature of the intracellular life cycle of *Legionella pneumophila* is the interaction between the vacuole in which *L. pneumophila* resides and the endoplasmic reticulum of the host cell. This interaction is crucial for *L. pneumophila* to establish a niche in which the bacteria can replicate intracellularly. Microscopic analysis of endoplasmic reticulum (ER) markers during infection yields information regarding the nature of the recruited vesicles as well as the kinetics of their recruitment. The recruitment of YFP-KDEL, GFP-p58, calnexin, and myc-Sec22b to the *L. pneumophila* – containing vacuole can be assessed by fluorescence microscopy. Methods for detection of these various ER markers during infection of mammalian cells by *L. pneumophila* are described.

Key Words: *Legionella pneumophila*; KDEL; p58; calnexin; Sec22b; phagosome; immunofluorescence microscopy.

1. Introduction

Legionella pneumophila are gram-negative bacteria abundant in the environment as intracellular parasites of freshwater amoeba ***(1)***. When *L. pneumophila* are phagocytosed by human alveolar macrophages, growth of the bacteria within these cells can lead to the severe pneumonia known as Legionnaire's disease ***(2–4)***. In order to survive and replicate within these eukaryotic cells, *L. pneumophila* modulate the transport of the phagocytic vacuole in which they reside to create a compartment conducive to *L. pneumophila* growth ***(5)***.

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After internalization of the bacteria by phagocytosis, the *L. pneumophila*–containing vacuole (LCV) circumvents the degradative endocytic pathway by avoiding fusion with late endosomes and lysosomes ***(6–8)***. Subsequently, endoplasmic reticulum (ER)–derived vesicles are recruited to the LCV membrane. The vesicles surround the LCV, fuse, flatten, and become studded with ribosomes such that *L. pneumophila* reside in a compartment resembling the rough ER ***(4,5,9–11)***. Within this ER-like organelle, the bacteria remain protected from host cell defenses and are able to replicate.

Visualization of the intracellular life cycle of *L. pneumophila* by various microscopy techniques has been fundamental to the understanding of the formation of this replication-competent organelle. While electron microscopy has been essential in defining the morphology and composition of the LCV during its establishment, more recently, the use of light microscopy has allowed additional characterization of the interaction of LCV with components of the ER ***(5,6,10,12–14)***. The kinetics of ER recruitment to the LCV and the nature of the recruited vesicles have been examined using markers that label specific subsets of early secretory compartments ***(12,14)***. These markers include yellow fluorescent protein-conjugated KDEL (YFP-KDEL), which cycles between the ER and Golgi, p58, a protein abundant in the ER – Golgi intermediate compartment (ERGIC) and transitional ER, and calnexin, an integral membrane ER-resident protein. In addition, these methods have suggested host factors and mechanisms mediating the interactions between the LCV and ER. For example, specific acquisition of the individual soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), Sec22b, is indicative of its role in LCV development ***(13,14)***. Here, techniques allowing visualization of these markers in mammalian cells during infection are described.

2. Materials

2.1. Preparation of Primary Murine Macrophages

1. A/J mice (Jackson Laboratories, Bar Harbor, ME).
2. Cell strainers are 100-μm nylon strainers (BD, Franklin Lakes, NJ).
3. L-cell conditioned medium: L929 fibroblasts (L-cells; ATCC, Manasassas, VA) are grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) for 10 d after the cultures have become confluent. The supernatant from these cells containing secreted macrophage colony-stimulating factor (MCSF) is filtered using a 0.22-μm filter flask and can be stored in aliquots at –20°C.
4. Bone marrow macrophage medium: 50% RPMI (Invitrogen), 20% FBS, 30% L-cell conditioned medium, 1% penicillin, and streptomycin (Invitrogen).
5. Bone marrow macrophage infection medium: 85% RPMI, 10% FBS, 5% L-cell conditioned medium.

6. Optilux 10 cm Petri dishes (BD).
7. Phosphate-buffered saline (PBS; Invitrogen).
8. Sterilization of 12-mm cover slips is achieved by autoclaving (Fisher Scientific, Pittsburgh, PA).

2.2. Transduction of Bone Marrow–Derived Macrophages

1. HEK 293T cells (ATCC) are propagated in DMEM supplemented with 10% FBS.
2. pCLXSN plasmids encoding YFP-KDEL and GFP-p58 are available from the Roy laboratory ***(12)***.
3. Carrier DNA: sonicated salmon sperm DNA (Stratagene, Cedar Creek, TX).

2.3. Infection of Bone Marrow–Derived Macrophages

1. Wild-type *L. pneumophila* CR39 (Lp01) and Δ*dotA L. pneumophila* CR58 strains are available from the Roy laboratory.
2. ACES–buffered yeast extract (AYE): Use 10 g yeast extract (BD) and 10g of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma, St. Louis, MO) per liter of media. Adjust pH to 6.9 with 1 *N* KOH before adjusting final volume. After sterilization by autoclaving, allow media to cool to at least 55°C. Add 10 mL each of filter-sterilized supplements, L-cysteine (0.4 g/10 mL water) and ferric nitrate (0.135 g [Fe(NO3)3 - 9H20]/10 mL water) (Sigma), to the sterilized media.
3. Charcoal yeast extract (CYE): Prepare as AYE with the addition of 15 g bacto-agar (BD) and 2 g activated charcoal (Sigma) prior to autoclaving. Pour into sterile 10-cm Petri dishes after addition of supplements.

2.4. Infection of CHO FcγRII Cells

1. CHO cells expression FcγRII were obtained from the laboratory of Dr. Ira Mellman and grown in minimal Eagle's media alpha (MEMα; Invitrogen) with 10% FBS ***(15)*** (*see* **Note 1**).
2. Primary antibody: α-*L. pneumophila* rabbit antibody is available from the Roy laboratory.

2.5. Fixation and Staining of Infected Cells

1. Paraformaldehyde (PFA): To prepare a 2% stock solution, dissolve 2 g of paraformadehyde (Mallinckrodt Baker, Phillipsburg, NJ) in 80 mL of PBS while heating and stirring in the fume hood. Heating to 55°C and adjusting the pH to 8.0 with NaOH is required for paraformadehyde to go into solution. Add $MgCl_2$ and KCl to final concentrations of 1 m*M* each.
2. Saponin (Sigma): 0.2% in PBS.
3. Blocking buffer: 2% goat serum (Invitrogen) and 50 m*M* ammonium chloride in PBS.
4. Antibody dilution buffer: 2% goat serum in PBS.

5. Primary antibody: rabbit α-calnexin (Stressgen, Ann Arbor, MI).
6. Primary antibody: α-myc: supernatants of the 9E10 hybridoma cell line were used to detect the myc epitope ***(16)***.
7. Secondary antibodies: Alexa488 α-mouse IgG, Alexa594 α-rabbit, and Cascade blue α-rabbit (Invitrogen).
8. 4,6-Diamidino-2-phenylindole (DAPI; Sigma) is diluted in water to 10 μg/mL.
9. MOWIOL Mounting Media: Combine 6 g glycerol (AR grade, EMD, San Diego, CA) and 2.4 g MOWIOL4-88 (EMD) and vortex. Add 6 mL dH_20 and incubate at 22°C for 2 h. Add 12 mL 0.2 *M* Tris-Hcl, pH8.5, and incubate at 53°C 12–16 h until Mowiol is dissolved. Centrifuge 5000*g* for 20 minutes; store supernatant as 1-mL aliquots at –20°C.

3. Methods

As the LCV develops in a biphasic manner, a series of ER markers localizing to this compartment can be detected. GFP-p58, YFP-KDEL, and myc-Sec22b can be detected on the LCV early after infection as ER-derived vesicles are intercepted by the LCV ***(12–14)***. When visualizing the LCV within host cells, calnexin, a resident ER marker, can be observed later during the maturation of the LCV ***(12)***. To visualize the tagged markers, cells must be made to express the tagged protein—YFP-KDEL, GFP-p58, or myc-Sec22b—prior to infection with the bacteria. Murine bone marrow–derived macrophages (BMM) can be transduced using a retroviral expression system. However, this method is unsuccessful for expression of proteins that disrupt secretory transport due to interference with retrovirus production. In the case of myc-Sec22b, transient transfection of a phagocytic cell line, CHO FCγRII, can be used to achieve expression of the marker prior to infection (*see* **Note 2**). Host cells are infected with *L. pneumophila*, fixed, and stained to allow visualization of both the specific ER marker and the bacteria by fluorescence microscopy (*see* **Note 3**).

3.1. Preparation of Murine Bone Marrow–Derived Macrophages

1. Bone marrow cells are prepared from the femurs of A/J mice as described by Celada et al. ***(17)***. Femurs are removed from the mouse using a sterile scissors, and remaining tissue is removed from bones with a sterile scalpel and gauze.
2. Bones are submerged in 70% ethanol for 10 min followed by rinsing with RPMI.
3. While holding the femur with a forceps, the ends of the bone are removed using a sterile scissors. The marrow is then expelled from the center of the bone with RPMI from a syringe with a 25-gauge needle. Pipet the cell suspension vigorously to achieve homogeneity.
4. Cells are centrifuged at 350*g* for 10 min and resuspended in bone marrow macrophage media. Plate cells at a density of 4×10^6 cells in 20 mL of media

on 10 cm non-tissue culture–treated Optilux dishes and incubate at 37°C in 5% carbon dioxide.
5. Four days after preparation, an additional 10 mL of bone marrow macrophage media is added to the cells.
6. To harvest the differentiated bone marrow macrophages, remove and save the media and incubate the plate with 10 mL of ice-cold PBS for 5 min. Cells are lifted from the plate by vigorous repeated pipetting of cold PBS. Pool and collect cells by centrifugation at 350*g* for 10 min.
7. Plate cells onto sterile cover slips in 24-well dishes and incubate at 37°C. Macrophages not being transduced with retrovirus should be replated 6–9 d after cells are prepared from the mouse and one day prior to infection at 1×10^5 cells per well in bone marrow macrophage infection media.

3.2. Transduction of Bone Marrow–Derived Macrophages

1. Retrovirus harboring the gene of interest, GFP-p58 or YFP-KDEL, is prepared in 293T cells as described by Naviaux et al. ***(18)***. 293T cells are cotransfected with pCL-eco and pCLXSN GFP-p58 or pCLXSN YFP-KDEL using Fugene-6 transfection reagent. Cells grown in DMEM supplemented with 10% FBS to 75% confluence in a 10-cm tissue culture–treated dish are transfected with 4 μg of each plasmid using 24 μL transfection reagent in 1 mL DMEM without serum. Two days following transfection, the DMEM growth media containing recombinant retrovirus is collected.
2. Macrophages are transduced 5 d after cells are prepared from the mouse during replating (*see* **Note 4**). Seed 4×10^4 cells per cover slip in 0.6 mL of a 2:1 mixture of bone marrow macrophage media and 293T supernatant.
3. Two to three days posttransduction, cells are assayed visually for expression of the GFP or YFP fusion proteins.
4. If transduction efficiency is appropriate, replace the cell medium with bone marrow macrophage infection media and perform infection the following day.

3.3. Infection of Bone Marrow–Derived Macrophages

1. *L. pneumophila* strains CR39 and CR58 are cultured in AYE broth for 18 h prior to infection. These cultures are started from bacteria freshly grown on CYE agar for 2–3 d that are resuspended in AYE broth at an OD_{600} of 0.1. After 18 h shaking at 37°C, these cultures should be in postexponential phase with an OD_{600} of approximately 3.4 (*see* **Note 5**).
2. Cell media should be replaced with 0.5 mL bone marrow infection media at least 30 min prior to infection. Postexponential *L. pneumophila* cultures are diluted with sterile PBS such that cells can be infected at a multiplicity of infection (MOI) of 50 based on 1×10^5 cells per well. Prepare *L. pneumophila* assuming an OD_{600} of 1 is equal to 10^9 bacteria/mL (*see* **Note 6**).
3. After addition of the bacteria to the macrophages, plates are centrifuged at 200*g* for 5 min. Cells are immediately warmed to 37°C by floating plates in a water

bath for 5 min. Plates are transferred to a 37°C humidified CO_2 incubator for an additional 5 min.

4. After this synchronized infection period, cells are washed three times with cold PBS. Subsequently, fresh warmed bone marrow infection media is added and cells are returned to the CO_2 incubator for the remainder of the infection.

3.4. Infection of CHO FcγRII Cells

1. CHO FCγRII cells are plated on sterile cover slips in 24-well dishes at 2×10^4 cells per well in MEMα supplemented with 10% FBS.
2. After incubation for 1 day at 37°C in 5% CO^2, cells are transfected with plasmid encoding myc-Sec22b. Each well is transfected with 0.2 μg of the plasmid encoding the marker and 0.3 μg carrier DNA using 1.5 μL Fugene-6 transfection reagent diluted in 25 μL MEMα. Cells are incubated for 14–20 h to allow for expression of the tagged proteins.
3. For *L. pneumophila* to be phagocytosed efficiently by this cell line, infection must be carried out in the presence of IgG to opsonize the bacteria. Therefore, before infection the cell media is replaced with MEMα with 10% FBS containing anti-*L. pneumophila* antiserum diluted 1:1000.
4. CR39 and CR58 strains are grown and prepared as in **Subheading 3.3.** and added to the transfected cells at an MOI of 1.
5. The 24-well dishes are centrifuged at 200*g* for 5 min and transferred to a 37°C water bath for efficient and synchronized uptake of the bacteria. Cells are then washed three times with cold PBS, refreshed with warm MEMα with 10% FBS, and returned to the 37°C CO^2 incubator.

3.5. Fixation and Staining of Infected Cells

3.5.1. Visualizing GFP-p58 or YFP-KDEL on LCV

1. Transduced or transfected cells infected with CR39 and CR58 strains of *L. pneumophila* can be fixed at any desired point in the infection process as early as 10 min postinfection. To fix infected cells, wash cells two times with PBS followed by incubation with 2% PFA for 20 min at 25°C.
2. Cells are washed once with PBS followed by incubation with DAPI at 0.2 μg/mL in PBS for 10 min. DAPI will stain the DNA of both the bacteria and the host cell allowing for visualization of the bacteria. After DAPI staining, wash cover slips three times with PBS.
3. Cover slips are mounted on glass slides with 3 μL of MOWIOL and allowed to dry overnight at room temperature (*see* **Note 7**). p58 or KDEL acquisition on the LCV can then be assessed by fluorescence microscopy. At 30 min postinfection, YFP-KDEL can be detected on approximately 60% of wild-type LCV, and GFP-p58 is present on approximately 20% (*see* **Note 8**). Vacuoles containing CR58 should not display either of these markers.

3.5.2. Visualizing Calnexin on LCV

1. Infected cells are fixed with 2% PFA as described in **Subheading 3.5.1**.
2. After washing cover slips once with PBS, cells are permeablized with 0.2% saponin for 5 min at 25°C. Cover slips are then incubated for 15 min in blocking buffer.
3. Dilute anti-Calnexin 1:250 in antibody dilution buffer. Spot 35 μL of diluted antibody per cover slip onto parafilm and invert cover slips onto individual drops. Incubate for 40 min at room temperature.
4. Return cover slips to 24-well dish and wash three times with PBS, allowing 5 min for each wash.
5. Invert cover slips onto 35- μL drops of secondary antibody Alexa594 conjugated anti-rabbit IgG that has been diluted 1:250 in antibody dilution buffer.
6. Samples should be stained with DAPI as described in **Subheading 3.5.1.** followed by three washes of PBS for 5 min each.
7. Mount cover slips using MOWIOL as in **Subheading 3.5.1**. Calnexin staining can be visualized on LCV by fluorescence microscopy by this method as early as three hours postinfection. At this time point, 30–40% of vacuoles containing wild-type bacteria will stain positive for calnexin as assessed by this method and almost all vacuoles containing replicating bacteria will have calnexin present at later time points after infection.

3.5.3. Visualization of myc-Sec22b in L. pneumophila–Infected CHO-FcγRII Cells

1. Infected cells are fixed with 2% PFA as described in **Subheading 3.5.1**. Prior to permeablization, uninternalized bacteria attached on the cell surface are stained by inverting fixed washed cover slips onto a 35-μL drop of secondary antibody, cascade blue anti-rabbit IgG, diluted 1:250 in antibody dilution buffer.
2. Cover slips are washed three times with PBS followed by a second fixation with 2% PFA. Cells are then permeablized after one wash with PBS by incubation for 5 min with 0.4% saponin in PBS.
3. After a 15-min incubation with blocking buffer, cells are incubated with primary antibody against myc (9E10) for 40 min at 25°C.
4. Cells are washed with PBS three times for 5 min each followed by incubation for 40 min with secondary antibodies. Use Alexa594 anti-rabbit IgG to stain *L. pneumophila* and Alexa488 anti-mouse IgG to recognize the antibody against myc 1:250 in antibody dilution buffer.
5. Wash cells four times with PBS allowing 5 min per wash and mount cover slips as in **Subheading 3.5.1**. By fluorescence microscopy, internalized bacteria will be visible in the red channel, but will not be stained with the cascade blue-conjugated antibody. Sec22b recruitment as visualized by staining against the myc epitope should be present on 50–70% of LCV containing CR39 as determined 30 min postinfection.

4. Notes

1. Penicillin and streptomycin may be included in the cell growth media when propagating the CHO FcγRII cells, but should be removed at least 12 h prior to infection to ensure no deleterious effect on *L. pneumophila*.
2. CHO FcγRII cells can also be used to visualize the YFP-KDEL and GFP-p58 association with LCV by transfecting cells with plasmids encoding these tagged proteins prior to infection as is described for myc-Sec22b analysis.
3. The methods described here allow visualization of ER markers associating with the LCV within the host cell during infection. Alternatively, ER markers can be successfully detected on intact LCV purified from host cells during infection ***(14)***. Analyzing these markers on purified LCV is a more sensitive method. For example, calnexin can be detected on purified LCV as early as 30 min postinfection, whereas calnexin cannot be seen when staining LCV within cells until 2 h postinfection. However, these methods described here examine these markers in the context of the host cell. This attribute allows comparison of the levels of the specific ER-associated protein on the LCV to the cellular levels of the protein.
4. Transduction of BMM is most successful when the BMM are rapidly growing. Monitor cells visually after initially plating the bone marrow–derived cells. On day 5 cells should be 50–60% confluent; otherwise, transduce cells either earlier or later after plating to achieve optimal efficiency.
5. The OD_{600} at which *L. pneumophila* reach postexponential phase will depend on how recently the AYE growth media has been prepared. Preparing several overnight cultures with a series of starting concentrations may be useful to ensure a postexponential phase culture with which to infect.
6. Cells should be infected at an MOI that results in only one internalized bacteria per infected cell. Approximately 10–20% of cells will be infected under conditions that achieve this outcome. Infecting multiple cover slips at various MOIs may be useful to ensure samples with ideal infectivity.
7. After drying, slides can be stored in the dark at either 4°C or –20°C for up to 2 wk.
8. The percent of LCV on which the various markers can be detected can vary among experiments by approximately 20%.

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