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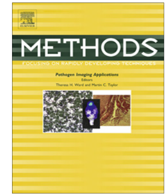

Imaging macropinosomes during *Shigella* infections

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ABSTRACT

Macropinocytosis is the uptake of extracellular fluid within vesicles of varying size that takes place during numerous cellular processes in a large variety of cells. A growing number of pathogens, including viruses, parasites, and bacteria are known to induce macropinocytosis during their entry into targeted host cells. We have recently discovered that the human enteroinvasive, bacterial pathogen *Shigella* causes *in situ* macropinosome formation during its entry into epithelial cells. These infection-associated macropinosomes are not generated to ingest the bacteria, but are instead involved in *Shigella*'s intracellular niche formation. They make contacts with the phagocytosed shigellae to promote vacuolar membrane rupture and their cytosolic release. Here, we provide an overview of the different imaging approaches that are currently used to analyze macropinocytosis during infectious processes with a focus on *Shigella* entry. We detail the advantages and disadvantages of genetically encoded reporters as well as chemical probes to trace fluid phase uptake. In addition, we report how such reporters can be combined with ultrastructural approaches for correlative light electron microscopy either in thin sections or within large volumes. The combined imaging techniques introduced here provide a detailed characterization of macropinosomes during bacterial entry, which, apart from *Shigella*, are relevant for numerous other ones, including *Salmonella*, *Brucella* or *Mycobacteria*.

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1. Introduction

Macropinocytosis describes the non-selective uptake of extracellular molecules into large, liquid-filled vesicles termed macropinosomes. Their formation requires extensive membrane ruffling, a process different from micropinocytosis, which comprises clathrin-dependent and -independent endocytosis of small vesicles [1–5]. One unique feature of macropinosomes in contrast to other endosomes is their varying size. They encompass a diameter range from 100 nm up to 5 μm compared to the approximately 100 nm of endosomes formed during clathrin-mediated endocytosis [6,5,7]. At the molecular level, macropinosome formation depends on the regulation of actin cytoskeletal rearrangements by small Rho GTPases, such as Rac1. In addition, dynamic changes in their membrane phospholipid composition and Rab GTPase association orchestrate macropinosome maturation. Phosphatidylinositol 3-phosphate (PI(3)P) for instance has been found to be essential for the closure of macropinocytic cups after ruffling [8]. Furthermore,

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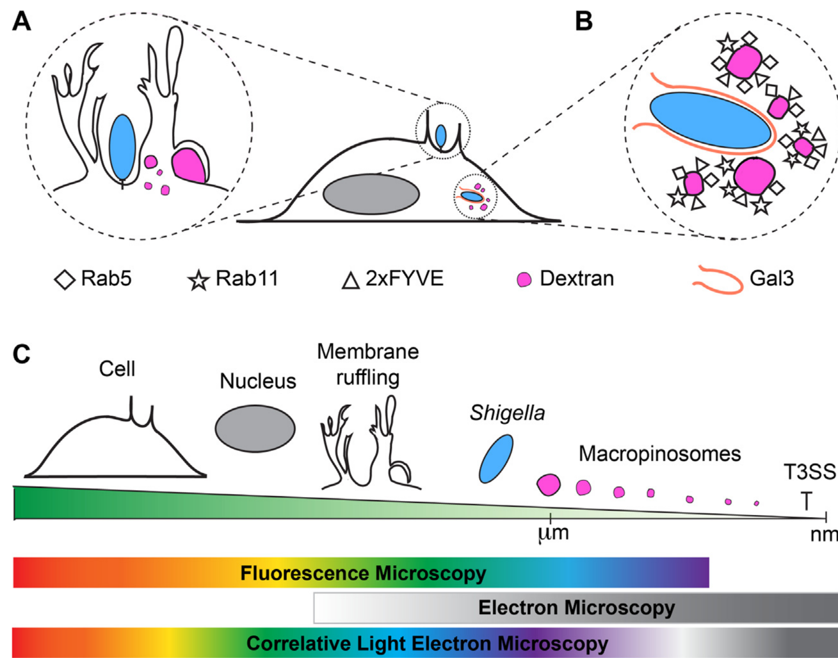

Fig. 1. Macropinosome imaging during early *Shigella* infection with different approaches. **A** and **B**) Schematic illustration of an epithelial cell infected by *Shigella flexneri* with membrane ruffling and macropinocytosis at the entry foci (**A**). The bacteria (blue) enters the cell in a tight bacteria-containing vacuole (BCV) and induces the formation of macropinosomes (pink) filled with extracellular liquid. Macropinosomes border the BCV in the moment of vacuolar rupture and *Shigella*'s release into the host cytoplasm (**B**). The genetically encoded biomarkers and chemical probes for macropinosome detection described in this review are shown. Rab5, Rab11, as well as 2xFYVE (for PI(3)P) (see chapter 2) are depicted as symbols, while dextran-labeling of unspecific uptake of extracellular liquid (see chapter 3) is illustrated in pink. The marker Galectin-3 (Gal3) labels the vacuolar membrane immediately after rupture. **C**) Represented are all components of the scheme arranged according to their size (from nm to µm range). T3SS: Type III secretion system. The spatial resolution of the described imaging modalities are below.

Rab5, Rab7, Rab20, and Rab21 are dynamically recruited to macropinosomes during their successive stages of their life cycle.

An increasing number of pathogens has been described to induce macropinocytosis during their infection processes, like several viruses (Vaccinia, Respiratory syncytial, Dengue, Ebola, Adeno 3 and 35, Kaposi sarcoma; reviewed in [9]) or bacteria (such as *Salmonella* [10], *Shigella* [11], *Brucella* [12] or Mycobacteria [13]). The macropinosomes formed during the infection process either engulf the entering pathogen, or they are observed in the vicinity of the infection site. Even though such infection-associated macropinosomes have been widely declared to directly promote the internalization of invading pathogens, their precise role in pathogenesis remains poorly characterized. For example in the case of mycobacteria, conflicting reports have been made on their epithelial cell uptake either through macropinosomes or through a phagocytic process [14,15].

One of the macropinosome-forming bacteria is the Gram-negative, enteroinvasive bacterium *Shigella flexneri* that has been a major model for the investigation of intracellular pathogenesis. It causes bacterial dysentery in humans and represents a major public health threat [16,17]. Key to *Shigella's* pathogenesis is its ability to induce endocytosis by injecting bacterial type 3 secretion system (T3SS) effectors for invasion into epithelial, non-phagocytic host cells (Fig. 1). *Shigella* enters the cell within a tight bacteria-containing vacuole (BCV) and subsequently escapes into the cytoplasm for replication. There, the bacterium propels itself forward by inducing actin polymerization at one of its poles [18], also enabling its subsequent cell-to-cell spread [19,20]. The current model of early invasion has focused on the subversion of the host actin cytoskeleton at the entry site [21–24]. More precisely, *Shigella* T3SS effectors target the host actin regulating machinery including Rho, Rac, Cdc42, and Src. This induces massive actin rearrangements at the entry foci and forms extensive, actin-rich membrane protrusions like ruffles. These membrane ruffles collapse to form macropinosomes, which are capable to ingest large particles including bacteria [10,25–27]. The next step of *Shigella*'s invasion process after entry is vacuolar rupture, which takes place rapidly within 10 min upon the onset of the entry ruffles. Until recently, little was known about the molecular processes involved in BCV membrane destabilization [28–30].

Studies of our laboratory have contributed to propose a novel view of *Shigella* invasion that takes into account two parallel processes: First, *Shigella* enters the targeted cells in a tight, newly formed endocytic compartment or BCV - this event does not require macropinocytosis. Second, the bacteria simultaneously induce macropinosomes of heterogeneous morphology in their surrounding that are structurally distinct from the BCV (Figs. 2 and 3). The infection-associated macropinosomes make direct contacts with the BCV, though remarkably without membrane fusion (Figs. 4D, 5B), and these contacts are required for efficient vacuolar rupture [11,31]. Therefore, the role of macropinosomes appears to be related with the formation of *Shigella*'s intracellular niche and not with its entry into host cells as previously thought.

With this review we portray current methods that we applied to investigate macropinosomes during *Shigella* infection. We summarize our experience using different approaches based on fluorescence imaging with genetically encoded biomarkers as well as small chemical probes, and correlative light electron microscopy. In each case, we discuss the advantages and limitations of these complementary approaches.

2. Genetically encoded biomarkers and lipid probes to monitor macropinocytosis

One widespread and frequently applied tool for the analysis of different biological processes is the use of fluorescence-based, genetically encoded biosensors expressed and monitored in living cells. They allow the detection of the dynamic subcellular localiza-

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