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Cell Labeling with Magneto-Endosymbionts and the Dissection of the Subcellular Location, Fate, and Host Cell Interactions

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Abstract

Purpose—The purposes of this study are to characterize magneto-endosymbiont (ME) labeling of mammalian cells and to discern the subcellular fate of these living contrast agents. MEs are novel magnetic resonance imaging (MRI) contrast agents that are being used for cell tracking studies. Understanding the fate of MEs in host cells is valuable for designing *in vivo* cell tracking experiments.

Procedures—The ME's surface epitopes, contrast-producing paramagnetic magnetosomal iron, and genome were studied using immunocytochemistry (ICC), Fe and MRI contrast measurements, and quantitative polymerase chain reaction (qPCR), respectively. These assays, coupled with other

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common assays, enabled validation of ME cell labeling and dissection of ME subcellular processing.

Results—The assays mentioned above provide qualitative and quantitative assessments of cell labeling, the subcellular localization and the fate of MEs. ICC results, with an ME-specific antibody, qualitatively shows homogenous labeling with MEs. The ferrozine assay shows that MEs have an average of 7 fg Fe/ME, ~30 % of which contributes to MRI contrast and ME-labeled MDA-MB-231 (MDA-231) cells generally have 2.4 pg Fe/cell, implying ~350 MEs/cell. Adjusting the concentration of Fe in the ME growth media reduces the concentration of non-MRI contrast-producing Fe. Results from the qPCR assay, which quantifies ME genomes in labeled cells, shows that processing of MEs begins within 24 h in MDA-231 cells. ICC results suggest this intracellular digestion of MEs occurs by the lysosomal degradation pathway. MEs coated with listeriolysin O (LLO) are able to escape the primary phagosome, but subsequently co-localize with LC3, an autophagy-associated molecule, and are processed for digestion. In embryos, where autophagy is transiently suppressed, MEs show an increased capacity for survival and even replication. Finally, transmission electron microscopy (TEM) of ME-labeled MDA-231 cells confirms that the magnetosomes (the MRI contrast-producing particles) remain intact and enable *in vivo* cell tracking.

Conclusions—MEs are used to label mammalian cells for the purpose of cell tracking *in vivo*, with MRI. Various assays described herein (ICC, ferrozine, and qPCR) allow qualitative and quantitative assessments of labeling efficiency and provide a detailed understanding of subcellular processing of MEs. In some cell types, MEs are digested, but the MRI-producing particles remain. Coating with LLO allows MEs to escape the primary phagosome, enhances retention slightly, and confirms that MEs are ultimately processed by autophagy. Numerous intracellular bacteria and all endosymbiotically derived organelles have evolved molecular mechanisms to avoid intracellular clearance, and identification of the specific processes involved in ME clearance provides a framework on which to develop MEs with enhanced retention in mammalian cells.

Keywords

Magnetic resonance imaging (MRI); Magnetotactic bacteria; Cell tracking; Autophagy; Endosymbiosis

Introduction

Endosymbiosis is a driver of evolution and is responsible for defining events in the biogenesis of eukaryotes. The mitochondria and chloroplast are the two best known examples of endosymbiotic organelles [1]. The ancestors of endosymbiotically derived organelles and many endosymbionts cluster in α -proteobacteria, suggesting that some aspect of this taxon predisposes its members for intracellular life [2]. Previous work using *Synechococcus elongatus* bacteria provides insight into the steps required to make chloroplasts and illustrates the potential of this type of platform for synthetic biology [3]. Existing models for the origin of eukaryotes generally propose that prokaryotes enter cells via endocytosis [4] and many intracellular pathogens also utilize this entry route.

Figure 1 presents a schematic representation of how some bacteria interact with host cells and illustrates the subcellular mechanisms that pathogenic bacteria use to survive. Non-pathogenic bacteria are endo/phagocytosed, and the bacteria-containing vacuole is processed sequentially by fusion of early endosomes (EEA-1, Rab5), maturation into late endosomes (Rab7/Rab9), and finally acidified by fusion with the lysosomes (LAMP-2) (Fig. 1). The lysosomes digest bacteria using low pH and a high concentration of proteases, lipases, and DNases [5]. These processes will kill most bacteria that challenge the cell; however, endosymbionts and facultative or obligate intracellular pathogens have developed strategies to subvert these processes [6, 7]. Examples of strategies that pathogenic bacteria have developed to survive and replicate within host cells include direct escape from the phagosomal compartment (*Listeria monocytogenes*), inhibition of the phagosomal-lysosomal fusion step (*Salmonella enterica*), or survival within the phagolysosomes (*Coxiella burnetii*) [7] (Fig. 1). In some cases, when a bacterium is taken up by phagocytosis and remains in an intact vacuole, an autophagic process termed LC3-associated phagocytosis (LAP) can promote the maturation of autophagosomes into autolysosomes [8]. To evade this process, *L. monocytogenes* expresses proteins AktA and InlK to interfere with the host mammalian cell's ubiquitination process, which disguise the bacteria from the host cell's autophagic machinery. *L. monocytogenes* also inhibits autophagosome acidification by producing the pore-forming toxin listeriolysin O (LLO) [9, 10] (Fig. 1). Each of these mechanisms relies on the manipulation of the host cell processes to enable survival and long-term persistence within the cell. Understanding the subcellular fate of the invading organism thus provides a necessary framework for engineering endosymbioses [11].

Magnetospirillum magneticum is a magnetotactic α -proteobacterium that synthesizes ordered chains of magnetite (Fe_3O_4) crystals, called magnetosomes, which produce strong MRI contrast [12, 13]. The notion of magnetic endosymbiosis has been around for many years [14], and recently, magneto-endosymbionts (MEs) derived from *M. magneticum*, strain AMB-1, have been used to magnetize host cells and enable visualization of the cells *in vivo* [15, 16]. A key advantage of MEs relative to other MRI contrast agents is signal clearance upon transplanted cell death [15]. Another potential advantage of any living contrast agent is the theoretical ability to persist as the host cells divide, allowing longitudinal cell tracking. Here, a variety of assays utilizing genetic, biochemical, and spectroscopic methods were used to assess subcellular processing and the MRI contrast potential of MEs, which inform ME usage for *in vivo* cell tracking. In their present form, MEs are not retained long term in most cell types and understanding the eukaryote-prokaryote interactions is required to inform future experimental undertakings for ME development.

Methods

ME Labeling of Mammalian Cells by Magnetofection

One day prior to labeling, mammalian cells were plated into six-well plates in culture media without antibiotics (Suppl. Methods 1). The following day, MEs were added to cells at varying ME per cell (MPC) concentrations and labelings were carried out by magnetofection overnight unless otherwise mentioned, using the MagTag-6TM system (Bell Biosystems).

After labeling, the cells were washed three times with media and incubated with gentamycin (50 µg/ml) to remove any extracellular MEs not taken up by the cells.

ICC Staining and Microscopy

Cells labeled on glass coverslips were fixed with 4 % paraformaldehyde and permeabilized with 0.25 % Triton-X. Samples were blocked with 1 % BSA for 10 min at room temperature and stained with an anti-ME primary antibody (generated by Thermo Fisher Scientific, Rockford, IL) diluted 1:1000, for 20 min at 37 °C. EEA-1 (Santa Cruz Biotechnology) staining was performed after a 90-min label; LAMP-2 (DSHB) and LysoTracker™ Red (Thermo Fisher Scientific) were used at 48 h post labeling. Alexa Fluor 594 or 488 secondary antibodies (Invitrogen) were used at 1:500, and Alexa Fluor 488 Phalloidin (Invitrogen) 1:100 was used to stain the cell cytoplasm. Coverslips were mounted with VECTASHIELD containing DAPI (Vector Laboratories). Imaging was performed using an ECLIPSE Ti-S microscope (Nikon, Japan) or a Zeiss laser scanning microscope (LSM) 710 for confocal imaging.

NMR Relaxivity Measurements and MRI

T2 relaxation, of MEs resuspended in 1× phosphate-buffered saline (PBS) pH 7.4, was measured at 60 MHz (1.4 T) and 37 °C using a Bruker Minispec mq60 spectrometer (Bruker, Billerica, MA). For MRI of ME-labeled MDA-231 cells, 0.5×10^6 cells were pelleted in PCR tubes in PBS. A 7-T Pharmascan (Bruker) small animal MRI scanner was used with a RARE pulse sequence and a RARE factor = 2. Scan parameters included TR 4000 ms; TE 16, 48, and 80 ms; slice thickness 1.0 mm; three slices; FOV 70×70 mm²; matrix 256×256 ; two averages; and scan duration 17 min 4 s.

Iron Quantification Using the Modified Ferrozine Assay

ME and ME-labeled cell samples (Suppl. Methods 2) in 200 µl of PBS were digested with 100 µl of acetone for 2 h followed by 100 µl of 2.4 M HCl at 75 °C for 2 h. The ferrozine reagent was then added to each sample and incubated for 1 h at room temperature. Absorbance was measured at 570 nm using a plate reader (Epoch, BioTek). To measure Fe in the free state (component A), the ferrozine reagent was added directly to the undigested sample in PBS. Fe not in the free state, such as in Fe oxide crystals (component B), was calculated by subtracting free Fe (component A) from the total Fe values of digested samples (see Suppl. Methods 3 for Fe quantification by ICP).

gDNA Isolation and Quantification of MEs by qPCR

ME and MDA-231 cell genomic DNA (gDNA) was isolated using QIAamp DNA Mini kit according to the manufacturer's protocol (Qiagen) and quantified using a NanoDrop 2000 (Thermo Scientific). Quantitative polymerase chain reaction (qPCR) measurements of the *mms6* gene for MEs and *β-actin* for mammalian cells, as described previously [17], were performed with SYBR GreenER qPCR SuperMix (Invitrogen), on an Eco Real-Time PCR System (Illumina) with the following cycling program: 95 °C for 8 min followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. ME/cell measurements were

calculated using a standard curve generated from cycle threshold (Ct) values of known numbers of MEs with MDA-231 cells (Suppl. Methods 4).

TEM Sample Preparation and Imaging

Labeled MDA-231 cells on ACLAR discs (Ted Pella) coated with 0.1 % poly-L-lysine were fixed for 1 h with 2 % glutaraldehyde in 0.1 M sodium cacodylate (Electron Microscopy Sciences). Sample processing, embedding, and sectioning onto transmission electron microscopy (TEM) grids was carried out by the UC Berkeley's Electron Microscope Lab. All images were acquired using an FEI Tecnai 12 (FEI, Hillsboro, OR) microscope.

His-LLO Coating of MEs for Labeling

MEs were resuspended in diluted PBS (1:5) containing 1 mM nickel(II) chloride. After 10 min, MEs were pelleted and resuspended in diluted PBS with 0.5 μ M His-LLO (96 Proteins) from a 4 mg/ml stock of purified six-HIS-tagged LLO and incubated for 10 min at room temperature [18]. The MEs were washed twice in 10 ml of diluted PBS, resuspended in DMEM media, and added to cells using a ratio of 500 MPC. The labeling was carried out for 1 h, followed by a wash with media containing gentamycin. Cells were collected at 2, 4, and 20 h post labeling for further analysis.

Microinjection and Staining of NRK Cells with MEs

Normal rat kidney (NRK) cells in microinjection medium (DMEM without bicarbonate, 25 mM HEPES/KOH pH 7.4, and 10 % calf serum) were microinjected with MEs and 2.5 mg/ml of a Dextran, Texas Red (Invitrogen) injection marker, using a Transjector 5246 and Micromanipulator 5171 (Eppendorf) attached to an Eclipse TE300 microscope (Nikon). After a 2 or a 24-h incubation, the cells were fixed with 3.7 % formaldehyde for 15 min at room temperature (RT) and permeabilized for 10 min in methanol at -20°C . Cells were blocked in 1 % BSA and incubated for 30 min at 37°C with the anti-ME antibody (1:1000). Samples were washed and incubated for 30 min at 37°C with 0.65 mg/ml Alexa Fluor 488 (Invitrogen). DNA was stained with Hoechst 33342 (Invitrogen) and mounted in Mowiol 4-88 (Calbiochem) [19]. Images were acquired on an Axiovert 200 M microscope (Zeiss).

ME Injections into Embryos

All animal work adhered to the standards set forth by the NIH and was approved by the Jackson Laboratories Animal and Use Committee. GFP⁺-MEs were loaded into glass needles by capillary action or with a microloader tip on a P20 pipettor and injected into one blastomere of each two-cell embryo (Suppl. Methods 6), using Eppendorf NK2 micromanipulators in conjunction with Narashige IM-5A injectors. After injection, the embryos were placed back into culture and allowed to develop until day 3.5 post copulation prior to imaging (Suppl. Methods 6).

Results

Qualitative Assessment of ME Labeling by Immunocytochemistry

To evaluate the labeling efficiency, potential heterogeneity in labeling, and to identify the ME's subcellular location, an ME-specific polyclonal antibody (anti-ME) was created. Figure 2a presents representative immunocytochemistry (ICC) results using the anti-ME antibody of cells labeled with two different concentrations of MEs and unlabeled control cells. MEs appear as punctate dots primarily in the perinuclear region. Careful inspection of the anti-ME (red), phalloidin (green), and DAPI (blue) composite ICC images shows relatively homogeneous labeling of cells. A slight increase in the number of red dots (MEs) for 5000 MPC (Fig. 2a, middle) demonstrates a qualitative increase in labeling between the 1000 and 5000 MPC. In unlabeled cells, there is no detectable background ICC signal confirming that the antibody staining is specific to MEs (Fig. 2a). Both labeling conditions produced strong MRI contrast as pelleted cell phantoms (far right images); although, no difference in the MRI contrast can be distinguished between the two labeling concentrations due to contrast saturation as the signal approaches background.

To confirm MEs are intracellular, GFP⁺ME-labeled cells were stained with the anti-ME antibody and analyzed by confocal microscopy (Fig. 2b). The orthogonal section of the z-series shows co-localization of GFP (green) and anti-ME antibody (red) resulting in yellow dots co-planar with the nucleus (blue) indicative of intracellular MEs. Some red anti-ME signal is present without the GFP signal, which suggests that the MEs may be digested inside of the cells and, as a result, are no longer expressing GFP.

Quantification of Iron Content of MEs and ME-Labeled Cells Using the Ferrozine Assay

In order to rapidly measure the Fe content of MEs and ME-labeled cells, a modified sample preparation for the ferrozine-based colorimetric assay was developed and compared to ICP measurements. MEs show statistically equivalent Fe content, ~7 fg Fe/ME by both methods (Fig. 3a). The optimized sample preparation for ME-labeled cells shows equivalent results with both methods at ~2.4 pg Fe/cell (Fig. 3a). An increase in the standard deviation for the absorption method, relative to ICP, is reproducibly observed and is likely due to cellular debris absorption and scattering.

To verify the reliable cell concentration range for ME-labeled cells, samples were prepared at different cell concentrations (1×10^5 to 1×10^6 in 200 μ l), all containing 2.4 pg Fe/cell as determined by ICP. Suppl. Fig. 1 shows a slight, though not statistically significant, overestimation of the Fe content at 2.6 pg Fe/cell for 10^6 labeled cells and a slight underestimation of the Fe content at 2.0 pg Fe/cell for 10^5 labeled cells. However, for 5×10^5 and 2.5×10^5 labeled cells, the ferrozine-measured Fe content is consistent with ICP measurements. Although there are some discrepancies for the highest and lowest cell concentrations, all cell concentrations give a reasonable estimation of the amount of Fe loading.

By modifying the sample preparation of MEs in the ferrozine assay as described in the methods, two different forms of Fe (components A and B) can be distinguished. The ratio of these components changes when the Fe concentration in the ME culture media is varied

(Fig. 3b). MEs grown with an Fe concentration of 10–35 μM in the media show a saturation of component B Fe (at approximately 2.2 fg Fe/ME), whereas the total Fe continues to increase due to component A (Fig. 3b). The transverse relaxation rate, R2, of these samples shows a plateauing that mimics the Fe content of component B (black in Fig. 3b), suggesting that the component B Fe pool in MEs contains the magnetosomal, contrast-producing Fe (Fig. 3c). This is further validated using a theoretical calculation of Fe in which the known cubo-octahedral shape of the magnetite crystals [20] was used to measure the volume and calculate the mass of Fe. TEM micrographs were used to measure magnetite crystals of MEs cultured in 35 μM Fe and resulted in an average of 2.33 fg Fe/ME (Suppl. Fig. 2).

Intracellular Fate of MEs

In order to understand the intracellular dynamics of MEs, MDA-231 labeled cells were co-stained with the anti-ME antibody and markers of the endocytic pathway. Figure 4a shows co-labeling data of anti-ME with EEA-1 (an early endosomal marker, left panel, immediately following a 90-min labeling), LAMP-2 (a late endosome, lysosomal marker, mid panel, 48 h post labeling), and LysoTracker (a pH-dependent dye indicative of fully developed lysosomes, right panel, 48 h post labeling). After a 90-min labeling, MEs co-localized with EEA-1, while at 48 h post labeling, MEs were found to co-localize with LAMP-2 and LysoTracker, which are markers of the late endosome and phagolysosome (Fig. 4a). These results suggest that in MDA-231 cells, MEs are processed through the phagocytic pathway.

Various intracellular bacteria escape phagocytosis through the release of protein factors, such as LLO (Fig. 1). Previous studies have shown that non-pathogenic bacteria can be coated with LLO, allowing escape from the primary phagosome [18, 21]. Thus, MEs were coated with LLO. qPCR was used to quantify of the *mms6*/ β -actin ratio over time—note that *mms6* is an ME-specific gene, and a standard curve was used to calculate the number of MEs/cell (Suppl. Fig. 3). For MDA-231 cells that have been labeled with uncoated MEs, a 60 % decrease in the *mms6*/ β -actin ratio was observed in the first 5 h, followed by a further decrease to essentially zero at 20 h. However, MEs coated with LLO show a short-term enhancement of survival (~5 h) suggesting escape from the primary phagosome. This is corroborated by the ICC results in Fig. 4b, which shows that MEs coated with LLO do not counterstain with LAMP-2 at 2 h post labeling. At 20 h, the number of MEs/cell for both coated and uncoated MEs is equivalent suggesting eventual processing of LLO-coated MEs. Interestingly, phagocytosis of MEs does not result in digestion of the magnetosomes, which are the contrast-producing agents. Figure 4c shows TEM micrographs of cells at 48 h post ME labeling, and the yellow arrows illustrate intact magnetosomes. The image also shows that the magnetosomes are in short chains with two to three magnetosomes.

To determine if ME digestion could be avoided using an alternative method of labeling, MEs were microinjected to circumvent phago/endocytosis and provide MEs with direct cytosolic access. Figure 5a shows MEs (green) microinjected into NRK epithelial cells. At 2 h, MEs retain their spirilla morphology (Fig. 5a, top). However, at 24 h, green punctate dots are observed, suggestive of autophagocytosis (Fig. 5a, bottom).

Autophagy is a catabolic host defense process against intracellular microbes that targets intracellular materials to the lysosomal pathway for degradation [22] and thus is an obvious candidate for digestion of MEs which circumvented the primary phagocytic event, either through microinjection or LLO coating. LC3 is an autophagy-specific protein that is recruited to the autophagosomal membrane. When GFP⁺LC3 BMDM cells are labeled with LLO-coated MEs, the anti-ME signal overlaps with the green LC3 signal, indicating that the MEs are taken up into the autophagy pathway (Fig. 5b). This is consistent with the results of GFP⁺LC3 BMDM cells infected with *L. monocytogenes*, which is known to co-localize with LC3 [23] (Fig. 5b).

To further validate ME association with autophagy, BMDM lacking Atg5 (Atg5^{-/-}BMDM), which is responsible for the formation of autophagosomes [24], were labeled with MEs. Figure 5c shows qPCR quantification of MEs/cell over time, for ME-labeled Atg5^{-/-} BMDM compared to ME-labeled control Atg5^{+/+} BMDM cells. The Atg5^{+/+} BMDM trace shows a steady decrease in the number of MEs/cell over time. However, Atg5^{-/-} BMDM - labeled cells show a short-term enhancement of ME survival (~5 h), followed by a steady decrease in signal. By 20 h, the number of MEs/cell for both cell types is equivalent, indicating the eventual digestion of MEs.

Embryos are known to have the autophagocytic process transiently suppressed during early development [25], and Fig. 5d shows the results of Balb/c mouse embryos injected with GFP⁺MEs into one cell of the two-cell stage embryos, the other cell serving as an internal control. Here a strong increasing GFP signal is detected up to the blastocyst stage, where the experiment was discontinued (Suppl. Video 1). Approximately half of the cells in the developing embryos fluoresced in the green channel (compare the two z-slices from the same embryo in Fig. 5d). This suggests that MEs are unable to be transmitted to non-daughter cells (*i.e.*, to the un-injected cell), but are able to reproduce and be passed to progeny in early embryos where autophagy is suppressed. Quantification of GFP fluorescence further supports this conclusion, as an approximate 40× increase, on a per embryo basis, is observed from the two-cell to the eight-cell stage (1.5 days from injection) and a further 40× increase from the eight-cell to blastula stage after another 1.5 days (Fig. 5e).

Discussion

Bacterial-based biomedical technologies are increasing in prevalence and importance [26–29]. Of particular interest are MEs, based on magnetotactic bacteria that enable *in vivo* MRI-based cell tracking of cells that contain them [15, 30]. Here results from various assays are presented that provide both a qualitative and quantitative understanding of ME labeling and information on subcellular processing and fate.

The anti-ME antibody detects epitopes on the surface of the bacteria and was used as a qualitative method for assessing labeling efficiency. Both labeling concentrations resulted in strong MRI contrast. Direct visualization using the anti-ME antibody showed homogenous labeling of cells, which is important for cell tracking as it provides information on whether you are visualizing all cells or only a subset of cells that were successfully labeled. The

ferrozine assay was used to quantify the labeling efficiency by measuring the Fe content in MEs and ME-labeled cells. Given a value of 7 fg Fe/ME for MEs and 2.4 pg Fe/cell for ME-labeled MDA-231 cells, the labeling can be quantified as ~350 MEs/cell.

MRI contrast fundamentally derives from paramagnetic Fe [31], and modifying the sample preparations for the ferrozine assay allows quantification of non-contrast-producing and contrast-producing Fe, described above as components A and B, respectively. This is in contrast to ICP which only measures total Fe content and cannot distinguish between different Fe states. Understanding the amount of free Fe (i.e., non-contrast-producing) is valuable as this form of Fe could result in reactive oxygen species in labeled mammalian cells and elicit a negative subcellular response when MEs are digested. Using the speciation information from the Fe assay allowed for the identification of an optimal ME growth condition, 10 μ m of Fe in the media, which leads to low levels of component A, without impacting the relaxivity properties. This reduces the Fe stress that MEs could have on cells when they are digested. Additionally, quantification of the contrast-producing fraction is valuable as this correlates to MRI contrast and more accurately informs *in vivo* expectations.

The co-localization and time evolution of the anti-ME signal with EEA-1, LAMP-2, or LysoTracker indicate that MEs introduced *via* magnetofection are processed through the phagocytic pathway. qPCR which follows the ME's genome shows a rapid decrease in the ME/cell ratio, consistent with processing of the bacteria. Intact MEs have a single chain with around 16–26 highly organized magnetosome crystals (Suppl. Fig. 2) [32], while TEM micrographs of ME-labeled cells show chains with only 2–3 magnetosomes. This further suggests that the ME bacterial proteins are degraded allowing for separation of the magnetosome chains. Despite the digestion of bacterial cell proteins through this pathway, the magnetosome crystals remain intact and are retained for extended periods. This observation that magnetosomes are not digested explains the ability to track ME-labeled cells for weeks *in vivo* in a non-dividing cell type where there is no dilution of the magnetosome particles [15, 33], despite *in vitro* assessments with ICC or qPCR that suggest digestion of MEs.

For further confirmation that ME digestion occurs the phagocytic pathway, MEs were coated with LLO, a protein known to help other bacteria escape phagocytosis. ICC results show that MEs coated with LLO do not co-localize with LAMP-2, indicating escape from the lysosome. qPCR confirms that coating MEs with LLO allows escape from the primary phagosome as seen in the increased ME/cell ratio at early time points. However, MEs are eventually digested, possibly due to autophagocytosis of MEs that have shed the LLO porin coating after evading the primary phagocytic event. This is supported by the observation that MEs coated with LLO co-localize with LC3, a protein that is known to be associated with autophagy [23]. Additionally, direct introduction of MEs into the cytoplasm by microinjection, where the MEs avoid the phagocytic/lysosomal pathways, still resulted in ME digestion over time.

To show that autophagy is involved in ME digestion, GFP⁺MEs were introduced by microinjection into mouse embryos, in which autophagy is transiently suppressed [25]. In this cell type, MEs are able to survive, replicate, and transfer to progeny cells. Additionally,

in BMDM cells in which Atg5 (a component of autophagosome formation machinery) is suppressed, MEs show a short-term enhancement of survival. The eventual clearance of the MEs in this cell type is likely because the deletion of Atg5 does not completely suppress autophagy in the cell [34, 35]. Although it is well known that Atg5 is critical for autophagy [36, 30], other studies have shown that LC3-positive autophagosomes can be found in Atg5-deficient cells [34, 35]. These results taken together implicate autophagy as the subcellular process that eventually clears MEs.

Intracellular bacteria have evolved mechanisms to avoid or suppress both phago- and autophagocytosis [6, 7, 11]. Utilizing the mechanisms that intracellular pathogens subvert these processes provides a framework for developing MEs with enhanced capacity to replicate in cells. Future studies to genetically engineer MEs that express autophagy suppression genes, enabling longer-term cell tracking are underway.

Conclusions

This work characterizes MEs as a novel MRI contrast agent for cell tracking studies. Various assays including ICC, ferrozine, and qPCR were used to qualitatively and quantitatively dissect cell labeling and the subcellular fate. The ICC images show sufficient cell labeling to produce MRI contrast and that cells are labeled homogeneously. The ferrozine assay allows quantification of total Fe as well as the discrimination of non-contrast-producing and MRI contrast-producing fractions. Together, these provide assurance of the labeling process and inform *in vivo* MRI cell tracking expectations. In addition, a detailed picture of the intracellular localization, fate, and processing of MEs is experimentally defined, which provides valuable insight for future development of the ME. As anticipated from knowledge of bacterial processing by eukaryotic cells, MEs engage with phagocytosis and autophagocytosis machineries in the mammalian cell (see Fig. 1). Phagocytosis can be subverted by coating with porin proteins, such as LLO. However, autophagy eventually clears MEs. The various mechanisms by which organelles, endosymbionts, and intracellular pathogens subvert this process provide a rich framework for genetically engineering the next generation of MEs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Schematic representation of bacterial interactions with mammalian host cells. Depicted are phagocytosis of non-pathogenic bacteria and strategies used by pathogenic bacteria to survive in the host cell. Indicated are host proteins (Rab, EEA-1, LAMP2, LC3) that are found on pathogen-occupied vacuoles and host early endosomes (*blue dotted compartment*), late endosomes (*brown dotted compartment*), and the lysosomes (*red dotted compartment*) with intact (*rod shaped*) and digested bacteria. After internalization within a vacuole, *L. monocytogenes* escape from the vacuole using LLO, escape autophagy using actin-based motility, and survive in the host cytosol. *S. enterica* and *C. burnetii* survive inside vacuoles.

Fig. 2.

Validation of ME labeling by ICC. **a** MDA-231 cells labeled with 1000 MPC, 5000 MPC, or unlabeled control cells. Images were acquired with a $\times 40$ objective, with the anti-ME antibody (*red*, perinuclear dots), Phalloidin 488 (*green*) to visualize cell borders, and DAPI in *blue* to visualize the nucleus. Corresponding cell pellet MRI phantoms are shown to the *right*. **b** ME-labeled cells imaged by confocal microscopy with GFP⁺-ME (*green*), anti-ME (*red*), and nuclei (*blue*). Orthogonal section of the z-series shows the co-localization of GFP and anti-ME, in line with the nuclei stain (*white arrows*), indicating intracellular localization of MEs.

Fig. 3.

Iron quantification. **a** Benchmarking the ferrozine (Fz) assay to ICP measurements, for total iron content per ME agent (Fe/ME (fg)) (*left*) and ME-labeled MDA-231 cell (Fe/cell (pg)) (*right*). **b** Measurements of different iron components for MEs cultured in media supplemented with 5, 10, 15, or 35 μM Fe. Component A represents the non-contrast-producing iron (*gray*) and component B, the magnetosomal or contrast-producing iron (*black*). **c** NMR relaxation rates (R2) of MEs alone, cultured with the same iron concentrations shown in **b**. All data represents mean ± SD from three independent experiments.

Fig. 4.

Intracellular fate of MEs in MDA-231 cells. **a** ICC of ME-labeled cells, anti-ME (red) and EEA-1 (green) after a 90-min label (left), and LAMP-2 (green, middle) and LysoTracker (red, right) 48 h post labeling. Yellow color indicates ME co-localization with the endocytic pathway. Images were acquired with a $\times 40$ objective. **b** ICC with LAMP-2 (red) and anti-ME (green) for cells labeled with uncoated or LLO-coated MEs (ME + LLO) at 2 h post labeling. Yellow color shows MEs co-localizing with the lysosome, while MEs + LLO evades the lysosome. qPCR data (graph) shows processing kinetics for cells labeled with uncoated MEs and LLO-coated MEs (ME + LLO) at 2, 4, and 24 h post labeling. All data represents mean $\pm$ SD from three independent experiments. **c** TEM images of ME-labeled cells, 48 h post labeling at $\times 12,000$ magnification. Intact ME magnetosome crystals are shown with yellow arrows (enlarged portion).

Fig. 5.

Intracellular processing of MEs by autophagy. **a** Microinjection of MEs into NRK cells, at 2 h (*top*) and 24 h (*bottom*) post injection. ICC with anti-ME (*green*), injection marker (*red*), and nuclei (*blue*), imaged with a $\times 40$ objective. The *white arrow* shows intact MEs. **b** GFP⁺-LC3 BMDMs after a 2-h labeling with LLO-coated MEs (ME + LLO) (*top*) with anti-ME (*red*) or RFP⁺ *Listeria* (*red*, *bottom*), with GFP-LC3 (*green*) and DAPI (*blue*). *White arrows* show co-localization of MEs and *Listeria* with LC3. **c** qPCR data for ME processing kinetics in Atg5^{-/-} and Atg5^{+/+} BMDM at 2, 5, and 20 h post labeling. All data represents mean $\pm$ SD from three independent experiments. **d** Live cell imaging of embryos microinjected with GFP⁺MEs (*green*) at the eight-cell stage, slice 1 (*left*), slice 2 (*middle*), and blastocyst stage (*right*), with a $\times 20$ objective. **e** Quantification of GFP signal (arbitrary fluorescent units, AU) at the two-cell, eight-cell, and blastula stage.