RESEARCH ARTICLE

Extrusions are phagocytosed and promote *Chlamydia* survival within macrophages

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Abstract  
The precise strategies that intracellular pathogens use to exit host cells have a direct impact on their ability to disseminate within a host, transmit to new hosts, and engage or avoid immune responses. The obligate intracellular bacterium *Chlamydia trachomatis* exits the host cell by two distinct exit strategies, lysis and extrusion. The defining characteristics of extrusions, and advantages gained by *Chlamydia* within this unique double-membrane structure, are not well understood. Here, we define extrusions as being largely devoid of host organelles, comprised mostly of *Chlamydia* elementary bodies, and containing phosphatidylserine on the outer surface of the extrusion membrane. Extrusions also served as transient, intracellular-like niches for enhanced *Chlamydia* survival outside the host cell. In addition to enhanced extracellular survival, we report the key discovery that chlamydial extrusions are phagocytosed by primary bone marrow-derived macrophages, after which they provide a protective microenvironment for *Chlamydia*. Extrusion-derived *Chlamydia* staved off macrophage-based killing and culminated in the release of infectious elementary bodies from the macrophage. Based on these findings, we propose a model in which *C. trachomatis* extrusions serve as “trojan horses” for bacteria, by exploiting macrophages as vehicles for dissemination, immune evasion, and potentially transmission.

KEYWORDS  
*Chlamydia*, exit, extrusion, macrophage, phagocytosis

1 INTRODUCTION

*Chlamydiae* are gram-negative obligate intracellular bacteria responsible for different diseases of clinical and public health importance. Among these are the causative agents of trachoma, the leading cause of infectious blindness worldwide (Burton & Mabey, 2009), and the most prevalent bacterial sexually transmitted disease in the United States (Gerbase, Rowley, Heymann, Berkley, & Piot, 1998). Infection is predominantly asymptomatic, and disease sequelae result from long-term infections or re-infections that can induce tissue damage and scarring (Darville & Hiltke, 2010). In the absence of diagnosis and treatment, *Chlamydia trachomatis* infection can have severe outcomes, including infertility, ectopic pregnancy, and pelvic inflammatory disease (Bruinen & Rey-Ladino, 2005; Hafner, Wilson, & Timms, 2014). Cellular exit mechanisms for *Chlamydia*, as for other intracellular pathogens, are poorly understood, yet have a fundamentally important role in infection (Hybiske & Stephens, 2008; Friedrich, Hagedorn, Soldati-Favre, & Soldati, 2012). Little mechanistic understanding exists for how *C. trachomatis* disseminate within a host, or transmit to new hosts, yet these mechanisms are likely complex given *Chlamydia*’s intracellular confinement and lack of inherent motility.

The ability to exit the host cell upon completion of intracellular developmental growth is a critical step in bacterial pathogenesis (Hybiske & Stephens, 2008; Friedrich et al., 2012), and *Chlamydia* has evolved two mutually exclusive mechanisms for this task—lysis and extrusion (Hybiske & Stephens, 2007). Lysis is a destructive process, characterized by a sequential rupture of vacuole, nuclear, and plasma membranes culminating in the release of free infectious bacteria (Neeper, Patton, & Kuo, 1990). Extrusion is a markedly different molecular mechanism of cellular exit, utilizing a packaged release strategy of *Chlamydia* that is defined by unique interactions between the bacteria and host cell (Hybiske & Stephens, 2007). Extrusion begins with the invagination of the *Chlamydia*-containing inclusion, followed by a furrowing of the host cell plasma membrane to allow the pinching of the cell, resulting in the release of a membrane-encased compartment containing *Chlamydia*, inclusion membrane, host cell cytoplasm,
a meshwork actin network, and plasma membrane (Doughri, Storz, & Altera, 1972; Chin, Kirker, Zuck, James, & Hybiske, 2012; Hybiske & Stephens, 2007). This process leaves the original host cell intact and often with a residual chlamydial inclusion.

Extrusions are novel pathogen-containing structures, and although there exists some understanding of the molecular pathways that govern the extrusion process (Chin et al., 2012; Hybiske & Stephens, 2007; Lutter, Barger, Nair, & Hackstadt, 2013; Volceanov et al., 2014), very little is known about their structure, internal composition, or functional roles in infection. The conservation of extrusion among chlamydiae is strongly suggestive of important roles in pathogenesis, such as dissemination within a host, transmission to new hosts, and may contribute to chronic infection through immune evasion (Hybiske & Stephens, 2007). In this study, we performed single-cell characterization of the internal composition of extrusions. We found that extrusions are formed without the accompaniment of any host organelles that are otherwise associated with the infected host cell. Importantly, extrusion structures were found to be largely stable up to 4 hr post-extrusion, and C. trachomatis derived from extrusions demonstrated enhanced extracellular survival compared to C. trachomatis released from host cells by lysis. Furthermore, we describe a novel process wherein C. trachomatis extrusions are engulfed by macrophages and promote subsequent survival of bacteria within primary bone marrow macrophages to eventually escape the macrophage while still retaining infectivity.

2 | RESULTS

2.1 | Isolation and enrichment of Chlamydia extrusions

In order to better characterize extrusions, and potential functional benefits for extrusion-derived Chlamydia, a method for enrichment and separation of extrusions from host cells and extracellular Chlamydia is necessary. We developed an extrusion collection method by investigating a series of physical and pharmacological manipulations, and differential centrifugation strategies, to empirically derive a reliable centrifugation method for the isolation and enrichment of extrusions from infected monolayers, which resulted in an enriched suspension of extrusions, with reduced extracellular Chlamydia, host cells, and cellular debris (Figure S1). Extrusions were routinely identified as circular, low-contrast cellular objects that lacked host nuclei and contained numerous bacteria (Figure 1a–b). There was visible heterogeneity in the appearance of extrusions by light microscopy, and some extrusions with high granularity were also typically present (Figure 1a).

2.2 | Cellular composition of Chlamydia extrusions

Because the mechanism of extrusion consists of the contraction of the chlamydial inclusion and infected host cell (Hybiske & Stephens, 2007), it was unknown whether extrusions additionally contained host organelles and other cellular structures. We probed the composition of live extrusions by labeling them with specific fluorescent markers for key host structures, followed by live fluorescence microscopy. HeLa cells were infected with a C. trachomatis LGV serovar L2 strain transformed with a GFP expression plasmid (Wang et al., 2011), and extrusions were collected at 48 hr post-infection (hpi). Enriched extrusions were typically circumscribed by host plasma membrane (Figure 1c), contained GFP-expressing Chlamydia (Figure 1d), and without host nuclei (Figure 1e). These data are in contrast to well-defined cell morphology of C. trachomatis-infected monolayers at 48 hpi (Figure 1g–j).

Chlamydia extrusions are morphologically similar to extracellular merosomes that are produced by malaria-infected hepatocytes, which contain thousands of Plasmodium sporozoites (Baer, Klotz, Kappe, Schneider, & Frevert, 2007; Sturm et al., 2006; Tarun et al., 2006). The external surface of merozoite membranes were shown to lack phosphatidylserine (PS), a marker recognized by phagocytes to engulf apoptotic bodies, thus providing a potential mechanism by which extracellular malaria evade clearance by macrophages (Baer et al., 2007; Graewe et al., 2011; Sturm et al., 2006). Given Chlamydia's similar need to avoid immune clearance, we investigated whether PS was absent on Chlamydia extrusions, by labeling extrusions with fluorophore-conjugated annexin-V, which specifically binds to PS. In contrast to Plasmodium merosomes, PS was frequently present on the surface of extrusions, typically as discrete puncta on the extrusion outer membrane (Figure 2a–d). A punctate PS pattern was present on 59.6% of extrusions (Figure 2m). In 15.3% of extrusions, extensive surface PS was found on extrusion membranes, appearing as PS rings surrounding the extrusion membrane (Figure 2m). Because PS is typically found on the inner leaflet of the host plasma membrane, this subpopulation may represent extrusions with permeabilized outer membranes. About 24.4% of extrusions exhibited no surface PS (Figure 2m). Little PS exposure was observed on the surface of HeLa cells infected with C. trachomatis for 24 and 48 hr (data not shown), consistent with data described previously (Goth & Stephens, 2001).

Historical data suggests that mitochondria may localize in proximity to Chlamydia inclusions (Peterson & de la Maza, 1988) and were therefore strong candidates to potentially associate with extrusions. We probed live extrusions with a mitochondrion-specific dye and determined that 20.3% of extrusions contained mitochondria, in cytosolic regions between vacuole and host plasma membranes (Figure 2e–h,m). HeLa cells infected with mKate2-expressing L2 were also probed at 48 hpi and revealed very little mitochondria staining in infected cells, compared to uninfected cells with robust mitochondria staining within the cell (Figure S2). This may be due to the late stage of infection and the large size of the chlamydial inclusion, making mitochondria harder to spot. Next, we probed for the endoplasmic reticulum (ER) using an ER-specific retroviral transfection reagent specific to identify this organelle inside or on the perimeter of extrusions. We found that 8.3% of extrusions contained ER markers, with a punctate staining pattern seen usually on the cytosolic periphery of extrusions, similar to that seen with mitochondria staining (Figure 2i–m). We additionally probed for the presence of the Golgi apparatus in extrusions, and like nuclei, this organelle was never observed (data not shown).

Transmission electron microscopy (TEM) of isolated extrusions was performed, and 30% of extrusions were found to contain mitochondria, similar to the 20.3% of extrusions quantified by fluorescence microscopy (Figure S3). We additionally observed extrusions with
vesiculated rough ER and secondary lysosomes (Figure S3). TEM confirmed the absence of Golgi apparatus and other discernible organelles within extrusions (Figure 2n). TEM further revealed that extrusions contained \textit{C. trachomatis} encased by a remnant of the chlamydial inclusion, as shown by the presence of the extrusion’s double membrane (Figure 2n, inset). Consistent with other TEM images, the extrusion image in Figure 2n shows a distribution of chlamydial inclusion on the left portion of the extrusion (marked with an “I” in Figure 2n), and host cell cytoplasm collected on the right half portion of the extrusion (marked with a “C”). The lack of organelles, and a similar inclusion distribution, was reported for \textit{in vivo} sections containing \textit{C. pecorum} extrusions (Doughri et al., 1972). Extrusions are thus defined as unique pathogen-containing structures that contain Chlamydia, host cytoplasm, chlamydial inclusion membrane, and plasma membrane that frequently contain externalized PS.

2.3 Extrusions enhance the extracellular survival of \textit{Chlamydia}

We next investigated whether extrusions were capable of delivering infectious \textit{C. trachomatis} to epithelial cells, to inform if extrusions are viable vehicles for facilitating the cell-to-cell spread and dissemination of \textit{Chlamydia}. Isolated extrusions were used to infect HeLa cells, and an evaluation of inclusion formation at 18 hpi revealed many infected

**FIGURE 1** Isolation and enrichment of \textit{Chlamydia} extrusions. Top row: Representative field of extrusions following enrichment procedures, at 20× magnification. (a) Brightfield image showing 14 extrusions (white arrowheads). Inset: enlargement of the extrusion marked with white asterisk. (b) Micrograph of the same field of extrusions shown in (a), labeled with DAPI (blue) to show the lack of nuclei in extrusions. A single host cell with nucleus is shown (yellow arrow). Middle row: Representative micrographs of an isolated extrusion, visualized live with fluorescent probes for (c) host plasma membrane (PM) (FM4-64, red), (d) GFP-expressing \textit{Chlamydia trachomatis} (GFP, green), (e) nuclei (DAPI, blue), (f) merge of (c)–(e). Bottom row: For comparison, micrographs of HeLa cells infected with GFP-expressing \textit{C. trachomatis} L2 for 48 hr post-infection, and visualized live with fluorescent probes for: (g) plasma membrane (FM4-64, red), (h) GFP-expressing \textit{C. trachomatis} (green), (i) nuclei (DAPI, blue), (j) merge of (g)–(i). Scale bar, 10 μm
cells that contained inclusions equivalent in size to cells infected with free C. trachomatis elementary bodies (EB) (Figure S4a–b). These inclusions were likely formed from lysed extrusions, as prematurely large inclusions in cells, which would be indicative of epithelial phagocytosis of extrusions, were never observed. Rather, it is likely that the exposure of C. trachomatis to epithelial cells occurred through the breakdown of extrusions. To ensure that there was no inhibition of subsequent infectivity by proteases or other factors released from broken-down extrusions, samples of extrusions, sonicated extrusions, and purified EB were infected onto HeLa cell monolayers before and after centrifugation to pellet Chlamydia and remove supernatants. Both raw and filtered suspensions of all samples were plated onto HeLa cells for inclusion-forming units (IFU) determination. All samples tested displayed similar IFU counts as purified EB, confirming no loss of infectivity due to factors released by extrusions (Figure S4c).

Outside the nurturing host cell environment, Chlamydia rapidly lose viability—within minutes to hours. We rationalized that extrusions may provide important supportive benefits to Chlamydia, conceptually as an “inclusion-like” environment for these bacteria to inhabit as they traverse the extracellular milieu on their way to infect new epithelial cells. We tested whether extrusions were capable of transiently preserving C. trachomatis survival and EB infectivity during culture outside of host cells. Extrusions were collected from infected HeLa cells at 72 hpi and incubated at 37°C up to 24 hr. As a control,
equivalent suspensions of extrusions were briefly sonicated to disrupt extrusion membranes, yielding an equivalent dose of EB ("free Chlamydia"). At indicated time points, the infectivity of C. trachomatis from both groups (extrusions and free Chlamydia) was quantified by IFU determination.

Free Chlamydia lost viability rapidly, with only 40% viable following 4-hr incubation at 37°C, and 3% of original infectivity present after 24 hr (Figure 3a). C. trachomatis within extrusions lost viability at a much slower rate; 76% viable EB were obtained from extrusions after 4 hr at 37°C, and 32% infectivity of the extrusion-derived EB were obtained after 24 hr (Figure 3a). Extrusion stability assays determined that extrusions remained largely intact for several hours, with 53% of extrusions retaining their structure and shape at 4 hr post-extrusion (Figure 3b). Extrusions continued to break down over time, but this early stability appears to be vital for retaining the prolonged infectivity seen for extrusion-derived Chlamydia (Figure 3a). These results suggest that extrusions may provide a niche environment that more closely mimics intracellular life within an inclusion, to allow enhanced survival of Chlamydia and protection from extracellular stresses.

2.4 Composition of developmental forms within extrusions remains unchanged

We postulated that one mechanistic basis for how extrusions could preserve Chlamydia infectivity is by facilitating continued RB–EB conversion, even while these bacteria are no longer host cell-associated. The conversion of Chlamydia reticulate bodies (RB) to infectious EB requires bacteria to be within an inclusion and host cell, which may be due to contact of the RB with the inclusion membrane (Wilson, Timms, McElwain, & Bavoil, 2006; Wilson, Whittum-Hudson, Timms, & Bavoil, 2009), and therefore would not be expected to occur for Chlamydia released by lysis of the host cell.

To experimentally investigate whether the microenvironment of extrusions was sufficient to enable continued RB–EB conversion, we developed a high-magnification fluorescence microscopy strategy to accurately quantify distinct populations of Chlamydia RB and EB from extrusion samples. Computational imaging algorithms were designed to accurately identify and separate RB and EB into distinct populations. An example of this algorithm is shown in Figure 4, where the RB (white arrows) are separated from EB (yellow arrows), by measurement parameters showing different populations in different colors (Figure 4a–b). The fidelity of measurement parameters was determined in advance using C. trachomatis preparations from sonicated 24 and 48 hpi infected cells that were enriched for RB and EB, respectively (Figure 4c, left columns).

The Chlamydia developmental body composition of extrusions revealed a mean composition of 7% RB and 93% EB immediately after extrusions were collected from infected cultures, at 0 hr. No significant increase in the percentage of EB was detected in extrusions from 0 to 8 hr at 37°C (Figure 4c, right columns). These data indicate that C. trachomatis RB–EB conversion did not noticeably occur in extrusions, or else happened at a level below the threshold of detection for this assay.

2.5 Engulfment of Chlamydia extrusions by macrophages

The presence of externalized PS on the outer surface of extrusion membranes raised the possibility that C. trachomatis extrusions might be readily recognized as apoptotic bodies by professional phagocytes. We explored this hypothesis by infecting primary bone marrow-derived murine macrophages with chlamydial extrusions (derived from infections with GFP-expressing C. trachomatis), and the uptake of extrusions was determined by immunofluorescence analysis. Sonicated extrusions were incubated onto macrophages as...
a control. After the early stages of incubation (0–4 hr), intact extrusions were frequently found inside macrophages (Figure 5a). The size of Chlamydia clusters within macrophages at <4 hr far exceeded what would be expected from a nascent infection, and these clusters were never found in macrophages that were inoculated with sonicated extrusions (Figure 5d) or high multiplicity of infection of Chlamydia EB (data not shown). Thus, it can be concluded that these objects resulted from the engulfment of extrusions. Similar fates were found for extrusions derived from other cell lines, of mouse or human origin, including L929, HeLa, and McCoy cell lines (data not shown).

At later times post-engulfment, 24–72 hr, Chlamydia from broken-down extrusions were commonly seen dispersed within large areas inside the macrophages (Figure 5b–c). Although engulfed Chlamydia from sonicated extrusions were readily visible at the early stages of infection, they were altogether cleared from macrophages at these later times (Figure 5e–f). For macrophages that had engulfed extrusions, no gross morphological changes were apparent. TEM analysis of extrusion-engulfed macrophages confirmed the presence of intact extrusions within the cytoplasm of macrophages at 1 hpi, shown by the yellow arrow (Figure 5g).

Other rare phenotypes were occasionally observed in macrophages following engulfment of extrusions. In some instances at 24 hpi, macrophages appeared to contain fully formed C. trachomatis inclusions as evidenced by the presence of the chlamydial inclusion protein CT223 that was only observed at late times after extrusion engulfment (Figure S5a). Of note, the presence of CT223 was never detected in macrophages containing intact, engulfed extrusions at early times of infection (0–4 hpi), and was diffuse, or altogether absent from all other samples (Figure S2b). This data suggests the capacity of extrusions to productively infect primary macrophages, although infrequently. Additionally, extrusions with enlarged Chlamydia, resembling aberrant bodies, were occasionally observed (Figure 5c). In rare cases of macrophages infected with free Chlamydia, bacteria could still be seen at late stages (Figure 5d), though no inclusions were ever seen from this infection group, which suggests that a productive infection was not common. This result is consistent with previous literature that demonstrated a decreased ability of C. trachomatis to infect primary macrophages (Steele, Steele, Balsara, & Starnbach, 2004).

Intracellular pathogens are often internalized through receptor-mediated phagocytosis, by interaction with host cell surface molecules, including actin cytoskeleton rearrangement (Swanson, 2008). We assessed phagocytosis as the mechanism of extrusion uptake utilized by macrophages by pre-treating the macrophages with an F-actin inhibitor, latrunculin B. The pre-treatment of latrunculin B reduced extrusion uptake by 92%, compared to mock-treated macrophages incubated with extrusions (Figure 5h). These results suggest that macrophages phagocytose extrusions in an actin-dependent manner.

With the majority of extrusions displaying PS, and similar mechanisms utilized by other pathogens for phagocytosis by immune cells (Czuczman et al., 2014), it is possible that the recognition of PS is one of the mechanisms of extrusion engulfment by bone marrow macrophages. To assess this, extrusions were pre-treated with Annexin V prior to incubation onto macrophages, to sterically interfere with receptor binding. Annexin V pre-treatment showed a 40% decrease in extrusion phagocytosis by macrophages (Figure 5h). This partial phenotype is not surprising, given the redundancy in receptor pairs for induction of phagocytosis, variation in the degree of PS exposure by extrusions, and previous reports for incomplete disruption of phagocytosis by Annexin V (Krahling, Callahan, Williamson, & Schlegel, 1999). These data indicate that extrusions enter macrophages through phagocytosis, and partially by receptor-mediated mechanisms through PS recognition.
FIGURE 5  Engulfment of *Chlamydia* extrusions by primary macrophages. Top row: Isolated *Chlamydia* extrusions were incubated with bone marrow-derived macrophages for 1 hr, then rinsed, and fresh macrophage media were plated onto cells for duration of assay; 0–72 hr at 37°C. At stages indicated, cells were fixed and stained to visualize: GFP-expressing *C. trachomatis* (green), macrophage nuclei (DAPI, blue), and actin (phalloidin-647 purple). Representative images of macrophages containing *C. trachomatis* extrusions show distinct stages of their interaction: (a) early, 0–4 hr, presence of intact extrusions; (b) middle, 4–24 hr post-infection, broken-down extrusions, with *Chlamydia* still visible within macrophage; (c) late, 48–72 hr post-infection, no visibly intact extrusions, but *Chlamydia* still visible within macrophage. In other experiments, isolated extrusions were briefly sonicated to release *Chlamydia*, and bacteria were incubated with macrophages for 0–72 hr at 37°C. Representative examples are shown at similar stages as for extrusions: (d) early, some bacteria present in macrophages; (e) middle, very few to no bacteria seen within macrophage; (f) late, no bacteria seen in macrophages. (g) Transmission electron micrograph of a representative macrophage containing an engulfed *C. trachomatis* extrusion following 1 hr co-incubation (shown by yellow arrow). Scale bar, 1 μm. (h) Macrophages were pretreated with 1 or 10-μM latrunculin B, or annexin V, to disrupt phagocytosis and phosphatidylserine recognition, respectively. Bars represent the number of macrophages positive for extrusion phagocytosis. Phagocytosis of *Mycobacterium smegmatis* was evaluated as a control, showing inhibition of uptake with latrunculin (b) treatment. Data show mean percentages ± SEM, n = 3. *** denotes a p value < 0.001, ** denotes a p value < 0.01. Scale bars, 10 μm for (a)–(f); 1 μm for (g).
FIGURE 6  Preferential survival of extrusion-encased Chlamydia within primary macrophages. Extrusions, or sonicated extrusions (free Chlamydia), were incubated onto murine bone marrow derived macrophages and incubated at 37°C for up to 8 hr. At times indicated, cells were sonicated to release bacteria, and Chlamydia trachomatis infectivity was quantitatively measured by performing inclusion-forming units assays on McCoy cells. Data points show mean ± SEM, n = 3. Statistics were performed using two-way analysis of variance with Tukey's multiple comparisons post-test. **** denotes a p value < 0.0001, ** denotes a p value < 0.01.

2.6 Extrusion-derived Chlamydia avoid killing by macrophages

Because Chlamydia were routinely present inside macrophages that had engulfed extrusions compared to free bacteria, and at late times post-engulfment (e.g., >24 hr), we hypothesized that extrusion-derived Chlamydia may benefit from preferential survival inside phagocytic cells. To test this hypothesis, extrusions and free Chlamydia of the same dose were infected onto macrophages for 1 hr, rinsed, then incubated for 8 hr at 37°C. Equivalent doses were prepared by separating enriched extrusions into equal volumes, and one sample sonicated to release Chlamydia from extrusions. IFU analysis was performed to confirm that sonication did not affect infectivity of EB. Following the 1-hr incubation, macrophages were lysed to release bacteria, and Chlamydia infectivity was quantitatively measured by IFU analysis. From macrophages infected with free Chlamydia, EB were only recoverable at 1 hr (3 × 10² IFU/mL), with no IFU detected at 4 or 8 hr (Figure 6). In contrast, significantly more infectious EB were recoverable from extrusion-containing macrophages at 1 hr (1.3 × 10³ IFU/mL), and even out to 8 hr. These data indicate that extrusions were able to transiently protect Chlamydia from phagolysosomal killing by macrophages, thus enabling Chlamydia survival up to 8 hr, and even out to 24 hr (data not shown). It is our interpretation that extrusion-bound Chlamydia are not establishing a productive infection, but delaying clearance from macrophages while retaining infectivity. One predicted in vivo outcome would be the migration of these extrusion-containing macrophages across tissues or to lymph nodes, potentially to elicit differential immune pathway activation and cytokine secretion.

2.7 Escape and infectivity of extrusion-derived Chlamydia from macrophages

The discovery that extrusion-derived C. trachomatis were capable of surviving inside macrophages raised intriguing questions about the beneficial outcomes Chlamydia might gain from this phenomenon. One possibility is that chlamydial extrusions induce eventual death of the host macrophage, thereby releasing Chlamydia into the extracellular milieu. To test whether extrusion-derived C. trachomatis were capable of eventually escaping the macrophage, we infected primary macrophages with extrusions, or sonicated extrusions, (free Chlamydia) for 1 hr, then rinsed macrophages (Figure 7a). Immediately after infection and rinsing, less than 1 IFU/field of C. trachomatis EB were recoverable from macrophages (~40 IFU/mL), indicating that most extracellular bacteria and extrusions had either been engulfed by macrophages or efficiently removed by rinsing (Figure 7b). Macrophages were allowed to incubate for 48 hr at 37°C, then supernatants were collected and subsequently plated onto HeLa cells to determine infectious Chlamydia that had escaped the macrophage. A significant amount of extrusion-derived EB (1.5 × 10³ IFU/mL) were present in macrophage supernatants 48 hr after extrusion engulfment (Figure 7b). This was in marked contrast to macrophages that were infected directly with free Chlamydia; with 83.6 IFU/mL present in culture supernatants at 48 hr (Figure 7b). These results provide compelling evidence that unlike phagocytosed free Chlamydia, extrusions provide a temporary niche for the transient survival inside primary macrophages. Furthermore, this trafficking strategy does not lead to a “dead end” for C. trachomatis; after avoidance of phagocytic killing, these extrusion-derived EB are able to ultimately leave the macrophage and infect new epithelial cells.

3 DISCUSSION

Since the discovery of extrusion as a Chlamydia exit mechanism, its functional role and contribution to chlamydial pathogenesis remains unknown. The engulfment and transient persistence of the chlamydial extrusion within a macrophage represents a novel discovery for bacterial pathogenesis (Figure 8). Early after internalization, extrusions existed as intact structures containing dozens to hundreds of bacteria. Within hours, most extrusions began to break down and bacteria were routinely found dispersed throughout the macrophage, and not in a vacuole. Coincident with this progression, a significant percentage of extrusion-derived C. trachomatis were able to withstand intracellular killing by macrophages, as evidenced by the continued (yet declining) presence of infectious C. trachomatis EB at all times of their residence in macrophages. This was in stark contrast to the fate of phagocytosed free C. trachomatis by macrophages. Consistent with the literature (Steele et al., 2004), engulfed free bacteria were rapidly killed by primary macrophages, and only a small percentage were capable of establishing an infection. With 4 results demonstrating that EB to RB conversion is not occurring within extrusions, we hypothesize that the extrusion-bound Chlamydia are uniquely suited to retain infectivity while delaying clearance from the macrophages.
At the late stages of extrusion-derived *C. trachomatis* survival in macrophages, infectious bacteria ultimately emerged in macrophage supernatants, suggesting that these *Chlamydia*, or undefined factor(s) contained within extrusions, can lead to macrophage lysis. We hypothesize that after their dissolution inside macrophages, extrusions present a high number of *C. trachomatis* and *Chlamydia*-derived pathogen-associated molecular pattern(s) to cytoplasmic pathogen-associated molecular pattern-sensing pathways in the macrophage that ultimately trigger macrophage cell death. Ongoing work will define the unique cellular responses of macrophages to chlamydial extrusions, as it carries important consequences for the host response to *C. trachomatis* infection *in vivo*. Although we have observed similar downstream fates of extrusions containing *C. muridarum*, or other *Chlamydia* species and strains, in primary murine macrophages, the outcome of *C. trachomatis* extrusions in primary macrophages from human reproductive tract tissues will ultimately need to be tested.

We determined that extrusion quality was an important factor and goal for downstream functional studies using extrusions. Chlamydial extrusions were durable enough to withstand successive rounds of centrifugation, and were relatively stable for several hours post-extrusion, but they intrinsically permeabilized over time. No deviations in the collection and handling strategy were able to completely prevent this phenomenon. The addition of supplemental bovine serum albumin (Matsumoto, 1981) and protease inhibitors were attempted previously (Hybiske & Stephens, 2007); however, our derived approach resulted in the least amount of breakdown among procedures tested. Collectively, these findings point to an innate tendency for extrusion membranes to weaken and permeabilize. As it pertains to the natural history of *C. trachomatis* infections, this outcome is probably a beneficial one for the bacteria. In this manner, extrusions can provide a temporary, protective niche for extracellular *Chlamydia* while also ensuring that *Chlamydia* ultimately escape to spread to new epithelial cells.

The successful method for isolating *Chlamydia* extrusions from host cells enabled the detailed examination of the internal composition of these structures. Our data demonstrate that the chlamydial extrusion is essentially an extracellular, pathogen-containing compartment. This indicates that the mechanism of extrusion formation is not due to wholesale division or cleavage of the infected host cell—wherein half of the cell gets released as an extrusion along with whatever host cell organelles that happened to accompany it. Instead, the extrusion mechanism appears to have evolved to produce a compartment of *Chlamydia* that are encased by vacuole and host plasma membranes, and little more. One can speculate that the small numbers of mitochondria that occasionally, albeit rarely, accompany extrusions may provide

FIGURE 7 Release of *Chlamydia* elementary bodies from extrusion-infected macrophages. (a) Overview of the experimental strategy depicting extrusion isolation, infection times in macrophages, and collection of released *Chlamydia trachomatis* elementary bodies. (b) Extrusions, or sonicated extrusions, were used to infect macrophages, and cell supernatants were collected immediately after rinsing (t = 0) and at 48 hr. The infectivity of *Chlamydia* in macrophage supernatants was determined by inclusion-forming units assays on HeLa cells, at 24 hr. Data points show mean ± SEM, n = 3. Statistics were performed using two-way analysis of variance with Tukey’s multiple comparisons post-test. ** denotes a p value < 0.01.

FIGURE 8 Model for the role of extrusions and macrophages in the dissemination of *Chlamydia trachomatis* in the upper female genital tract. Upon release from infected epithelial cells, *Chlamydia*-containing extrusions are engulfed by macrophages. Migration of these macrophages, followed by eventual escape of *Chlamydia* from them, can result in the dissemination of infectious *C. trachomatis* to more distant sites, for example, away from inflammatory foci surrounding the primary site of infection, to draining lymph nodes, or to new hosts. Extrusions may alternatively mediate some of these outcomes without the need for hijacking macrophages.
a temporary source of energy or oxidative buffering for these extracellular Chlamydia.

Although Chlamydia extrusions represent unique structures in the bacterial world, they share morphological similarities with merosomes that are produced by Plasmodium spp. (Baer et al., 2007; Graewe et al., 2011; Sturm et al., 2006; Tarun et al., 2006). After their liver stage conversion from sporozoites to merozoites, Plasmodium exit hepatocytes in membrane-bound compartments containing thousands of parasites. Interestingly, phosphatidylserine externalization was commonly associated with extrusion outer membranes, and blocking PS recognition resulted in a decrease of extrusion phagocytosis. Unlike Chlamydia extrusions, Plasmodium merosomes suppress externalization of PS, and therefore evade interactions with macrophages to efficiently transition to the blood stage of malaria infection (Baer et al., 2007; Sturm et al., 2006; Tarun et al., 2006). It is fascinating to consider both the convergent evolution of the extrusion-like structure for exiting host cells, and also its variable roles for disseminating within a host.

Our data demonstrate tangible benefits for Chlamydia that exit epithelial cells within extrusions. These bacteria are able to survive in the extracellular environment much longer than free C. trachomatis EB, providing intracellular-like benefits to Chlamydia even while they navigate the extracellular environment. These benefits may include enhancing retention of nutrients, energy, or other factors vital to chlamydial viability maintenance of EB infectivity. In this manner, we view the Chlamydia extrusion as a transient, extracellular niche, from which Chlamydia can break out to infect new epithelial cells—likely through delivery of a bolus of infectious EB—and also be protected from extracellular immune defenses. It is conceivable that the release of “packages” of EB onto nascent epithelial cells results in more efficient infection in vivo. Moreover, our data suggest that a major outcome of chlamydial exit within extrusions is to facilitate their uptake by and survival within macrophages. If macrophage killing and Chlamydia escape were to occur after macrophage migration, it could provide an opportunity for C. trachomatis to disseminate in the female genital tract (i.e., ascend to upper genital tract tissue), or evade clearance by innate immune cells. We propose a model in which C. trachomatis exploits macrophages for the advantage of dissemination within a host (away from inflammatory foci), and potentially host–host transmission, as has been proposed for Neisseria gonorrhoeae (Cris & Seifert, 2012) and Staphylococcus aureus (Thwaites & Gant, 2011).

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell culture, Chlamydia propagation and infections

HeLa 229 and McCoy cells, were routinely grown in RPMI 1640 media supplemented with 10% FBS (HyClone, Thermo Fisher Scientific, Rockford, IL) and 2 mM L-glutamine (HyClone), at 37°C with CO₂. For all microscopy experiments, cells were subcultured and plated onto chambered coverglass slides (Lab-Tek II; Nunc, Rochester, NY), or glass bottom culture dishes (MatTek, Ashland, MA) or 6-well and 24-well plates (BD Falcon).

C. trachomatis serovar L2 (LGV 434) was propagated in L929 cells grown in suspension culture or HeLa cells grown in T75 flasks and purified as previously described (Lipkin, Moncada, Shafer, Wilson, & Schachter, 1986). Chlamydial EB were isolated by sonic disruption of L929 suspensions and purification by centrifugation. The final L2 pellet was resuspended in sucrose phosphate buffer (SPG; 5 mM glutamine, 0.2 M sucrose, 0.2 M phosphate buffer) and stored at −80°C.

Primary bone marrow-derived murine macrophages were prepared and frozen from femurs of 6 to 8-week old C57BL/6 female mice (Jackson Laboratory, Bar Harbor, ME). Macrophages were grown in RPMI 1640 media supplemented with 20% FBS (HyClone, Thermo Fisher Scientific, Rockford, IL), 10% M-CSF, and 2 mM L-glutamine (HyClone), at 37°C with CO₂. For all experiments, cells were subcultured and plated onto chambered coverglass slides (Lab-Tek II; Nunc, Rochester, NY), glass bottom culture dishes (MatTek, Ashland, MA), or 24-well plates (BD Falcon). On average, extrusions were found inside approximately 5% of confluent macrophages for any given experiment and incubated onto macrophages at a dose of ~0.1 extrusions for every macrophage. For each macrophage infection experiment, approximately 2.5 × 10⁴ extrusions were incubated onto 3 × 10⁵ macrophages, corresponding to roughly 0.1 extrusions per macrophage. It is important to note, however, that the same dose of extrusions onto McCoy cells for IFU analysis corresponded to a multiplicity of infection ~1.

4.2 | Macrophage assays

To assess survival in macrophages, extrusions were collected and infected onto macrophages for 1 hr at 37°C, with sonicated, free Chlamydia as a control. Following infection, cells were rinsed twice with Hank’s balanced saline solution (HBSS, HyClone) and incubated at 37°C for up to 24 hr. At indicated time points, macrophages were rinsed with HBBS, then scraped and lysed with a 26 and ½ gauge needle and resuspended in RPMI, to release any infectious Chlamydia within macrophages from all samples. Supernatants were subsequently plated onto HeLa cells in dilutions to count surviving IFU of extrusion-derived Chlamydia or sonicated Chlamydia over time.

To assess macrophage escape, extrusions were collected and infected onto macrophages for 1 hr at 37°C, with sonicated, free Chlamydia as a control. Cells were rinsed twice with HBSS and incubated at 37°C for up to 48 hr. Daily rinses of cultures were performed to ensure no carryover of infectious C. trachomatis that had escaped prior to the indicated time points. At 0 and 48 hpi, supernatants were collected from macrophages and infectious EB in supernatants was determined by IFU analysis.

Infections were performed by washing cells with HBSS and incubating cells with Chlamydia EB diluted in HBSS to a multiplicity of infection ≤1, for 2 hr at 25°C. Following static incubation, cells were rinsed twice with HBSS, re-immersed in growth media, and incubated at 37°C for 24 hr, then fixed with 100% methanol and stained for IFU analysis.

4.3 | Extrusion isolation

C. trachomatis serovar L2 was grown in semiconfluent HeLa cells for 72 hr, or in McCoy cells for 48 hr in RPMI supplemented with 10% FBS, L-glutamine and cycloheximide (2 μg/mL). Media on cell
monolayers was removed, rinsed, and new media added to infected cultures at 72 or 48 hpi (for McCoy cells). Infected cell cultures were allowed to proceed with infection in new media for 2–4 h to endogenously collect extrusions, then media was centrifuged at 75 × g for 5 min, followed by the removal of supernatant and a second centrifugation spin at 1200 rpm for 5 min. The extrusion pellet was immediately resuspended in fresh RPMI. To enumerate the number of extrusions obtained from a cell monolayer, resuspended extrusions were stained with SYTOX Green (1:2000, Molecular Probes) and Hoechst (1:2000, Molecular Probes) for 5 min at 25°C, plated as 10–20-μL drops onto glass slides and imaged immediately on an inverted fluorescence microscope. Intact extrusions were identified as having chlamydial inclusions, lacking nuclei and being the appropriate size.

4.4 Sonication of extrusions

Extrusions were collected using the method described above and separated into two populations. One population was sonicated using a hand sonicator set at 20 A and placed into a conical tube of extrusions for 2–3 s, 10 times, while on ice. This setting and brief sonication method was used to rupture the extrusion membranes to release free Chlamydia, and also keep the viability of the sonicated and extrusion population equal.

4.5 Supernatant centrifugation assay

Extrusions were collected using the method described above and resuspended in fresh RPMI media, then separated into two conical tubes of equal volumes. One tube was sonicated (as described above) for the “sonicated extrusion” control, then 500 μL volumes of both samples were plated onto HeLa cell monolayers to infect at room temperature for 2 hr, as a “raw” control. Both sets of samples were centrifuged at 16,000 × g for 30 min to pellet all Chlamydia. Supernatants were removed and the pellets were resuspended in the same volume as the supernatant that was removed to determine if any infectivity was lost due to potential neutralization by proteases in the supernatants. IFU analysis was performed to evaluate any difference in infectivity between samples and controls.

4.6 Extrusion stability assay

Extrusions were collected using the procedure mentioned above then the extrusion pellet was resuspended in 100 mL fresh RPMI and incubated up to 4 hr at 25°C. Extrusions were plated in 25-μL drops onto poly-D-Lysine-coated glass-bottom dishes and imaged at 60× magnification using brightfield microscopy to show overall shape and intactness, and fluorescence microscopy to confirm the absence of nuclei. Ten fields per time point were imaged, and images were taken every 30 min. Images were counted for intact extrusions.

4.7 Immunofluorescence and live fluorescence microscopy

All live microscopy and immunofluorescence was performed on a Nikon Eclipse Ti inverted fluorescence microscope. Image capturing was performed using the Hamamatsu camera controller C10600 and Volocity imaging software, version 6.3 (PerkinElmer; Waltham, MA). Infected cells were fixed in 3.7% paraformaldehyde (Ted Pella) for 15 min, then permeabilized with 0.1% Triton-X-100 (Fisher), blocked with 1% BSA-PBS (Fisher), and stained. Antibodies/dyes were obtained from the following sources: CellLight ER-RFP from ThermoFisher (Waltham, MA), Phalloidin 633, donkey anti-goat 488 from Invitrogen (Waltham, MA), DAPI, goat anti-mouse 488 from Thermo Fisher (Waltham, MA), anti-GFP 488, FM4-64 from Molecular Probes (Eugene, OR), MitoTracker Green, Annexin-V 568 from Life Technologies (Carlsbad, CA), mouse anti-Chlamydia FITC conjugate from Meridian Diagnostics (Cincinnati, OH), goat anti-C. trachomatis MOMP from Virostat (Portland, ME), mouse anti-C. trachomatis LPS and mouse anti-CT223 donated by Bob Suchland (University of Washington, WA).

Microscopy to determine Chlamydia developmental forms was performed as follows: Extrusions were collected and incubated at 37°C from 0 to 8 hr, gently sonicated and subsequently plated onto coated coverglass for fluorescence microscopy at 100× magnification to visually distinguish RB and EB within the population. Imaging algorithms using Volocity software were established using control samples of 24- and 48-hr infected cells that have been sonicated and processed similarly, to be enriched for RB and EB respectively.

4.8 Image processing and analysis

Three-dimensional image stacks were further processed in Volocity by performing illumination correction (in z dimension) and deconvolution (25 iterations). Individual xy and xz slices were obtained from image stacks for figure assembly. Three-dimensional opacity renderings of fluorescent image stacks were generated in Volocity. Minor retouching of all micrographs—for example, color assignment, contrast adjustment, RGB merges, and cropping—were performed with Volocity and Photoshop CS6 (Adobe). Photoshop CS6 (Adobe) was used to assemble all figures into their final form. Quantitation of RB and EB forms was performed with the following parameters: (a) Find objects function, using the GFP filter, was used to identify all fluorescent objects within the image, then (b) “Filter population” using surface area (μm²) as the filter was used to separate RB from EB. The surface area values used to separate RB and EB are larger than actual RB and EB surface areas, due to a “halo” effect of the “find objects” function. While these values are not accurate to RB and EB sizes, the values used accurately separate the two developmental forms in extrusions and controls tested.

4.9 Electron microscopy

Extrusions, or macrophages containing extrusions, were collected and cold fixative added, and centrifuged to form tight pellet. Supernatant was removed and cold fixative was added on top of a cell or extrusion pellet, and centrifuged once more. All samples were kept at 4°C. All samples were fixed in 2% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4, post-fixed in 2% osmium tetroxide in the same buffer, then block stained with 2% aqueous uranyl acetate, dehydrated in acetone, infiltrated, and embedded in LX-112 resin (Ladd Research Industries, Burlington, VT). Samples were ultrathin sectioned on a Reichert Ultracut S ultramicrotome and counter stained with 0.8% lead citrate. Grids were examined on a JEOL JEM-1230 transmission electron microscope (JEOL USA, Inc., Peabody, MA) and photographed with the Gatan Ultrascan 1000 digital camera (Gatan Inc., Warrendale, PA).
4.10 Statistical analysis

Statistical evaluation of data was performed by calculating the standard error of the mean or using linear regression, one way, or two-way analysis of variance, using Bonferroni’s post-test (Figure 3), or Tukey’s post-test (Figures 4, 6, and 7). P values <0.05 (*) were considered statistically significant. P values of <0.001 (**), <0.0001 (***) were marked as indicated. Calculations were performed in Prism (Graphpad) and Microsoft Excel.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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