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Report

Phagosome Extrusion and Host-Cell Survival after *Cryptococcus neoformans* Phagocytosis by Macrophages

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Summary

***Cryptococcus neoformans* (Cn) is an encapsulated yeast that is a facultative intracellular pathogen and a frequent cause of human disease. The interaction of Cn with alveolar macrophages is critical for containing the infection [1, 2], but Cn can also replicate intracellularly and lyse macrophages [3–5]. Cn has a unique intracellular pathogenic strategy that involves cytoplasmic accumulation of polysaccharide-containing vesicles and intracellular replication leading to the formation of spacious phagosomes in which multiple cryptococcal cells are present [3]. The Cn intracellular pathogenic strategy in macrophages and amoebas is similar, leading to the proposal that it originated as a mechanism for survival against phagocytic predators in the environment [6]. Here, we report that under certain conditions, including phagosomal maturation, possible actin depolymerization, and homotypic phagosome fusion, Cn can exit the macrophage host through an extrusion of the phagosome, while both the released pathogen and host remain alive and able to propagate. The phenomenon of "phagosomal extrusion" indicates the existence of a previously unrecognized mechanism whereby a fungal pathogen can escape the intracellular confines of mammalian macrophages to continue propagation and, possibly, dissemination.**

Results

All strains of Cn evaluated in this study were efficiently phagocytosed by and replicated inside macrophages-like cells (Figure S1 and S2 in the Supplemental Data online). After the commencement of intracellular replication, we frequently witnessed the extrusion of the Cn-containing phagosome from the macrophage to the extracellular space, an event we have simply termed "phagosomal extrusion." This process involved the formation of a large phagosome filled with cryptococcal cells followed by ejection of the phagosome and subsequent survival of the macrophage, as evidenced by the continuation of host-macrophage pseudopodial movement and subsequent replication (Figure 1, Movie S1). The phagosomal extrusion event was observed with macrophages infected by cells of all three Cn varieties (*neoformans*, *gattii*, and *grubii*), although the frequency varied with the strain and was most prevalent with the Cn variety *gattii* strain NIH 198 (Figure 2). Phagosomal extrusion was observed as early as 2 hr after macrophage infection, and the likelihood of extrusion was temporally dependent on the initial ratio of pathogens to host cells, with higher ratios resulting in earlier phagosomal extrusion events (data not shown). Phagosomal extrusion was independent of the activated state of macrophages because nonactivated macrophages equally extruded Cn cells (Figure 2). Additionally, fusion of Cn-containing phagosomes led to the formation of a giant compartment that appeared to be a prerequisite for the complete extrusion of all Cn cells (Figure 1 and Figure S3).

Phagosomal extrusion was not observed with macrophages that ingested polystyrene beads (data not shown) or acapsular Cn (cap67), but on rare occasions, incomplete extrusion of the latter was observed, where only small numbers of Cn escaped the macrophages, with most remaining intracellular. Addition of capsular polysaccharide to cap67 cells triggered complete phagosomal extrusion (Figure 2). When heat-killed encapsulated Cn was used for phagocytosis, phagosomal extrusion occurred, albeit at a much lower frequency than for live encapsulated Cn (Figure 2). These extrusion events were noted with both J774.16 cells and primary macrophages from 129SV and BALB/C mice, representing mouse strains with and without the natural-resistance-associated macrophage protein (NRAMP) [7]. Phagosomal extrusion following complement opsonization was observed, but less frequently than for antibody-mediated phagocytosis.

To determine whether phagosomal extrusion was a macrophage- or Cn-dictated event, we incubated macrophages with Cn, then with FITC-labeled beads, and observed the outcome by time-lapse imaging. We found that in macrophages that ingested both Cn and FITC beads, only the Cn-containing phagosomes were extruded from the macrophages (Movie S2).

To assess the role of the cytoskeleton in phagosomal extrusion, we incubated Cn with macrophage-like cells and then added Cytochalasin D and Colchicine in feeding media at inhibitory, but sublethal, concentrations. Phagosomal-extrusion rates were enhanced in cells treated with these drugs (Figure 2). Addition of Cytochalasin D alone enhanced phagosome extrusion, suggesting that the effect was due mostly to inhibition of actin polymerization (data not shown).

To test whether phagosomal extrusion was preceded by phagosomal maturation, we investigated the percent colocalization of lysosomal-associated membrane protein (LAMP-1) to the cryptococcal phagosomes at 5, 15, 30, 60, and 120 min. By 1 hr, almost all Cn-containing phagosomes were LAMP-1 positive. These results were confirmed by using macrophages that were preloaded with Texas red (TR) dextran to label lysosomes (Figure 3 and Figure S4). Given that extrusion occurs after 2 hr, this result indicates that phagosome-lysosome fusion occurs prior to extrusion of Cn phagosomes (Movie S3).

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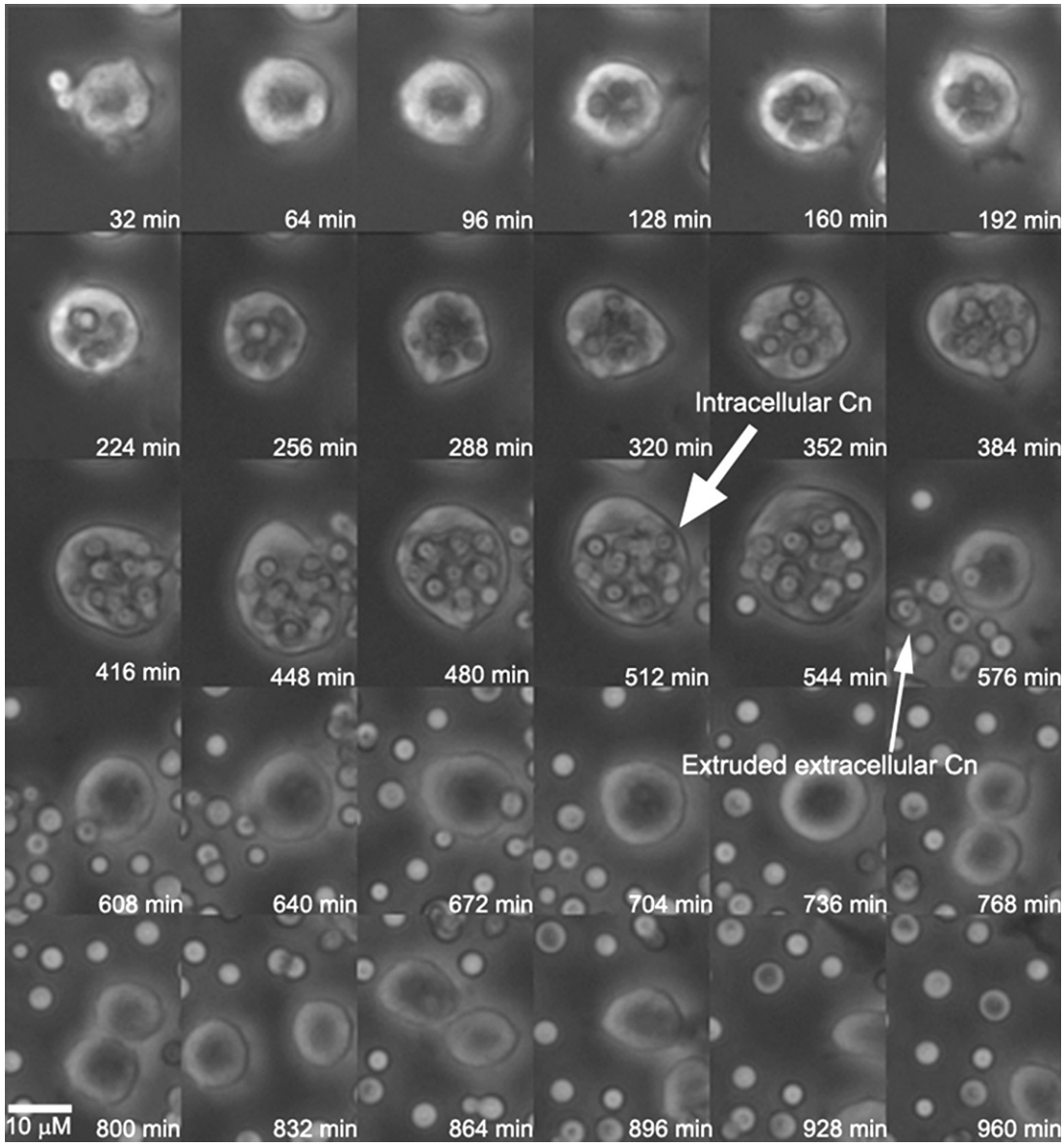

Figure 1. Phagosome Extrusion by *C. neoformans*-Infected Macrophages

Macrophages expel the Cn-containing phagosome and subsequently survive and propagate. Expelled Cn could propagate as well. Frames are labeled according to the start of the imaging process, which is approximately an hour after phagocytosis was initiated. Arrows indicate intracellular Cn and extruded extracellular Cn.

Complete phagosome extrusion was usually preceded by formation of a large phagosome, suggesting the occurrence of homotypic phagosome fusion. To investigate whether Cn-containing phagosomes could fuse with one another, we labeled cryptococcal cells with FITC or TRITC and sequentially infected macrophages with FITC-labeled Cn and then with TRITC-labeled Cn. We found that the FITC and TRITC signals from phagosomal vesicles overlapped, suggesting that these phagosomes had fused (Figure S3).

Discussion

Phagosomal extrusion was triggered by microbial components and involved only the phagosomes containing Cn. Complete phagosomal extrusion was preceded by the formation of a giant phagosome that could have resulted from homotypic phagosome fusion. Capsular polysaccharide is a major virulence factor of Cn [8] and appeared to be required for this phagosomal extrusion event because it was not observed with acapsular cells or polystyrene beads. We speculate that Cn may alter the membrane properties of phagosomes and promote their fusion. Given that the major component of Cn polysaccharide, glucuronoxylomannan, affects signaling processes in macrophages [9], it is conceivable that these contribute to phagosome extrusion. The lower frequency of extrusion after macrophage ingestion of heat-killed encapsulated Cn may reflect lower polysaccharide inside macrophages, as a result of no intracellular Cn replication and polysaccharide shedding. This might explain why the frequency of extrusion of heat-killed Cn

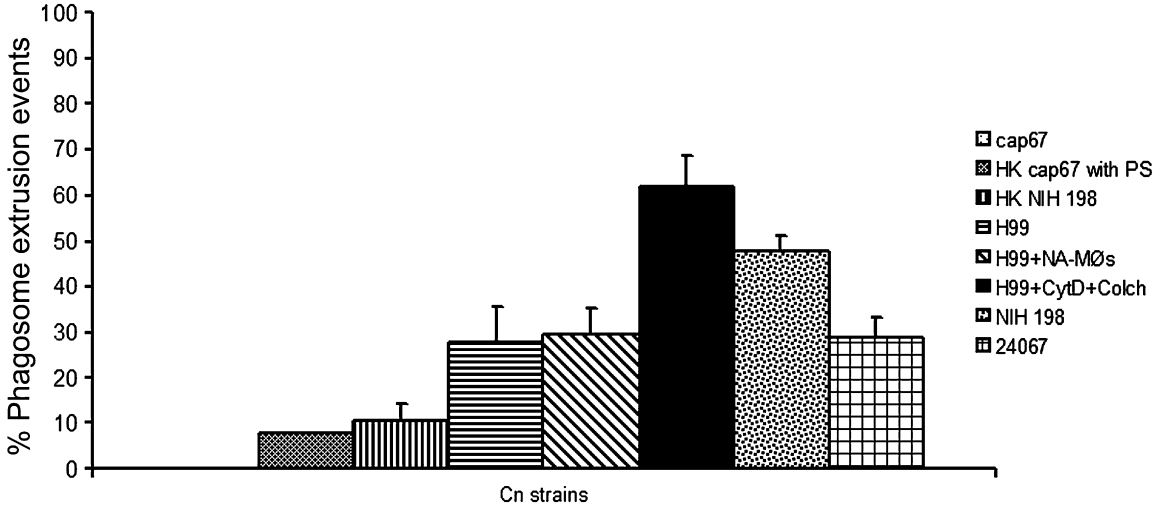

Figure 2. Phagosome Extrusion Rates of Live and Heat-Killed Cn Strains by Activated and Nonactivated Macrophages and Macrophages Treated with Cytoskeletal Inhibitors

Macrophage-like cells were incubated with live encapsulated (H99, 24067, and NIH198), acapsular (cap67), heat-killed (HK) encapsulated (HK NIH198), and HK acapsular (HK cap67) Cn with incorporated polysaccharide (PS). Additionally, macrophage-like cells were treated with Cytochalasin D and Colchicine (H99+CytD+Colch) after incubation with Cn, as indicated in the Supplemental Experimental Procedures. Percent phagosome extrusion was determined as indicated in Supplemental Experimental Procedures and was also determined in macrophages that were not activated with LPS and IFN-γ that were incubated with H99 (H99+NA-MØs). Primary-cell phagosome extrusion of Cn (NIH198) was comparable to extrusion in macrophage-like cells (approximately 47%) (data not shown). Bars represent experimental data carried out in triplicate, ± standard deviation (SD).

and heat-killed cap67 with added polysaccharide were similar. The observation that in macrophage-like cells coinfected with Cn and FITC beads, only the Cn-containing phagosomes were extruded suggests that this phenomenon is dictated by Cn and not the host macrophage. However, the observation that phagosomal extrusion occurs more frequently and rapidly when Cn is opsonized with mAb than complement suggests that this phenomenon also involves host factors. It is also possible that mAb opsonization induces a structural change on the capsule, allowing it to be recognized more efficiently by pattern-recognition receptors and other signaling receptors, thus enhancing the extrusion effect [10]. Because phagosomal maturation has previously been observed in cells infected with Cn, it is not surprising that extrusion follows phagosome-lysosome fusion [5, 11]. However, the observation that cytoskeletal inhibitors increased the phagosomal extrusion rate was particularly interesting given that actin depolymerization is linked to exocytosis in mammalian cells and plays a role in a postlysosomal exocytic pathway in amoebas [12, 13]. This suggests that an intact cytoskeleton might be necessary to retain Cn within the macrophage. Perhaps these drugs potentiate a mechanism Cn uses to induce depolymerization of actin, culminating in the exocytosis of Cn. Future studies are necessary to fully characterize the mechanism(s) involved in phagosomal extrusion of Cn and to establish a possible connection between polysaccharide and actin in this process.

A precedent for microbial escape from a phagocytic cell is provided by the observation that *Legionella pneumophila* can escape from protozoa [14, 15]. Interestingly, a parallel exists between Cn and *L. pneumophila* in that both microbes can replicate in macrophages and amoebas [16, 17]. Studies of the interaction between bacteria and protozoa also provide a precedent for the observation that Cn promotes homotypic phagosome fusion in mammalian macrophages. Homotypic phagosome fusion was described for *Helicobacter pylori* in amoebas, and the phenomenon is extended here to a fungal intracellular pathogen in mammalian macrophages [18, 19]. A study of *D. discoideum* that ingested bacteria revealed that phagosome-phagosome fusion occurred and led to the formation of spacious phagosomes [19, 20]. This sequence of events might also be occurring in our system. Formation of a spacious phagosome might be required for complete phagosome extrusion of Cn, given that most of the events observed occurred when a phagosome vesicle containing all intracellular Cn was extruded from the host macrophage.

In summary, we make three novel observations. First, we describe a macrophage phagosomal extrusion event that allows viable fungal cells to escape without killing the host cells. We speculate that this is a cellular exit strategy that has been selected in evolution as an escape hatch from phagocytic predators. Second, we provide evidence that phagosome maturation precedes extrusion and homotypic phagosome fusion occurs in macrophages with a fungal pathogen resulting in the formation of giant phagosomes. Third, we document that cytoskeleton serves to retain Cn intracellularly and that depolymerization of actin is a possible means of escape for Cn. The observations described here support the view that Cn has a uniquely different intracellular pathogenic strategy with no counterpart on previously investigated bacterial, fungal, and parasitic intracellular pathogens.

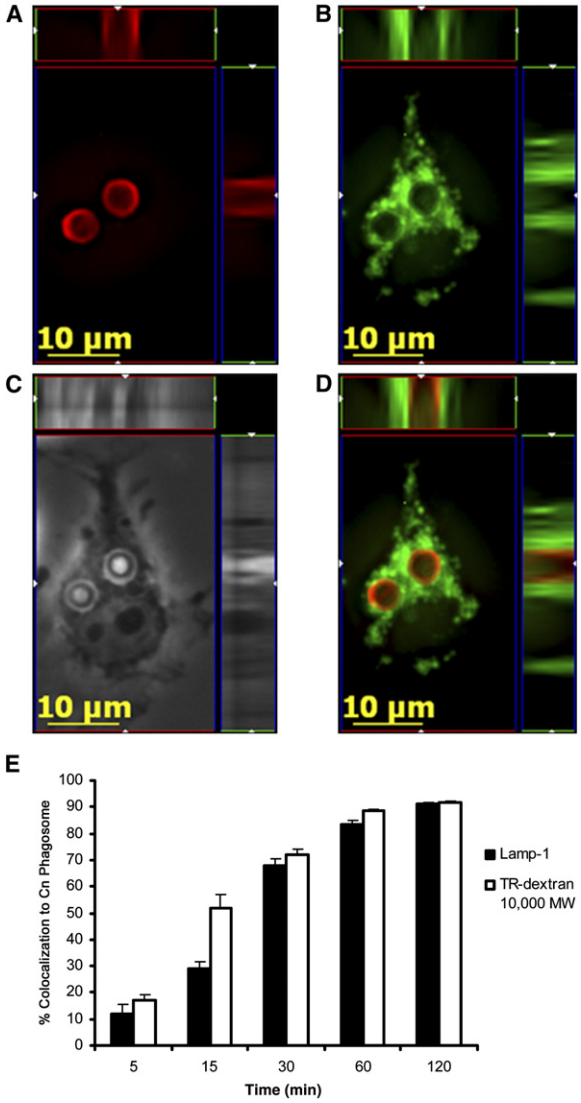

Figure 3. Phagosome-Lysosome Fusion after Ingestion of *C. neoformans* by Macrophages

Macrophage-like cells were challenged with Cn for various time points followed by staining for LAMP-1 and Cn's capsule, as indicated in Supplemental Experimental Procedures. Images were collected after the 1 hr time point.

(A) Cn capsule (red) within the macrophage.

(B) LAMP-1 (green) surrounding Cn's capsule.

(C) Phase image showing internalized Cn.

(D) Merged red and green channels showing LAMP-1-positive phagosomes.

(E) Percent colocalization of LAMP-1 and TR-dextran to the Cn-containing phagogomes was determined as indicated in the Supplemental Experimental Procedures. Bars represent experimental data carried out in triplicate, ± SD.

Supplemental Data

Supplemental Data include Experimental Procedures, five figures, and three movies and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/21/2161/DC1/.

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