

Fc- and Complement-Receptor Activation Stimulates Cell Cycle Progression of Macrophage Cells from G₁ to S¹

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Phagocytosis of microorganisms by macrophages is an important host defense mechanism. While studying the phagocytosis of the human pathogenic fungus *Cryptococcus neoformans*, we noted that macrophage-like J774 cells with ingested fungal cells had frequent mitotic figures. By analyzing the relative proportion of phagocytic cells as a function of cell cycle phase, we observed an increase in S phase cells after Fc-mediated phagocytosis of polystyrene beads, live or heat-killed *C. neoformans*. This result was confirmed by increased nuclear BrdU incorporation after Fc-mediated phagocytosis. The induced progression to S phase was observed after both Fc- and complement-mediated phagocytosis of live yeasts. Fc-mediated stimulation of cell division did not require ingestion, because it could be triggered by incubating cells in IgG1-coated plates. Phagocytosis-mediated stimulation of replication was confirmed in vitro using primary bone marrow macrophages and in vivo for peritoneal macrophages. We conclude that phagocytosis of microbes or inert particles can stimulate macrophages to enter S phase and commence cell division. This observation suggests a potential mechanism for increasing the number of effector cells after microbial ingestion, but can also promote the spread of infection. *The Journal of Immunology*, 2005, 174: 7226–7233.

Phagocytosis of microorganisms by host phagocytic cells is an important host defense mechanism. The process of phagocytosis involves a rearrangement of the cytoskeleton and cell membrane to ingest the microbial particle (1, 2). Microbial phagocytosis can be followed by cellular activities that result in the death of the microbe, including lysosomal fusion with the phagosome and oxidative burst (2, 3). Alternatively, phagocytosis may not lead to the killing of the microbe because many pathogens have strategies to evade cellular microbicidal mechanisms. Examples of microbes that replicate inside macrophages include *Mycobacterium tuberculosis* (4), *Listeria monocytogenes* (5), and *Cryptococcus neoformans* (Cn)⁴ (6). Given that the outcome of phagocytosis is uncertain for the host cell, it is likely that immune cells have additional safeguards for the eventuality that phagocytosis does not lead to microbial killing.

Most mature macrophages, such as those in the peritoneum, originate from blood monocytes, which are derived from bone marrow. Generally, these cells are considered to be postmitotic (7). However, peritoneal exudate macrophages have been reported to form colonies when placed in suitable culture conditions in vitro

(8, 9). Several studies have shown that a significant proportion of macrophages in the peritoneum and other organs can undergo mitosis (10–15). Cell division would lead to a doubling of the phagocytic cells, an event that could conceivably benefit the host by increasing the number of effector cells at the site of infection. However, division of cells infected with a live microbe that is replicating intracellularly could also lead to a doubling of infected cells, an event that could potentially harm the host. Hence, one can posit theoretical reasons for the supposition that phagocytosis could alter cell division through programmed host cell effects or subversion of the process by microbial actions. Although there are examples of microbial toxins impacting cell division (16, 17), we could find no reports in the literature that explored direct links between phagocytosis and cell division effects.

Cn is a major cause of life-threatening infections in patients with impaired immunity (18). This microbe is a facultative intracellular pathogen that replicates inside macrophages by using an unusual strategy that includes accumulation of polysaccharide-containing vesicles and induction of phagosome leakiness (6). While studying the phagocytosis of this organism, we noted that macrophages with ingested fungal cells frequently appeared to be dividing, as indicated by mitotic figures. Interestingly, in the late 1960s, Mackaness (10) reported that peritoneal macrophages manifested numerous mitotic figures after in vivo injection of *Listeria monocytogenes* into the peritoneum. Hence, we hypothesized that the phagocytosis of microbes could affect macrophage cell division. This phenomenon may have been due to the stimulation, synchronization, or arrest of cell cycle. We report in this work that the phagocytic ability of macrophages is cell cycle dependent and, most interestingly, that phagocytosis of microbes or inert particles may allow macrophages to re-enter the cell cycle and start replication. We believe these observations could herald a general phenomenon with important implications for both host defense and microbial pathogenesis.

Materials and Methods

Mammalian cell lines

Phagocytosis assays were performed with the standard macrophage-like cell line J774.16, which is derived from a reticulum sarcoma and has phenotypic characteristics similar to murine peritoneal macrophages (19).

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⁴ Abbreviations used in this paper: Cn, *Cryptococcus neoformans*; cdk, cyclin-dependent kinase; FSC, forward light scatter; Rb, retinoblastoma tumor suppressor protein.

Cells were cultured at 37°C with 10% CO₂ in DMEM containing 10% heat-inactivated FCS, 10% NCTC-109 medium, and 1% nonessential amino acids. The cell lines for the experiments were no older than 15 passages.

Primary mouse bone marrow macrophage culture

Three-month-old male C57BL/6 mice were purchased from The Jackson Laboratory, and bone marrow cells were harvested from the hind leg bones. After lysis of erythroid cells, bone marrow cells containing monocytes were cultured at 37°C with 10% CO₂ in RPMI 1640 medium with 10% heat-inactivated FCS. Both IL-3 and M-CSF (R&D Systems) were added to the medium for the first week. The second week, M-CSF was added alone. The medium was replaced every 3 days to remove floating B cells.

Yeast strains

Cn strain 24067 (serotype D) was obtained from the American Type Culture Collection. Strain H99 was obtained from J. Perfect (Department of Medicine, Division of Infectious Disease, Duke University Medical Center, Durham, NC). Cn was cultured for 2–3 days in Sabouraud dextrose broth at 30°C with moderate shaking at 150 rpm. Cells were collected by centrifugation, washed with PBS three times, and counted in a hemocytometer. Heat-killed Cn were prepared by incubating cultures in a water bath at 65°C for 30 min. Cultures were plated for CFU to verify that cell killing occurred. Strain 24067 was used in all experiments, except for the complement-mediated phagocytosis experiments, because this strain is not phagocytosed when opsonized with complement. For the complement experiment strain H99 was used (20).

Phagocytosis assay

Macrophages cultured in petri dishes were harvested, and the cell number was counted with hemocytometer. The cells were seeded into six-well plates to a density of 5×10^5 to 2×10^6 cells/well, and after studying the efficacy of phagocytosis as a function of macrophage cell density, a working density of 1×10^6 cells/well was chosen because this produced optimal results on phagocytosis efficiency and cell cycle analysis. Phagocytosis assays were done when the seeded cells were attached to the bottom of the plates. At the indicated times, polystyrene beads (FACS AccuDrop beads, 6 μ m in diameter, conjugated with allophycocyanin, which is excited at 670 nm and emits at 750 nm; BD Biosciences) or Ab-opsonized Cn strain 24067 were added at an E:T ratio of 1:1 or as indicated. Cn capsule-specific mAb, 18B7, was used as an opsonin at 10 μ g/ml. Incubation was conducted in 10% CO₂ at 37°C. In complement-mediated phagocytosis assays, FITC-labeled Cn strain H99 was added at an E:T ratio of 1:1 and 20% guinea pig serum (Calbiochem) was added to promote phagocytosis, as described (20). After incubating for 1.5 h, any remaining extracellular yeast cells or beads were removed with three washes of PBS. The resulting monolayer was fixed with ice-cold methanol for 30 min and stained with 1/20 diluted Giemsa dye for 30 min. Monolayers were then washed with PBS and examined under the microscope at a magnification of $\times 600$. Phagocytic index is defined as the percentage of macrophages with internalized particles multiplied by the average number of internalized particles. The percentage of phagocytosis is defined as the percentage of macrophages with internalized particles. At least 5 fields and 200 macrophages were counted for each well, and 4–6 repetitions were analyzed for each condition.

Flow cytometric analysis (FACS)

Ab-opsonized Cn or unopsonized, allophycocyanin-conjugated beads were incubated with macrophages using the conditions described for the phagocytosis assay. As for phagocytosis of Cn, the opsonic mAb 18B7 conjugated to Alexa 488 dye was used as above at 10 μ g/ml. To reduce the noise of sampling by FACS, extracellular Cn or beads were removed by washing the monolayer with PBS. The removal of extracellular Cn was optimized by adding consistent wash volumes and shaking the plates three to six times, a protocol that was previously determined to be effective at eliminating extracellular particles. The macrophage monolayer was gently scraped from the six-well plates and suspended in 1 ml of PBS for each well. The cells were dispersed with 1 ml of pipette 5–10 times to insure that single cell suspension was obtained. Cells were fixed by the addition of 5 ml of ice-cold 70% ethanol and 2-h incubation on ice. In preparation for FACS analysis, cells were centrifuged at 600 rpm for 10 min. DNA content was labeled by incubating the pellets in a 0.5-ml solution of propidium iodide (Molecular Probes) at 20 μ g/ml in PBS, containing RNase at a final concentration of 200 μ g/ml. Samples were stained at room temperature for 30 min and analyzed by FACSscan (BD Biosciences). Before FACS analysis, cell samples stained with propidium iodide were observed under the

microscope. The DNA of macrophage cells stained well with propidium iodide, whereas the DNA of intracellular Cn was not stained by this dye. Hence, there was no contribution from the DNA of yeast cells to the FACS analysis. When the FACS data were collected, cell debris from the sample preparations and any remaining beads/Cn particles that were not successfully removed by washing were excluded using the forward light scatter (FSC)-height-side light scatter-height scale. Any doublets that might have been formed in the cell preparations were excluded by the FSC-height-FSC-pulse width scale. J774 cells incubated with particles were sorted into the nonphagocytic population and the phagocytic population according to absence or presence of intracellular FITC (from 18B7 conjugated with Alexa 488) or allophycocyanin (from AccuDrop beads) signal. Data were analyzed by ModFit 3.0 software (Verity Software House) for cell cycle distribution. The distribution of cell cycle stages in each population was compared.

In vivo phagocytosis assay

Three-month-old male C57BL/6 mice were purchased from The Jackson Laboratory. Cn strain 24067 was opsonized with FITC-conjugated 18B7 at 100 μ g/ml for 1 h at room temperature. A 1-ml suspension containing 10^7 Cn particles opsonized with mAb 18B7 was injected into the peritoneum of each mouse. After 2 h, the mice were sacrificed and peritoneal cells were collected by lavage with PBS. After lysis of erythroid cells, peritoneal cells were fixed with ice-cold 70% ethanol overnight, and cell cycle distribution was analyzed by FACSscan.

BrdU incorporation

J774 cells were seeded at 5×10^4 cells per chamber in eight-well chamber slides (Nalge Nunc International) 12 h before phagocytosis assays. Cn or beads were incubated with macrophages using the conditions described for the phagocytosis assay. Cn or beads were added to the monolayer simultaneously with 0.1 mM BrdU (Sigma-Aldrich). After a 1.5-h incubation, monolayers were washed three times with PBS and fixed in 100% methanol for 2 min at room temperature. A volume of 500 μ l of DNA denaturing solution (2 N HCl and 0.5% Triton X-100 in ddH₂O) was added to each chamber for 10 min at room temperature. Monolayers were rinsed with immunofluorescence buffer (0.2% BSA and 0.1% Triton X-100 in PBS/0.1 mM CaCl₂, 1 mM MgCl₂), and 100 μ l of anti-BrdU Ab conjugated with Alexa 594 (Molecular Probes) was added per chamber (1/100 diluted in immunofluorescence buffer) for 1 h at room temperature. Cell monolayers were washed three times with PBS and stained with 10 μ g/ml 4',6'-diamidino-2-phenylindole (Sigma-Aldrich). The incorporation index was defined as the percentage of macrophages with positive BrdU staining. Digital images were taken with an Olympus AX70 microscope (Olympus).

Coating six-well plates with 18B7

The mAb 18B7 (1.98 mg/ml) was added at a volume of 1 ml/well into six-well plates to cover the bottom of wells. Plates were incubated at 37°C for 1 h before seeding 1×10^6 J774 cells/well into the plates.

Statistics

Pairwise comparison between groups was done by *t* test using Excel. Value of *p* < 0.05 was considered significant.

Results

Phagocytosis is a cell cycle-dependent process

To investigate the relationship between phagocytic efficacy and cell cycle phase, we evaluated the proportion of J774 cells with ingested yeast cells relative to their cell cycle stage. The growth curve showed that J774 cells duplicated every 12 h in nutritional culture medium (Fig. 1A). The phagocytic efficacy of 3T3 cells was reported to depend on cell cycle phase, with cells in G₁ phase being most efficient in their ability to phagocytose particles (21). Consistent with that report, our results showed that macrophages in G₁ phase have higher phagocytic ability than those in S and G₂ phase. J774 cells were synchronized to G₁ phase by starvation in serum-depleted medium for 72 h (Fig. 1B). More than 80% of J774 cells were synchronized to G₁ phase by this procedure, as analyzed by FACS. In the G₁-enriched fractions, the percentage of phagocytosis rose to 70%. While under normal conditions, the percentage of J774 cells in G₁ was from 40 to 60%, and the percentage of

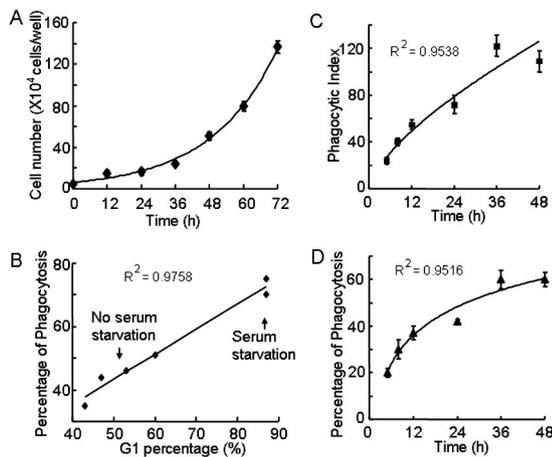


FIGURE 1. Relationship between J774 cell growth and phagocytic efficacy. *A*, J774 cell growth with time. J774 cells were seeded on six-well dishes and incubated at 37°C. The cells were harvested and cell numbers were counted in 12-h intervals. *B*, J774 cells were synchronized in G₁ by serum starvation before phagocytosis assay of live Cn opsonized by mAb 18B7. Percentage of phagocytosis was determined by FACS analysis. *C* and *D*, Phagocytic efficacy of J774 cells grown for different times in culture before the phagocytosis assay. Live Cn was opsonized by mAb 18B7. Percentage of phagocytosis and phagocytic index was determined as defined in *Materials and Methods*.

phagocytosis was below 50%. There was a strong correlation between the percentage of phagocytosis and the proportion of J774 cells in G₁ phase (Fig. 1*B*). In a separate experiment, the phagocytic index of J774 cells cultured for 48 h in serum-depleted medium increased >5-fold and the percentage of phagocytosis increased 3-fold, relative to cells cultured for 5 h in fresh medium (Fig. 1, *C* and *D*). These experiments confirmed that J774 cells, as 3T3 cells, are more phagocytic in G₁ phase.

Fc-mediated phagocytosis alters the proportion of cells in G₁ and S phase

FACS was used to analyze the cell cycle distribution of J774 cells after phagocytosis of Cn strain 24067 or inert polystyrene beads (Fig. 2). Cn were labeled by Alexa 448-conjugated mAb 18B7, which specifically binds to capsular glucuronoxylomannan and also functions as a powerful opsonin for phagocytosis of Cn. The potential for factors derived from live Cn cells to actively affect host cell cycle was evaluated by comparing the macrophage cell cycle distribution after ingestion of live or dead Cn cells.

Because mAb 18B7 is an IgG1, opsonized Cn is phagocytosed by FcγR. To elucidate the role of FcγR in the pathway that activates cell cycle, fluorescent allophycocyanin-conjugated polystyrene beads, which are phagocytosed without a need for opsonin, were fed to J774 cells for comparison. FACS analysis was accomplished by the fact that the fluorescence of Cn or bead particles

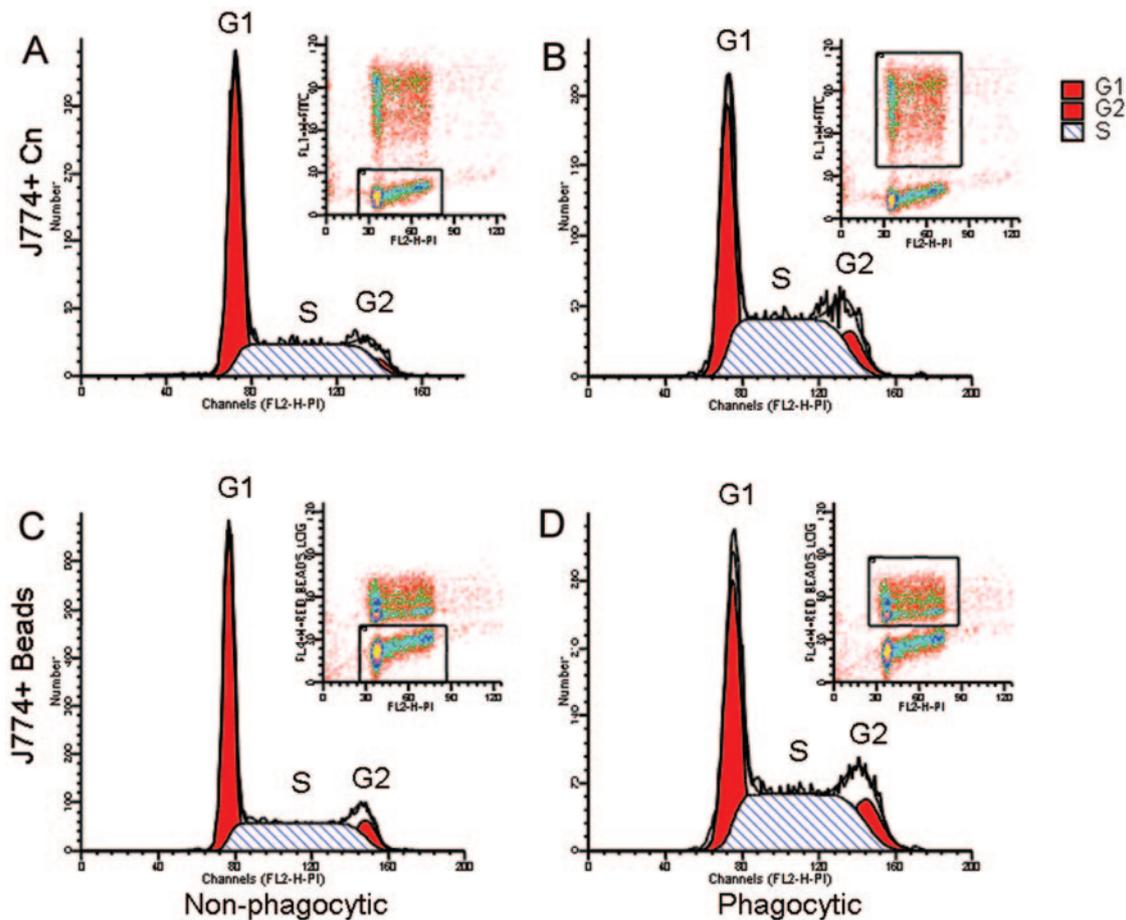


FIGURE 2. Cell cycle analysis of J774 cells by FACS after phagocytosis assays. *A* and *B*, Show an analysis of the percentage of cells in G₁, S, and G₂ cell cycle phases after phagocytosis of Cn. *C* and *D*, Show an analysis of the percentage of cells in G₁, S, and G₂ cell cycle phases after phagocytosis of beads. *A* and *C*, Show an analysis of the percentage of cells in G₁, S, and G₂ cell cycle phases for the nonphagocytic J774 population (within the square of the inset of the upper right panels). *B* and *D*, Show an analysis of the percentage of cells in G₁, S, and G₂ cell cycle phases for the phagocytic J774 population (within the square of the inset of the upper right panels). All cell cycle data were analyzed by Modfit software and showed similar results. Each experiment was repeated at least three times, and the data shown here are representative of the results obtained.

inside J774 cells was sufficient to differentiate J774 cells into two types of populations, one with ingested (live/dead) Cn or beads and another without Cn or beads (Fig. 2). These two populations were then subjected to cell cycle analysis individually, and the percentages of G₁, S, and G₂ phases in each population were ascertained by quantitating cellular DNA content using propidium iodide staining. We noted a difference in the phagocytic efficacy of live and dead Cn cells by J774 cells, which may reflect interference of phagocytosis by live cryptococci, which are known to produce an antiphagocytic protein (22). The percentages of J774 cells that phagocytosed live and dead Cn were 35 and 47%, respectively (Fig. 3). The percentage of phagocytosis was even higher for beads. Interestingly, we consistently found that the J774 cell population that phagocytosed live Cn, heat-killed Cn or beads had a lower percentage of cells in G₁ phase and higher percentage of cells in S phase relative to the population that did not phagocytose particles or to control cells not exposed to particles (Fig. 3A). To ascertain whether this difference was due to toxicity by internalized live Cn, J774 cells were incubated with live Cn for 1.5 and 4 h, and the same cell cycle distribution was found in both conditions (data not shown). This suggested that the difference was not due to the Cn-mediated cytotoxicity. To test the possibility that the

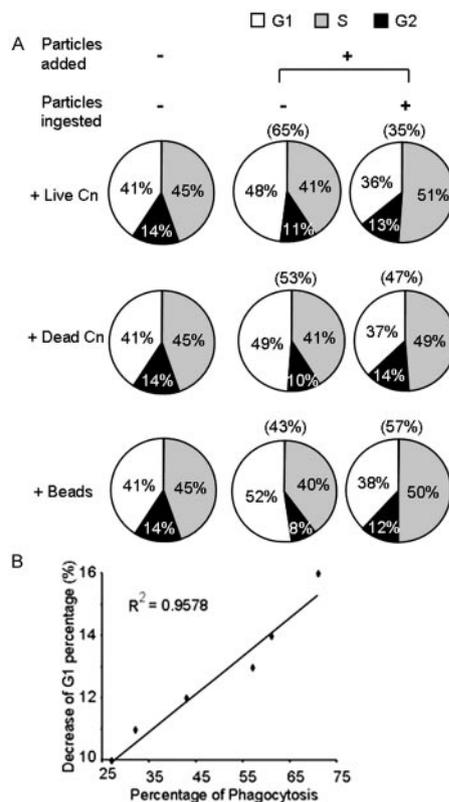


FIGURE 3. Cell cycle distribution of J774 cells after phagocytosis of polystyrene beads, 18B7-opsonized living Cn, or 18B7-opsonized heat-killed Cn. *A*, Percentage of G₁, S, and G₂ cells is indicated in the nonphagocytic (Cn added + and Cn ingested -) and phagocytic (Cn added + and Cn ingested +) groups. Comparison of G₁, S, and G₂ percentages between nonphagocytic and phagocytic groups revealed statistically significant differences between nonphagocytic and phagocytic groups was given in parenthesis. *B*, Difference of the percentage of G₁ between the nonphagocytic and the phagocytic groups correlated with the percentage of phagocytosis after phagocytosis of polystyrene beads. The experiments of phagocytosis of polystyrene beads, 18B7-opsonized living Cn, or 18B7-opsonized heat-killed Cn were repeated for six, five, and three times, respectively, and produced similar results.

E:T ratio or the host cell density in dishes might influence the results, we incubated J774 cells that were seeded at varying densities (0.5, 1, or 2×10^6 cells/well) at E:T ratios of 2:1, 1:1, and 1:2 for each density, with beads as the effector. The percentage of phagocytosis increased from 27 to 71% with higher cell density and higher bead to macrophage ratio. The decrease of G₁% of phagocytic group compared with that of nonphagocytic group was positively correlated with the percentage of phagocytosis (Fig. 3B). To ascertain whether the phenomenon was the result of increased phagocytosis by individual cells or a population/quorum effect caused by the release of a soluble factor upon phagocytosis, we repeated the experiments using conditioned medium generated by incubating macrophages with live Cn for 1.5 h. The cell cycle distribution of naive macrophages did not change after incubation with conditioned cell medium relative to control conditions.

As noted above, cells in G₁ had a higher capacity to carry out phagocytosis than cells in other phases. However, the FACS study did not show this result. Hence, the most likely explanation for our results is that cells in G₁ do carry out phagocytosis, but are then driven into S and G₂. Consistent with this conclusion, we observed that the population of cells with ingested Cn cells included a higher percentage of cells in S phase and a lower percentage in G₁ phase. The most straightforward interpretation of the data is that phagocytosis promoted cell division, an explanation that was supported by BrdU incorporation of J774 cells during phagocytosis.

Phagocytosis enhances BrdU incorporation

BrdU, an analog of thymidine, is incorporated into the nucleus by DNA synthesis and can be used to detect S phase cells. At the onset of the phagocytosis assay, BrdU was added simultaneously to cells in combination with either beads, live Cn, or dead Cn. BrdU incorporation into the cell nucleus was detected by immunohistochemistry. As predicted, cells in the phagocytosis groups had a higher BrdU incorporation index over nonphagocytic cells, confirming the results observed with FACS that cells are induced to replicate after phagocytosis of particles (Fig. 4).

Complement-mediated phagocytosis alters the proportion of J774 cells in G₁ and S phase

To test whether this phenomenon was opsonin dependent, we examined complement-mediated phagocytosis of Cn strain H99, which were labeled with FITC. The FITC fluorescence of Cn inside J774 cells allowed us to differentiate J774 cells into two populations by FACS, with and without ingested Cn. These two populations were then subjected to cell cycle analysis individually, and

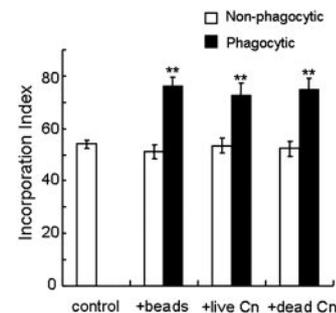


FIGURE 4. BrdU incorporation index after phagocytosis of beads, live Cn or heat-killed Cn. Incorporation index for nonphagocytic and phagocytic J774 cell groups after phagocytosis of beads, live Cn or heat-killed Cn. Differences in incorporation index were statistically significant with $p < 0.001$. The experiment was repeated three times and produced similar results.

the percentages of G₁, S, and G₂ phases in each population were ascertained by cellular DNA content using propidium iodide staining. Again, we found that the J774 cell population that phagocytosed Cn had a lower percentage of cells in G₁ phase and higher percentage of cells in S phase relative to the population that did not phagocytose Cn and to control cells not exposed to Cn (Fig. 5).

Incubation of J774 on IgG1-coated plate alters cell cycle distribution

To determine whether FcR activation by Ab was sufficient to drive cells into the cell cycle, J774 cells were plated on culture dishes coated with IgG1. BSA was coated on the plates as the control. We found that after plated for 2 h, J774 cells grown on plates coated with IgG1 had fewer cells in G₁ phase and higher percentage of cells in S phase compared with J774 cells that were incubated with BSA (Fig. 6). When IgG1 was added to cells in suspension, no difference in cell cycle distribution was observed after 2-h incubation, showing that IgG in solution did not mediate this effect (data not shown).

Cell cycle distribution of bone marrow-derived macrophages is altered after Fc- and complement-mediated phagocytosis

After establishing that phagocytosis enhanced replication in J774 cells, we investigated whether the same phenomenon occurred in primary macrophages. As expected, the overwhelming majority of bone marrow-derived macrophages were in G₁ phase in our culture conditions. Just as in cultured J774 cells, mature bone marrow-derived macrophages phagocytosed Cn strain 24067 opsonized with mAb 18B7 and H99 opsonized with guinea pig serum, although not to the same degree with the latter. Both Fc- and complement-mediated phagocytosis resulted in cell populations that had an appreciable shift in cell cycle such that the bone marrow macrophages with ingested Cn had a higher percentage of cells shifted into S phase relative to the population that did not phagocytose Cn or relative to control cells that were unexposed to Cn (Fig. 7).

Cell cycle distribution of peritoneal macrophages is altered after Fc-mediated phagocytosis in vivo

We investigated whether the phenomenon of enhanced cell cycle progression observed in the J774 cell line and in cultured primary macrophages would occur in vivo. Analysis of peritoneal macrophages from uninfected mice revealed that 90% of the cells collected expressed macrophage marker MAC-3 and all of these peritoneal macrophages were in G₁ phase. After mice were infected i.p. with Ab-opsonized Cn, peritoneal macrophages were harvested and analyzed. Although phagocytosis of Ab-opsonized Cn by peritoneal macrophages in vivo was not as robust as in the optimized

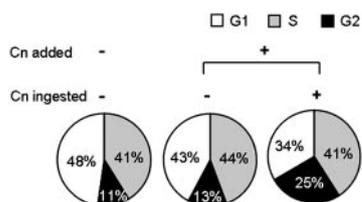


FIGURE 5. Cell cycle distribution of J774 cells after phagocytosis of Cn strain H99 opsonized with guinea pig serum. Percentage of G₁, S, and G₂ cells is indicated in the nonphagocytic (Cn added + and Cn ingested -) and phagocytic (Cn added + and Cn ingested +) groups. Comparison of G₁ percentages between nonphagocytic and phagocytic groups revealed statistically significant differences $p < 0.001$. The experiment was repeated three times and produced similar results.

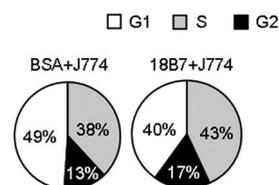


FIGURE 6. Cell cycle distribution of J774 cells incubated in plates coated with BSA or 18B7. Percentage of G₁, S, and G₂ cells is indicated. Comparison of G₁ percentages between nonphagocytic and phagocytic groups revealed statistically significant differences $p < 0.05$. This experiment was repeated three times and produced similar results.

J774 culture assay, it nevertheless revealed that those peritoneal macrophages, which had internalized Cn, were shifted to S and G₂ just as in the previous assays. This was observed despite the variability between individual mice (Table I). This experiment was done eight times, and the effect was observed in four independent experiments.

Discussion

This study originated from two observations. First, we made the serendipitous observation that cells with ingested Cn were more likely to have morphological features consistent with cell division. Second, we noted great interexperimental variation in the phagocytic index of macrophage-like cells for Cn. After considering and excluding such explanations as inadequate time for phagocytosis, opsonin quantity, and yeast cell to macrophage ratio, we combined these observations to hypothesize that the interexperimental differences reflected the state of the phagocytic cells and focused on the cell cycle phase. Our results establish that the efficiency of phagocytosis is a cell cycle-dependent process, and demonstrate for the first time that phagocytosis in turn promotes cell division. J774 cells are more efficient in phagocytosis when they reside in G₁ phase of the cell cycle compared with other phases. This result is consistent with, and confirmatory of, a report that phagocytosis in 3T3 cells occurred primarily in G₁ phase cells (21). Most importantly, we found that phagocytosis can drive the cell cycle of macrophages from G₁ into S phase, which has numerous implications. The FACS results showed that phagocytosis changed the percentage of distribution of cells in the different cycle phases; namely, that in the nonphagocytic population, there were more cells in G₁ than in S phase, and in the phagocytic population there were fewer cells in G₁ and more in S phase. The conclusion that phagocytosis stimulated cell division was supported by the BrdU incorporation experiments, which revealed increased DNA synthesis in cells undergoing phagocytosis. These observations with cultured J774 cells were confirmed with bone marrow-derived macrophages and peritoneal cells in vivo, demonstrating for the first time that induction of cell cycle progression is enhanced by phagocytosis regardless of the type of ingested particles.

Cell replication is affected by external stimuli such as growth factors, cell-cell contact, and cell adhesion to the extracellular matrix. Despite the fact that both cell division and phagocytosis are basic processes of cell biology that have been exhaustively studied for decades, we could not find prior studies in the literature that directly explored connections between phagocytosis and cell division. However, certain observations hint the two processes may be connected. It was recently reported that cyclin D1 deficiency impaired the motility and guided migration of bone marrow macrophages and reduced membrane ruffles (23), in which the ability to make ruffles has been shown to be important for internalizing particles. This suggests an explanation for the result that macrophages in G₁ phase have the optimal ability to carry out phagocytosis

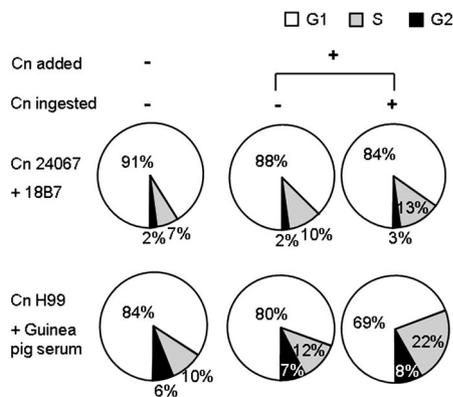


FIGURE 7. Cell cycle distribution of bone marrow-derived macrophages after phagocytosis of Cn strain 20467 mediated by 18B7 and Cn strain H99 mediated by guinea pig serum. Percentage of G₁, S, and G₂ cells is indicated in the nonphagocytic (Cn added + and Cn ingested -) and phagocytic (Cn added + and Cn ingested +) groups. Comparison of G₁, S, and G₂ percentages between nonphagocytic and phagocytic groups revealed statistically significant differences $p < 0.001$. The experiments of phagocytosis of Cn strain 20467 and Cn strain H99 were repeated four and three times, respectively, and produced similar results.

because cyclin D dominates in the G₁ phase of cell cycle. Although the relationship between cytoskeletal changes and cyclins has also been explored (24, 25), a direct link between phagocytosis and cell cycle has not been made. Our findings in this study show that phagocytosis is potentially a powerful stimulus to promote macrophage replication.

Phagocytosis-induced stimulation of cell cycle progression of J774 cells was repeatedly observed after the phagocytosis of polystyrene beads, live Cn or dead Cn. We observed the same phenomenon with bone marrow macrophages, thus extending this to another cell type and to primary macrophages. In the Cn experimental system, IgG1-mediated opsonization can trigger phagocytosis through the FcR or through complement receptor in an unusual complement-independent mechanism whereby the capsule interacts directly with CD18 (26). Because both mechanisms occur simultaneously, we conducted additional experiments to discriminate whether Fc- or complement-mediated opsonization was responsible for the observed stimulation of cell division. Furthermore, we were interested in establishing whether phagocytosis was necessary for the effect. Incubating J774 cells on a plate coated with IgG1 stimulated cell cycle progression, indicating that FcR activation could induce the phenomenon. Furthermore, we noted cell cycle progression after C3-mediated phagocytosis, indicating that the phenomenon could follow activation of the complement receptors. Hence, engaging either Fc or complement receptors can trigger cell cycle progression, and at least the Fc-mediated process does not require ingestion. However, the observation that J774 cells incubated with naked polystyrene beads stimulated cell cycle progression indicates that additional receptors may feed into this signal transduction pathway. The fact that multiple surface receptors central to the phagocytic process can stimulate cell cycle progression, combined with the observation that it occurs in both primary and continuous cell lines, suggests a basic link between two physiological pathways.

A 10–15% progression from G₁ to S phase was consistently observed after the phagocytosis of polystyrene beads, live Cn or dead Cn, in 20 independent experiments. Although at first glance it may appear that only a small percentage of the cells in any one experiment progressed to S phase, this percentage is relatively

large when one considers that approximately half the cells are in a resting state (40–50% for J774 cells and bone marrow macrophages). Incidentally, we obtained similar results with longer incubation time (as long as 4 h). Considering that phagocytosis is a rapid event that is largely completed by 15 min, the relatively short duration of activation of Fc/complement receptors might lead to the measured change of the cell cycle progression.

In the past two decades, the signaling cascades responsible for and resulting from phagocytosis and cell division have been elucidated. Therefore, it is possible to identify shared pathways that may explain our findings. The cell cycle is divided into G₁, S, G₂, and M phases in eukaryotic cells. The regulation of this cycle is primarily controlled by periodic synthesis and destruction of cyclins, which in turn bind to cyclin-dependent kinases (cdks) and activate them. To enter S phase, cells must pass a restriction point in late G₁ phase. This process is conducted by cyclin D, which phosphorylates retinoblastoma tumor suppressor protein (Rb) through the binding of cyclin D with cdk4 and cdk6. Cyclin D expression is induced by mitogenic stimuli instead of oscillating during cell cycle, as the other cyclins. Phosphorylation of Rb prevents it from binding to E2F factors and thus switches E2F from a repressor to an activator of gene expression of cell cycle proteins, including cyclins E and A. Cyclin E participates in a positive feedback control of Rb by maintaining Rb in a hyperphosphorylated state, and thus drives the cell cycle through S and G₂ (27). Intensive studies showed that the cell cycle is under subtle regulation by signaling molecules such as PI3K and Rho GTPases (25, 28–30). The activation of PI3K and Rho GTPases is required for the process of macrophage phagocytosis (2, 3). This leads us to posit that activation of known signaling pathways involved in phagocytosis could potentially influence cell proliferation. For instance, activation of PI3K in phagocytosis could participate not only in the molecular events in phagocytosis, but also in the cell cycle regulation such as the inhibition of p27^{kip1}, which in turn increases the activity of cyclin E/cdk2 and thus enhances the G₁ to S transition (29, 31, 32). Hence, phagocytosis could activate PI3K and stimulate cells to progress in the cell cycle progression via cyclin E.

Phagocytosis by macrophages is an important process in host defense mechanisms. Microbial killing of phagocytosed microbes is beneficial to the protection of organisms from infection. Macrophage local proliferation was an efficient way to replenish the damaged macrophage after killing. Although most hemopoietic cells mature after leaving the bone marrow and cannot proliferate, local proliferation of mononuclear phagocytes has been observed (9, 33). For example, macrophages resident in spleen and peritoneum were renewed by local proliferation (14, 15). Furthermore, macrophages can replicate upon the stimulation of growth factors such as CSFs, and especially during inflammation (11). Thus, the linkage of phagocytosis and cell division could have important consequences for our understanding of microbial pathogenesis and host defense. On one hand, phagocytosis-induced cell division could increase the number of effector cells in response to infection, and also provides a novel mechanism for macrophages to cure themselves after ingesting microbes by the generation of new cells that have the potential to be noninfected. In fact, a study on persistent infection of host cells with *Coxiella burnetii* showed that heavily infected host cells had the ability to restrain intracellular *Coxiella* in one large parasite-containing vacuole, which was passaged into one of the two daughter cells of dividing host cells. Thus, a pathogen-free companion daughter cell was generated (34, 35). This would be one of the mechanisms that infected cells could cure themselves by cell replication. In contrast, many microbes are intracellular pathogens that replicate preferentially inside phagocytic cells and use intracellular residence as a mechanism to evade

Table 1. Cell cycle of peritoneal macrophages after Fc-mediated phagocytosis in vivo^a

Cell Cycle	-			+					
	Cn Added			- ^b			+ ^c		
	G ₁	S	G ₂	G ₁	S	G ₂	G ₁	S	G ₂
Experiment 1	100	0	0	100	0	0	77	15	8
Experiment 2	100	0	0	100	0	0	96	2	2
Experiment 3	100	0	0	99	0	1	91	3	6
Experiment 4	100	0	0	100	0	0	91	7	2

^a Percentage of G₁, S, and G₂ cells is indicated in the control (Cn added -, Cn ingested -), nonphagocytic (Cn added + and Cn ingested -), and phagocytic (Cn added + and Cn ingested +) groups.

^b $p < 0.01$, compared between the Cn added +/Cn ingested + group and the Cn added -/Cn ingested - group.

^c $p < 0.01$, compared between the Cn added +/Cn ingested + group and the Cn added +/Cn ingested - group.

the immune system. Consequently, this phenomenon could also undermine host defenses by generating new infected cells in which microbes can proliferate. Clearly, the benefit or debit of this phenomenon for host defense would be dependent on the type of pathogen. The linkage of phagocytosis-induced cell division with the need for nutrients is intriguing given that phagocytosis of bacteria and other microbes is used by unicellular eukaryotes for food acquisition. Hence, phagocytosis may be linked to cell division through ancient pathways that signal the acquisition of food, which in turn would imply favorable conditions for replication.

In summary, our results indicate that phagocytosis occurs preferentially in G₁ phase, and that particle ingestion stimulates the progression of cell cycle. Remarkably, this phenomenon is unknown despite the fact that both processes have been studied extensively. Cellular division following phagocytosis could have major implications for the outcome of macrophage infection. For some pathogens, we anticipate that the effect would help the host to generate additional uninfected effector cells at the site of infection. However, for other pathogens, this effect could contribute to pathogenesis. If that is the case, it is possible that future studies could identify the precise signaling pathway involved in phagocytosis-associated cell division and evaluate it as a target for antimicrobial therapy of certain infectious diseases. Furthermore, the findings suggest the need to investigate the phylogenetic origins of this phenomenon, which may have ancient roots in eukaryotic evolution.

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Disclosures

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