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Phagocytosis Inhibits F-Actin-Enriched Membrane Protrusions Stimulated by Fractalkine (CX3CL1) and Colony-Stimulating Factor 1[▽]

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Cryptococcus neoformans **is the only encapsulated human-pathogenic fungus and a facultative intracellular pathogen that can reside in macrophages without host cell lysis. In the present study, we investigated how phagocytosis of *C. neoformans* affected the macrophage response to chemoattractants such as fractalkine (FKN) (CX3CL1) and colony-stimulating factor 1 (CSF-1). Phagocytosis of immunoglobulin G (IgG)-opsonized *C. neoformans* and IgG- or C3bi-opsonized sheep erythrocytes was performed using a RAW 264.7 subline (LR5 cells) and bone marrow-derived macrophages (BMM). The chemotactic response to FKN or CSF-1 was quantitated by measurement of the formation of F-actin-enriched membrane protrusions (ruffles), which showed that FKN or CSF-1 stimulated strong transient ruffling in both LR5 cells and BMM. This stimulated cell ruffling was inhibited by phagocytosis in an intracellular-pathogen-number-dependent manner. The inhibition of ruffling was not simply a result of reduced membrane availability since membrane sequestration by sucrose treatment did not inhibit the ruffling response. The phagocytosis process was required to inhibit ruffling as BMM from FcγR$^{-/-}$ mice that bound *C. neoformans* but did not ingest it retained the ability to ruffle in response to chemoattractants. These results imply that the inhibition of FKN- or CSF-1-stimulated cell ruffling was a direct consequence of the phagocytosis process. Since cell ruffling is a prelude to chemotaxis, this observation links two functions of macrophages that are critical to host defense, chemotaxis and phagocytosis. Phagocytosis-induced chemotactic suppression may enhance host defense by keeping these antimicrobial effector cells at infected sites and reduce the likelihood of microbial spread by wandering macrophages containing infectious cargo.**

Macrophages are professional phagocytic cells that are critical effector cells of the immune system. Phagocytosis is a process by which certain cells ingest large foreign particles, typically 0.5 μm or larger. Phagocytosis begins with the recognition of foreign particles by cell surface receptors on the macrophages. Two types of these receptors, Fcγ receptors (FcγRs) and complement receptors, bind antibody-opsonized and complement-opsonized microbial particles, respectively (1, 49). Phagocytosis includes rearrangement of the cytoskeleton and cell membrane to ingest and kill microbes (19, 49). The process of opsonization and ingestion involves many cellular activities, such as receptor engagement, signaling transduction, actin rearrangement, membrane ruffling, reactive oxygen species generation, cytokine release, and antigen presentation.

Macrophages are capable of chemotaxis, which is defined as the directional migration of cells along a chemical gradient. Cell migration plays a critical role in such diverse processes as innate immunity, embryogenesis, and angiogenesis, as well as in pathological conditions such as wound healing, inflammation, and metastasis of cancer cells (22). In particular, the chemotaxis of macrophages into tissues is an important step in host defense against infection by microbial pathogens as the capacity of macrophages to migrate to infection loci enables the host to remove infectious microbes effectively (43). In general, chemoattractants bind to cell surface receptors that can belong either to the tyrosine kinase receptor family or to a large family of G-protein-coupled receptors (GPCRs) and subsequently trigger rapid reorganization of the actin cytoskeleton. The chemoattractant gradient biases F-actin formation and thus guides cell movement toward the source of chemoattractants (22, 46). F-actin enrichment under the cell membrane generates morphological changes, such as membrane protrusions or ruffles (23).

More than 50 chemokines (chemoattractants and chemorepellents) have been identified, and these molecules are classified into the C, CC, CXC, and CX3C subfamilies according to the number and arrangement of the cysteine residues in their conserved N-terminal cysteine motifs (36, 37). Functionally, chemokines are classified as "inflammatory" or "homeostatic" chemokines depending on whether they are generated at an inflammation site to recruit cells or they are physiologically involved in basal trafficking and homing of cells (34). Fractalkine (FKN), also known as CX3CL1 or neurotactin, was originally identified as a transmembrane-anchored molecule on the surface of endothelial cells in various tissues, acting as an adhesion molecule. The soluble form of FKN, which is generated by proteolytic cleavage from the cell membrane-anchored form, functions as a chemokine for monocytes, NK cells, and T cells (4, 40). Previous studies revealed that FKN has a significant role in targeting mononuclear phagocytes during the pathogenesis of chronic inflammatory conditions, including rheumatoid arthritis, atherosclerosis, and brain inflammation (12, 28, 31, 35, 38, 42), as well as in host defense against

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microbial pathogens (21, 47). Colony-stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor, is an extensively studied factor with functional heterogeneity for macrophages. It enhances chemotaxis and regulates proliferation, differentiation, and survival of macrophages (41). It also augments other critical functions of monocytes and macrophages for the control of microbial infections, such as phagocytosis, cytotoxicity, superoxide production, and secondary cytokine production (39). In addition, CSF-1 plays a role in various inflammatory conditions, including those of the brain (10).

This study began with the observation that phagocytosis of the human-pathogenic fungus *Cryptococcus neoformans* by macrophages reduced the cell membrane ruffling response to chemoattractants, which is a prelude to chemotaxis. *C. neoformans* is a major cause of life-threatening infections, such as pulmonary cryptococcosis and meningoencephalitis in patients with impaired immunity (8, 32). *C. neoformans* is a facultative intracellular pathogen, and macrophages are the first line of defense in controlling pulmonary infection and maintaining the state of latency (18, 48). Extrapulmonary dissemination often results in meningoencephalitis, a condition where *C. neoformans* invades the central nervous system after crossing the blood-brain barrier. There is evidence that cerebral infection occurs when yeast cells are shuttled across the barrier by carrier macrophages by a "Trojan horse" mechanism (9). Hence, the observation that phagocytosis of *C. neoformans* reduced the ruffling response was intriguing because it associated two macrophage properties that are implicated in cryptococcal pathogenesis, phagocytosis and chemotaxis. In the present study, we showed that phagocytosis inhibited FKN- and CSF-1-induced macrophage ruffling. The association of phagocytosis with inhibition of chemotaxis has important potential implications for our views of macrophage function in host defense.

MATERIALS AND METHODS

Reagents. FKN and CSF-1 were obtained from R&D Systems (Minneapolis, MN). Phalloidin conjugated with Alexa Fluor 568 was obtained from Invitrogen (Carlsbad, CA).

Culture of *C. neoformans*. Acapsular *C. neoformans* strain Cap67, its parental strain B3501 (serotype D), and *C. neoformans* strain 24067 (serotype D) were obtained from the American Type Culture Collection (Manassas, VA). *C. neoformans* was cultured for 2 to 3 days in Sabouraud dextrose broth at 30°C with moderate shaking at 150 rpm. Cells were collected by centrifugation, washed with phosphate-buffered saline three times, and resuspended with phosphate-buffered saline at the appropriate concentration after counting with a hemocytometer. Dead *C. neoformans* cells were prepared by incubating *C. neoformans* strain 24067 cultures in a water bath at 65°C for 60 min.

Culture of RAW/LR5 cells. LR5 is a subline derived from the RAW 264.7 cell line as previously described (13). The cells were cultured at 37°C with 5% CO_2 in RPMI media (Mediatech Inc., Herndon, VA) with 10% fetal bovine serum.

Culture of murine BMM. To obtain wild-type bone marrow-derived macrophages (BMM), 3-month-old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). FcγRII$^{-/-}$ and Fc$\gamma^{-/-}$ mice, which were generated from C57BL/6 mice and were a similar age, were obtained from the animal facility at the Albert Einstein College of Medicine (Bronx, NY). Mice were euthanized, and bone marrow cells were harvested from the hind leg bones as previously described (45). The harvested cells were cultured at 37°C with 5% CO_2 in αMEM media with 15% fetal bovine serum and 360 ng/ml recombinant human CSF-1 (Chiron, Emeryville, CA). The media were replaced after 3 days to remove floating cells, and the attached cells (mature macrophages) were used in the subsequent experiments.

Phagocytosis assay. Approximately 10^5 macrophages were plated on round glass coverslips in a 24-well plate and cultured overnight at 37°C with 5% CO_2 in RPMI media with 10% fetal bovine serum. Prior to the phagocytosis assay, the cells were serum starved with BWD buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 5 mM dextrose, 10 mM $NaHCO_3$, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.4) for 2 h. For the phagocytosis assay with *C. neoformans* as the target, washed *C. neoformans* strain 24067 cells were added at an effector-to-target ratio of 10:1. *C. neoformans* capsule-specific immunoglobulin G1 (IgG1) monoclonal antibody 18B7 was used as an opsonin at a concentration of 10 μg/ml. Incubation was carried out in the presence of 5% CO_2 at 37°C for 1.5 h. Alternatively, sheep erythrocytes opsonized with either IgG (EIgG) or C3bi (EC3bi) as described previously (14) were used as phagocytic particles. Macrophages were incubated with opsonized erythrocyte suspensions for 0.5 h unless otherwise indicated, and any remaining extracellular particles were removed by three washes with BWD buffer after incubation. For quantification of phagocytosis, macrophages were examined with a microscope at a magnification of $\times$600, and the number of ingested particles was determined. The phagocytosis index was determined by multiplying the percentage of macrophages with internalized particles by the average number of internalized particles, and the attachment index was determined by multiplying the percentage of macrophages with attached particles by the average number of attached particles.

Cell ruffling assay. Approximately 10^5 macrophages were plated on round glass coverslips in a 24-well plate and were cultured overnight at 37°C with 5% CO_2 in RPMI media with 10% fetal bovine serum. Prior to stimulation with FKN or CSF-1, the cells were serum starved with BWD buffer for 2 h and then incubated with or without phagocytic particles as described above. Cells were then stimulated with 50 ng/ml FKN for 1 min or with 20 ng/ml CSF-1 for 5 min. Stimulation was promptly stopped by fixing the cells with 3.7% formaldehyde, and the cells were permeabilized with 0.2% Triton 100 and then stained to determine the presence of F-actin using phalloidin conjugated with Alex Fluor 568. All images were taken using the 60$\times$ oil/1.40 phase3 objective of an Olympus IX71 microscope coupled to a Sensicam cooled charge-coupled device camera. Cell ruffles were quantified as described previously (26). Briefly, the cell ruffles were scored using a scale from 0 to 3, with 0 indicating no protrusion, 1 indicating protrusions in one area of the cell, 2 indicating protrusions in two distinct areas of the cell, and 3 indicating protrusions in more than two distinct areas of the cell. The ruffling index was calculated by determining the average of the ruffling scores of at least 50 cells.

Membrane sequestration. Vacuolization was induced as described by Cannon and Swanson (6) by incubating BMM in 20 mg/ml sucrose in αMEM media with 15% fetal bovine serum for 16 h. Sucrose-treated cells either were used immediately for phagocytosis assays or were incubated further in serum-free media containing sucrose prior to stimulation with either FKN or CSF-1 as described above.

Statistical analysis. The Mann-Whitney test was used to compare the ruffling indexes of different groups. A P value of <0.05 was considered statistically significant. Standard errors of the means were also determined. The analysis was performed by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

RESULTS

***C. neoformans* phagocytosis inhibits the macrophage chemotactic response to FKN and CSF-1.** To investigate the chemotactic response of macrophages, BMM were stimulated with two commonly used chemoattractants, FKN and CSF-1, that have been shown to mediate migration of monocytes to the brain (10, 12). The macrophage response was documented by quantification of F-actin-enriched membrane protrusions (cell ruffling) with a microscope after fixation and staining of F-actin with phalloidin. We typically observed robust cell ruffling on lateral and dorsal sides of macrophages after treatment with FKN for 1 min or after treatment with CSF-1 for 5 min (Fig. 1). Using this system, the effect of phagocytosis of *C. neoformans* on the chemotactic responses to FKN and CSF-1 was studied by comparing cell ruffling before and after phagocytosis. The chemotactic response of macrophages to FKN and CSF-1 was significantly inhibited after ingestion of *C. neoformans* compared to the chemotactic response of cells before phagocytosis

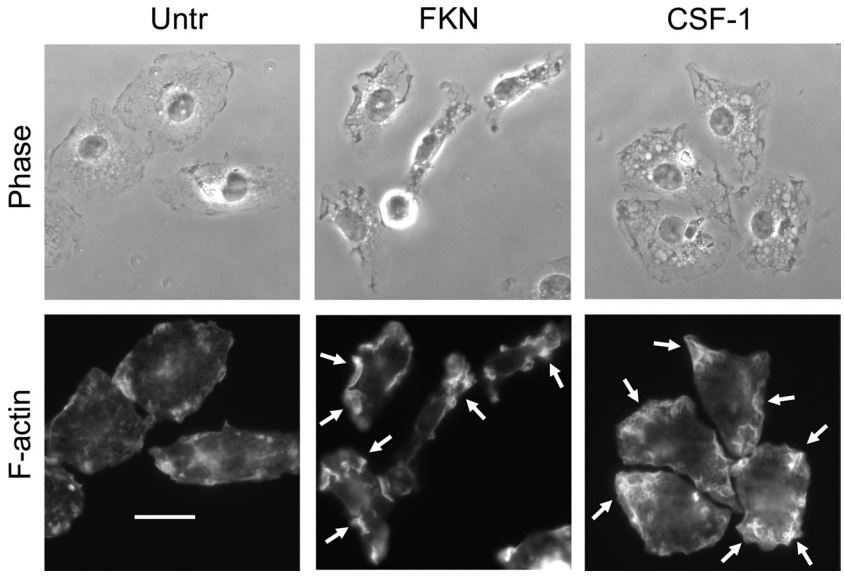

FIG. 1. Macrophage cell ruffling stimulated by FKN and CSF-1. BMM were stimulated with FKN or CSF-1, and untreated cells (Untr) were used as a negative control. After fixation, F-actin in the cells was stained using phalloidin conjugated with Alexa Fluor 568. BMM cell ruffling was determined microscopically. The images are fluorescence images (F-actin) and corresponding phase-contrast images (Phase). The arrows indicate F-actin-enriched membrane protrusions (ruffles) stimulated by FKN and CSF-1 treatment. Scale bar = 10 μm. The images are representative of at least 10 independent experimental repeats.

(Fig. 2). In the untreated group, BMM had a low basal level of ruffling (ruffling index, 0.3). Following treatment with FKN for 1 min or with CSF-1 for 5 min, BMM showed a significantly greater cell ruffling response, as the ruffling indexes of both treated groups were around 2.5 (Fig. 2B and C). However, after phagocytosis of IgG-opsonized *C. neoformans*, the indexes of cell ruffling induced by FKN and CSF-1 decreased significantly to 0.2 and 0.4, respectively, and were 6- to 12-fold lower than the ruffling index for BMM without addition of *C. neoformans* (Fig. 2B). To investigate whether this phenomenon could be generalized to other cell types, the experiment was repeated using a macrophage cell line (LR5), and similar results were observed (Fig. 2C). While LR5 cells had a higher baseline ruffling index than BMM, treatment of the LR5 cells with FKN for 1 min or with CSF-1 for 5 min increased the cell ruffling index to ~2.5. Similar to the results obtained with BMM, phagocytosis of *C. neoformans* significantly reduced the ruffling indexes of both treated groups to 1.2. This result showed that phagocytosis of *C. neoformans* significantly inhibited the macrophage chemotactic response to FKN and CSF-1.

Phagocytosis-induced inhibition of the macrophage chemotactic response was dependent on the number of ingested yeast cells. We considered whether the inhibition of the chemotactic response to FKN and CSF-1 by phagocytosis of *C. neoformans* occurred via two independent mechanisms. One possibility was that the inhibition of ruffling was due to the phagocytosis of *C. neoformans* and subsequent intracellular events. Alternatively, the inhibition could have been a result of inhibitory signals, such as soluble factors secreted from the macrophages undergoing phagocytosis affecting nearby macrophages. To differentiate these two possibilities, we correlated the ruffling index for the untreated LR5 cells, the LR5 cells treated with FKN for 1 min, or the LR5 cells treated with CSF-1 for 5 min after phagocytosis of *C. neoformans* with the number of intracellular ingested *C. neoformans* cells (0, 1, 2, and ≥3 *C. neoformans* cells) (Fig. 3). The ruffling index before phagocytosis was the control. The results showed that the ruffling index was inversely correlated with the number of ingested intracellular *C. neoformans* cells in both untreated LR5 cells and LR5 cells treated with FKN and CSF-1. This in turn implied that the inhibition of the chemotactic response caused by phagocytosis of *C. neoformans* was most likely due to the phagocytosis of *C. neoformans* and subsequent intracellular events, given that inhibition was dependent on the number of ingested *C. neoformans* cells. LR5 cells that did not ingest *C. neoformans* after phagocytosis had a ruffling index similar to that of the LR5 cells in the control group, which were not infected with *C. neoformans*. This finding provided evidence that soluble factors were not involved. In addition, the correlation lines for LR5 cells treated with FKN and LR5 cells treated with CSF-1 had similar slopes, and this suggested that the mechanisms underlying the phenomenon are similar.

Phagocytosis of dead *C. neoformans* and an acapsular strain of *C. neoformans* inhibits the macrophage chemotactic response to FKN and CSF-1. It is possible that the ingested *C. neoformans* cells within the macrophages could inhibit the macrophage chemotactic response to FKN and CSF-1 through toxic activities or their capsular components that are major virulent factors. To investigate this possibility, dead *C. neoformans* strain 24067 cells prepared by heat killing or acapsular strain Cap67 cells were used in our experiments; live strain 24067 cells and cells of the parental strain of Cap67, *C. neoformans* strain B3501, were used as the controls, respectively. Our results showed that, similar to the phenomenon that we observed previously, phagocytosis of these yeasts also inhibited the macrophage ruffling response to FKN (Fig. 4) and CSF-1 (data not shown).

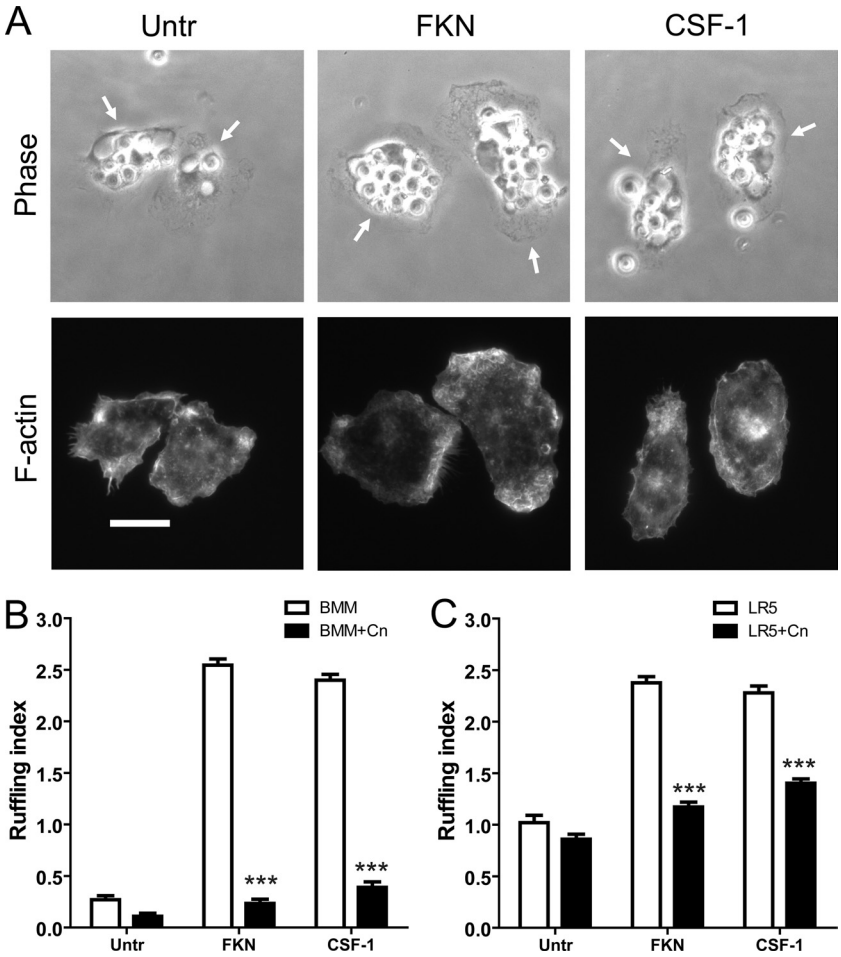

FIG. 2. Inhibition of FKN- and CSF-1-stimulated macrophage cell ruffling by phagocytosis of *C. neoformans*. (A) After phagocytosis of IgG-opsonized *C. neoformans*, BMM were stimulated with FKN or CSF-1, and untreated cells (Untr) were used as a negative control. After fixation, F-actin in the cells was stained using phalloidin conjugated with Alexa Fluor 568. BMM cell ruffling was determined microscopically. The images are fluorescence images (F-actin) and corresponding phase-contrast images (Phase). Scale bar = 10 μm. The images are representative of at least five independent experimental repeats. (B and C) Cell ruffling of BMM (B) or LR5 cells (C) stimulated by FKN and CSF-1 was quantified by determining the ruffling index (see Materials and Methods). Untreated cells were used as a negative control. Data for cells after phagocytosis of IgG-opsonized *C. neoformans* (BMM+Cn, LR5+Cn) were compared to data for cells without phagocytosis (BMM, LR5) by the Mann-Whitney test (***, $P < 0.001$). The error bars indicate standard errors of the means. The data were obtained from three independent experimental repeats.

Erythrocyte phagocytosis inhibits the macrophage chemotactic response to FKN and CSF-1. Given that all the initial observations were made with phagocytosis of IgG-opsonized *C. neoformans*, we investigated whether the phenomenon was specific to phagocytosis of *C. neoformans* or was an outcome of activation of the FcγR-mediated phagocytosis pathway. Consequently, we investigated ruffling after phagocytosis of EIgG or EC3bi and observed similar results in both experiments (Fig. 5). For both groups, FKN and CSF-1 stimulation induced cell ruffling of the LR5 cells at levels that were three- to fivefold greater than the levels observed for untreated cells (ruffling indexes, 0.3 or 0.4 to 1.3 or 1.4). Phagocytosis of EIgG for 0.5 h significantly reduced the cell ruffling index to that of untreated cells, 0.2 to 0.3 (Fig. 5A). The suppression of FKN- and CSF-1-stimulated cell ruffling persisted long after phagocytosis was completed since cell ruffling by FKN and CSF-1 was still inhibited even after 1.5 h of incubation.

Given that the mechanisms of Fc- and complement-mediated phagocytosis are quite different, we tested the inhibitory effect of complement-mediated phagocytosis on cell ruffling using phagocytosis of EC3bi (Fig. 5B). To enhance complement-mediated phagocytosis, LR5 cells were pretreated with phorbol myristate acetate (PMA) prior to phagocytosis; hence, the impact of PMA on cell ruffling was included in the data for comparison. While PMA treatment slightly inhibited cell ruffling in response to FKN and CSF-1 (ruffling indexes, 1.3 or 1.4 to 0.7 or 0.9), phagocytosis of EC3bi significantly reduced cell ruffling to a much lesser extent (ruffling index, 0.13 to 0.16).

Phagocytosis-induced inhibition of the macrophage chemotactic response was not due to membrane sequestration. Phagocytosis results in a decrease in the cell surface membrane along with internalization of ingested particles. This can result in membrane sequestration, especially with phagocytosis of either a large number of particles or large particles. Conceivably, internalization of *C. neoformans* could lead to movement of a large portion of the cell membranes into phagosomes,

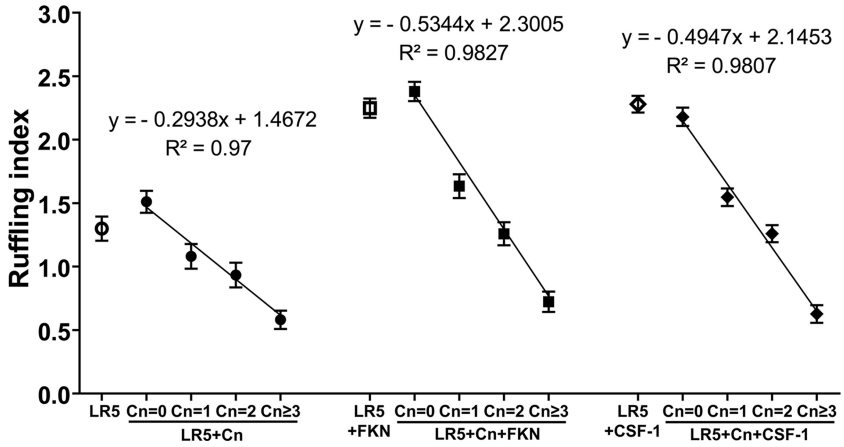

FIG. 3. Correlation of FKN- and CSF-1-stimulated macrophage cell ruffling with the number of ingested intracellular *C. neoformans* cells. The ruffling indexes for untreated LR5 cells and for LR5 cells treated with FKN or CSF-1 after phagocytosis of *C. neoformans* (LR5+Cn, LR5+Cn+FKN, and LR5+Cn+CSF-1, respectively) were correlated with the number of ingested *C. neoformans* cells (Cn=0, Cn=1, Cn=2, Cn≥3). LR5 cells with no addition of *C. neoformans* were used as a control (LR5, LR5+FKN, and LR5+CSF-1). The data were analyzed by linear regression, and the equations generated and R^2 values are shown. The error bars indicate standard errors of the means. The data were obtained from three independent experimental repeats.

which might in turn reduce cell ruffling. To test this possibility, BMM were treated with sucrose to induce membrane sequestration by maximally expanding the endocytic compartment (6). As expected from previous reports (6), sucrose-treated BMM exhibited reduced phagocytic capacity for EIgG (Fig. 6A). However, the cell ruffling response to FKN and CSF-1 was not inhibited by sucrose treatment (Fig. 6B and C). These results indicate that the inhibition of the chemotactic response to FKN and CSF-1 was not due to membrane sequestration caused by particle uptake.

Phagocytosis-induced inhibition of the macrophage chemotactic response was partially mediated by FcγR signaling. To investigate whether FcγRs were involved in the inhibitory effect of phagocytosis on cell ruffling described above, we investigated this process using BMM from Fcγ subunit double-knockout mice ($Fc\gamma^{-/-}$ BMM), FcγRII double-knockout mice ($Fc\gamma RII^{-/-}$ BMM), and wild-type C57BL parental mice (WT BMM) (Fig. 7). The efficacies of phagocytosis for BMM and attachment of *C. neoformans* to BMM in these experiments were quantified using the phagocytosis index and the attachment index, respectively (Fig. 7B and C). As expected, *C. neoformans* cells were largely bound to $Fc\gamma^{-/-}$ BMM, and there was less internalization compared to the results for WT BMM and $Fc\gamma RII^{-/-}$ BMM as the γ subunit is required for activation of the internalization process (Fig. 7B and C). In the control group, in which no *C. neoformans* was added to the system, $Fc\gamma^{-/-}$ and WT BMM responded similarly to FKN and CSF-1 (Fig. 7D). However, after *C. neoformans* attach-

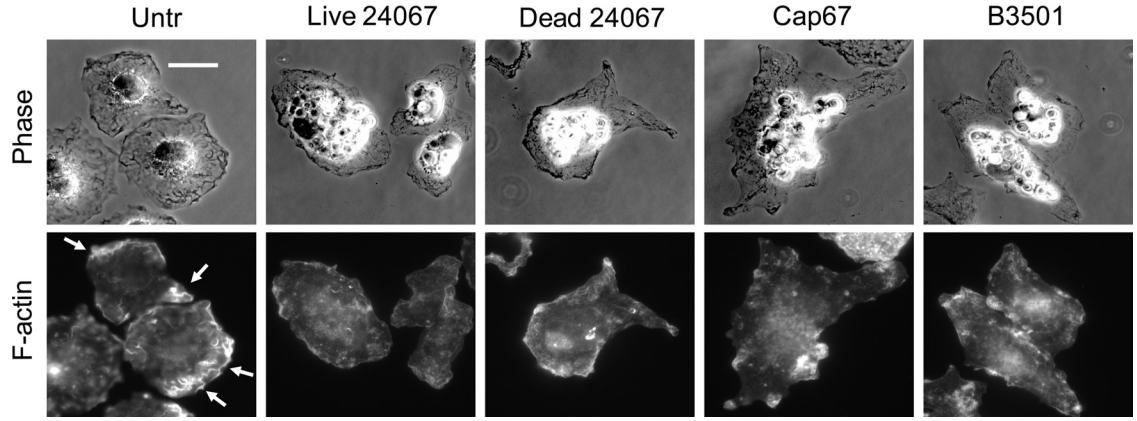

FIG. 4. Inhibition of FKN- and CSF-1-stimulated macrophage cell ruffling by phagocytosis of dead *C. neoformans* cells and acapsular *C. neoformans* cells. After phagocytosis of IgG-opsonized dead *C. neoformans* cells (Dead 24067) (live 24067 cells were used as the control) and acapsular *C. neoformans* cells (Cap67) (IgG-opsonized B3501 cells were used as the control), BMM were stimulated with FKN or CSF-1 (data not shown). Untreated cells (Untr) were used as a negative control. After fixation, F-actin in the cells was stained using phalloidin conjugated with Alexa Fluor 568. BMM cell ruffling was determined microscopically. The images are fluorescence images (F-actin) and corresponding phase-contrast images (Phase). The arrows indicate F-actin-enriched membrane protrusions (ruffles) stimulated by FKN treatment. Scale bar = 10 μm. The images are representative of three independent experimental repeats. Data obtained with CSF-1 are not shown but were similar to the data obtained with FKN.

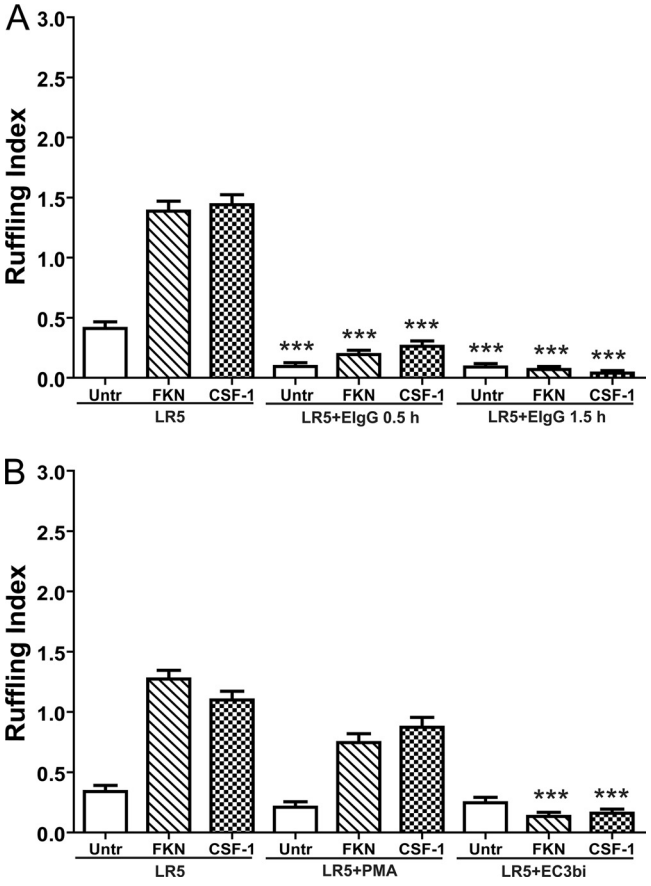

FIG. 5. Inhibition of FKN- and CSF-1-stimulated macrophage cell ruffling by phagocytosis of EIgG or EC3bi. (A) LR5 cells with no erythrocytes added (LR5) and LR5 cells after phagocytosis of EIgG for 0.5 h (LR5+EIgG 0.5 h) and 1.5 h (LR5+EIgG 1.5 h) were stimulated with FKN or CSF-1. Untreated LR5 cells (Untr) were used as a control. Data for LR5 cells after phagocytosis of EIgG were compared to data for the same treatment for LR5 cells by Student's *t* test (***, $P < 0.001$). The data were obtained from three independent experimental repeats which produced similar results. (B) LR5 cells with no erythrocytes added (LR5), LR5 cells treated with PMA (LR5+PMA), and LR5 cells after phagocytosis of EC3bi (LR5+EC3bi) were stimulated with FKN and CSF-1. Untreated LR5 cells were used as a control. Data for LR5 cells after phagocytosis of EC3bi were compared to data for the same treatment for LR5 cells treated with PMA and for LR5 cells with no erythrocytes added by the Mann-Whitney test (***, $P < 0.001$). The error bars indicate standard errors of the means. The data were obtained from three independent experimental repeats.

ment, the cell ruffling response to FKN and CSF-1 of Fc$\gamma^{-/-}$ BMM was significantly higher than that of the C57BL control and FcγRII$^{-/-}$ BMM. These results indicated that the Fcγ subunit partially mediated the inhibition of the macrophage chemotactic response to FKN and CSF-1.

DISCUSSION

Phagocytosis and chemotaxis are two fundamental macrophage functions that share many cellular mechanisms. Despite their importance for host defense, the connection between phagocytosis and chemotaxis has not been extensively explored. In the present study, we investigated the effect of phagocytosis on the macrophage response to chemoattractants such as FKN and CSF-1, as quantitatively measured by the formation of cell ruffles, an event that heralds the onset of chemotaxis. The FKN- or CSF-1-stimulated cell ruffling was significantly inhibited by phagocytosis of IgG-opsonized *C. neoformans* in an intracellular-pathogen-number-dependent manner. Similar results were observed after phagocytosis of EIgG by both Fc-mediated and complement-mediated pathways. The inhibition of ruffling was not simply a result of reduced membrane availability since membrane sequestration by sucrose treatment did not inhibit the ruffling response. The inhibition of cell ruffling was partially mediated by the γ subunits of FcγR as the inhibition was largely attenuated in macrophages obtained from Fc$\gamma^{-/-}$ mice. These results imply that the inhibition of FKN- or CSF-1-stimulated cell ruffling was a direct consequence of the phagocytosis process. These observations establish a functional link between macrophage phagocytosis as a host defense mechanism and the chemotactic responses of macrophages to various factors during inflammatory processes. Compared to previous observations related to inhibition of neutrophil chemotaxis specifically caused by ingestion of *Legionella micdadei* (17) and capsular mannoprotein 4 of *C. neoformans* (11), the present study demonstrated the link with macrophages, another important type of professional phagocytes, on a broader basis, which is phagocytosis per se, not phagocytosis related to the phagocytic target.

Membrane ruffles are important components of the machinery of cell migration, which is initiated by the rapid reorganization of actin around the cell edge. The reorganization of actin leads to the formation of lamellipodia and membrane ruffles. Lamellipodia are characterized by the formation of focal adhesions between the cell membrane and substratum, and they serve as the major locomotory apparatus of the cells by generating traction force (23). Membrane ruffles are curled structures which have no focal adhesions underneath them that are often located on the edge of cell surfaces in lamellipodia. These ruffles are commonly believed to participate in various motile functions of the cells, including phagocytosis and macropinocytosis, as well as receptor tyrosine kinase signaling (27, 33, 44). In the present study, membrane ruffles were used as a visual indicator of the macrophage chemotactic response to chemoattractants, which was well established previously (13). Therefore, a reduction in cell ruffles was inferred to indicate a reduced chemotactic capacity of macrophages induced by phagocytosis.

We hypothesize that the reduced chemotactic capacity of macrophages induced by phagocytosis could contribute to macrophage retention at sites of infection. Our results showed that a rapid decline in cell ruffling was related to an increase in the number of phagocytosed *C. neoformans* cells. Phagocytosis of two and three *C. neoformans* particles decreased the cell ruffling index to values that were one-half and one-third, respectively, of the value observed for stimulated cells with no ingested particles. The usual phagocytosis capacity of macrophages is far greater than two or three *C. neoformans* particles, depending on the effector/target ratio at the sites of infection. A fully activated macrophage may phagocytose 30 to 50 *C. neoformans* cells, as we commonly observed. Therefore, phagocytosis-mediated reduced chemotactic capacity provides an effective mechanism to retain activated macrophages at the sites

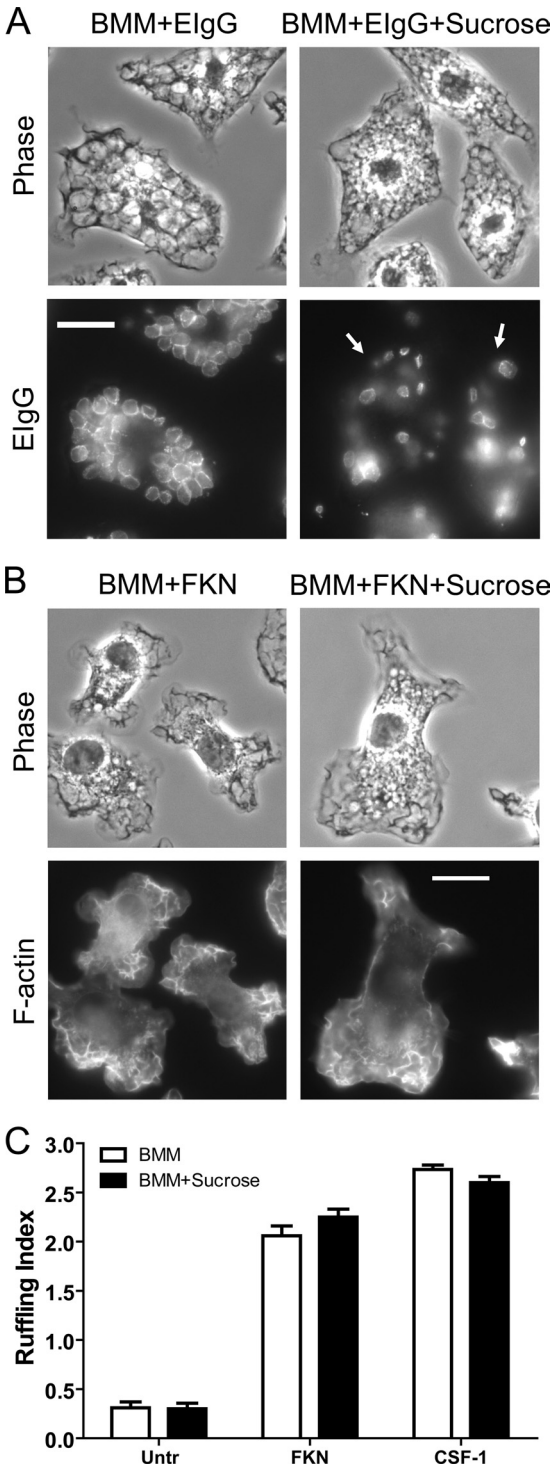

FIG. 6. Cell ruffling stimulated by FKN and CSF-1 in macrophages following vacuolization using sucrose. (A) Phagocytosis of EIgG with untreated BMM (BMM+EIgG) and sucrose-treated BMM (BMM+EIgG+Sucrose). After fixation, EIgG were labeled with anti-IgG antibody conjugated with Alexa Fluor 488 (EIgG). The images are immunofluorescence images and corresponding phase-contrast images (Phase). Scale bar = 10 μm. The images are representative of three independent experimental repeats with similar results. The arrows indicate significantly decreased phagocytosis of EIgG by macrophages treated with sucrose. (B) Untreated BMM and sucrose-treated BMM were stimulated with FKN (BMM+FKN and BMM+FKN+Sucrose, respectively). After fixation, F-actin in the cells was stained using phalloidin conjugated with Alexa Fluor 568. The images are fluorescence images (F-actin) and corresponding phase-contrast images (Phase). Scale bar = 10 μm. The images are representative of three independent experiments. (C) FKN- and CSF-1-stimulated cell ruffling of BMM or sucrose-treated BMM (BMM+Sucrose) was quantified by determining the ruffling index. BMM and sucrose-treated BMM were compared. No statistical difference was revealed by the Mann-Whitney test. The error bars indicate standard errors of the means. The data were obtained from three independent experiments. Untr, untreated BMM.

of infection, which in turn could result in better control of microbial infection. In addition, this mechanism may also reduce the likelihood of microbial spread due to wandering macrophages containing infectious cargo. In this regard, it has been proposed that *C. neoformans* disseminates from the lung and across the brain barrier inside migrating infected macrophages (Trojan horse mechanism) (9, 25). Inhibition of macrophage migration after phagocytosis, therefore, may be particularly important for *C. neoformans* given that dissemination of *C. neoformans* across the blood-brain barrier in the pathogenesis of cryptococcal meningoencephalitis can follow carriage by infected macrophages (9). However, this inhibition is probably only partially effective, and given that the degree cell membrane ruffling inhibition correlated with the number of ingested cryptococci, it is possible that extrapulmonary and meningeal dissemination occur primarily with phagocytic cells containing relatively few ingested yeast cells.

Phagocytosis and chemotaxis are both complex biological processes that involve the activation of numerous signaling pathways (1, 22, 23, 49). Phagocytosis of *C. neoformans*, which requires opsonins, is generally classified into Fc-mediated phagocytosis and complement-mediated phagocytosis, depending on whether the opsonins are antibodies or complement components, respectively. Binding of antibody-opsonized particles results in cross-linking of FcγRs on the macrophage cell surface. This induces the phosphorylation of tyrosine residues on immunoreceptor tyrosine-based activation motifs of the receptors by Src tyrosine kinases (16). The downstream signaling molecules involved in the subsequent phagocytosis process include the Rho family of small GTPases (Rac, CDC42), phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC), and protein kinase C (5, 7, 15, 30, 51). Complement-mediated phagocytosis shares similar signaling pathways, which are based on the activation of intracellular tyrosine kinases and downstream molecules, including PLC, PI3K, and ERK1/2 (2), but there are differences between complement-mediated phagocytosis and Fc-mediated phagocytosis. The small GTPases Rac and Cdc42 are required for phagocytosis of antibody-opsonized phagocytosis, whereas only Rho is needed for phagocytosis of C3b-opsonized particles (7). Similarly, chemotaxis is also a complicated process, and the signaling transduction pathways responsible for chemotaxis have significant overlap with those of phagocytosis. Macrophages can respond to chemoattractants through both GPCR and receptor tyrosine kinases, to which FKN and CSF-1 receptors belong, respectively (23, 29). Binding of GPCR to its ligand leads to activation of heterotrimeric G proteins, whereas binding of the

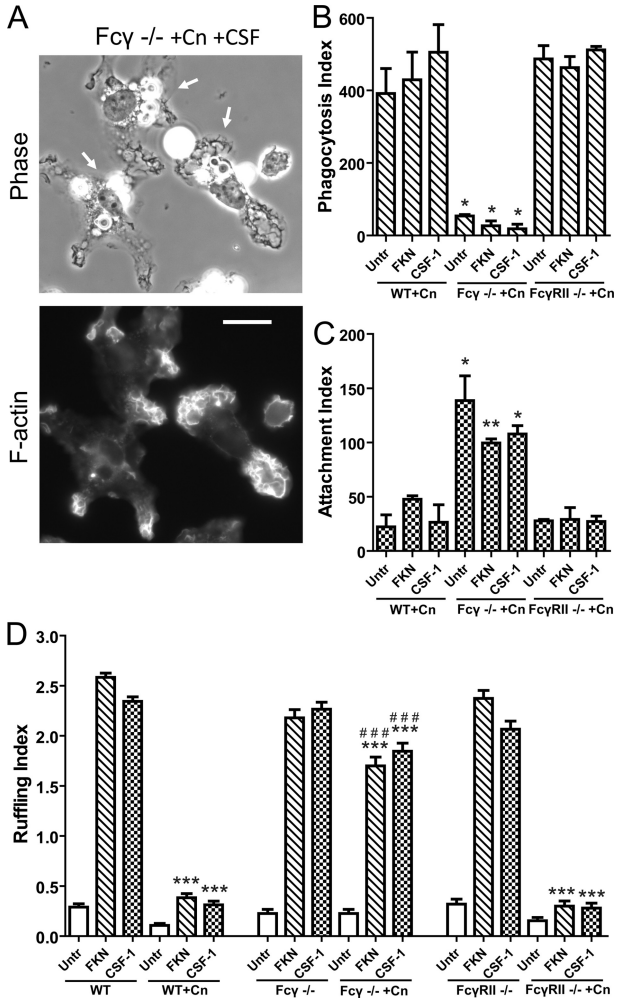

FIG. 7. Cell ruffling of macrophages from FcγR-competent or -deficient mice stimulated by FKN and CSF-1 after phagocytosis of *C. neoformans*. (A) BMM from Fcγ$^{-/-}$ mice were stimulated with FKN (data not shown) and CSF-1 after incubation with IgG-opsonized *C. neoformans* (Cn). After fixation, F-actin in the cells was stained using phalloidin conjugated with Alexa Fluor 568. The images are fluorescence images (F-actin) and corresponding phase-contrast images (Phase). The arrows indicate BMM with attached *C. neoformans* cells. Scale bar = 10 μm. The images are representative of three independent experimental repeats with similar results. (B and C) Phagocytosis of IgG-opsonized *C. neoformans* was carried out with BMM from wild-type C57BL mice (WT), Fcγ subunit double-knockout mice (Fcγ$^{-/-}$), and FcγRII double-knockout mice (FcγRII$^{-/-}$). The cells were then stimulated by FKN and CSF-1, and untreated macrophages were used as a negative control (Untr). For each group, phagocytosis of *C. neoformans* and attachment of *C. neoformans* were quantified using the phagocytosis index (B) and the attachment index (C), respectively, as described in Materials and Methods. Data for Fcγ subunit double-knockout mice (Fcγ$^{-/-}$+Cn) were compared to data for the same treatment for wild-type C57BL mice (WT+Cn) by the Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$). The data were obtained from three independent experimental repeats in which similar results were obtained. (D) BMM from wild-type C57BL mice (WT), Fcγ subunit double-knockout mice (Fcγ$^{-/-}$), and FcγRII double-knockout mice (FcγRII$^{-/-}$) were stimulated by FKN and CSF-1, with untreated macrophages as a control, after phagocytosis of *C. neoformans* (+Cn) or with no phagocytosis. Data for Fcγ$^{-/-}$ mice with *C. neoformans* or for FcγRII$^{-/-}$ mice with *C. neoformans* were compared to data for the same treatment for wild-type mice with *C. neoformans* by the Mann-Whitney test (***, $P < 0.001$). Data for Fcγ$^{-/-}$ mice with *C. neoformans* were compared to data for the same treatment for Fcγ$^{-/-}$ mice by the Mann-Whitney test (###, $P < 0.001$). The error bars indicate standard errors of the means. The data were obtained from three independent experiments.

CSF-1 receptor triggers tyrosine phosphorylation cascades. Like phagocytosis, chemotaxis involves the activation of small GTPases (Rho, Rac, Cdc42), PI3K, and PLC, which are responsible for the actin cytoskeleton reorganization and cell membrane rearrangement during chemotaxis (3, 20, 22, 23). Given that the signaling transduction responsible for chemotaxis follows a precise temporal and spatial regulation, phagocytosis could conceivably interfere with the similar signaling transduction pathways in chemotaxis and generate inhibitory effects. Alternatively, signals that result in the resolution of phagocytic responses, such as the negative regulators of PI3K, SHIP, and PTEN (14, 24), can suppress chemotactic responses (50). The mechanism underlying the possible interference is an intriguing subject for future study.

In conclusion, we observed that macrophage cell ruffling stimulated by FKN and CSF-1 was significantly inhibited by phagocytosis of *C. neoformans* and EIgG. We hypothesize that this phenomenon could contribute to macrophage retention at sites of infection, leading to increased effector-target cell interaction and restriction of microbial spread by wandering macrophages containing infectious cargo. The association of phagocytosis with inhibition of mobility has important potential implications for our views of the function of macrophages in host defense.

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