Cryptococcus neoformans (Cn) is a common facultative intracellular pathogen that can cause life-threatening fungal meningitis in immunocompromised individuals. Shortly after infection, Cn is detectable both as extra- and intracellular yeast particles, with Cn being capable of establishing long-lasting latent infections within host macrophages. While recent studies have shown shed capsular polysaccharides and intact extracellular Cn can compromise macrophage function through modulation of NF-κB signaling, it is currently unclear whether intracellular Cn also affects NF-κB signaling. Utilizing live cell imaging and computational modeling, we find extra- and intracellular Cn support distinct modes of NF-κB signaling in cultured murine macrophages. Specifically, in RAW 264.7 murine macrophages treated with extracellular glucuronoxylomannan (GXM), the major Cn capsular polysaccharide, LPS-induced nuclear translocation of p65 is inhibited, while in cells with intracellular Cn, LPS-induced nuclear translocation of p65 is both amplified and sustained. Mathematical simulations and quantification of nascent protein expression indicate this is a possible consequence of Cn-induced ‘translational interference’, impeding IκBα resynthesis. We also show long-term Cn infection induces stable nuclear localization of p65 and IκBα proteins in the absence of additional pro-inflammatory stimuli. In this case, nuclear localization of p65 is not accompanied by TNFα or iNOS expression. These results demonstrate that capsular polysaccharides and intact intracellular yeast manipulate NF-κB via multiple distinct mechanisms and provide new insights into how Cn might modulate cellular signaling at different stages of an infection.
Intracellular C. neoformans modulates macrophage NF-κB signaling

glucuronoxylomannan (GXM, ~80% by mass (4)), is produced and shed during growth, and is essential for virulence (5,6). It promotes Cn survival during the initial stage of infection by acting as an anti-phagocytic coating, thereby preventing ingestion of yeast in a naïve host during the short-lived acute stage of infection (~7 days post exposure (7)). An escalating humoral response then leads to the phagocytosis of Cn. Unfortunately, the macrophage phagolysosome is a surprisingly permmissive environment for Cn growth (8), making Cn a facultative intracellular pathogen (reviewed in (9)). Intracellular Cn continue to produce and shed GXM, resulting in the accumulation of polysaccharide-filled vesicles within the host cell cytosol (8). While the effects of intracellular GXM are unclear, extracellular GXM is known to disrupt macrophage behaviors and limit inflammation (10,11). These effects can be explained, at least in part, by the ability of GXM to subvert the activity of the nuclear factor-kappa B (NF-κB) signaling pathway (reviewed in (12)).

NF-κB is a family of dimeric transcription factors that regulate the inflammatory response (reviewed in (13)), and includes the ubiquitously expressed p65:p50 heterodimer. The activity of p65-containing dimers is controlled by cytoplasmic sequestration through association with inhibitor kappa B (IκB) proteins, primarily IκBα (14). NF-κB activity in macrophages can be triggered by a range of different stimuli, including lipopolysaccharides (LPS) from gram-negative bacteria. LPS is detected by toll-like receptor-4 (TLR4) at the macrophage cell surface, stimulating a cascade of intracellular signaling events that culminate in the phosphorylation of NF-κB-IκB complexes by IκB kinase (IKK). This leads to the proteasomal degradation of IκB proteins and subsequent nuclear accumulation of p65-containing transcription factors (15,16). Within the nucleus, these regulate the expression of genes associated with the immune response, including pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α; (17-19)) and inducible nitric oxide synthase (iNOS; (20,21)), the latter of which is associated with M1 or ‘classical’ activation of macrophages. Crucially, p65 also stimulates re-synthesis of IκBα. This action constitutes a delayed auto-regulatory loop that promotes re-localization of NF-κB dimers to the cytosol and eventually restores the pathway to an inactive state once stimuli are withdrawn (22,23). The behaviors produced by this and the various other feedback loops that regulate NF-κB activity in macrophages are highly non-linear and show substantial cell-to-cell variation (24). This has led to increased utilization of complementary computational modeling and live cell imaging to study NF-κB signaling dynamics (23-26).

Previous investigations into the effects of GXM on inflammatory NF-κB signaling, which have predominantly utilized extracellular Cn or purified GXM, have produced conflicting results. Through interaction with exogenous TLR4 receptors in Chinese hamster ovary (CHO) cells, GXM was able to promote NF-κB nuclear localization and chromatin binding (27). Meanwhile, others have shown that GXM can block TLR4-induced inflammation in a LPS-induced mouse model of endotoxic shock. When studied further in cultured murine macrophages, GXM was shown to interact with surface FcγRIIb receptors, blocking LPS-induced Akt and IKK activity via SHIP, thereby abrogating NF-κB induction and downstream inflammation. (11). No studies to date have addressed the effects of phagosomal Cn or intracellular GXM on NF-κB signaling. This constitutes a significant gap in our understanding of the Cn:macrophage relationship and Cn-associated disease as Cn is predominantly intracellular during chronic infection.

In this current study, we present detailed single-cell analysis of NF-κB signaling dynamics in cultured macrophages and show that purified extracellular GXM and intracellular GXM-positive Cn have contrasting effects on the behavior of the pathway in the context of LPS-induced activation. Our results show that GXM pretreatment inhibits the nuclear accumulation of p65 proteins in response to LPS. We also show for the first time that GXM-producing phagosomal Cn modulate host NF-κB signaling in a manner that differs from that of extracellular GXM. Specifically, the duration of LPS-induced p65 nuclear accumulation is significantly increased in macrophages containing relatively low numbers of ingested GXM-expressing Cn but not GXM-null Cn. A combination of computational modeling and experimental methods indicated that this extended response was caused by translational interference...
in cells infected with GXM-expressing Cn, which acts to decrease the strength of negative feedback. We also found that extended intracellular occupancy of Cn in the absence of LPS promotes the accumulation of both p65 and IkBα in the nucleus leading to the repression of the NF-κB-regulated target genes TNFα and iNOS. These results suggest that Cn may employ distinct mechanisms of NF-κB modulation at different stages of an infection to promote its own survival within the host.

RESULTS
GXM attenuates LPS-induced nuclear translocation of NF-κB. The anti-inflammatory capacity of Cn capsular polysaccharide was first illustrated by its ability to block LPS-induced TNFα production by monocyte-derived macrophages (10). Subsequent studies showed that GXM activates SHIP via surface FcγRIIB receptors. Active SHIP then blocks the recruitment of MyD88 and Akt, thereby impeding IkBα phosphorylation and degradation (11,12). These data imply that GXM would block LPS-induced nuclear accumulation of p65-containing NF-κB dimers, thereby preventing the NF-κB-dependent production of pro-inflammatory cytokines. To investigate this in detail, we employed a previously described murine macrophage NF-κB reporter cell line comprising RAW 264.7 cells stably expressing p65-EGFP from the RelA (p65) promoter and a destabilized mCherry reporter of TNFα gene transactivation (NF-κB reporter cells) (24). These cells were cultured in the presence or absence of disease-relevant concentrations of GXM and the temporal dynamics of p65 nuclear translocation were determined by live cell fluorescence imaging (Fig. 1A-D).

We observed that GXM significantly attenuated LPS-induced nuclear accumulation of p65-EGFP and caused it to occur slightly later than in control cells without affecting the overall duration of the response (Fig. 1E). However, treatment with GXM alone had no effect on p65 localization (Fig. 1D). To test whether these data were consistent with GXM blocking MyD88-dependent IkBα phosphorylation and degradation via SHIP activation we utilized a mathematical model of LPS-induced NF-κB signaling developed by Sung et al (24) from the original Hoffmann NF-κB model (36). We found that modest reductions (25%) in the magnitude of the initial wave of MyD88-dependent IKK activity, parameter 19 (P19) in the model, recapitulated the decreased amplitude and delay in nuclear p65 accumulation observed in our experiments.

Development of a strategy to infect macrophages with C. neoformans without perturbing NF-κB signaling. Having verified that GXM pretreatment can attenuate LPS-induced nuclear translocation of p65 in macrophages, we then hypothesized that GXM produced by phagocytosed, intracellular Cn may have a similar effect. To test this, we developed a methodology to infect macrophages with Cn without compromising measurements of NF-κB activity.

Typically, the phagocytosis of encapsulated Cn by macrophages in vitro is inefficient unless i) the macrophages are ‘activated’ and ii) Cn have been opsonized. Treatments that induce macrophage activation (typically co-treatment with LPS and IFN-γ) can confound studies involving NF-κB as these will also induce significant NF-κB activity (Fig. 2A). To circumvent this problem, we transiently stimulated the reporter cells with IFN-γ alone (16 h; Fig. 2B), which enhances activation and Cn clearance in vivo (37) without inducing NF-κB in macrophages (38). This was confirmed by live imaging of NF-κB reporter cells exposed to 500 U/mL IFN-γ (Fig. 2A). As expected, complement, which is commonly used to opsonize Cn, was found to induce NF-κB activity in our reporter cell line. We therefore used 18B7 mouse monoclonal antibodies to capsular GXM (39) as our opsonization agent as these do not induce NF-κB signaling (Fig. 2A).

Intracellular C. neoformans delays and extends LPS-induced NF-κB signaling. Since chronic Cn infection primarily involves intracellular Cn, we investigated whether Cn can modulate the NF-κB pathway post-phagocytosis by infecting macrophages with a virulent strain of Cn (H99S) that produces significant quantities of GXM (28).

The phagocytosis of individual Cn by our macrophage reporter cell line did not elicit an immediate NF-κB response (data not shown). However, we found that phagosomal Cn altered the temporal dynamics of LPS-induced NF-κB.
signaling (Fig. 3A-C). Notably, intracellular Cn appeared to alter NF-κB signaling in a manner that differed from that of purified, extracellular GXM. While pretreatment with purified GXM resulted in a ~2-fold decrease in the amplitude of the LPS-induced p65 response (as defined by the maximal nuc:cyto p65 ratio), cells containing intracellular Cn showed a slight but significant increase in amplitude (Fig. 3C). This was accompanied by a pronounced delay in the time taken to achieve maximal nuclear p65-EGFP levels (Fig. 3C), which also increased with Cn-burden (the number of intracellular Cn per macrophage). Furthermore, and in contrast with GXM-dependent effects, the duration of LPS-induced p65 nuclear occupancy was substantially extended in macrophages containing intracellular Cn compared to controls (Fig. 3C). Similar results were obtained in macrophages infected with 24067, a serotype D strain of GXM-expressing Cn (Fig. 3D), with infected cells also exhibiting a delayed and extended NF-κB response to LPS stimulation.

To test whether these effects were GXM-dependent, we infected macrophages with Cap59, a serotype A mutant Cn strain that has a defect in capsule synthesis and does not produce capsular GXM (40), and then treated with LPS. In this case no differences were seen in the amplitude, timing, or duration of p65-EGFP nuclear localization (Fig. 3E), suggesting that the ability of intracellular Cn to modulate LPS-induced NF-κB signaling is GXM-dependent.

**Intracellular C. neoformans-induced changes in macrophage NF-κB dynamics can be simulated in a mathematical model of macrophage LPS:NF-κB signaling.** To investigate the possible causes of intracellular Cn-induced changes in the dynamics of the NF-κB response to LPS, we again utilized our modified Sung et al NF-κB mathematical model (24). This enabled us to simulate possible known and suspected effects of intracellular Cn proliferation (Fig. 4A), including (i) translational interference (P9), (ii) inhibition of the rate of IκB nuclear import or export of p65:IκB complexes (P14 and P12, respectively), and (iii) an increase in TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent IKK activation at Cn-containing phagosomes (P20). Regarding mechanism (i), live (but not heat-killed) intracellular Cn has been shown to alter protein translation rate in both murine peritoneal macrophages and J774.16 macrophage-like cells (33). We hypothesized that this could prolong the length of time that NF-κB spends in the nucleus by reducing the rate of IκBα synthesis. Regarding mechanism (ii), we hypothesized that the large Cn-containing phagosome, which tends to be positioned at the nuclear periphery, and Cn-induced organelle crowding around the nucleus (41,42), could potentially reduce the rate of p65 and IκBα trafficking across the nuclear membrane, as suggested by spatial models of NF-κB signaling (43), thereby altering the temporal dynamics of NF-κB signaling. Regarding mechanism (iii), although GXM has been shown to reduce MyD88-induced IκBα phosphorylation at the cell surface (11), there is a potential for TLR4 to signal through TRIF from the Cn-containing phagosomes, possibly enhancing the NF-κB response to LPS (44).

We evaluated these mechanisms by performing numerical experiments in which the associated parameters were varied individually (Fig. 4B-D). Our results support the hypotheses that a reduction in the rate of IκBα synthesis (P9), possibly caused by translational interference, or an increase in the strength of TRIF-dependent IKK activation could explain the sustained nuclear localization of NF-κB that we have observed in Cn-infected cells post LPS stimulation (Fig. 4B+C). Specifically, both a 50% reduction in the rate of IκBα synthesis and a 50% increase in the strength of TRIF-dependent IKK activation were able to extend the duration of NF-κB in the nucleus, while modestly increasing and delaying the peak response (Fig. 4B+C). In both cases, the sustained nuclear localization of p65 may be accomplished through the merging of the early MyD88- and late TRIF-dependent responses. On the other hand, 50% reductions in the rate of nuclear import of IκB or that of the export of the IκB-p65 complex did not significantly alter the dynamics of p65 nuclear localization in response to LPS (Fig. 4D+E).

Partial inhibition of macrophage protein translation caused by intracellular C. neoformans...
or cycloheximide can increase the duration of the p65 nuclear occupancy post-LPS. To investigate the possibility that translational interference was responsible for the change in the dynamics of LPS-induced p65-EGFP nuclear translocation we first tested whether partial inhibition of protein translation using the ribosome inhibitor, cycloheximide (CHX), could recapitulate the extended response observed in Cn-containing cells. We observed that while complete inhibition of protein synthesis in RAW 264.7 cells with high doses of CHX (10 µM) caused stable nuclear localization of p65-EGFP, partial inhibition achieved using lower doses (1 µM), produced an extended response, similar to that observed in cells infected with GXM-positive Cn (Fig. 5A).

Having established that partially blocking translation could produce an extended NF-κB response to LPS we measured changes in nascent protein synthesis in RAW 264.7 cells infected with GXM-positive H99S and GXM-negative Cap59 Cn yeast. We found that live but not heat killed H99S Cn infection caused a statistically significant decrease in nascent protein production in two out of three experiments, as measured using Click-iT O-propargyl-puromycin (OPP) staining (Fig. 5B+C), and the magnitude of this effect increased with intracellular Cn burden. Since statistically significant decreases in translation were also obtained using ribopuromycylation assays (Fig. 5D), this suggests that Cn is somehow interfering with translation. However, infection with Cap59 had no significant effect on OPP staining indicating that this GXM-negative strain was not capable of causing translational interference (Fig. 5E), suggesting that translational interference is GXM-dependent and possibly explaining the inability of intracellular Cap59 to alter the dynamics of LPS-induced NF-κB signaling in macrophages (Fig. 3E).

Proliferation of intracellular C. neoformans alone can promote nuclear accumulation of p65-containing dimers. During our initial experiments where we investigated altered TLR4 signaling in Cn infected cells, we noticed that a small number of macrophages containing Cn were found to exhibit nuclear p65-EGFP (nuc:cyto>1) within 2 h of initial exposure to Cn, prior to LPS addition. This effect was not seen in surrounding uninfected cells. Therefore, these cells were classified as showing ‘Cn-induced nuclear accumulation of p65’ (CIN-p65). The proportion of cells showing CIN-p65 increased over time and cells with more intracellular Cn were more likely to exhibit nuclear p65.

To further investigate the effects of proliferating phagosomal Cn alone on NF-κB, we infected macrophages with opsonized Cn, allowed 2 h for phagocytosis of Cn, washed off extracellular Cn (and polysaccharide-containing media), and imaged macrophages containing phagosomal Cn for an additional 24 h.

Consistent with our previous results, we found that phagosomal proliferation of Cn alone sometimes promoted nuclear accumulation of p65-EGFP (seen in 2.8% of Cn-containing cells at 2 h and 6.8% of Cn-containing cells at 24 h post Cn exposure, with cells containing an average of 2.7 and 5.6 Cn, respectively). Unlike the transient nuclear localization produced by LPS, which occurs rapidly and is followed by the swift return of p65-containing dimers to the cytosol, Cn-induced nuclear accumulation of p65-EGFP occurred gradually and produced long-lasting nuclear localization (Fig. 6A+B). This slow accumulation of p65-containing dimers within the nucleus appeared to be influenced by macrophage intracellular Cn ‘burden’, which gradually increased over time as phagocytosed Cn began to proliferate within individual macrophages (Fig. 6F-G). Unexpectedly, the nuclear accumulation of p65-EGFP was not accompanied by mCherry expression from the Tnf promoter, in spite of abundant nuclear p65 (Fig. 6A+C). This effect was observed in multiple cells (Fig. 6D-G).

Phagosomal C. neoformans suppresses TNFα and iNOS production in cells exhibiting nuclear p65-EGFP. As Cn-induced nuclear p65 accumulation was not accompanied by NF-κB-regulated gene expression, we speculated that intracellular Cn may also render these cells incapable of responding to additional NF-κB-activating stimuli. To test this, we exposed non-infected and CIN-p65 cells to 100 ng/mL LPS and measured both mCherry levels (reporting TNFα gene transactivation), nuc:cyto p65-EGFP, and total p65-EGFP levels, as the RELA promoter is also positively regulated by NF-κB at high LPS doses (24).
Our data indicate CIN-p65 macrophages were largely refractory to exogenous NF-κB-activating stimuli, as LPS treatment failed to promote either the return of p65-EGFP to the cytosol or expression of TNFα (Fig. 7A). While neighboring non-infected macrophages exhibited a ‘normal’ NF-κB response to LPS (i.e. a rapid but transient nuclear accumulation of p65-EGFP), LPS seemingly had little to no effect on p65-EGFP distribution in CIN-p65 macrophages (Fig. 7A+B). Additionally, and in stark contrast to non-infected control cells, the NF-κB-dependent transcriptional output in CIN-p65 macrophages was seemingly negligible with no significant increase observed in either total p65-EGFP or mCherry levels as measured at the population level (Fig. 7C+D).

As mCherry expression is regulated by an exogenous copy of the the Tnf promoter and may not accurately reflect the regulation of endogenous NF-κB target genes, we also immunostained control and CIN-p65 NF-κB reporter cells for iNOS, a bona fide NF-κB target gene and marker of M1 activation in macrophages. As expected, LPS stimulation of uninfected macrophages induced a small increase in iNOS protein levels at 5 h and high levels of expression by 15 h. However, in Cn-containing macrophages, iNOS was found to be consistently at basal levels 24 h post infection despite stable nuclear localization of p65 (Fig. 7E). Taken together, these data indicated that CIN-p65 was either transcriptionally incompetent or was capable of repressing NF-κB target genes.

Stable nuclear localization of p65 in C. neoformans-containing macrophages is caused by altered trafficking of IκBα. To investigate how intracellular Cn accumulation may cause stable nuclear localization of p65 we again turned to the mathematical model. We simulated the effect of varying parameters (Fig. 4A) in the absence of LPS (Fig. 8A-C). As before, we noticed that a sustained decrease in the rate of IκBα synthesis (P9; Fig. 8A), as might be caused by translational interference, could promote stable nuclear localization of p65. However, the model also suggested that a decrease in the rate of nuclear export of NF-κB:IkBα complexes could have a similar effect (Fig. 8B). Changes to other model parameters, such as the rate of IκBα nuclear import (P14; Fig. 8C), did not cause stable nuclear localization of NF-κB.

To discriminate between the two possibilities that stable nuclear p65 was caused by inhibition of either (i) IκBα synthesis or (ii) nuclear export of NF-κB:IkBα complexes, we measured IκBα levels in CIN-p65 cells. We found that all CIN-p65 cells exhibited high levels of nuclear IκBα staining (Fig. 8D), which was consistent with our second hypothesis. Furthermore, as interaction between IκBα and p65 in the nucleus prevents effective chromatin binding (45-48), these data are also consistent with the absence of TNFα and iNOS expression in these cells.

DISCUSSION
The nature of host:pathogen interactions are typically highly dynamic, a metaphorical arms-race of move and counter-move in which the host and pathogen employ a changing repertoire of strategies to destroy or evade the other. As a key regulator of immune cell function and survival, it is perhaps unsurprising that the NF-κB pathway is a frequent target of human pathogens. While Yersinia inhibits NF-κB in naive macrophages (49), triggering their apoptosis, the intracellular pathogens Mycobacterium tuberculosis and Shigella both exploit pro-survival activities of the NF-κB pathway during the intracellular phase of infection, ‘buying time’ to replicate before either actively triggering host cell apoptosis or permitting cell death in order to escape and disseminate (reviewed in (50)).

Over the past 15 years, a number of studies utilizing both purified capsular components and intact yeast have shown that Cn may also subvert immune cell NF-κB signaling during acute, extracellular infections (10,11,27,51). Perhaps due to differences in experimental design, there is still disagreement within the field, on the precise mechanism(s) and consequences of NF-κB modulation.

Because gram-negative bacteremia can occur in small numbers of Cn infections (52) and GXM has been shown to block LPS-induced inflammation (11), we utilized a simple cell culture model to study Cn-dependent NF-κB modulation in the context of LPS activation. We
assumed that during extracellular infections, macrophages would be more likely to encounter free GXM than intact Cn. This would seem plausible as GXM is shed by proliferating yeast in vivo, accumulates in the serum and tissue of infected individuals to μg/mL levels, and remains for weeks or months before it is cleared (53). Our data suggest that GXM attenuates LPS-induced NF-κB activation by inhibiting the accumulation of p65-containing dimers in the nucleus. This is consistent with reports indicating GXM suppresses phosphorylation and degradation of IκB by promoting SHIP activity (11).

While the chronic, intracellular phase of Cn infection may last for years or even decades, it has remained unclear whether phagosomal Cn continue to modulate NF-κB signaling. This important question has remained open due to numerous technical challenges. Of these, perhaps the most fundamental is the low rate at which Cn phagocytosis occurs in vitro unless macrophages are first ‘activated’ by pro-inflammatory stimuli (typically IFN-γ in combination with LPS or TNFα), which could mask the effects of ingested Cn on NF-κB signaling or at least make it extremely difficult to measure using standard biochemical techniques and end-point assays.

In this current study, by utilizing a live cell imaging approach, we identified and tracked those cells within an ‘un-activated’ macrophage population that had ingested Cn in order to measure the dynamics of canonical NF-κB signaling and downstream gene expression. In contrast to the effects of purified GXM, we saw that infection of macrophages with GXM-expressing Cn strains (H99S and 24067) significantly increased the duration of the canonical NF-κB response to LPS. However, this effect was absent when cells were infected with the GXM-deficient Cn strain, Cap59, suggesting that the ability of intracellular Cn to modulate NF-κB signaling was GXM-dependent and that the modulatory effects for GXM differ depending on the mode of presentation (i.e., whether it is extra- versus intracellular).

To determine how intracellular Cn might perturb the kinetics of the p65-response to LPS, we employed a mathematical model of NF-κB signaling (24). Simulations indicated that a small reduction in the rate of IκBα synthesis, effectively decreasing the strength of negative feedback, would extend the duration of p65 nuclear occupancy post-LPS. This model-generated hypothesis was plausible as (i) intracellular Cn has been previously shown to affect protein translation in murine peritoneal macrophages and 3774.16 macrophage-like cells (33) and (ii) we were able to demonstrate that partial inhibition of translation using CHX extended p65 nuclear occupancy post-LPS in RAW 264.7 macrophages. Measurement of nascent protein production in Cn-infected cells using two different methods showed that only live, GXM-expressing Cn were able to partially inhibit protein synthesis, providing a possible explanation for the differences in LPS-induced NF-κB signaling dynamics observed between GXM-positive and --negative Cn strains.

In this study, we were also able to show that phagosomal proliferation of Cn to high-burden was sufficient to induce stable nuclear localization of p65 in the absence of exogenous pro-inflammatory stimuli. Contrary to expectations, this was not accompanied by expression from the NF-κB-regulated Tnf promoter or expression of iNOS, a well-studied NF-κB target gene and a marker of M1 macrophage activation. As before, we utilized the mathematical model to identify potential mechanisms to explain the stable nuclear localization of p65 in these cells in the absence of stimuli and again found that this could be caused by decreases in the rate of IκBα synthesis but also by a reduction in the rate of nuclear export of NF-κB:IkBα complexes. These two competing hypotheses were tested by immunostaining cells for IkBα. We found that CIn-p65 cells contained large quantities of nuclear IkBα, which was consistent with the second hypothesis. This finding, while unexpected, is not without precedent, and may help to explain the absence of TNFα and iNOS expression in these cells.

We and others have previously shown that extended nuclear occupancy of p65 may not necessarily result in effective interactions with chromatin (22,47,48,54). Trapping of p65 in the nucleus through the use of nuclear export inhibitors (i.e. leptomycin B) may be accompanied by the loss of p65 post-translational modifications such as phosphorylation of ser536, that are associated with activity (22). Newly synthesized IkBα in these cells will complex with nuclear p65, decreasing association with chromatin (47,48). It has also been shown in LPS and leptomycin B co-
treated macrophages that IκBα is selectively recruited with p65 to various NF-κB-regulated promoters, including the Tnf, IL-1β, and IL-6 gene promoters, repressing gene transcription (54). While these data are consistent with our findings, the causes of nuclear p65 and IκBα accumulation and the absence of TNFα and iNOS expression in Cn-infected macrophages, remains unclear. One possible explanation is that p65: IκBα complexes are being trapped in the nucleus as a consequence of Cn-induced post-translational modifications or through inhibitory interaction with other nuclear proteins. One such candidate would be protein methyltransferase 2 (PRMT2). PRMT2 has been shown to control inflammatory TLR4:NF-κB signaling in macrophages by directly associating with IκBα in the nucleus, blocking its chromosome region maintenance 1(CRM1)- dependent nuclear export and decreasing TNFα and IL-6 expression (55, 56). It is also possible that in Cn-containing cells, nuclear p65 may associate with co-repressors such as histone deacetylases (HDAC) 1+2 or other negative regulators of p65 activity like SIRT1 (57, 58). Indeed, other pathogens have been shown to utilize SIRT1 activity in macrophages as part of intracellular survival strategies (59). These and other possible mechanisms will be explored in future studies.

In summary, in the context of LPS-induced activation, our data shows that Cn is able to alter the way in which macrophages encode information about the inflammatory signaling environment in the dynamics of p65 transcription factors, ultimately suppressing the ability of macrophages to participate in an effective immune response through the production of proinflammatory cytokines or the expression of genes required for M1 activation. We also propose that the mechanisms utilized by Cn to modulate NF-κB signaling change as the pathogen transitions from an extracellular to an intracellular lifestyle.

**EXPERIMENTAL PROCEDURES**

**Culture and opsonization of Cn**

Cn serotype A, strains H99S (28), Cap59 and serotype D strain 24067 were grown in yeast peptone dextrose (YPD) broth for 48 h at 37 °C with shaking (Cap59 was grown at 30 °C for similar amounts of time). After 48 h, cells were washed 3x with PBS and counted. For GXM-expressing strains, 1 x 10^7 cells were opsonized with 10 μg/mL 18B7 mAb (a kind gift from Dr. Arturo Casadevall, John Hopkins School of Public Health) at 37 °C for either 30 min (H99S) or 1 h (24067) then washed again (3x with PBS) to remove excess antibody and infected at a 10:1 MOI. For experiments requiring heat-killed Cn, yeast were incubated at 60 °C for 30 min prior to opsonization. All cultures were started from frozen stock to ensure no microevolution occurred.

**GXM purification and validation**

To ensure there was no contaminating galactoxylomannan (GalXM) present, GXM was prepared using the Cn serotype A, GalXM-negative strain Uge1 (29). Uge1 was cultured in 200 mL of defined minimal media (15 mM glucose, 10 mM MgSO4-7H2O, 29.4 mM KH2PO4, 0.13 mM glycine, 3 µM thiamine HCl; pH = 6.0) for 5 days. Yeast cells were centrifuged using an ultracentrifuge (Beckman Coulter Inc., Pasadena, CA) at 11,000 × g (8000 rpm) for 20 min. GXM was purified from the supernatant by precipitation with 0.2 M NaCl and 0.05% cetyltrimethylammonium bromide (CTAB) as described by Cherniak et al (30) followed by additional washing steps with 2% acetate in ethanol, 90% ethanol, and absolute ethanol (31). While not tested specifically in these experiments, this GXM purification protocol has been shown to preserve the O-acetylation of GXM (30). The solution was centrifuged again at 11,000 × g (8000 rpm) for 10 min, the supernatant was removed, and the remaining pellet was dried and weighed. LPS was removed from all samples using a Polymyxin B column in LPS-free distilled water. The samples were then lyophilized and re-suspended in LPS-free water. The purified GXM was validated by ELISA using IgG1 mAb 18b7 and a colorimetric sugar assay employing H2SO4 and phenol (32). Purified GXM was negative for endotoxin/LPS contamination, as tested by Limulus Amebocyte Lysate (LAL) assay (<0.017 EU/mL).

**Macrophage culture and phagocytosis conditions**

RAW 264.7 (TIB 71) murine macrophages were obtained from ATCC (Manassas, VA). RAW 264.7 stably expressing mCherry reporter of Tnf promoter activation (‘NF-κB reporter cells’) were a kind gift from Dr. Iain...
Fraser (NIH) and have been previously described (24). Both cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS), 200 mM L-glutamine, 100 units/mL penicillin and streptomycin, and 50 μg/mL gentamicin, and maintained at 37°C in a humidified 5% CO2 atmosphere.

For live cell microscopy, 2x10^5 NF-κB reporter cells were seeded into 35 mm glass-bottom dishes (Cellvis, USA) 24 h prior to imaging in 2 mL of medium. Approximately 16 h prior to Cn infection, the cells were ‘primed’ with interferon-gamma (IFN-γ; 500 units/mL) to increase the proportion of macrophages capable of phagocytosing Cn.

**Time-lapse microscopy and immunofluorescence**

Cells plated in 35 mm glass-bottom dishes were imaged using a Nikon Ti-Eclipse wide-field microscope equipped with a CFI Plan Fluor 40x oil immersion NA 1.30 objective, Intensilight Epi-fluorescence illuminator, computer-controlled stage (Nikon, USA), CoolSNAP MYO camera (Photometrics, USA), and a full environmental enclosure with CO2, humidity, and temperature control (InVivo Scientific, USA). The microscope was controlled using Nikon Elements Software (Nikon, USA).

Images were acquired at 3 min intervals for the indicated durations. Typically, cells were imaged for up to 1 h prior to infection with Cn and then an additional 1 h before cells were exposed to pro-inflammatory stimuli (i.e. LPS). EGFP was excited through a 465-495 nm excitation filter, and emitted light was detected through a 515-555 nm barrier filter reflected from a 505 nm dichroic mirror. mCherry was excited through a 535-550 nm excitation filter, and emitted light was detected through a 610-675 nm barrier filter reflected from a 655 nm dichroic mirror.

In cases where Cn infection induced stable nuclear localization of p65-EGFP, cells were fixed with ice cold methanol for 5 minutes and then immunostained with a primary antibody raised against either iNOS (1:100 dilution, #13120S; Cell Signaling, USA) or IκBα (1:100 dilution; sc-371, Santa Cruz Biotechnology, USA), followed by an anti-rabbit Alexa Fluor 647-labeled secondary antibody (1:200 dilution, ab150075; Abcam, USA), then stained with Hoechst 33342. Stained cells were also imaged using a Nikon Ti-Eclipse wide-field microscope. Alexa Fluor 647 was excited through a 590-650 nm excitation filter, and emitted light was detected through a 663-738 nm barrier filter reflected from a 660 nm dichroic mirror. Hoechst 33342 was excited through a 340-380 nm excitation filter, and emitted light was detected through a 435-485 nm barrier filter reflected from a 400 nm dichroic mirror.

**Protein translation assays**

RAW 264.7 cells were seeded at a density of 2x10^5 per well into tissue culture treated 6 well plates (USA Scientific, Ocala, FL) containing No. 1.5 (0.17 mm thick) glass coverslips. Cells were activated with 500 units/mL IFN-γ and 1 μg/mL LPS ~16 h prior to infection with Cn and again with 1 μg/mL LPS concurrent with Cn infection. Cells were allowed to phagocytose Cn for 2 h (10:1 MOI). Extracellular Cn was washed away with RAW264.7 culture media and macrophage ribosome activity was determined by two separate methods, ribopuromycylation, adapted from Coelho et al 2015 (33), and staining with O-propargyl-puromycin using the Click-iT Plus OPP Protein Synthesis Assay Kit Alexa Fluor 647 (Life Technologies, Carlsbad, CA), in accordance with manufacturers guidelines. For the ribopuromycylation (RPM) assay, cells were incubated for 15 min at 37°C in a humidified 5% CO2 atmosphere with culture media supplemented with 91 μM puromycin and 208 μM emetine, with one well not including puromycin as a negative control. Cells were permeabilized with 50 mM Tris-HCl, 10 U/mL RNaseOut, 0.015% digitonin, cOmplete™ Mini Protease Inhibitor Cocktail tablet (Sigma-Aldrich, St. Louis, MO), fixed with 3% paraformaldehyde, and blocked with 1X PBS, 0.05% saponin, 10 mM glycine, and 5% goat serum. Cells were immunostained with a primary antibody raised against puromycin, PMY-2A4 (1:100 dilution, developed by Dr. Jonathan Yewdell (NIH) (34), and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA), detected using Alexa Fluor 555-labelled anti-mouse secondary antibodies (1:200).and the cells were co-stained with Hoechst 33342 nuclear stain. With the Click-iT Plus OPP kit, one well was not infected with Cn and treated with 100 μM of the ribosome inhibitor, cycloheximide (CHX)
cells were analyzed. Control cells in every field containing A previous model of NF-κB signaling modulates macrophage NF-κB signaling.

**Image analysis**

Post-acquisition, 14-bit Nikon nd2 images were analyzed using FIJI (35). Typically, individual field time-courses were thresholded for each recorded fluorescence channel. Mean nuclear and cytoplasmic p65-EGFP fluorescence was quantified for individual cells and expressed as the nuclear:cytoplasmic (N:C) ratio. For each cell analyzed, this ratio was used to generate values for i) amplitude, reported as the maximum N:C ratio reached during the time-course, ii) time to max, and iii) duration, which was quantified for each cell for the Click-iT Plus OPP staining. To avoid bias, all non-infected control cells in every field containing Cn-infected cells were analyzed.

**Numerical Experiments**

A previous model of NF-κB signaling in macrophages (Sung et al 2014 (24)) was adapted to investigate potential mechanisms through which Cn modulates LPS-induced NF-κB signaling. Specifically, we adapted the original model to account for the ratio of nuclear to cytoplasmic volume, which we took as .3 (25). For example, if a chemical species, X, is present in both the nucleus and the cytoplasm then the mathematical model tracks the concentration in both compartments. Let $X_n$ be the concentration in the nucleus, $X_c$ be the concentration in the cytoplasm, $V_n$ be the volume of the nucleus, and $V_c$ be the volume of the cytoplasm. If $a$ is the rate at which $X_c$ decreases due to the movement of $X$ out of the cytoplasm and into the nucleus, so that $\frac{dX_c}{dt} = \ldots - a$, then $X_n$ increases at rate $\frac{V_c}{V_n}a$, so that $\frac{dX_n}{dt} = \ldots + \frac{V_c}{V_n}a$. Similarly, when $X$ moves from the nucleus to the cytoplasm, the resulting rate of change of $X_c$ is $-\frac{V_n}{V_c}$ that of $X_n$. In order to provide a better qualitative fit to our data, we also lowered the rate of IκB synthesis by a factor of .2, and the maximum rate at which IKK is activated through the MyD88- and TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathways by factors of 1/2 and 1/6, respectively. Aside from these adjustments, the equations are as in the original model (24). A Matlab program was developed in order to (i) vary the model parameters that control the initial (p19) and delayed (p20) waves of IKK activity, the rate of IκB translation (p9), the rate of nuclear import of IκB (p12), and the rate of nuclear export of IκB-p65 (p14) and (ii) visualize how the timing and magnitude of the p65 response varies with these parameters. Because the mathematical model consists of a system of delay differential equations, we generated a solution history for each numerical experiment: The solver was initialized using the constant history provided in Sung et al. The solution was then simulated over a long time interval (20 h) in order to eliminate transient behavior. In case our intent was to investigate the effect of changing a select kinetic parameter from the downstream pathway (i.e. p9, p12, or p14), at the end of this time interval the parameter of interest was altered (in order to reflect a potential effect of Cn infection) and the solution was simulated over an additional time interval (20 h) before LPS stimulation was initiated by changing the associated parameters (p19 and p20) to nonzero values. Post LPS stimulation the solution was simulated for an additional 10 hours.
Statistical analysis
For the purified GXM experiments, differences in time to maximum amplitude were analyzed using analysis of variance (ANOVA) while differences in the maximum amplitude were analyzed using Wilcoxon Rank sums because the data did not fall into a normal distribution and Wilcoxon Rank sums is a more conservative test. For the intracellular Cn experiments, the data for time to maximum amplitude, maximum amplitude and length of time p65 was in the nucleus (duration) were transformed into a normal distribution and then analyzed using multivariate ANOVA with simple effects to test for differences between different amounts of intracellular Cn. Differences in translation were analyzed using ANOVA for assays using the Click-iT O-propargyl-puromycin (OPP) staining kit and Wilcoxon Rank sums for the ribopuromycylation assays. To determine if macrophages with high intracellular burden had decreased TNFα expression after the addition of LPS, the data were analyzed using an unpaired student t-test. For all tests, \( p<0.05 \) was considered significant.

ACKNOWLEDGMENTS

We appreciate the gift of the RAW 264.7 NF-κB reporter cell line from Iain Fraser (NIH). We thank Jonathan Logan Bowling (MTSU) for his technical assistance, Kirsten Cunningham and Devyn Hayes for help with data analysis, and Drs. Anthony Farone (MTSU) and Eric Batchelor (NIH) for useful discussions. This work was supported by start-up funds from Middle Tennessee State University to D.E.N and E.E.M. D.E.N was also supported by MTSU FRCAC and MTSU Foundation (Special Projects) funds. LEH was supported by scholarships from MTSU URECA and Sigma Xi GIAR.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article

AUTHOR CONTRIBUTIONS

LEH performed and analyzed the experiments shown in Figure 1. JBH performed and analyzed the experiments in Figures 2, 3, and 6-8. WD and RNL performed and interpreted the numerical experiments in Figures 1, 4, and 8. LMS performed and analyzed the experiments in Figure 5. EEM and DEN conceived, designed, and coordinated the study. JBH, WD, RNL, EEM, and DEN wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

ABBREVIATIONS

The abbreviations used are: AP-1, activator protein 1; CHO, Chinese hamster ovary; CHX, cyclohexamide; Cn, Cryptococcus neoformans; EGFP, enhanced green fluorescent protein; FcγRIIb, Fc gamma receptor IIB; GXM, glucuronoxylomannan; HDAC, histone deacetylase; IκB, inhibitor kappa B; IKK, IkB kinase; IFN-γ, interferon-gamma; iNOS, inducible nitric oxide synthase; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharides; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor-kappa B; PRMT2, protein methyltransferase 2; SHIP, SH2-containing inositol phosphatase 1; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; TRIF, TIR-domain-containing adapter-inducing interferon-β; WT, wild-type.
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Intracellular C. neoformans modulates macrophage NF-κB signaling

FIGURE LEGENDS

Fig. 1: Exposure to GXM inhibits LPS-induced nuclear accumulation of p65. (A) Fluorescence microscopy images of p65-EGFP (green) localization in RAW 264.7 NF-κB reporter cells exposed to 100 ng/mL LPS with and without a 1 h pre-treatment with 200 µg/mL GXM. Scale bar represents 20 µm. (B-D) Single cell trajectories of the nuc:cyto ratio of p65-EGFP fluorescence post LPS (B) without and (C) with GXM pretreatment for 4 representative cells and (D) the population average for GXM, LPS, and GXM+LPS cells. (E) Quantification of the average maximum amplitude, time to achieve maximum amplitude, and response duration. Error is represented as the SE. Statistical significance is indicated as follows, *p<0.05, **p<0.01, and ***p<0.001. Data from > 100 cells were collected per condition across 3 independent biological repeats. (F) A diagram linking model parameters to biological events. (G) P19, the parameter describing the magnitude of MyD88-dependent IKK activity was decreased to 0.75 and 0.50 of the nominal value and the model was simulated in MATLAB as described in Numerical Experiments. The predicted ratio in the concentration of nuclear to cytoplasmic p65 was plotted as a function of time post LPS stimulation.

Fig. 2: Effect of macrophage-activating stimuli and Cn opsonins on p65-EGFP localization in RAW 264.7 cells. (A) RAW 264.7 NF-κB reporter cells were treated as indicated and imaged by live cell microscopy for periods of up to 5 h. Conditions that induced p65-EGFP nuclear localization (nuc:cyto p65-EGFP ≥ 1) in cells were classified as ‘Activating’. Conditions that caused no apparent accumulation of p65-EGFP were classified as ‘Not Activating’. (B) Schematic of the Cn:macrophage infection protocol. Timeline for the preparation of Cn-infected RAW 264.7 NF-κB reporter cells for live cell imaging.

Fig. 3: Macrophages containing intracellular Cn exhibit a delayed and sustained NF-κB response to LPS stimulation. (A) Fluorescence microscopy images of p65-EGFP (green) localization in RAW 264.7 NF-κB reporter cells exposed to 100 ng/mL LPS 2 h post infection with Cn. Scale bar represents 20 µm. Intracellular Cn are indicated with arrows. (B) Single cell trajectories of the nuc:cyto ratio of p65-EGFP fluorescence post LPS for cells that do not (Ctrl) and do contain >3 intracellular H99S Cn (3+ H99S) for 4 representative cells. The population average trajectory of the nuc:cyto ratio of p65-EGFP fluorescence post LPS for cells that do not (Ctrl) and do contain intracellular Cn with quantification of the average maximum amplitude, time to achieve maximum amplitude, and response duration for (C) H99S, (D) 24067, and (E) Cap59-infected macrophages. Error is represented as the SE. Statistical significance is indicated as follows, *p<0.05, **p<0.01, and ***p<0.001 (ANOVA, p<0.05). Data from > 35 cells were collected per condition across a minimum of 6 independent biological repeats.

Fig. 4: Numerical experiments using the Sung et al model to evaluate potential mechanisms through which intracellular Cn modulates NF-κB signaling. (A) A diagram linking model parameters to biological events. (B-E) Select model parameters were varied about a nominal value as indicate in the figure legends and the model was simulated in MATLAB as described in Numerical Experiments. The predicted ratio in the concentration of nuclear to cytoplasmic p65 was plotted as a function of time post LPS stimulation.

Fig. 5: Live GXM-positive Cn causes translational interference in RAW 264.7 macrophages (A) Quantification of the nuclear:cytoplasmic ratio of p65-EGFP fluorescence in LPS-treated live RAW 264.7 NF-κB-reporter cells pre-treated with vehicle (Ctrl), or the indicated doses of CHX. Data from > 40 cells were collected per condition across a minimum of 2 independent biological repeats. (B) Fluorescence microscopy images of RAW 264.7 cells treated with 100 µM CHX or infected with live or heat killed H99S Cn. Nascent protein synthesis was detected by staining with OPP-647 (red in merge) and genomic DNA was stained using NuclearMask Blue (blue in merge). Arrows indicate Cn-infected macrophages. (C) Quantification of OPP-647 staining in non-infected RAW 264.7 cells (‘No Cn’) and cells infected with live (‘Live’); also separated in to low (1-2 Cn; ‘LB’) and high burden (3+ Cn; ‘HB’) infected cells) or heat killed (‘HK’) H99S Cn. (D) Quantification of RPM staining of H99S-infected RAW264.7 cells. (E) OPP-647 staining was quantified as described in (B+C) for Cap59 infected RAW 264.7 cells. Error is represented as the SE. Statistical significance is indicated as follows, *p<0.05, **p<0.01, and ***p<0.001
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(ANOVA, *p*<0.05). Data from > 40 cells were collected per condition across a minimum of 3 independent biological repeats. Scale bar represents 20 µm.

**Fig. 6:** High intracellular Cn burden stimulates nuclear accumulation of p65 without gene expression. (A) Fluorescence microscopy images of p65-EGFP (green) localization and destabilized mCherry (red) expression in RAW 264.7 NF-κB reporter cells after phagocytosis of opsonized H99S Cn. Scale bar represents 20 µm. Intracellular Cn are indicated with arrows. (B) Quantification of the nuc:cyto ratio of p65-EGFP fluorescence and (C) mCherry fluorescence for the cell depicted in (A). The approximate number of intracellular Cn is represented by the dotted line. (D-G) Representative single cell trajectories of the nuc:cyto ratio of p65-EGFP fluorescence and mCherry fluorescence for 3 representative cells.

**Fig. 7:** High intracellular Cn burden alters the NF-κB response to LPS. (A) Fluorescence microscopy images of p65-EGFP (green) localization and destabilized mCherry (red) expression in RAW 264.7 NF-κB reporter cells. Cells with Cn (H99S)-induced nuclear p65-EGFP and control cells were imaged for 2 h prior to treatment with 100 ng/mL LPS (T= 0 min). Intracellular Cn are indicated with arrows. (B) Single cell trajectories of the nuc:cyto ratio of p65-EGFP fluorescence pre- and post LPS for representative cells with (Cn +ve) and without (Cn –ve) intracellular Cn as indicated in (A). Change in (C) total p65-EGFP and (D) mCherry fluorescence post LPS for cells exhibiting Cn-induced nuclear p65 (Cn +ve) and control cells that do not contain Cn (Cn –ve). Error is represented as the SE. Statistical significance is indicated as follows, *p*<0.05, **p*<0.01, and ***p*<0.001. Data from > 8 Cn-infected cells (containing an average of 4.5+/−2.8 Cn) were collected across 5 independent biological repeats. (E) RAW 264.7 NF-κB reporter cells were fixed at the indicated times post-stimulation with 100 ng/mL LPS or after infection with H99S and stained with Hoechst 33342 (blue) and immunostained with anti-iNOS antibodies (red). Fluorescence from p65-EGFP is represented in green. The large white circle demarcates a representative Cn-infected macrophage and the smaller white circle indicates the nucleus of this cell. This result was consistent across all Cn-infected cells (12 cells) from 4 independent biological repeats. These cells contained an average of 4.3+/−1.6 Cn. Scale bars represent 20 µm.

**Fig. 8:** Numerical experiments using the Sung et al model to evaluate potential mechanisms through which intracellular Cn can induce stable nuclear localization of p65. (A-C) Select model parameters were varied about a nominal value as indicate in the figure legends and the model was simulated in MATLAB as described in Numerical Experiments. The predicted ratio in the concentration of nuclear to cytoplasmic p65 was plotted as a function of time in the absence of LPS stimulation. (D) RAW 264.7 NF-κB reporter cells were fixed 24 h post-infection with H99S and stained with Hoechst 33342 (blue) and immunostained with anti-iκBα antibodies (red). Fluorescence from p65-EGFP is represented in green. Arrow indicates the nucleus of a representative Cn-infected macrophage. This result was consistent across all Cn-infected cells (11 cells) from 6 independent biological repeats. These cells contained an average of 6.0+/−2.2 Cn. Scale bars represent 20 µm.
Figure 1
**Figure 2**

**A**

<table>
<thead>
<tr>
<th>Time (min) post treatment</th>
<th>p65-EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

**Treatment:**
- LPS (100 ng/mL)
- Complement (20%)
- 18B7 (10 mg/mL)
- IFNγ (500 U/mL)

**Effect on NF-κB:**
- Activates

---

**B**

1. Plate macrophages
2. IFNγ Treatment
3. Wash off IFNγ
4. Infect with Cn
5. Wash off extracellular Cn

Time (hours): 0, 8, 24, 26, 28

**Figure 2**
Figure 3
Figure 4
Figure 5

**A**

![Graph showing Time (min) post CHX vs. N:C p65-EGFP intensity for Ctrl, 1 uM, and 10 uM treatments. The graph demonstrates a significant increase in intensity over time for the treatments compared to control.](image)

**B**

**Brightfield DNA OPP-467 Merge**

- **CHX**
- **Live Cn**
- **HK Cn**

*H99S (GXM Positive)*

**C**

**H99S (GXM Positive)**

- No Cn
- Live Cn
- HB
- LB
- HK

Mean OPP-467 Intensity

- Control: *

**D**

**Mean RPM-555 Intensity**

- Control: *

**E**

**Cap59 (GXM Negative)**

- Control
- Live
Figure 6
Figure 7

Note: 20 um scale bar
Figure 8
Modulation of macrophage inflammatory NF-κB signaling by intracellular Cryptococcus neoformans

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J. Biol. Chem. published online May 26, 2016

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