Modulation of Macrophage Inflammatory Nuclear FactorκB (NF-κB) Signaling by Intracellular Cryptococcus neoformans

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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 as both extra- and intracellular yeast particles, with Cn being capable of establishing long-lasting latent infections within host macrophages. Although recent studies have shown that 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 that 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, whereas 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 that this is a possible consequence of Cn-induced “translational interference,” impeding IκBα resynthesis. We also show that 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 inducible NOS (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.

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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 (myeloid differentiation primary response gene 88) and Akt, thereby impeding IκBα 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. 1, A–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 IκBα 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 IκBα phosphorylation 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 because they 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 units/ml IFN-γ (Fig. 2A). As expected, complement, which is commonly used to opsonize
**FIGURE 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 pretreatment with 200 μg/ml GXM. The scale bar represents 20 μm. B–D, single cell trajectories of the nuc/cyt ratio of p65-EGFP fluorescence after LPS stimulation without (B) and with (C) GXM pretreatment for four 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 S.E. Statistical significance is indicated as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001. The data from >100 cells were collected per condition across three 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 under "Numerical Experiments." The predicted ratio of the concentration of nuclear to cytoplasmic p65 was plotted as a function of time after LPS stimulation.
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C. neoformans 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 because they do not induce NF-κB signaling (Fig. 2A).

Intracellular C. neoformans Delays and Extends LPS-induced NF-κB Signaling—Because chronic Cn infection primarily involves intracellular Cn, we investigated whether Cn can modulate the NF-κB pathway after 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. 3, A–C). Notably, intracellular Cn appeared to alter NF-κB signaling in a manner that differed from that of purified, extracellular GXM. Although 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 pronouced delay in the time taken to achieve maximal nuclear p65-EGFP levels (Fig. 3, A–C). In this case, intracellular Cn burden (the number of intracellular Cn per macrophage). Furthermore, in contrast with GXM-dependent effects, the duration of LPS-induced p65 nuclear occupancy was substantially extended in macrophages containing intracellular Cn compared with controls (Fig. 3C). Similar results were obtained in macrophages infected with 24067, a serotype D strain of C. neoformans, 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 NF-κB mathematical model modified from that of Sung et al. (24). This enabled us to simulate possible known and suspected effects of intracellular Cn proliferation (Fig. 4Aa), 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) above, 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) above, 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) above, 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. 4, B–E). 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 after LPS stimulation (Fig. 4, B and 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. 4, B and E). 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. 4, C and D).

Partial Inhibition of Macrophage Protein Translation Caused by Intracellular C. neoformans or Cycloheximide Can Increase the Duration of the p65 Nuclear Occupancy after LPS Stimulation—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
Because statistically significant decreases in translation were also obtained using ribopuromycylation assays (Fig. 5D), this suggests that Cn somehow interferes 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 CIN-p65 (Cn-induced nuclear accumulation of 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 after 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. 6, A and B). This slow accumulation of p65-containing dimers within the nucleus appeared to be influenced by macropage intracellular Cn “burden,” which gradually increased over time as phagocytosed Cn began to proliferate within individual macrophages (Fig. 6B). Unexpectedly, the nuclear accumulation of p65-EGFP was not accompanied by mCherry expression from the Tnf promoter, despite abundant nuclear p65 (Fig. 6, A and C). This effect was observed in multiple cells (Fig. 6, D–G).

Phagosomal C. neoformans Suppresses TNFα and iNOS Production in Cells Exhibiting Nuclear p65—Because 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, because 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, because LPS treatment failed to promote either the return of p65-EGFP to the cytosol or expression of TNFα (Fig. 7A). Although neighboring

**FIGURE 4.** Numerical experiments using the model of Sung et al. (24) to evaluate potential mechanisms through which intracellular Cn modulates NF-κB signaling. **A,** diagram linking model parameters to biological events. **B–E,** select model parameters were varied about a nominal value as indicated under "Numerical Experiments." The predicted ratio in the concentration of nuclear to cytoplasmic p65 was plotted as a function of time after LPS stimulation.

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 although 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 of three experiments, as measured using Click-iT O-propargyl-puromycin (OPP) staining (Fig. 5, B and C), and the magnitude of this effect increased with intracellular Cn burden.
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. 7, A and 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. 7, C and D).

Because mCherry expression is regulated by an exogenous copy of 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

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FIGURE 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 pretreated with vehicle (Ctrl) or the indicated doses of CHX. Data from >40 cells were collected per condition across a minimum of two 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 (3–5 Cn; HB) infected cells) or heat-killed (HK) H99S Cn. D, quantification of ribopuromycylation (RPM) staining of H99S-infected RAW264.7 cells. E, OPP-647 staining was quantified as described in B and C for Cap59 infected RAW 264.7 cells. Error is represented as the S.E. Statistical significance is indicated as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001 (ANOVA, p < 0.05). Data from >40 cells were collected per condition across a minimum of three independent biological repeats. The scale bar represents 20 μm.
basal levels 24 h after 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. 8, A–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:IκBα complexes could have a similar effect (Fig. 8B). Changes to other model parameters, such as the rate of IκBα nuclear import (P12; 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:IκBα 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, because 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 countermove 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

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**FIGURE 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. The scale bar represents 20 μm. Intracellular Cn are indicated with arrows. B and C, quantification of the nuc:cyto ratio of p65-EGFP fluorescence (B) and mCherry fluorescence (C) 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 three representative cells.
The NF-κB pathway is a frequent target of human pathogens. Although *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 to escape and disseminate (reviewed in Ref. 50).

Over the past 15 years, a number of studies utilizing both purified capsular components and intact yeast have shown that *C. neoformans* may also subvert immune cell NF-κB signaling during acute, extracellular infections (10, 11, 27, 51). Perhaps because of 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 infection, the *Cn* burden alters the NF-κB response to LPS.

**FIGURE 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 before and after LPS stimulation for representative cells with (Cn +ve) and without (Cn −ve) intracellular Cn as indicated in A. C and D, change in total p65-EGFP (C) and mCherry fluorescence (D) after LPS stimulation for cells exhibiting Cn-induced nuclear p65 (Cn +ve) and control cells that do not contain Cn (Cn −ve). Error is represented as the S.E. 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 five independent biological repeats. E, RAW 264.7 NF-κB reporter cells were fixed at the indicated times after 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 four independent biological repeats. These cells contained an average of 4.3 ± 1.6 Cn. The scale bars represent 20 μm.

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GXM is shed by proliferating yeast suggest that GXM attenuates LPS-induced NF-κB signaling. This important question has remained open because of 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 “unactivated” macrophage population that had ingested Cn 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- or 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 after LPS stimulation. This model-generated hypothesis was plausible because (i) intracellular Cn has been previously shown to affect protein translation in murine peritoneal macrophages and J774.16 macrophage-like cells (33), and (ii) we were able to demonstrate that partial inhibition of translation using CHX extended p65 nuclear occupancy after LPS stimulation. 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-IκBα complexes. These two competing hypotheses were tested by immunostaining cells for IκBα. We found that CIN-p65 cells contained large quantities of nuclear IκBα, which was consistent with the second hypothesis. This finding, although 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). 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 IκBα in these cells will complex with nuclear p65, decreasing association with chromatin (47, 48). It has also been shown in LPS and leptomyacin 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). Although 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 remain 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, which 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 1 and 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 show 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 pro-inflammatory 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.
C. neoforms modulates macrophage NF-κB signaling

Nikon Ti-Eclipse wide field microscope equipped with a CFI Plan Fluor 40× oil immersion NA 1.30 objective, Intensilight epifluorescence illuminator, computer-controlled stage (Nikon), CoolSNAP MYO camera (Photometrics), and a full environmental enclosure with CO₂, humidity, and temperature control (InVivo Scientific). The microscope was controlled using Nikon Elements Software (Nikon).

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 660-nm dichroic mirror.

In cases where Cn infection induced stable nuclear localization of p65-EGFP, the cells were fixed with ice cold methanol for 5 min and then immunostained with a primary antibody raised against either iNOS (1:100 dilution; catalog no. 13120S, Cell Signaling) or IκBα (1:100 dilution; catalog no. sc-371, Santa Cruz Biotechnology), followed by an anti-rabbit Alexa Fluor 647-labeled secondary antibody (1:200 dilution; catalog no. ab150075, Abcam) and 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 2 × 10⁵/well into tissue culture-treated 6-well plates (USA Scientific, Ocala, FL) containing no. 1.5 (0.17-mm thick) glass coverslips. The cells were activated with 500 g/ml LPS for 16 h prior to infection with Cn. The cells were allowed to phagocytose Cn and then an additional 1 h before cells were treated with 100 μM of the ribosome inhibitor CHX for 30 min. Stained cells were imaged using a Zeiss LSM700 laser scanning confocal microscope. Alexa Fluor 647 fluorescence was excited using a 555-nm laser and detected through a LP640 filter. NuclearMask Blue fluorescence was excited using a 405-nm laser, and fluorescence was detected through a SP 555-nm filter.

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 nuc:cyto ratio. For each cell analyzed, this ratio was used to generate values for (i) amplitude, reported as the maximum nuc:cyto ratio reached during the time course; (ii) time to maximum, reported as minutes after LPS stimulation until the first maximum nuc:cyto ratio was achieved; and (iii) duration, which utilized nuc:cyto normalized to the maximum value and was reported as minutes between nuc:cyto > 0.5 to nuc:cyto < 0.5. Whole cell integrated mCherry fluorescence was determined for each cell and normalized to the pretreatment or initial value. For experiments involving infection of cells with Cn, data were produced for both Cn-containing (Cn +ve) and an equal or greater number of cells that did not contain Cn (Cn −ve) as internal controls. The initial number of Cn present within the cell was recorded and in some cases tracked throughout experiments. For the protein translation assays, 16-bit Zeiss czi images were also analyzed using Fiji. The images were background subtracted, and mean whole cell Alexa Fluor 555 was quantified for each cell for ribopuromycylation, and mean nuclear Alexa Fluor 647 fluorescence 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. (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 0.3 (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 Xn be the concentration in the nucleus, and Xc be the concentration in the cytoplasm, Vn be the volume of the nucleus, and Vc be the volume of the cytoplasm. If α is the rate at which Xc decreases because of the movement of X out of the cytoplasm and into the nucleus, so that dXc/dt = −αXc, then Xn increases at rate (Vn/Vc)α, so that dXn/dt = + (Vn/Vc)α. Similarly, when X moves from the nucleus to the cytoplasm, the resulting rate of change of Xc is −Vn/Vc that of Xn. To provide a better qualitative fit to our data, we also lowered the rate of IkB synthesis by a factor of 0.2 and the maximum rate at which IKK is activated through the MyD88- and 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 to (i) vary the model parameters that control the initial (p19) and delayed (p20) waves of IKK activity, the rate of IkB translation (p9), the rate of nuclear import of IkB (p12), and the rate of nuclear export of IkB-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. (24). The solution was then simulated over a long time interval (20 h) 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 (to reflect a potential effect of Cn injection), 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. After LPS stimulation the solution was simulated for an additional 10 h (model driver file in supplemental materials).

Statistical Analysis—For the purified GXM experiments, differences in time to maximum amplitude were analyzed using analysis of variance (ANOVA), whereas 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 OPP staining kit and Wilcoxon Rank sums for the riboporumucylation assays. To determine whether macrophages with high intracellular burden had decreased TNFa expression after the addition of LPS, the data were analyzed using an unpaired Student’s t test. For all tests, p < 0.05 was considered significant.

Author Contributions—L. E. H. performed and analyzed the experiments shown in Fig. 1. J. B. H. performed and analyzed the experiments in Figs. 2, 3, and 6–8. W. D. and R. N. L. performed and interpreted the numerical experiments in Figs. 1, 4, and 8. L. M. S. performed and analyzed the experiments in Fig. 5. E. E. N. and D. E. N. conceived, designed, and coordinated the study. J. B. H., W. D., R. N. L., E. E. M., and D. E. N. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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References


function [nuc_cyt, nuc_cyt0, tint_stim_plot, tint0] = kappamacrophage_2016
%This code simulates the effect of changing one of a collection of select parameters of an augmented version of the model of NF-κB signaling by Sung et al. The model variables are as in Sung et al.

% p19: a vector of magnitudes of initial wave of IKK activation,
% p20: a vector of magnitudes of delayed IKK activation
% p12: a vector of rates of transport of IkB into nucleus
% p14: a vector of rates of export of IkB-p65 from the nucleus
% p9: a vector of rates of IkB synthesis

% Numerical values for the parameters are from Sung et al, however, the rate of synthesis is reduced from Sung et al to provide better qualitative match to data.

% Additional parameters for augmented model include the strength of late and early phase LPS signaling (ck(19) and ck(20) respectively) and the ratio of nuclear to cytoplasmic volume.

% set parameters
kappamacrophage_parameters

% NC=nuclear volume : cytoplamic volume
NC=1/CN;

% id = 9, 12, 14, 15, 17, 19, 20
id=19; % index of parameter to vary
N=3; % number of parameter choices

%factors by which a select parameter is varied
vry=zeros(21,3);
vry(9,:)=[.4,.5,1];
vry(12,:)=[.25,.5,1];
vry(14,:)=[.25,.5,1];
vry(15,:)=[1,2,3];
vry(17,:)=[.5,.75,1];
vry(19,:)=[.5,.75,1];
vry(20,:)=[1,1.5, 2];

% Tf: final time for simulation,
Tf=600;

% set time at which parameter value is changes (StimTIme1) and time at which LPS stimulus is added (StimTime2)
StimTime1=1200;
StimTime2=2*StimTime1;

%absolute times at which to get solution
tint_stim=(StimTime2:.1:Tf+StimTime2);
tint0=(StimTime1:.1:StimTime2);

%corresponding times post StimTime2 for plotting
tint_stim_plot=(0:.1:Tf);

%all times at which solution is available
tint=(0:.1:StimTime2+Tf);

n=length(tint_stim_plot);
n0=length(tint0);

nuc=zeros(N,n);
cyt=zeros(N,n);
nuc0=zeros(N,n0);
cyt0=zeros(N,n0);

%ck and ck0 are vectors of baseline model parameters with and without
LPS
%stimulation.  k and k0 are vectors of parameters, with parameter
%values
%varied from their baseline.

k=ck;
k0=ck0;

%simulate model with select parameters varied
for i=1:N
%vary select parameter after LPS stim
k(id)=vry(id,i)*ck(id);
%vary select parameter before LPS stim
k0(id)=vry(id,i)*ck0(id);

%simulate model with select parameter varied
opts = ddeset('Jumps',1200);
sol3=dde23(@(t,y,z)KappaMacrophage_StimTime(t,y,z,k,k0,ck0,StimTime1,StimTime2),[Lag1,Lag2,Lag3],y0,[0,StimTime2+Tf],opts);

%get full solution
yint3 = deval(sol3,tint);
plot(tint,yint3)
%get solution after stim
yint = deval(sol3,tint_stim);
plot(tint_stim,yint)
% get nuclear and cytoplasmic concentrations of p65 after stim
nuc(i,:) = yint(7,:) + yint(9,:) + yint(14,:);
cyto(i,:) = yint(1,:) + yint(3,:) + yint(6,:) + yint(11,:) + yint(12,:);
tot = nuc*(CN+1)^-1 + cyt*(NC+1)^-1;

% get concentrations before stim
yint0 = deval(sol3,tint0);
% get nuclear and cytoplasmic concentrations of p65 before stim
nuc0(i,:) = yint0(7,:) + yint0(9,:) + yint0(14,:);
cyto0(i,:) = yint0(1,:) + yint0(3,:) + yint0(6,:) + yint0(11,:) + yint0(12,:);
end
% get nuc : cyt for p65 after and before LPS stimulus.
nuc_cyt = nuc./cyt;
nuc_cyt0 = nuc0./cyt0;

% plot response vs. parameter

% plot time series
plot(tint_stim_plot(1:3001),nuc(:,1:3001),'LineWidth',4);
xlabel('time (min)','FontSize', 20);
ylabel('nuclear NF-$\kappa$B','FontSize',20);
legend(sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,1)),
sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,2)),
sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,3)));
legend('boxoff')
saveas(gcf,sprintf('NFkBnP%d',id));

plot(tint0(1:6001),nuc0(:,1:6001),'LineWidth',4);
xlabel('time (min)','FontSize', 20);
ylabel('nuclear NF-$\kappa$B','FontSize',20);
legend(sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,1)),
sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,2)),
sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,3)));
legend('boxoff')
saveas(gcf,sprintf('NFkBnP%d_0',id));
plot(tint0(1:6001),nuc_cyt0(:,1:6001),'LineWidth',4);
xlabel('time (min)','FontSize', 20);
ylabel('nuc NF-kB : cyt NF-kB','FontSize',20);
legend(sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,1)),
      sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,2)),
      sprintf('p_{%d}:p_{%d}^*=%.2f',id,id,vry(id,3)));
legend('boxoff')
saveas(gcf,sprintf('NFkBnP%d_nc0',id));

plot(tint_stim,tot);
saveas(gcf,sprintf('NFkBtotP%d',id));

end
Modulation of Macrophage Inflammatory Nuclear Factor κB (NF-κB) Signaling by Intracellular Cryptococcus neoformans

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