Intracellular Cryptococcus neoformans disrupts the transcriptome profile of M1- and M2-polarized host macrophages

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Abstract

Macrophages serve as a first line of defense against infection with the facultative intracellular pathogen, *Cryptococcus neoformans* (*Cn*). However, the ability of these innate phagocytic cells to destroy ingested *Cn* is strongly influenced by polarization state with classically (M1) activated macrophages better able to control cryptococcal infections than alternatively (M2) activated cells. While earlier studies have demonstrated that intracellular *Cn* minimally affects the expression of M1 and M2 markers, the impact on the broader transcriptome associated with these states remains unclear. To investigate this, we used an *in vitro* cell culture model of intracellular infection together with RNA sequencing-based transcriptome profiling to measure the impact of *Cn* infection on gene expression in both polarization states. The gene expression profile of both M1 and M2 cells was extensively altered to become more like naive (M0) macrophages. Gene ontology analysis suggested that this involved changes in the activity of the Janus kinase-signal transducers and activators of transcription (JAK-STAT), p53, and nuclear factor-κB (NF-κB) pathways. Analyses of the principle polarization markers at the protein-level also revealed discrepancies between the RNA- and protein-level responses. In contrast to earlier studies, intracellular *Cn* was found to increase protein levels of the M1 marker iNos. In addition, we identified common gene expression changes that occurred post-*Cn* infection, independent of polarization state. This included upregulation of the transcriptional co-regulator *Cited1*, which was also apparent at the protein level. These changes constitute a transcriptional signature of
macrophage Cn infection and provide new insights into how Cn impacts gene expression and the phenotype of host phagocytes.

Introduction

The basidiomycetous yeast Cryptococcus neoformans (Cn) is a common facultative intracellular pathogen and the causative agent of cryptococcosis, a pulmonary infection that predominantly affects immune-compromised individuals that can disseminate to the central nervous system, resulting in life-threatening fungal meningitis [1, 2]. Cryptococcal infections occur when propagules, typically from bird excreta-contaminated soils, are inhaled into the lungs. Here, they encounter alveolar macrophages, innate phagocytes that act as a first line of defense against the pathogen. This interaction between host macrophages and Cn is perhaps the most important in determining the course and outcome of an infection [3-5], and there is substantial evidence to show that macrophages are essential for the successful control of cryptococcosis [6, 7].

The ability of macrophages to efficiently kill ingested Cn is greatly influenced by the polarization state of these cells [8-10]. Macrophage polarization is a continuum of phenotypes of differing function and microbicidal activity requiring the altered expression of >1000 genes [11, 12]. These do not represent terminal differentiation states as macrophages can rapidly repolarize over the course of hours from one state to another in response to microbe and immune cell-derived signals (i.e. cytokines) [13].
Interferon-gamma (IFNγ) or lipopolysaccharide (LPS) stimulates classical activation or M1 polarization and is associated with proinflammatory cytokine expression and metabolic shifts that increase production of microbicidal reactive oxygen and reactive nitrogen species [14]. This is partially achieved through increased expression of the Nos2 gene, which encodes inducible nitric oxide synthase (iNOS) and catalyzes the production of nitric oxide (NO) from L-arginine [15]. As infections are resolved, elevated levels of interleukin-4 (IL-4) and IL-13 promote the repolarization of macrophages to an anti-inflammatory alternative or M2 polarization state, which is accompanied by increased expression of arginase-1 (Arg1) [16]. This enzyme competes with iNOS for substrate, thereby reducing NO production and macrophage microbicidal activity.

As M2 cells typically present a less hostile environment for the intracellular growth and replication of microbes, a variety of pathogens have evolved mechanisms to interfere with host cell polarization as a survival strategy (reviewed in [17]). While previous studies have suggested that Cn minimally affects the expression of Nos2 and Arg1, the principle markers of the M1 and M2 states [13], the impact on the broader transcriptome associated with these states remains unclear and is not captured in earlier microarray or RNA sequencing-based analyses of Cn-infected macrophages where polarization state is not explicitly considered [18-20].

Here, we present data showing that intracellular Cn infection resulted in extensive changes to the transcriptome of host macrophages, involving altered activity of the Janus kinase-signal transducers and activators of transcription (JAK-STAT), p53, and nuclear factor κB (NF-κB) pathways. Infection with Cn also shifted the gene
expression profiles of both M1 and M2 cells to a more M0-like state, while causing relatively small changes in the expression of Nos2 and Arg1. Additionally, we identified a transcriptional signature of Cn infection, common to both polarization states, which included upregulation of the transcriptional co-regulator Cited1. Collectively, these results provide new insights into how Cn reshapes gene expression and the phenotype of host cells.

Materials and methods

Culture and opsonization of Cn

The H99S strain [21] of Cn serotype A was grown in yeast peptone dextrose (YPD; ThermoFisher Scientific) broth, shaking at 37 °C for 36 h prior to infection. After 36 h, 1x10⁷ Cn cells were washed 3X with phosphate-buffered saline (PBS), pelleted by centrifugation at 750 x g for 5 min, and then counted. The cells were opsonized with 18B7 (a kind gift from Dr. Arturo Casadevall; previously described in [22]) using 20 µg per 1.5 x 10⁶ cells in 1 mL 20% goat serum (GS, Sigma Aldrich, St. Louis, MO) for 30 min. The opsonized cells were washed 3X with PBS to remove excess 18B7 and were recounted. A total of 2.25 x 10⁶ cells/mL were necessary to ensure a 3:1 multiplicity of infection (MOI; Cn:macrophage).
Macrophage culture, polarization, and infection

RAW 264.7 cells, a murine macrophage-like cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; VWR, Radnor, PA), 200 mM L-glutamine, 1% penicillin and streptomycin, and 50 µg/mL gentamicin (all from Sigma Aldrich). Macrophages were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

Prior to infection, RAW 264.7 cells were seeded into 6-well plates at a density of 7.5 x 10⁵ cells/well and incubated overnight with 200 U/mL of recombinant murine IFNγ (Biolegend, San Diego, CA) to promote M1-polarization and then infected with opsonized Cn for 2 h at 3:1 MOI or mock infected with PBS. After 2 h, extracellular Cn were removed by washing 2X with PBS and the macrophages were cultured for a further 24 h in fresh growth media containing either IFNγ to maintain the M1 polarization state or 100 ng/mL recombinant interleukin-4 (IL-4; Sigma-Aldrich, St. Louis, MO, and Peprotech, Rocky Hill, NJ) to promote repolarization to the M2 state. Cells were washed and growth medium containing IFNγ or IL-4 was replaced every 6 h to prevent nutrient depletion, remove extracellular Cn, and maintain the appropriate polarizing environment.
Glucose assays

RAW264.7 cells were grown in phenol red-free DMEM adjusted to contain the indicated concentration of glucose at the start of the experiment. Growth medium from the cultures was sampled (1 mL) at the indicated times post-treatment and centrifuged to remove Cn and cellular debris. Glucose concentration was measured using a glucose oxidase assay kit (GAGO20, Sigma Aldrich) according to manufacturer’s instructions.

Immunoblotting

Macrophages were lysed using radioimmunoprecipitation assay (RIPA) buffer containing 1x protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich). Harvested samples were vortexed and centrifuged at 13,000 × g for 15 min at 4 °C to remove cell debris. Sample protein concentrations were measured using a bicinchoninic acid (BCA) assay kit according to manufacturer’s instructions. Normalized protein lysates were boiled at 95 °C for 10 min in Laemmli sample buffer, electrophoresed on 10 or 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline/Tween 20 (TBS/T) containing 5% non-fat milk powder for 1 h and incubated overnight at 4°C with the appropriate primary antibodies. These included β-actin (A2066, Sigma), iNos (D6B6S, Cell Signaling Technology, Danvers, MA), Arg1 (ab124917, Abcam, Cambridge, MA), MSG1 (Cited1; sc-393585, Santa Cruz Biotechnology, Dallas, TX), and VDUP1 (TXNIP; sc-271238, Santa Cruz Biotechnology). Primary antibody binding
was detected using mouse anti-rabbit IgG-horseradish peroxidase (HRP; sc-2357, Santa Cruz Biotechnology) or anti-mouse m-IgG kappa binding protein (BP)-HRP (sc-516102, Santa Cruz Biotechnology), as appropriate. Membranes were incubated with enhanced chemiluminescent (ECL) reagents and bands were visualized using a ChemiDoc MP Imaging System with Image Lab Software (Bio-Rad, Hercules, CA).

**RNA extraction and cDNA library preparation**

Macrophages were homogenized in lysis reagent (RLT buffer, Qiagen, Germantown, MD) using a 20-gauge needle and remaining intact Cn were removed by centrifugation at 750 x g for 5 min at 4 °C. RNA was extracted from the lysates using an RNeasy® Mini Kit (Qiagen), according to the manufacturer’s instructions. Genomic DNA was then removed from the total RNA using a Message Clean kit (GenHunter, Nashville, TN) per the manufacturer’s instructions. Clean RNA was resuspended in 10 µL diethyl pyrocarbonate (DEPC)-treated water and RNA integrity and quality was appraised using a Qubit 2 fluorometer (ThermoFisher Scientific).

The cDNA libraries for RNA sequencing were prepared from 1 µg of isolated RNA using the NEBNext UltraTM Directional RNA Library Prep Kit for Illumina, the NEBNext Multiplex Oligos for Illumina Index Primers, and the NEBNext Poly(A) Magnetic Isolation Module (all from New England BioLabs, Ipswich, MA) in accordance with the manufacturer’s instructions.
Analysis of RNA sequencing data

RNA sequencing of each library sample was performed at Novogene (Sacramento, CA) using the HiSeq 2500 system to produce 150 bp transcriptome paired-end reads. FastQC (version 0.11.5; [23]) was used to check quality of the fastq data files. No trimming of the files was necessary based on the data quality. The STAR aligner (version 2.5.3a; [24]) was used to align reads to the version 38 mouse genome [25] with scaffolding provided by the mouse reference genome annotation (version 39.90, [25]) within the CyVerse Discovery Environment [26]. The resulting bam and the same mouse genome annotation were used to generate a read count table by gene using FeatureCounts [27] and multi-join [28] within the Galaxy platform [29]. These read counts were imported into R where they were used to cluster samples according to their whole genome gene expression profile using EdgeR [30], as detailed in [31]. Data were displayed as both a multi-dimensional scale plot and dendogram to aid in evaluating samples for inclusion or exclusion. Four samples were excluded from further analysis due to their failure to cluster within their replicate pool (M1_mock replicate 2, M1_Cn replicate 1, M2_mock replicate 3, and M0 replicate 1). Next, StringTie (version 1.3.3; [32]) was used to construct transcript annotations based on the bam data and the same mouse reference genome and genome annotation within the CyVerse Discovery Environment[26]. StringTieMerge (version 1.3.3;[32]) was then used to merge these sample-specific genome annotations into an experiment-specific genome annotation, also within the CyVerse Discovery Environment [26]. Finally, CuffDiff2 (version 2.2.1; [33]) was then used to make pairwise comparisons for differentially expressed genes.
(DEGs) among the replicate groups of aligned reads (bam files) based on the experiment-specific genome annotation within the Cyverse Discovery Environment [26]. From the pairwise comparisons, DEGs with fold change $\geq 2.0$ and $q \leq 0.05$ were considered biologically relevant and statistically significant. Functional categorization and pathway over-representation analysis of genes within the statistically significant DEGs were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resource tool (version 6.8; [34, 35]) to identify biological pathways specific to polarization state and infection state. Gene ontology (GO) terms were ranked by $P$-value, which was plotted as $-\log(P$-value). Finally, protein-protein interaction networks were identified among DEG sets using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, version: 11.0; [36]). The confidence interval was set at 0.700. Evidence based analysis was used with Markov Cluster (MCL) grouping. Common protein-protein interaction clusters were then visually identified for M0-M1mk compared to M0-M1Cn, M0-M2mk compared to M0-M2Cn, M0-M1Cn compared to M0-M2Cn, and M1-M1Cn compared to M2-M2Cn. Protein network changes based on polarization state and infection status were identified by determining the common and unique genes/proteins between the common clusters for each pair.
Results

Development of a strategy to obtain *Cn*-infected M1 and M2-polarized macrophages

To develop an *in vitro* system to accurately characterize the effects of intracellular *Cn* infection on the transcriptome of host macrophages in different polarization states, it was necessary to i) utilize a macrophage cell line that can adopt clear M1 and M2 phenotypes, ii) maintain a stable growth environment for the cells so that nutrient depletion did not impact gene expression, and iii) be able to infect these cells with high efficiency so that the vast majority of mRNA transcripts identified in the RNA sequencing analysis originated from *Cn*-infected cells.

For this study, we selected the murine macrophage-like cell line RAW 264.7, which is frequently used as an *in vitro* model to investigate how intracellular pathogens affect macrophage polarization and gene expression [37, 38], and how gene expression of pathogens is altered in host cell environments [39]. It has also been used by us and others to study macrophage:*Cn* interactions [13, 40].

While naive RAW 264.7 cells can be stimulated to adopt an M1-like phenotype through exposure to IFNγ alone, expressing various proinflammatory cytokines and detectable levels of the M1 marker, iNos [13], it is unclear whether these cells can be directly polarized to the M2 state. Stimulation of naive RAW 264.7 cells with IL-4 has been shown to induce transcriptional upregulation of the M2 marker, *Arg1*, but does not result in detectable levels of the Arg-1 protein [41]. This was also true in our hands.
and we were only able to stimulate a measurable increase in Arg-1 protein expression if RAW 264.7 cells were co-stimulated with IL-4 and the cAMP analog, 8-bromo-cAMP to activate C/EBPbeta, a co-regulator of the Arg1 promoter [41](Fig 1A).

**Figure 1. Optimization of murine macrophage intracellular Cn infection for transcriptome profiling.** Western blot analysis of RAW264.7 macrophages for M1 (iNos) and/or M2 (Arg-1) marker proteins after incubation for 24 h with **(A)** IL-4 and cAMP or **(B)** IFNγ and IL-4. **(C)** iNos and **(D)** Arg-1 levels in **(B)** were quantified by densitometry based on six discrete biological repeats. **(E)** Schematic to summarize data from **(A-D)**, which suggests that while RAW264.7 cells cannot be directly polarized from M0 to M2 by IL-4 treatment, they can be repolarized from M1 to M2. Expression of TXNIP, a marker of glucose levels, as measured in RAW264.7 macrophages by western blotting after 24 h **(F)** incubation in growth medium containing the indicated glucose concentrations or **(G)** infection with 18B7-opsonized Cn. LPS was added for 2 h during infection to promote phagocytosis of Cn. **(H)** Glucose concentration as measured in RAW264.7 macrophage growth medium using a glucose oxidase assay kit 24 h post-mock or Cn-infection. Complete replacement of growth medium at 6 h intervals to remove extruded Cn prevents glucose depletion. **(I and J)** The percentage of Cn-infected RAW264.7 cells was quantified by light microscopy 24 h post-infection using the indicated conditions. For all experiments, concentrations are as follows: 200 U/mL IFNγ, 100 ng/mL IL-4, 1 µg/mL LPS, cAMP is 0.5 mM 8-Br-cAMP, 1x 18B7 is 10 µg per 1.5x10^6 Cn, GS is goat serum. Error is represented as S.E. Statistical differences between samples.
were appraised by one-way analysis of variance (ANOVA) followed by a Tukey’s multiple comparison test. Statistical significance is indicated as follows: *, \( p < 0.05 \); **, \( p < 0.01 \).

With the exception of (B-D), data is from three biological repeats.

As macrophages will typically repolarize from an M1 to an M2 state rather than directly from M0 to M2 during the course of a normal infection, we reasoned that RAW 264.7 macrophages may operate in a similar fashion. To test this, we stimulated RAW 264.7 cells with IFN\(\gamma\) for 24 h followed by treatment with IFN\(\gamma\) or IL-4 for a further 24 h. As expected, continuous IFN\(\gamma\) treatment resulted in \(~300\) fold increase in iNos protein levels (Figs 1B and C). While Arg-1 protein levels were indistinguishable from untreated controls and samples that did not receive IFN\(\gamma\) prior to IL-4 stimulation, repolarization from M1 to M2 resulted in \(>100\) fold increase in Arg-1 protein levels (Fig 1E).

Collectively, these data suggested that RAW 264.7 cells could be used to model both the M1 and M2 state with the caveat that the M2 phenotype could not be reached directly from the naive state but via repolarization from M1 (Fig 1E).

Given that \(Cn\) can replicate within host cell phagolysosomes and can escape into the surrounding culture medium through non-lytic exocytosis or vomocytosis [42-45], we reasoned that the accumulation of live, extracellular \(Cn\) might result in the accelerated depletion of glucose and other nutrients from the culture medium. As this could potentially result in spurious changes in gene expression not directly caused by intracellular \(Cn\) growth, we used the expression of the glucose-regulated gene, thioredoxin–interacting protein (\(TXNIP\)) as a marker of glucose depletion-induced gene
expression changes in our experiments. TXNIP is a regulator of nitrosative stress and glucose metabolism in a variety of cell types and is itself regulated at a transcriptional level by heterodimers of the glucose-responsive transcription factors, MondoA and Mlx [46-48]. We found that decreasing glucose levels from 4.5 g/dL in the macrophage culture medium to 1.0 g/dL for 24 h resulted in a complete loss of TXNIP protein expression in RAW 264.7 cells, indicating that TXNIP expression was a suitable marker of glucose depletion in our system (Fig 1F).

In preliminary RNA sequencing experiments (unpublished), the expression of TXNIP transcripts was decreased 8.34 fold ($q = 0.0016$) in Cn versus mock-infected RAW 264.7. Additionally, in parallel immunoblotting experiments, we found that TXNIP proteins dropped to undetectable levels at 24 h post-Cn infection (Fig 1G). Although the decrease in glucose concentration in Cn-infected cultures at 24 h post-infection was relatively modest (~20%; Fig 1H), our data suggested that the loss of TXNIP expression was artifactual and that Cn-induced changes in culture conditions over the course of the 24 h experiments may impact the resultant transcriptome profiles. In an effort to mitigate this, in all subsequent experiments, macrophage cultures were washed and the medium was replaced at 6 h intervals to remove extracellular Cn and prevent large changes in culture conditions (Fig 1H).

As we utilized a standard RNA sequencing approach, harvesting RNA from large populations of cells, we optimized phagocytosis conditions to maximize the number of Cn-infected M1 and M2-polarized macrophages in our cultures. This was achieved by titrating the concentration of 18B7, a monoclonal antibody raised against Cn capsular
polysaccharide [22] used to opsonize the yeast prior to infection of M1-polarized macrophages (Fig 1I). We found that a concentration of 2× 18B7 (20 μg for 1.5 x 10^6 cells) was sufficient to routinely obtain infection efficiencies of ~80%, as determined at the time of macrophage harvest for mRNA extraction. While it is common to add LPS to macrophages to promote phagocytosis, we found that this did not affect the percentage of Cn-infected macrophages (Fig 1I). Furthermore, as co-infections with gram-negative bacteria are uncommon in cryptococcosis patients [49], LPS was excluded from all subsequent transcriptome-profiling experiments. Finally, we found that infection efficiencies of >50% could be achieved for M2 macrophages if the cells were IFNγ-stimulated 24 h prior to infection and then repolarized to M2. If the cells were not M1 polarized first, not only was Arg-1 protein expression lost (Figs 1B-D), but also the percentage of Cn-infected macrophages decreased to <20% (Fig 1J).

**Intracellular Cn increased iNos protein expression in M1-polarized macrophages**

Based on the data described in Figure 1, we developed a strategy to produce Cn-infected M1 and M2-polarized RAW 264.7 cells (Fig 2A). In brief, this involved M1-polarizing naive macrophages by incubation with IFNγ for 24 h, then mock or Cn-infection with complement- (provided by 20% goat serum) and 18B7-opsonized yeast at an MOI of 3:1. After a 2 h infection period, residual extracellular Cn were removed by washing and the cells were cultured for a further 24 h in growth medium containing...
either IFNγ to maintain the M1 polarization state or IL-4 to repolarize to M2, with the
growth medium and cytokines replaced at 6 h intervals.

**Fig 2. Expression of iNos is increased in Cn-infected M1-polarized macrophages. (A)**

Schematic to represent macrophage infection protocol used for subsequent western
blotting and transcriptome profiling experiments. **(B)** Western blot analysis of
RAW264.7 macrophages for M1 (iNos) and M2 (Arg-1) markers after the indicated
treatments. **(C)** iNos and **(D)** Arg-1 levels in **(B)** were quantified by densitometry based
on six discrete biological repeats. Error is represented as S.E. Statistical differences
between samples were appraised by one-way ANOVA followed by a Tukey’s multiple
comparison test. Statistical significance is indicated as follows: **, p < 0.01.

We utilized this protocol to perform RNA sequencing-based transcriptome
profiling of naive (M0), mock- (M1mk and M2mk) and Cn-infected (M1Cn and M2Cn)
macrophages in both polarization states. As an initial quality control step, we performed
pairwise comparisons of gene expression in M0 with M1mk or M2mk samples and
looked for changes in the expression of a small panel of known polarization markers [11,
12, 50]. As expected, Nos2, Stat1, a range of M1-associated cytokines and chemokines
(Illb, Cxcl9, Cxcl10, and Ccl5), and surface markers (Fcgr1 and Cd86) were strongly
upregulated (Table 1). A similar analysis was performed for cells repolarized from M1 to
M2, and in this case, we saw upregulation of the M2 markers, Arg1 and IL10, as well as
downregulation of M1 markers (Table 2).
Table 1. M0 vs. M1mk

| Gene   | FC    | Direction | q-value       |
|--------|-------|-----------|---------------|
| Cxcl9  | 682.63| UP        | 0.00626191    |
| Gbp2   | 369.19| UP        | 0.00626191    |
| Cd86   | 356.53| UP        | 0.0383977     |
| Nos2   | 150.18| UP        | 0.00626191    |
| Cxcl10 | 139.62| UP        | 0.00626191    |
| Stat1  | 46.87 | UP        | 0.0334391     |
| IL1b   | 46.41 | UP        | 0.0161112     |
| Fcgr1  | 17.61 | UP        | 0.00626191    |
| Ccl5   | 6.66  | UP        | 0.0370904     |

Expression of M1 macrophage markers (FC = Fold-change)

Table 2. M1mk vs. M2mk

| Gene   | FC    | Direction | q-value       |
|--------|-------|-----------|---------------|
| Arg1   | 623.88| UP        | 0.00626191    |
| Atp6v0d2 | 14.06| UP        | 0.0350194     |
| IL10   | 10.08 | UP        | 0.0413593     |
| Cxcl10 | 18.9  | DOWN      | 0.00626191    |
| Cxcl11 | 15.77 | DOWN      | 0.0200165     |
| Nos2   | 12.24 | DOWN      | 0.00626191    |
| Cxcl9  | 8.84  | DOWN      | 0.00626191    |
| GBP2   | 5.21  | DOWN      | 0.00626191    |

Consistent with previous reports [13], Cn infection appeared to have little effect on the expression of these core polarization markers. The expression of Nos2 was not significantly altered in M1- or M2-polarized cells during Cn infection, and amongst the remaining M1 markers, only Ccl5 was increased in M1mk vs. M1Cn (FC = 3.71, q = 0.046). Surprisingly, Arg1 was decreased in M2mk vs. M2cn (FC = 3.97, q = 0.006). To determine whether these effects were also apparent at the protein level, we measured
Arg-1 and iNos levels by western blotting. Surprisingly, while Arg-1 was seemingly unaffected by \( Cn \) infection in both polarization states (Figs 2B and C), iNos levels were increased by ~40% in M1-polarized cells (Figs 2B-D).

**Distortion of the M1 and M2 transcriptome in \( Cn \)-infected macrophages**

Although our initial analysis suggested that \( Cn \) infection minimally affected principle polarization state markers, we could not rule out the possibility that the broader transcriptome and key processes associated with each state were impacted. To comprehensively examine this, DEGs from each state were identified through pairwise comparisons of M0 to M1mk or M2mk RNA sequencing data. These were then compared to corresponding DEGs from M0 to M1\( Cn \) or M2\( Cn \) comparisons.

Of the 931 DEGs associated with M0 to M1 polarization, 332 (~36%) were common to the 460 DEGs from the M0:M1\( Cn \) comparison (Fig 3A). This suggested that the expression of the remaining 599 DEGs (~64%) associated with the M1 state became more M0-like post-\( Cn \) infection. The 128 DEGs (~28%) unique to the M0:M1\( Cn \) comparison were assumed to be \( Cn \)-induced changes in gene expression not associated with the normal M1 transcriptional profile. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the common pool of DEGs showed an enrichment of genes associated with core M1 processes, including ‘JAK-STAT signaling’, ‘Cell adhesion molecules’, and ‘Cytosolic DNA-sensing pathway’. Interestingly, a subset of these terms,
including ‘Phagosome’, ‘Antigen processing and presentation’, ‘Toll-like receptor signaling’, and ‘Tumor necrosis factor (TNF) signaling pathway’ also appeared significant for the M0:M1mk-exclusive DEG pool, suggesting changes to these M1-associated processes during Cn infection. Similarly, the ‘NF-κB signaling pathway’ term was significant for both the common and M0:M1Cn-exclusive pools of DEGs. This was consistent with the known function of the NF-κB pathway as a regulator of M1 polarization and prior studies by our and other groups showing that Cn infection modulates the activity of NF-κB transcription factors in host macrophages [40, 51].

Interestingly, B cell leukemia/lymphoma 2 related protein A1a (Bcl2a1a), the murine orthologue of the anti-apoptotic protein A1, was amongst the NF-κB-regulated DEGs present within the M0:M1Cn-exclusive pools of DEGs and was upregulated 4.95 fold (q = 0.006); Bcl2a1a was also upregulated in Cn-infected M2-polarized cells (M0:M2Cn, FC = 4.93, q = 0.006). To visualize changes to M1-associated gene interaction networks M0:M1mk and M0:M1Cn DEGs were analyzed using STRING (Figs 3B and C). Consistent with the notion of disrupted M1 polarization, the gene cluster associated with innate immune function was smaller for the M0:M1Cn compared to M0:M1mk DEGs (24 vs. 30 genes), as was the antigen processing and presentation cluster.

Fig 3. Gene ontology (GO) analysis of Cn-infected M1 macrophages. (A) Venn diagram to represent common and differentially expressed genes in M0:M1mk and M0:M1Cn comparisons. These are accompanied by GO analysis performed in DAVID on genes appearing in each division of the Venn diagrams. Relevant pathways are ranked by –
log(P-value). Differentially expressed genes from (B) M0:M1mk and (C) M0:M1Cn pairwise comparisons were analyzed in STRING. Boundaries enclosing gene clusters with common function are drawn based on gene GO term data and information from the literature. *Note: Many of the genes contained within the ‘Ribosome function’ boundary are pseudogenes.

Equivalent analyses were performed for the M2 data sets, yielding similar results. Here, of the 583 DEGs associated with M0 to M2 polarization, 234 (~40%) were common to the 340 DEGs from the M0:M2Cn comparison (Fig 4A). As before, this indicated that the expression of the remaining 349 (~60%) DEGs associated with the M2 state became more M0-like post-Cn infection and the 106 DEG unique to the M0:M2Cn comparison were assumed to be Cn-induced changes in gene expression not associated with either the normal M0 or M2 transcriptional profiles. Similar to the data for M1-polarized cells, genes associated with the KEGG pathway term, ‘Phagosome’ and ‘Antigen processing and presentation’ were enriched amongst the common and M0:M2mk unique pools of DEGs. In contrast, genes associated with ‘p53 signaling’ were only significantly enriched amongst the M0:M2mk pool in the M2 analysis and not in the equivalent M0:M1mk pool. These genes included the canonical p53 targets, Ccng1, Cdkn1a (p21), and Mdm2, all of which were upregulated >3-fold. The pool of M0:M2Cn-exclusive DEGs was relatively small and the ‘NF-κB signaling pathway’ GO term was one of the few that could be associated with this group. This short list included Plau, Cd40, Traf1, and, as previously mentioned, Bcl2a1a.
Fig 4. Gene ontology (GO) analysis of Cn-infected M2 macrophages. (A) Venn diagram to represent common and differentially expressed genes in M0:M2mk and M0:M2Cn comparisons. These are accompanied by GO analysis performed in DAVID on genes appearing in each division of the Venn diagrams. Relevant pathways are ranked by $-\log(P\text{-value})$. Differentially expressed genes from (B) M0:M2mk and (C) M0:M2Cn pairwise comparisons were analyzed in STRING. Boundaries enclosing gene clusters with common function are drawn based on gene GO term data and information from the literature. *Note: Many of the genes contained within the ‘Ribosome function’ boundary are pseudogenes.

STRING analysis using DEGs from M0:M2mk and M0:M2Cn pairwise comparisons also showed differences in the gene cluster associated with innate immune function (Fig 4B). However, in contrast to the analysis of M1-polarized cells, the cluster was larger for M0:M2Cn compared to M0:M2mk DEGs (23 vs. 17 genes). Additionally, the antigen processing and presentation cluster was more similar in size for M0:M2mk and M0:M2Cn than in the equivalent analysis for M1-polarized cells, suggesting that Cn infection possibly has smaller effects on this process in M2 macrophages.

A transcriptome signature of Cn infection

Having examined the effects of Cn infection on the M1 and M2 transcriptional profile, we sought to determine whether there was a common set of genes affected by
Cn infection, regardless of host cell polarization state. This was performed by identifying DEGs from M1mk:M1Cn and M2mk:M2Cn pairwise comparisons. These data were used to (i) produce a visual representation of gene networks in Cn-infected M1 and M2 cells using STRING analysis (S1 Fig), and (ii) identify concordant genes present in both M1mk:M1Cn and M2mk:M2Cn DEG lists (Table 3 and 4, respectively). Similar network structures were evident in both M1mk:M1Cn (S1A Fig) and M2mk:M2Cn (S1B Fig) with both containing clusters associated with the innate immune system and ribosome function, although there were differences in the identity and numbers of genes in these clusters. The importance of the ribosomal function cluster was also questionable as many of these were pseudogenes. The M1mk:M1Cn contained a cluster of three genes associated with cell cycle regulation, Klf4, Cdkn1a (p21), and Ccng1, all of which were downregulated in M1-polarized Cn-infected cells. Although Ccng1 was also downregulated in Cn-infected M2 cells, the corresponding cluster was absent, as the expression of Klf4 and Cdkn1a was seemingly unaffected. A cell adhesion cluster could not be identified amongst the M2mk:M2Cn DEGs for similar reasons.
Table 3. Common concordant up-regulated genes in *Cn*-infected cells

| Gene   | M1mk vs. M1Cn FC | q-value     | M2mk vs. M2Cn FC | q-value     |
|--------|------------------|-------------|------------------|-------------|
| Cited1 | 14.81            | 6.26E-03    | 20.49            | 1.61E-02    |
| Hsf3   | 7.93             | 2.86E-02    | 8.04             | 0.0200165   |
| Jarid2 | 5.45             | 2.52E-02    | 2.88             | 0.0389813   |
| Tmtc2  | 5.14             | 9.61E-03    | 3.94             | 0.00626191  |
| Ccl22  | 5.09             | 3.50E-02    | 4.1              | 0.00626191  |
| Sspn   | 3.66             | 4.28E-02    | 3.21             | 0.0251778   |
| Wdr89  | 3.09             | 3.34E-02    | 2.43             | 0.0389813   |
| Bcl2a1a| 2.68             | 4.68E-02    | 2.95             | 0.0171929   |

Top common concordant upregulated genes in M1 and M2 *Cn*-infected macrophages

(FC = Fold-change)
### Table 4. Common concordant down-regulated genes in Cn-infected cells

| Gene   | M1mk vs. M1Cn | M2mk vs. M2Cn |
|--------|---------------|---------------|
|        | FC          | q-value       | FC          | q-value       |
| Sorl1  | 37.12       | 3.90E-02      | 3.86        | 0.046023     |
| Dusp6  | 14.54       | 1.29E-02      | 6.72        | 0.0375289    |
| C5ar1  | 12.07       | 6.26E-03      | 5.45        | 0.00626191   |
| Ehd2   | 11.63       | 6.26E-03      | 5.08        | 0.00626191   |
| Grk5   | 8.83        | 6.26E-03      | 4.48        | 0.0128586    |
| Cd300ld| 7.24        | 6.26E-03      | 2.7         | 0.0389813    |
| Zfp146 | 6.31        | 1.61E-02      | 3.97        | 0.0304857    |
| Endod1 | 6.21        | 6.26E-03      | 2.57        | 0.027269     |
| Usp9x  | 6.11        | 2.00E-02      | 3.88        | 0.0383977    |
| Xdh    | 6.02        | 6.26E-03      | 3.21        | 0.0171929    |
| Stom   | 4.86        | 6.26E-03      | 2.84        | 0.00626191   |
| Pik3ap1| 4.48        | 6.26E-03      | 3.52        | 0.0389813    |
| Car5b  | 4.36        | 2.52E-02      | 3.91        | 0.034954     |
| Itgax  | 4.2         | 2.25E-02      | 3.73        | 0.0171929    |
| Gatm   | 4.06        | 6.26E-03      | 7.79        | 0.00626191   |
| Ms4a6b | 3.46        | 3.50E-02      | 4.81        | 0.0128586    |
| Fgd3   | 3.42        | 1.29E-02      | 2.64        | 0.0383977    |
| Man2b1 | 3.27        | 1.29E-02      | 3.37        | 0.00961334   |
| Lamic1 | 3.23        | 3.63E-02      | 3.69        | 0.0200165    |
| Tep1   | 3.1         | 4.60E-02      | 2.74        | 0.0375289    |
| Dhx40  | 3.07        | 4.77E-02      | 3.22        | 0.0322409    |
| Ank    | 3.07        | 2.00E-02      | 2.97        | 0.00626191   |
| Ccn1   | 2.9         | 2.00E-02      | 2.89        | 0.00626191   |
| Stt3a  | 2.9         | 2.73E-02      | 2.81        | 0.0249383    |
| Plekho2| 2.88        | 3.84E-02      | 3.36        | 0.0251778    |
| Cdc42se2| 2.82       | 2.86E-02      | 2.39        | 0.0383977    |
| Lrp1   | 2.75        | 4.77E-02      | 2.55        | 0.0357649    |
| Myo1e  | 2.7         | 3.63E-02      | 3.75        | 0.00626191   |
| Nckap1l| 2.63        | 3.75E-02      | 2.52        | 0.0251778    |
| Atp2a2 | 2.5         | 4.28E-02      | 2.23        | 0.0420558    |

Top common concordant downregulated genes in M1 and M2 Cn-infected macrophages

(FC = Fold-change)
We identified 204 and 254 DEGs from the M1mk:M1Cn and M2mk:M2Cn pairwise comparisons, respectively. Of these, 38 genes were common and concordant (~15-19%), with 8 upregulated and 30 downregulated (Table 3 and 4). Interestingly, a large number of these represented reversals or partial reversals of gene expression changes occurring when repolarizing from M0 to M1, M0 to M2, or both. However, seven genes from this set were not part of the M1 or M2 transcriptome profile and appeared unique to Cn-infected cells in our analysis. These included three upregulated (Cited1, Ccl22, and Bcl2a1a) and four downregulated (Itgax, Ank, Lrp1 and Atp2a2) genes.

**CITED1 is upregulated by Cn-infected M1 and M2 macrophages**

The CBP/p300-interacting transactivator with glutamic acid/aspartic acid-rich carboxyl-terminal domain (CITED) gene family encode transcriptional co-regulators that activate or repress gene expression through direct interaction with CBP/p300. Of the three CITED family members present in mammals (1, 2, and 4), only CITED2 appears to be abundantly expressed in human and murine macrophages [52].

While the expression of CITED2 transcripts were unchanged in the conditions tested in our study, CITED1 showed the largest fold-change of all concordant DEGs and was upregulated in both M1 and M2 Cn-infected cells (FC = 14.81 and 20.49 in M1 and M2 cells, respectively). To determine whether this change in transcript abundance was accompanied by a similar increase at the protein level, we used western blotting to measure CITED1 levels in M1 and M2-polarized Cn-infected cells. As expected, CITED1
protein levels were strongly increased in Cn-infected cells in both polarization states, but not mock-infected controls (Fig 5). Additionally, under conditions where macrophages exhibited much-reduced rates of phagocytosis (i.e. naive macrophages or cells treated with IL-4 alone without prior IFNγ stimulation), exposure to Cn did not stimulate increased CITED1 expression. Taken together, these data indicated that CITED1 was expressed in response to intracellular rather than extracellular Cn and was not affected by polarization alone.

Fig 5. Cn infection stimulates increased Cited1 expression in M1 and M2-polarized macrophages. Western blot analysis of RAW264.7 macrophages for Cited1 and the M1 (iNos) and M2 (Arg-1) markers after the indicated treatments.

Discussion

The polarization of macrophages is highly plastic, shifting between functionally distinct states during different stages of an infection. While these changes are largely driven by the milieu of cytokines and inflammatory regulators present within the microenvironment of the cell, a broad range of pathogens are known to subvert this process as part of intracellular survival strategies [17]. For example, the bacterial pathogen Salmonella enterica serovar Typhimurium (S. Typhimurium) utilizes secreted effectors to alter the activity of host cell STAT3 signaling to promote repolarization to an
M2-like state [53, 54], creating an environment more permissive for intracellular survival.

Whether or not Cn is able to exert similar effects on host cells remains an open question within the field. Prior in vitro studies have indicated that Cn co-culture can promote a weak M1 phenotype, which was readily reversible by cytokine stimulation, suggesting that Cn does not have this capacity [13]. However, macrophage polarization was interrogated in these studies by measuring the expression of a small number of transcripts as markers of M1 and M2 polarization rather than studying the broader gene expression networks associated with each state.

Our study is the first to comprehensively examine the effect of intracellular Cn infection on the transcriptome of M1- and M2-polarized macrophages using an RNA sequencing-based approach. In agreement with earlier studies, we also find that Cn infection has relatively modest effects on the principle polarization markers, including Nos2 and Arg1. We observed a small reduction in Arg1 transcript levels in Cn-infected M2 cells that did not extend to the protein level (Figs 2B and C). Conversely, although Nos2 transcript levels were not affected by Cn-infected M1 macrophages, there was a 40% increase in protein levels (Figs 2B-D). These discrepancies could possibly be explained by translational or post-translational mechanisms regulating the translation or stability of the two proteins.

However, our data revealed extensive disruption of both the M1 and M2 transcriptome in Cn-infected macrophages, shifting cells towards a more M0-like state. Given that culture conditions were tightly controlled throughout our experiments with
growth medium and cytokines replaced at regular intervals, we believe that these changes are genuine and not simply caused by relaxation back to an M0 state, as might happen on withdrawal of polarizing stimuli. Additionally, we identified a common set of genes that were affected in a similar fashion regardless of host cell polarization state and constitute a transcriptional signature of intracellular Cn infection (Table 3 and 4).

The effect of intracellular Cn on the host macrophage transcriptome has been investigated in a small number of earlier studies [18, 20]. While the overlap between the gene expression changes detected in these studies and ours were limited, possibly due to the use of differing cell models and older microarray technology [20], certain consistencies are evident. When regarded from the level of signaling pathways affected rather than individual genes, both our and the Coehlo et al study detect changes in the activity of the HIF-1 signaling pathway (Fig 3A), which has also been observed in pulmonary fungal infections [55]. Additionally, NF-κB, JAK-STAT, TNF, and Toll-like receptor signaling pathway were also amongst the highest ranked KEGG GO terms in both our and the Chen et al study [18] (Figs 3 and 4). This is both surprising and informative as Chen et al used heat-killed rather than live Cn in their study, which implies that many of the changes observed may not require Cn metabolic activity. One possible explanation for this might be the immunomodulatory effects of capsular polysaccharides, which would be present on the surface of live and dead ingested yeast [40, 56].

Our analysis also allowed us to identify common and differing cellular processes affected by Cn infection in M1 and M2 cells. The most notable common effect was the
reversal of transcriptome changes associated with phagocytosis and lysosomal function (Fig 3A and 4A). For example, transcripts encoding lysosomal components, including LAMP2 and various lysosomal hydrolases that were upregulated upon polarization to M1 or M2, were returned to M0-levels in Cn-infected cells. We also observed decreased expression of various subunits of the V-type proton pump required for lysosome acidification (e.g. Atp6v0a2 and Atp6v1d). However, Atp6v0d2, encoding a macrophage-specific component of the pump [57], was amongst the top upregulated genes in Cn-infected M1-polarized cells (M1mk:M1 Cn FC = 12.98, q = 0.036). This particular subunit has been shown to have a role in enhancing autophagosome-lysosome fusion as part of the response to S. Typhimurium infection. Therefore, it seems plausible that Cn may promote an exchange of lysosomal proton-pump components as part of the response to intracellular infection.

An additional commonality in the responses of M1 and M2 cells to Cn infection was altered activity of the NF-κB signaling pathway (Figs 3A and 4A). This was a common GO term enriched in the pool of DEGs associated with M1 and M2 Cn-infected cells. Indeed, it was the only one identified for M2 cells (Fig 4A). As the pathway plays a significant role in regulating polarization and is affected by Cn infection in macrophages [40, 51], this was unsurprising. However, with the exception of Bcl2a1a, there was no overlap between the genes associated with this term in the two states, suggestive of unique effects on the pathway in differing polarization states.

Another notable difference between the polarization states was the presence of ‘p53 signaling’ as an enriched GO term for M2mk but not M1mk-exclusive genes (Figs 3A
and 4A). This is significant as p53 suppresses the M2 phenotype in vivo by downregulating the expression of M2-associated genes [58]. While this might suggest that this inhibition was removed or weakened post-Cn infection, the relatively small difference in M2 marker expression (e.g. Arg1) and the re-establishment of a more M0-like state indicated this may not be the case.

Perhaps the most significant outcome of this study was the identification of a transcriptome signature of Cn-infection, a set of genes commonly and concordantly regulated in both polarization states. This included the downregulation of 30 genes and upregulation of a relatively small pool of 8 functionally diverse genes (Table 3 and 4). This latter group included the transcriptional regulators Cited1, Hsf3, and Jarid2, the cytokine Ccl2, and the anti-apoptotic factor Bcl2a1a. Of these, Cited1, which was previously known as melanocyte-specific protein 1 (Msg1), showed the largest fold change and was also upregulated at the protein level post-Cn infection (Fig 5).

As a transcriptional co-regulator, CITED1 proteins cannot bind DNA directly and are brought to gene enhancer elements by protein-protein interactions with other transcription factors, recruiting CBP/p300 to regulate gene expression [59]. To date, Cited1 has been shown to co-regulate estrogen receptor alpha [60], TGF-β4/Smad4 [61], and Wnt/β-catenin-responsive genes [60-63]. While the specific function of CITED1 in macrophages remains enigmatic and has yet to be thoroughly investigated, CITED2 has been shown to repress proinflammatory gene expression associated with M1-polarizing stimuli, is itself induced by IL-4 and IL-13, and enhances the expression of M2-associated genes [52]. This is likely achieved through destabilization of HIF1α proteins [58] and the
attenuation of canonical NF-κB transcription factor activity [64]. This raises the interesting possibility that Cited1 may serve a similar function during fungal infection. This is the subject of ongoing investigations in our lab and our findings will be described in detail as part of a future publication.

Despite the damage wrought by intracellular Cn growth, host macrophages exhibit lower rates of cell death and apoptosis than might be expected [20]. It is perhaps notable that the list of common and concordant Cn-responsive genes included Bcl2a1a, the murine orthologue of the human anti-apoptotic Bcl-2 family member A1. Upregulation of Bcl2a1a has also been identified in a prior transcriptome analysis of murine macrophages infected with Streptococcus pyogenes [65]. It is known to protect immune cells from TNFα-induced apoptosis, and its expression is directly regulated by NF-κB [66]. However, its significance in the context of our own study remains unclear, particularly as the ‘apoptosis’ GO term appeared only in the pools of DEGs common to mock- and Cn-infected cells of both polarization states.

Finally, our study also highlights the importance of carefully controlling nutrient levels in in vitro culture systems used to study the Cn:macrophage interaction, especially if live rather than heat-killed Cn are utilized and experiments last more than a few hours. In preliminary experiments that preceded this study, we detected a significant reduction in the expression of the glucose-responsive gene TXNIP at the transcript and protein level (Fig 1G). As TXNIP is a regulator of p53, NF-κB, and other pathways associated with the control of macrophage polarization, glucose depletion may impact macrophage phenotype and result in spurious gene expression changes that do not...
reflect the potential effects of intracellular Cn growth on the transcriptome of host macrophages in an in vivo setting [67-69]. Regular replacement of culture medium in our experiments prevented loss of TXNIP expression in the RNA sequencing experiments described in this publication, and we strongly recommend that future in vitro studies account for this during experiment design.

Author Contributions

Conceived and designed the experiments: EEM RLST DEN. Performed the experiments: AS PG JM JT LMS. Analyzed the data: AS NF JM RLST DEN. Funding acquisition: EEM RLST DEN. Contributed reagents and materials: EEM. Supervision and oversight: EEM RLST DEN. Wrote the paper: AS PG NF EEM RLST DEN.

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Supporting information

S1 Fig. STRING analysis of gene networks associated with Cn infection of M1- and M2-polarized macrophages DEGs from (S1A) M1mk:M1Cn and (S1B) M2mk:M2Cn pairwise comparisons were analyzed in STRING. Boundaries enclosing gene clusters with common function were drawn based on gene GO term data and information from the literature. *Note: Many of the genes contained within the ‘Ribosome function’ boundary are pseudogenes.

S1 File. RNA sequencing data – All conditions compared to M0 Pairwise comparisons of M0 to all other conditions.

S2 File. RNA sequencing data – Mock vs. infected Pairwise comparisons of mock- and Cn-infected samples.
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Figure 1
Figure 2

(A) Schematic representation of the experimental setup.

- **Initial**
  - M1-polarize (24 h)
  - IFNγ

- **Cn infection**
  - Infection period is 2 h,
  - Extracellular Cn removed

- **Secondary**
  - Repolarize (24 h),
  - Medium replaced every 6 h

(B) Western blots for Arg-1, iNOS, and Actin.

(C) Statistical analysis of Arg-1 expression.

(D) Statistical analysis of iNOS expression.

*Significance levels: **p < 0.01*
Figure 4
Figure 5

- IFN - IFN - IFN - IFN - IFN  Initial
- IFN  IL4  IL4  - IFN  IL4  IL4  Secondary

Cited1
Arg-1
iNos
Actin