**Cryptococcus Neoformans Infection and Immune Cell Regulation in Human Monocytes**

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**Key Words**
Cryptococcus neoformans infection • Immune cell regulation • Monocytes

**Abstract**

**Background/Aims:** *Cryptococcus neoformans* infections are becoming increasingly prevalent and remain a life-threatening clinical issue in immune-compromised hosts. The microorganism evades a variety of endogenous anti-fungal mechanisms of host immune cells. The signaling pathways in human immune cells that become activated in response to *Cryptococcus neoformans* infection have yet to be fully characterized. **Methods:** Human monocytes were incubated with *Cryptococcus neoformans*, and the whole transcriptome of monocytes was sequenced before and after exposure to *Cryptococcus neoformans* using mass parallel sequencing techniques. Based on the genes that demonstrated altered expression patterns, we performed GO and KEGG enrichment analysis to further characterize the pathways involved in monocyte activation by *Cryptococcus neoformans*. **Results:** We found that immune and inflammatory responses, as well as chemotaxis, were the most heavily activated cellular events. Specifically, the toll-like receptor, tumor necrosis factor, NF-κB and Jak-STAT pathways were the most active pathways in response to *Cryptococcus neoformans* infection. The sequencing data of selected genes from the transcriptome analysis were further validated by real-time polymerase chain reactions. **Conclusion:** Taken together, our study is the first characterization of the transcriptome alterations in human immune cells upon *C. neoformans* infection, providing additional information that may be helpful in discovering novel anti-fungal targets.

**Introduction**

*Cryptococcus neoformans* is a pathogenic fungus found worldwide, which is characterized by the presence of an anti-phagocytic capsule [1]. Clinical cases of *C. neoformans* infection have become increasingly more prevalent over the past several decades, and patient S. Chen, H. Yan and L. Zhang contributed equally to this work.
prognosis is highly dependent on the ability of hosts to mount an effective immune response [2]. In immunocompetent individuals, pulmonary infection by C. neoformans is generally asymptomatic [3]; however, the organism can cause life-threatening complications in immunocompromised states, such as in AIDS and in patients undergoing immunesuppressive therapy [4]. In the later stages of AIDS, the uncontrolled dispersal of C. neoformans in the lungs can cause severe and highly recurrent meningoencephalitis, which affects approximately 4-8% of AIDS patients [4]. Despite progress in the development of novel antifungal therapies, these treatments have not completely eradicated C. neoformans infection [5-7]. The difficulty in eradicating this organism lies in the intrinsic properties of its capsule, which can possibly explain the reasons behind its persistence and recurrence.

Host defense against C. neoformans infection is accomplished by the combined action of activated macrophages, NK cells and T cells [8]. In general, alveolar macrophages stand as the first-line innate defense against C. neoformans infection [9]. These cells phagocytose cryptococcal cells after host inhalation [9]. Monocytes are then recruited to the site of infection, playing an essential role in protective immunity of the host against C. neoformans [10]. Several studies have suggested that crosstalk between different types of immune cells may be crucial for an adequate anti-fungal response [11-13]. However, the signaling pathways mediating this highly coordinated process are not well understood.

On the other hand, we should note that C. neoformans might survive despite multiple immune barriers, as a result of an adaptive interaction of C. neoformans with host immune cells [14, 15]. For example, several studies have shown that C. neoformans are resistant to anti-fungal compounds secreted by activated macrophages and can even survive after phagocytosed by macrophages [16-18]. The specific intracellular signaling pathways in the immune cells that are activated in response to fungal infection and utilized by the microbes for microenvironment adaptions remain unknown.

Although previous studies have identified some of the genes involved in the host response to C. neoformans (e.g. MCP1, IL10 and IL17A) [19-23], they mainly describe infection in murine models and have fallen short of a systemic exploration of all altered signal pathways. An understanding of these pathways and their potential for crosstalk may be necessary for pragmatic understanding of high resistance C. neoformans infections. Herein, we described the de novo mapping of the whole-transcriptome scale alterations in human monocytes before and after exposure to C. neoformans using Next-Generation sequencing. We then systematically analyzed the altered signaling pathways based on the differentially regulated genes. Furthermore, we validated many of these genes that were possibly involved in the immune response using real-time polymerase chain reactions (PCRs). Thus, we describe the first characterization of the transcriptome features in human monocytes in response to C. neoformans infection, which may provide useful data for the discovery of effective novel antifungal targets.

**Materials and Methods**

**Reagents and media**

RPMI 1640 medium and fetal calf serum (FCS) were purchased from Gibco BRL (Paisley, Scotland). Human blood was obtained from healthy donors. All media and buffers were tested for endotoxin contamination by the limulus amebocyte lysate assay (Sigma), which has a sensitivity of approximately 0.05 – 0.1 ng of E. coli LPS/ml. All media and reagents tested were negative.

**Preparation of peripheral blood monocytes (PBM)**

Heparinized venous blood obtained from healthy donors was diluted with RPMI 1640, and peripheral blood monocytes (PBM) were separated by density-gradient centrifugation on Ficoll-Hypaque [24]. PBMC was recovered, washed twice in RPMI 1640 supplement, and incubated for 1 hour at a concentration of 2x10^7 to 3x10^7/ml in cell culture petri dishes (Nunc Inter Med, Roskilde, Denmark). Nonadherent cells were removed and adherent cells (PBM) were recovered as previously described [25].
Microorganisms and co-culture with *C. neoformans*

*C. Neoformans* H99 was cultured in YPD broth (1% yeast extract, 2% peptone and 2% dextrose) for 3-5 days at 30°C, harvested, washed with sterile saline, and inactivated by heating at 56°C for 60 min. The efficiency of heat-killing was assessed by culture in Sabouraud glucose broth, and negative cultures were defined as no growth after 5 days of incubation. Heat-killed *C. Neoformans* was washed with sterile saline, re-suspended in RPMI1640 medium, and adjusted to the appropriate concentration. Heat-killed *C. neoformans* (1×10^7/wall) was then added and incubated for 12 hours [26, 27].

RNA Extraction, Library Preparation, and Sequencing

Total RNA was extracted from the infected and un-infected monocytes using TRIzol reagent (Invitrogen, USA). The quality and quantity of the RNA was validated using a Qubit fluorometer (Invitrogen, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). The mRNA was enriched using oligo (dT) magnetic beads, and then fragmented into short fragments (approximately 200–700 nt) using fragmentation buffer (Invitrogen, USA). The cDNA synthesis was carried out according to the manufacturer’s instructions. Following cDNA synthesis, the product was purified using a QiaQuick PCR extraction kit (Qiagen, USA) and eluted with EB buffer for end repair and poly(A) addition. Finally, sequencing adapters were ligated to the fragments, and the fragments were purified, followed by enrichment via PCR amplification. The library products were sequenced using the IlluminaHiSeq™ 2000 system.

Analysis of Transcriptome Sequencing Results

The low-quality reads were rejected, and clean reads were mapped to the human genome (GRCh37/hg19) using the computer software, MapSplice. We screened differentially-expressed genes between the infected and uninfected group using EB-Seq. For GO and KEGG pathway enrichment analysis, all differentially expressed genes were mapped to terms in the KEGG and GO databases and queried for significantly enriched terms.

Validation of RNA-seq Data with qRT-PCR

Total RNA was extracted using TRIzol reagent and was pre-treated with DNase to avoid DNA contamination. qRT-PCR was performed to verify the data obtained by RNA-seq using SYBR Green (TaKaRa, Japan) using the ABI 7500 SDS system (Applied Biosystems, USA) as previously described [28-30]. Primer sequences (5’-3’, forward and reverse) used in the present study are as follows: CXCL1, F-TGC CAG CCA CTG TGA TAGAG; R-AGC CCC TTT GTT CTA AGG CAG; CXCL2, F-AGA TCA ATG TGA CGG CAGGG; R-TCT CTG CTC TAA CAC AGA GGG; CXCL3, F-CCG CCA AAC CGA AGT CATAG; R-GCT CCC CTT GTT CAG TAT CTTTT; CXCL5, F-TCT GCA AGT GTT CGC CATAG; R-TGT CTT CCC TGG GTT CAGAG; CCL1, F-CTC ATT TGC GGA GCA AGA GAT; R-GCC TCT GAA CCC ATC CAA CTG; CCL4, F-GCT TTT TTT CCT ACA CTG CGA GGA; R-CCA GGA TCT ACT GGG ATCAG; CCL7, F-AGA AGG ACC ACC AGT AGCCA; R-ACG AGT CCT ATT CCC TGC CCA; CCR2, F-TAC GGT GCT CCC TGT CAT AAA; R-TAA GAT GAG GAC GAC CAG CAT; IL1B, F-ATG GCT TAT TAC GAT GGCAG; R-GTC GGA GAT TCG TAG CTG GA; MET, F-GAG CGG TCC GTG AGC AGATG; R-AAC CAG TCG AGA AGT CAGCG. Beta-actin was selected as the endogenous control. Primer efficiency was validated using standard protocols. Briefly, a standard curve of 10 genes was generated using which serial dilutions of amplified cDNA. The melting curves were determined before each test to confirm the specificity of each primer. The relative expression value was calculated via the 2^-ΔΔCt method.

Results

Data Acquisition and Statistical Analysis

Human monocytes were co-cultured with *Cryptococcus neoformans* (1:0 and 1:10) as previously described [2], and total RNA was collected for comparative transcriptome analysis. Samples were sequenced by Illumina Hiseq 2000. After trimming the adapter and low quality reads, 51588294 and 52303788 high quality reads were obtained from the uninfected and infected libraries for uninfected and infected groups, respectively. Clean reads were mapped to the human genome (GRCh37/hg19) using MapSplice. The mapping rate of uninfected and infected samples was 82.8% and 84.1% respectively. The corresponding raw data files were deposited into the SRA Database (SRR1823063).
Differential expression analysis

To explore the differential expressed genes (DEG) involved in infection of C. neoformans, we calculated the normalized expression values (FPKM, fragments per kilobase per million mapped reads) of each gene between infected and uninfected monocytes. Genes with greater than 2-fold change with a false discovery rate (FDR) of less than 0.01 were considered as differentially expressed.

Ontologic Analysis of DEGs

Gene Ontology (GO) assignment was used to functionally categorize each of the differentially expressed genes. Of these, 701 unigenes were assigned to the 'biological process (BP)', 698 to the 'molecular function (MF)', and 751 to the 'cellular component (CC)'. Within the BP category, the cluster for 'inflammatory response' ranked the highest (70, 10.0%) followed by 'immune response' (60, 8.6%), 'cellular response to lipopolysaccharide' (24, 3.4%) and 'chemotaxis' (30, 4.3%) (Fig. 2A). Within the 'MF' category, the cluster for 'cytokine activity' represented the largest group (50, 7.2%) followed by 'chemokine activity' (18, 2.6%), 'heparin binding' (20, 2.9%) and 'growth factor activity' (21, 3.0%) (Fig. 2B). GO analysis for the 'CC' category indicated that genes related to the 'extracellular space'.
'extracellular region', 'cell surface', and 'interleukin-1 receptor binding' were enriched (Fig. 2C). Together, the results from this analysis confirmed that human monocytes stimulated by *C. neoformans* promote initiation of an inflammatory response by activating various cytokines.

**Pathway Enrichment Analysis of DEGs**

To further understand the functions of the DEGs, we performed KEGG pathway analysis on the transcriptome data. Among the unigenes described in the KEGG pathway annotation, 227 pathways were involved, containing more than 342 DEGs. The top five pathways were 'cytokine-cytokine receptor interaction' (56, 16.4%), 'rheumatoid arthritis' (23, 6.7%), 'TNF signaling pathway' (23, 6.7%), 'malaria' (15, 4.4%), and 'salmonella infection' (18, 5.3%) (Fig. 3A). Two additional key pathways, associated with the inflammatory and immune responses, were also involved in the top ten pathways: 'Toll-like receptor signaling pathway' (19, 5.56%) and 'Jak-STAT signaling pathway' (19, 5.56%). Meanwhile, these pathways appear to interact with each other as well as with the 'cytokine-cytokine receptors', 'Toll-like receptor signaling pathways', and the 'Jak-STAT signaling pathways' (Fig. 3B). Table 1 illustrates the genes that were differentially altered with respect to the fold-change and FDR values. These
Fig. 3. The pathway assignment based on KEGG. (A) The classification according to pathway analysis; (B) Interaction of pathways.

Table 1. Differential expression gene data

| Gene Name | Fold Change | Regulation | FDR       |
|-----------|-------------|------------|-----------|
| CCL1      | 51.8501171  | up         | 0         |
| CCL4      | 7.452992704 | up         | 0         |
| CCL7      | 4.417099497 | up         | 1.58687E-08 |
| CCR2      | 0.155591717 | down       | 8.59313E-14 |
| CXCL1     | 26.95733097 | up         | 0         |
| CXCL2     | 8.970335215 | up         | 0         |
| CXCL3     | 14.77853579 | up         | 0         |
| CXCL5     | 29.06301291 | up         | 0         |
| IL1B      | 19.97609576 | up         | 0         |
| MET       | 7.836893388 | up         | 0         |

Key signaling pathways may warrant attention by future research efforts, given their roles in the human monocyte response to *C. neoformans*, and may represent potential targets for intervention.

Relative qRT-PCR Analysis

Within the DEGs and pathways identified, we were particularly intere-
Fig. 4. Validation of differentially expressed genes in both uninfected and Cryptococcus neoformans-infected monocytes.

...ted in transcripts involved in cytokine-cytokine receptor interactions, as well as inflammatory and immune responses. Therefore, 19 randomly-selected DEGs and 35 DEGs related to...
inflammatory or immune response were validated in 6 pairs of cases with or without *C. neoformans* infection by qRT-PCR using the ABI 7500 SDS system with SYBR Green. As expected, genes involved in inflammatory or immune response were dramatically changed after infection. Fig. 4 illustrates the altered genes including: CXCL1, CXCL2, CXCL3, CXCL5, IL1B, CCL1, CCL4, CCL7, CCR2, and MET. Each of these genes, with the exception of CCR2, were found to be increased in human monocytes after stimulation by *C. neoformans*. Briefly, the expression levels of CXCL1, CXCL2, CXCL3, CXCL5, IL1B, CCL1, CCL4, and MET were sharply increased in 5 of 6 samples. CCL7 mRNA level was decreased in one sample but upregulated in the other 5 samples. CCR2 was ubiquitously decreased in human monocytes after stimulation by *C. neoformans*. Taken together, it appears that increased expression of CXCL1, CXCL2, CXCL3, CXCL5, IL1B, CCL1, CCL4, MET and down-regulation of CCR2 may be required for human monocytes to mount a response to *C. neoformans*.

**Discussion**

In the current study, we describe the differences seen in the transcriptomes of peripheral blood monocytes before and after stimulation with *C. neoformans* infection. A total of 1243 differentially-expressed genes were detected in this study. Gene ontologic analysis and pathway enrichment investigations were then performed for each of these genes. This study detected many genes involved in inflammatory response that were significantly altered during exposure to the fungus. Specifically, pathways of TNF-α, Jak-Stat and toll-like receptors were activated in PBM upon *C. neoformans* exposure. Further validation of the inflammatory genes was performed using RT-PCRs, which confirmed that many chemokine and cytokine genes were selectively expressed by PBM. This study has revealed a number of important genes and signaling pathways provide insight into the immune response that is generated upon exposure of monocytes to *Cryptococcus neoformans*. One important limitation of the current study was the use of heat-killed fungal cells during the incubation step of our experiments. Although this method is widely described in the current literature, such a preparation may potentially affect the final results, as cell surface immune-stimulatory molecules may be modified.

Many of the previous mechanistic studies concerning the host defense against *C. neoformans* have been based on animal models [31-34]. In contrast to these methods, we incorporated human monocytes as an immune cell model in this study. Consistent with previous findings [31-34], we found that the inflammatory and immune response ranked as the first and second most altered biological processes after exposure. It is important to note that, since these studies have not been performed previously in human monocytes, they offer proof-of-principle confirmation that this is a suitable cell model. The extensive immune response of PBM upon *C. neoformans* exposure was also reflected by up-regulation of many mRNAs encoding cytokines, including interferon and interleukin. These molecules have been reported contribute to host innate immune response to *C. neoformans* infection [31-34]. The fact that cytokine activity ranked the first largest cluster of differential genes is fully complimented by similar observations in clinical cases of *C. neoformans* infection [31-34].

Our mRNA sequencing data also revealed that many chemokines were selectively expressed in response to *C. neoformans*. Although increased chemotaxis has been reported in immune system stimulation [35-37], the exact chemokines and receptors involved have so far remained undescribed. Among those differentially-expressed genes revealed by mRNA sequencing, the up-regulation of specific chemokine and chemokine receptors were confirmed using real-time PCR, including CXCL1, CCL1, CXCL2, CCL4, CXCL5 and CCR2. Of note, CCL1 and CXCL5 have yet to be reported in the immune cell response to *C. neoformans* infection [35-37]. CCL1 has been reported the regulation of anti-bacterial innate immunity of macrophages and is also one of the major effectors involved in the eradication of *C. neoformans* [38]. CXCL5 may regulate host defense in response to bacterial infection in the lung, which is the most common site of *C. neoformans* infection [39, 40]. However, further
Another novel finding of the transcriptome analysis was the potential role of the Jak-STAT pathway in PBM after *C. neoformans* exposure. In addition to other previously-described pathways (i.e., toll-like receptor signaling, NF-κB signaling and the TNFα signaling) [41-44], the pathway interaction analysis revealed that Jak-STAT signaling has a central position that potentially bridges nine other pathways within the network. We found that 18 genes of Jak-STAT pathway were upregulated, including LIF, IL2RA, CISH, IL10, CSF3, PRLR, OSM, IL3RA, IL12B, CRLF2, IL6, IL24, PIM1, IL20, IL23A, CSF2 and SOCS3. Many of these genes have not been revealed by prior studies. These results indicate that putative fungicidal activity of the Jak-STAT pathway warrants additional studies.

In summary, this is the first report of a high-resolution transcriptome map of monocytes following exposure to *C. neoformans*. These findings update and contribute to the current understanding of host defenses involved in *C. neoformans* and may lead to novel mechanistic insight that will hopefully be useful in the development of novel anti-fungal strategies.

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Disclosure Statement

The authors declare there are no conflicts of interest.

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