The Presence of Capsule in *Cryptococcus neoformans* Influences the Gene Expression Profile in Dendritic Cells during Interaction with the Fungus[∇][†]

P. Lupo,[1,3] Y. C. Chang,[1] B. L. Kelsall,[2] J. M. Farber,[2] D. Pietrella,[3] A. Vecchiarelli,[3]
F. Leon,[2][‡] and K. J. Kwon-Chung[1]*

Laboratory of Clinical Investigation[1] and Laboratory of Molecular Immunology,[2] National Institute of Allergy and Infectious Diseases, National Institutes of Health Bethesda, Maryland 20892, and Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto, 06123 Perugia, Italy[3]

The aim of this investigation was to study the effect of polysaccharide capsule on the gene expression in dendritic cells (DC) during their interaction with *Cryptococcus neoformans*. To this end, we used an encapsulated virulent strain of *C. neoformans* and a *cap59* gene-disrupted acapsular avirulent strain derived from the same genetic background. DC were exposed to encapsulated and acapsular *C. neoformans* strains for 4 h and 18 h, and their transcriptional profiles were analyzed using the Affymetrix mouse gene chip U74Av2. A large number of DC genes were up-regulated after treatment with the acapsular strain. In particular, we observed the up-regulation of the genes involved in DC maturation, such as cell surface receptors, cytokines, and chemokines (interleukin-12 [IL-12], IL-2, IL-1α, IL-1β, IL-6, IL-10, tumor necrosis factor alpha, CCR7, CCL17, CCL22, CCL3, CCL4, CCL7, and CXCL10), membrane proteins, and the genes involved in antigen processing and presentation as well as cell cycle or apoptosis. The chemokine gene expression data were confirmed by real-time reverse transcription-PCR, while the expression of cytokine genes was correlated with their secretion. A completely different pattern of gene expression was observed for DC treated with an encapsulated strain of *C. neoformans*. In particular, no significant induction was observed in the expression of the genes mentioned above. Moreover, a number of genes, such as those coding for chemokines, were down-regulated. These results suggest that the polysaccharide capsule shrouding the cell wall of *C. neoformans* plays a fundamental role in inducing DC response, highlighting the molecular basis of the true nature of immune silencing exerted by capsular material.

Cryptococcus neoformans is an opportunistic encapsulated yeast that causes pulmonary, cerebral, and disseminated infections primarily in patients with defective T-cell immunity, such as those with AIDS, hematological malignancies, and organ transplants (33). The polysaccharide capsule is a major virulence factor of *C. neoformans*, the concept of which was initially established by studying several acapsular mutants obtained by chemical mutagenesis. These acapsular strains were avirulent in mice, and they were readily ingested by phagocytes, but their ingestion could be inhibited by the addition of purified polysaccharide (3). The effect of the *C. neoformans* polysaccharide capsule on the host cells can be summarized as follows: it interferes with phagocytosis, blocks the recruitment of inflammatory cells, increases costimulatory molecules, suppresses the delayed-type-hypersensitivity response, and reduces the antibody production in response to fungal infection (11, 43).

Dendritic cells (DC) are professional antigen-presenting cells that can initiate the innate and adaptive immune response against invading pathogens, thus enabling the decoding of microbe-associated information which then results in qualitatively different adaptive T-helper responses in vitro and in vivo (2, 8).

Mouse DC internalize *C. neoformans* cells via the mannose receptor and the Fc receptor for immunoglobulin G (FcγR). The cryptococcal antigens are then presented to T cells, which undergo proliferation and activation (40). The protective immunity against *C. neoformans* has been attributed to a T-helper 1 type response (17) with consequent phagocytic-cell activation and direct antifungal activity (33).

The role of capsular material in DC activation and maturation was also investigated using the encapsulated and acapsular strains of *C. neoformans*. Acapsular strains are easily phagocytosed by immature DC, triggering the up-regulation of several costimulatory molecules, thereby promoting DC maturation. Conversely, encapsulated yeast cells are unable to produce an increase in costimulatory molecules, thus inhibiting maturation (44). These results show how the capsule interferes with DC activation and maturation, thereby hampering an efficient T-cell response. In addition, the presence of the inflammatory process plays a key role in restricting *C. neoformans* to the local level, preventing dissemination (12). Accordingly, the absence of inflammation observed for immunocompromised hosts represents the negative outcome of cryptococcosis (12).

Microarray technology, which allows the simultaneous anal-

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ysis of the expression of thousands of genes, has been used to investigate immune system and pathogen interplay. These studies have included analyses of the responses of particular tissues and cells, e.g., macrophages, to a wide range of pathogens, such as bacteria, fungi, helminthes, and protozoan parasites. The majority of these studies have concentrated on the responses of human or mouse cell lines and primary cells to different challenges (30).

In this study, we examined the role of capsular material of *C. neoformans* in the gene expression profiles of DC. Our previous study pointed out that capsular material of *C. neoformans* limits DC maturation and activation (44). By analyzing the gene expression profiles, we attempted to characterize the diverse molecular events that are influenced by the presence or absence of capsular material during DC-*C. neoformans* interaction. To this end, we used mouse myeloid DC line D1 (37) and two strains of *C. neoformans*, an encapsulated virulent strain (JEC21) and an acapsular avirulent strain (C566) that was constructed by disruption of the *CAP59* gene. Mouse myeloid DC line D1 was challenged with both strains. Gene expression profiling revealed that the acapsular strain not only induces maturation of DC but also is an efficient inducer of genes involved in cytokine and chemotaxis activity and immune response as well as T-cell regulation. The presence of capsular material precludes or hampers activation of the genes involved not only in DC maturation but also in the induction of cytokines, chemotaxis, and inflammation that represent crucial events confining *C. neoformans* to the local level.

MATERIALS AND METHODS

Strains. The D1 cell line, a long-term growth-factor-dependent immature myeloid (CD11b$^+$ CD8α^+) DC line of mouse splenic origin, was provided by P. Ricciardi-Castagnoli (University of Milan—Bicocca, Milan, Italy). The cell line was cultured in Iscove's modified Dulbecco's medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD), 100 IU of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine (all from Sigma), and 50 μM β-mercaptoethanol, with 30% supernatant from R1 medium (supernatant from NIH 3T3 fibroblasts transfected with granulocyte-macrophage colony-stimulating factor). JEC21 is an encapsulated virulent strain of *C. neoformans*, and C566 is an acapsular prototrophic strain obtained from the F$_1$ progeny of a cross between the encapsulated strain JEC33 (*MATα lys2*), a JEC21 derivative, and an acapsular strain, TYCC33 (*MATa cap59 ura5*). TYCC33 was constructed by disruption of the *CAP59* gene in an *ade2 ura5* mutant, B-4530, derived from strain JEC20, which is isogenic to JEC21 (6). *C. neoformans* was cultured for 2 days on yeast extract-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, 2% glucose) agar at 30°C. *C. neoformans* (yeast) cells were harvested and heat inactivated at 60°C for 30 min before use.

Phagocytosis assay. Yeast cells were opsonized with 20% normal mouse serum in phosphate-buffered saline and 10 mM EDTA. Opsonization was performed by incubating the yeast cells at 37°C for 1 h, after which the cells were washed and used for phagocytosis assays. The D1 cell line, at a concentration of 2 × 10^5, was added to the eight-well Lab-Tek Chamber slide 2 h before stimulation. Adherent DC were then stimulated at a ratio of 1:2 with opsonized encapsulated or acapsular strains for 2 and 4 h. After incubation, the chambers were removed and the slides were washed with RPMI, air dried, fixed, and stained with the Hemacolor kit for cells (Merck). One hundred macrophages were examined per well, and the phagocytic indices (number of cells ingested per macrophage) were determined.

Stimulation of DC by *C. neoformans*. For microarray assays, the D1 cell line (3 × 10^6 cells) was either not stimulated or stimulated at a ratio of 1:2 with yeast cells of either encapsulated or acapsular strains (6 × 10^6 yeasts cells were first opsonized with normal mouse serum [obtained from naive C57BL/6 mice] at 37°C for 30 min, washed, and incubated with the D1 cell line). The samples were incubated for 4 h and 18 h at 37°C under 5% CO$_2$. For the cytokine assays, 10^6 D1 cells/ml and CD11c$^+$ DC from naive mice were either not stimulated or stimulated at a ratio of 1:4 with yeast cells of encapsulated and acapsular strains.

As a positive control, lipopolysaccharide (LPS) was used at a concentration of 10 μg/ml. Samples were incubated for 18 h at 37°C under 5% CO$_2$. For each experiment, three samples were included at each time point for each strain.

Isolation of CD11c$^+$ cells. C57BL/6 mice were obtained from Jackson Laboratory. Spleen cell suspensions were prepared by Liberase CI (Roche, Lewes, United Kingdom) and DNase I digestion. DC-enriched fractions were prepared by labeling splenocytes with anti-CD11c MACS beads (Miltenyi Biotec, Bisley, United Kingdom), followed by a positive selection using LS magnetic columns (Miltenyi Biotec). CD11c$^+$-enriched preparations were further checked for purity by flow cytometry.

RNA extraction, amplification, and labeling. After 4 h and 18 h of incubation, D1 cells and CD11c$^+$ cells from untreated mice were harvested with RNA extraction buffer and kept in a −80°C freezer until RNA was extracted. Antisense cRNA was prepared according to the recommendations of Affymetrix (Santa Clara, CA). Briefly, total RNA was extracted from frozen cell pellets by using the RNeasy mini kit (Qiagen, Chatsworth, CA). For the microarray assay, 5 μg of RNA was reverse transcribed by using a modified oligo(dT) primer with a 5′ T7 RNA polymerase promoter sequence and the Superscript Choice system for cDNA synthesis (Life Technologies, Gaithersburg, MD). Double-stranded cDNA (1 μg) was transcribed to cRNA with the ENZO kit (Affymetrix). cRNA was purified on an RNeasy affinity column (Qiagen, Chatsworth, CA) and then fragmented to an average size of 50 to 200 bp by incubation for 35 min at 94°C in 40 mM Tris-acetate at pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate.

Microarray experiments and analysis. Hybridization and scanning of arrays were performed according to the manufacturer's (Affymetrix) protocol. Labeled cRNA was hybridized to Affymetrix murine chip U74Av2. The murine genome chip U74Av2 consists of 36,000 mRNA transcripts from mouse and expressed sequence tag cluster sequences including 6,000 known mouse genes. The arrays were read at a resolution of 7.5 μm by a confocal scanner and analyzed with the Microarray Suite 5.0 Gene Expression analysis program (both from Affymetrix). The data for each microarray were initially normalized by scaling all signals to a target intensity of 500. Expression analysis was performed using the default parameter settings. Present calls required a *P* value of <0.05, and marginal calls required a *P* value of 0.065 for all probe sets. Probe sets with a *P* value of >0.065 were marked absent. Each array was analyzed based on the present, marginal, or absent call for each probe set. The data were then uploaded to the National Cancer Institute's mAdb microarray website (http://nciarray.nci.nih.gov) and the absolute expression analysis was performed using the mAdb bioinformatics system generated by the NCI/CCR μArray Center.

Three or four replicates for each array were performed, starting with 21 arrays and 7,582 genes. All data were centered to a signal median, and all absent calls or samples in which the signal was less than 30 were excluded. Signals were also floored at 1. Replicate samples at 4 h and 18 h were grouped and compared by statistical analysis. Transcriptional profiles of D1 cells exposed to encapsulated and acapsular strains were compared to those of nonstimulated cells (NS cells) at both 4-h and 18-h time points. The *t* test was used to analyze the results. All genes with a *P* value of <0.05 and with a group mean difference of ≥1 or ≤−1 were considered significantly modulated and used for the next analysis. The logical Boolean comparison analysis was used to compare the data from NS cells against data from the cells exposed to encapsulated strains and the data from NS cells against data from the cells exposed to acapsular strains at both 4 h and 18 h. The final output numbers of significantly modulated genes were 170 at 4 h and 330 at 18 h (see Table S1 in the supplemental material). Each set of data analyzed is statistically highly significant, since it represents the average of three or four replicates. Final data sets are expressed as log$_2$ ratios in signal intensity between the cells exposed to encapsulated strains and NS cells and between those exposed to acapsular strains and NS cells at both 4-h and 18-h time points.

Real-time PCR. The SuperScript first-strand synthesis system for reverse transcription-PCR (RT-PCR) (Invitrogen, Carlsbad, CA) was used to synthesize cDNA by using the Superscript reverse transcriptase. For PCR amplification, 5 μg of RNA was transcribed and 0.5 μl of cDNA was used per 25 μl of PCR mixture. The TaqMan probes (6-carboxyfluorescein dye labeled) for CCL3, CCL4, CXCL10, CCL7, CCL22, and CCR7 (Applied Biosystems) were used, and the relative changes in gene expression were analyzed by the 2$^{-\Delta\Delta CT}$ method and compared to the expression of β2-microglobulin.

ELISA. D1 cell line and CD11c$^+$ cells (10^6 cells/ml) were stimulated at a ratio of 1:4 with an encapsulated or acapsular strain of *C. neoformans*. After 18 h of stimulation, supernatant fluids were recovered and stored at −20°C. Cytokine presence in culture supernatant fluids was measured by enzyme-linked immunosorbent assay (ELISA) for mouse tumor necrosis factor alpha (TNF-α) and interleukin-12p40 (IL-12p40) (BD Biosciences-Pharmingen, San Diego, CA).

RESULTS

The mouse DC line D1 is a well-characterized cell line. In this paper, we show that D1 is able to recognize and phagocytize two isogenic strains of *C. neoformans* with or without a capsule. Following opsonization with normal mouse serum, acapsular and encapsulated strains were added to the D1 cell line and phagocytosis was evaluated after 2 and 4 h. The results from three experiments showed that, following 2 h of incubation, the acapsular strain is easily internalized. In particular, the phagocytic index at 2 h was 1.40 ± 0.20, and the phagocytic index after 4 h of incubation was similar. Moreover, the phagocytic index for the encapsulated strain was also appreciable (0.60 ± 0.04) after 2 and 4 h of incubation.

In order to investigate if the changes in the gene expression profile of DC during interaction with *C. neoformans* were related to the presence of capsular material, we examined DC gene expression profiles after treatment with two strains of *C. neoformans* from an isogenic background, one with a capsule and one without capsule. The D1 cell line was stimulated with *C. neoformans* at a ratio of 1:2 for 4 h and 18 h.

The RNA expression profiles were analyzed using Affymetrix murine chip U74Av2, and the resulting 21-array data set (7,582 genes) were filtered using a *P* value of <0.05 and a group mean difference in $\log_2$ ratio of ≥ 1 or ≤ -1 as described in Materials and Methods. From the data obtained after 4-h incubation, there were 18 and 156 genes which showed >2-fold differences in expression levels in cells stimulated with encapsulated *C. neoformans* and those stimulated with acapsular *C. neoformans*, respectively, in comparison to expression in unstimulated cells (see Table S1 in the supplemental material). Among these two groups of genes, four genes were commonly affected in DC exposed to both encapsulated and acapsular *C. neoformans* strains. In contrast, after 18 h of incubation, the numbers of genes that showed differential expression were 3 and 327 when the unstimulated DC were compared to the cells stimulated with either encapsulated or acapsular strains, respectively, and there was no overlap between these two groups of genes (see Table S1 in the supplemental material).

To evaluate whether the poor stimulatory activity of the encapsulated strain could be due to the suboptimal dose of yeast used in our experimental system, higher doses of both yeasts were tested (effector-to-target cell ratio, 1:5). Results obtained were comparable to those obtained by using lower doses of yeasts (effector-to-target cell ratio, 1:2).

We clustered the differentially expressed genes into different categories based on the proposed gene functions antigen presentation, cytokines, chemokines, transcription factors, and signaling (Table 1). We focused on the genes that are involved in antigen presentation, maturation of DC, cytokine production, and chemotaxis. Most of these genes were significantly up- or down-regulated by the acapsular strain, in contrast to the encapsulated strain, by which only a few genes were modulated.

Genes related to antigen presentation. Many major histocompatibility complex class II (MHC-II)-related genes, such as *H2-D1*, *H2-Q7*, and *H2-Q8*, were up-regulated by the acapsular strain at 18 h. *H2-DMb1* was the only gene in this group whose expression was down-regulated by both yeast strains after 4 h of incubation: the encapsulated strain induced almost a fourfold decrease (ratio of -1.78), and the acapsular strain induced a threefold decrease (ratio of -1.54).

In addition, the acapsular strain up-regulated the expression of the genes that are hallmarks of DC differentiation, such as the *c-Relb* gene (28, 46), as well as the genes involved in the up-regulation of costimulatory molecules. In particular, the *c-Relb* gene was up-regulated after 4 and 18 h with $\log_2$ ratios of 1.53 and 1.78, respectively. The expression of costimulatory molecule genes, such as the CD86 gene, was also up-regulated with a ratio of 1.02. Other genes involved in antigen presentation, such as the CD14 gene, were up-regulated by the acapsular strain at 4 h. CD83 and intracellular cell adhesion molecule (ICAM) were both up-regulated at 18 h with ratios of 3.42 and 1.36, respectively.

Cytokine- and receptor-related genes. Significant numbers of the genes encoding the innate host defense, which includes complement component C3, proinflammatory, and chemotaxis regulators were up-regulated only by the acapsular strain.

Genes expressing cytokines, such as TNF-α, interleukin 1 (*IL-1a* and *IL-1b*), colony stimulating factor 1 (*Csf1*), and cellular receptors (*Tnfsfr5*, *Tnfsf5*, *Tnfsf9*, and *Traf6*) were induced early at 4 h by acapsular strain. TNF-α was up-regulated almost 16-fold; IL-1α and IL-1β were up-regulated at 4 h with $\log_2$ ratios of 2.77 and 1.63, respectively. Other TNF-α-related genes were promptly induced: induction of the TNF ligand 9 (*Tnfsf9*) was up-regulated fourfold, the TNF receptor 5 (*Tnfsfr5*) was up-regulated threefold, and the TNF-α-induced protein 2 (*Tnfaip2*) was up-regulated more than threefold. At 18 h, the expression of TNF ligands 5 and 9 (*Tnfsf5* and *Tnfsf9*) was comparably up-regulated. Acapsular cells also induced up-regulation of the TNF receptor-associated factor (*Traf6*) with a ratio of 1.86. In addition, the complement component C3 was up-regulated after 18 h with a ratio of 1.94.

Other genes involved in the immune response, such as the gene encoding prostaglandin E receptor 4 (*Ptger4*), were up-regulated only by the acapsular strain at 18 h. Moreover, the genes encoding receptors, such as CD68, were down-regulated by the acapsular strain at 4 h, while the endothelin-1 gene (*Edn1*) and CD47 antigen were up-regulated. Acapsular cells also up-regulated several interferon-related genes, such as *Ifi203*, *Ifi204*, *Ifit1*, *Ifit2*, and *Ifit3*, which were induced at 4 h. In contrast, the encapsulated cells did not modulate the expression of these genes. The only gene that was regulated by the encapsulated strain in this category was *Tgfb3*, which was also up-regulated by the acapsular strain; the levels of up-regulation by the two types of strains were twofold and fourfold, respectively. It is known that transforming growth factor b3 (TGF-β3), a known inhibitor of cell proliferation (27), prevents cell cycle progression and leads to the accumulation of cells in G_1 or G_0 phase.

To verify whether the cytokine gene expression induced by the acapsular mutant corresponded to an induction of protein synthesis, we investigated the production of selected cytokines, such as IL-12p40 and TNF-α, after exposing DC to acapsular and encapsulated *C. neoformans* strains by using an ELISA. Figure 1A shows that acapsular cells induce significantly

TABLE 1. List of differentially expressed genes in DC upon challenging with *C. neoformans*

Function/product and gene	Affy idenitification no.[a]	Log₂ ratio[b]			
		4 h		18 h	
		Encapsulated/NS	Acapsular/NS	Encapsulated/NS	Acapsular/NS
Antigen presentation					
H2-D1	97541_f_at				1.06
H2-Q7	98438_f_at				1.02
H2-Q8	101658_f_at				1.67
H2DMb1	101868_i_at	−1.78	−1.54		
Relb	103091_at		1.53		1.78
CD86	102830_at				1.02
CD1d2	101897_g_at				1.18
CD14	98088_at		1.12		
CD83	103040_at				3.42
ICAM1	96752_at				1.36
Cytokines and receptor					
Tgfb3	102751_at	1.05	1.92		
Tgtp	102906_at		2.90		
TNF	102629_at		3.79		
Csf1	101450_at		2.50		
C3	93497_at				1.94
Tnfsf9	92415_at		2.04		
Tnfrsf5	92962_at		1.50		1.87
Tnfaip2	160489_at		1.58		
Tnfrsf11a	101632_at				1.04
Traf6	104189_at				1.86
IL-1a	94755_at		2.77		2.60
IL-1b	103486_at		1.63		
IL-1rn	93871_at				1.76
Ifi203	93321_at		2.03		
Ifi204	98465_f_at		2.39		
Ifit1	100981_at		4.63		
Ifit2	103639_at		4.73		
Ifit3	93956_at		3.78		
Ptger4	103362_at				1.84
Irg1	98773_s_at				2.63
CD47	95020_at				1.43
CD68	103016_s_at		−1.09		
Edn1	102737_at		3.37		
Chemokines					
CCL3	102424_at		4.23		
SCYA4 (CCL4)	94146_at		4.48		
SCYA7 (CCL7)	94761_at		3.49		
CXCL10	93858_at		5.02		
CCL6	92849_at		−1.71		
CXCL5	98772_at		−2.29		
CCL17	97783_at	−1.37			
CCRL2	93617_at		2.00		
CCL12	93717_at		1.48		
CCL22	102310_at		1.42		2.43
CCL5	98406_at				1.96
CCR7	104443_at				3.84
CXCL2	101160_at				1.85
DNA binding and transcription factors					
Elf1	160721_at	−1.19			
Sqstm1	101995_at	−1.28			
Irf1	102401_at		1.98		
Idb1	100050_at		−1.56		
Irf4	92737_at		1.36		
Irf7	162202_f_at		1.14		
Isg20	103432_at		2.41		
Nfkb2	103614_at		1.46		1.64
Nfkbia	101554_at		1.16		1.73
Nfkbiz	98988_at		2.51		
Ilf2	93823_at			1.05	
Signaling					
Sema4b	95387_f_at	−1.20			
Ifnra2	101014_at	1.13			
IL-17r	99992_at		−1.01		
Igtp	160933_at		2.18		
Ifih1	103446_at		2.70		
Socs3	162206_f_at		1.31		1.09
Stat5a	100422_i_at		1.31		1.28
Ikbkb	93706_at				1.39

[a] Affy identification numbers represent codes provided by Affymetrix.
[b] Each number in the columns represents gene expression as log₂ of the ratio of cells stimulated with an encapsulated strain to NS cells or of cells stimulated with an acapsular strain to NS cells. Blank entries indicate no significant changes in gene expression. Each data point is the average of three or four different experiments. Data shown have P values of <0.05 and represent a group mean difference of ≥ 1 or ≤ -1.
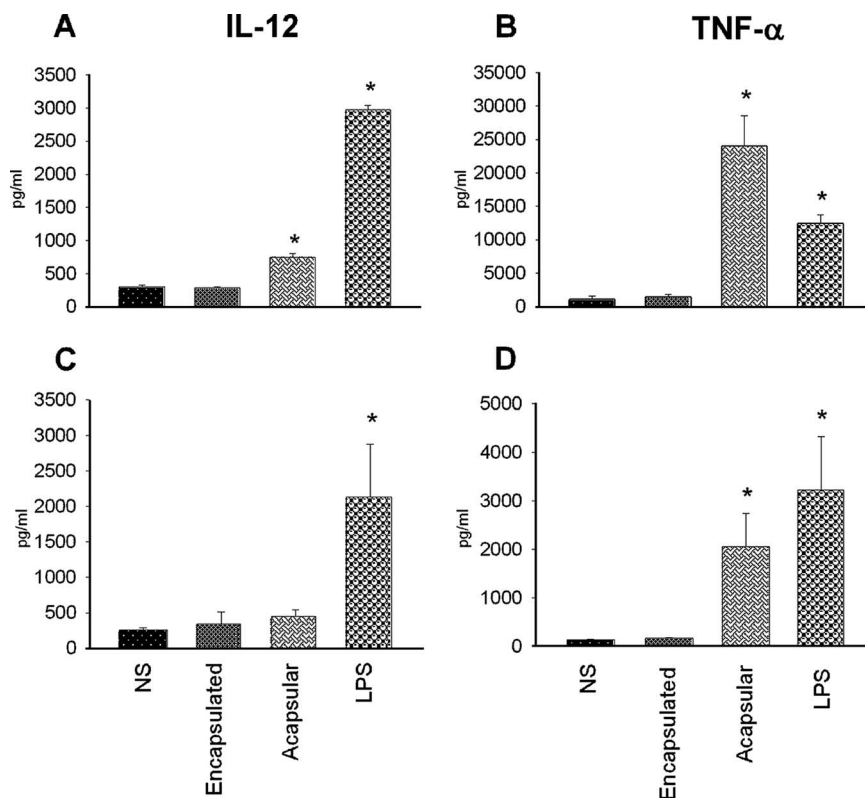
FIG. 1. IL-12p40 and TNF-α production by mouse DC. D1 cell line and CD11c^{+} cells (10^6 cells/ml) were stimulated at a ratio of 1:4 with an encapsulated or acapsular strain of *C. neoformans*. Supernatants of NS and stimulated cells were collected after 24 h and tested for the presence of IL-12p40 and TNF-α. (A and B) IL-12p40 and TNF-α production in the D1 cell line. (C and D) Cytokine production of CD11c^{+} cells. *, $P <$ 0.05 (cells treated with encapsulated and acapsular yeasts and LPS versus untreated cells).

greater production of IL-12p40 in D1 cells than in NS cells, although the IL-12p40 levels were not as high as those produced following LPS stimulation. On the other hand, IL-12p40 expression in D1 cells was unchanged when stimulated with cells of the encapsulated strain. Moreover, the acapsular strain strongly induced TNF-α production, while the encapsulated strain did not (Fig. 1B). To ascertain whether the results obtained with the D1 cell line were at least partially applicable to ex vivo cells, CD11c^{+} cells were isolated from the spleens of naive mice and stimulated in vitro with the two strains of *C. neoformans* and screened for production of the selected cytokines. After 18 h of incubation, the supernatants were recovered and the concentration of IL-12p40 and TNF-α were measured. No significant increase in IL-12p40 production was observed for CD11c^{+} cells treated with either encapsulated or acapsular strains compared to NS cells (Fig. 1C). Interestingly, acapsular cells stimulated the production of TNF-α significantly, while encapsulated cells did not (Fig. 1D).

Chemokine-related genes. Chemokines are chemotactic factors that are critical for the function of the immune system and can affect a range of additional biological processes, such as angiogenesis and hematopoiesis. We found that the encapsulated strain down-regulated chemokines, such as *CCL17*, and also did not modulate other chemokine-related genes (Table 1). Conversely, the acapsular strain induced the expression of genes encoding several chemokines. Genes such as *CCL3*, *SCYA4* (*CCL4*), *SCYA7* (*CCL7*), *CCL12*, and *CXCL10* were

highly induced (4 h). In particular, *CCL7*, *CCL3*, and *CCL4* genes and the *CXCL10* gene were up-regulated with ratios of 3.49, 4.23, 4.48, and 5.02, respectively. On the other hand, the chemokine genes, such as *CCL6* and *CXCL5*, were down-regulated with ratios of −1.7 and −2.29, respectively. After 18 h, other chemokines were highly up-regulated by the acapsular strain, in particular, *CCL22*, *CCL5*, and *CXCL2*, with ratios of 2.43, 1.96, and 1.85, respectively. The chemokine receptor *CCR7*, known to be up-regulated during DC activation/maturation in order for DC to traffic to lymph nodes (13, 15), was up-regulated with a ratio of 3.84.

To confirm the microarray data, we performed real-time PCR experiments on genes that showed a >4-fold increase at 4 h and 18 h after stimulation. The same batch of RNA that was used for the array study was transcribed into cDNA and amplified by using TaqMan real-time PCR. Figure 2A shows that the DC treated with acapsular yeast for 4 h showed expression of *CCL3* that was 8.56-fold higher than that of the NS cells. In contrast, the encapsulated strain did not influence the transcriptional level of this chemokine in DC. The up-regulation of *CXCL10*, *CCL4*, and *CCL7* at 4 h after challenging with *C. neoformans* was also confirmed by RT-PCR. We found that these chemokines were up-regulated by treating DC with the acapsular strain, and the increases were 10.63-, 23.44-, and 7.75-fold, respectively (Fig. 2B, C, and D). Higher expression of the genes for the chemokine *CCL22* and the chemokine receptor *CCR7* in DC challenged with the acapsular strain for
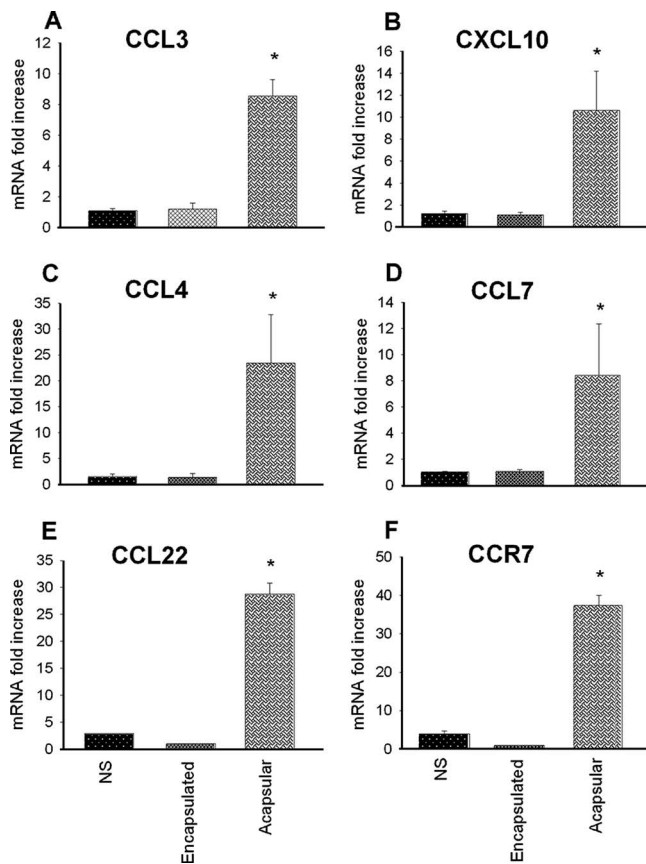
FIG. 2. Real-time PCR analysis of the chemokine gene expression from D1 cells. Expression levels of *CCL3* (A), *CXCL10* (B), *CCL4* (C), and *CCL7* (D) were compared to that of the β2-microglobulin gene from D1 cells 4 h after challenging with *C. neoformans*. Each data point represents an average of three independent experiments. The relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. Expression levels of *CCL22* (E) and *CCR7* (F) were also measured at 18 h in comparison to the expression level of the β2-microglobulin gene. *, $P < 0.05$ (cells treated with encapsulated and acapsular yeasts versus untreated cells).

18 h but not in DC challenged with the encapsulated strain was also confirmed (Fig. 2E and F).

Similar experiments using RT-PCR to quantitate mRNA levels were performed with the CD11c⁺ cells. These cells were isolated from the mouse spleen and incubated in the presence or absence of *C. neoformans* cells for 4 h. RNA was extracted from these cells, and the expression levels of *CCL3*, *CCL4*, *CCL7*, and *CXCL10* were determined. Only the acapsular strain induced a significant increase in the mRNA expression of *CCL3* and *CCL4* (Fig. 3). Albeit not significantly modulated, expression levels of *CXCL10* and *CCL7* were found to have been induced in all the experiments. The encapsulated cells also induced a slight increase in the expression of *CCL3*, *CCL4*, and *CCL7* genes compared to the expression in NS cells. The mRNA expression of *CXCL10* did not appear to be influenced by the encapsulated form compared with the NS sample.

DNA binding proteins and transcription factors. At the early time point of 4 h, the encapsulated strain induced the down-regulation of two genes: the E74-like factor 1 gene

(*Elf1*), which is involved in chemokine production, and the sequestosome 1 gene (*Sqstm1*), which regulates protein degradation. The acapsular strain, however, down-regulated the inhibitor of DNA binding-1 gene (*Idb1*), which is involved in TGF-β signaling. Other transcription factors, such as interferon-regulatory factors 1 and 7 (*Irf1* and *Irf7*) and interferon-stimulated protein (*Isg20*), were up-regulated by the acapsular strain. The acapsular strain also up-regulated the expression of genes such as the nuclear factor genes *Nfkb2* and *Nfkbia*. *Nfkb2* inhibits the transcriptional activity of NF-κB complexes by sequestering them in the cytoplasm and blocking their binding to DNA sequences (16). The expression levels of nuclear factors *Nfkb2*, *Nfkbiz*, and *Nfkbia* were still threefold higher at 18 h after interaction with the acapsular strain. In contrast, the only DC gene that was up-regulated after 18 h of incubation with an encapsulated strain was the IL enhancer binding factor 2 (*Ilf2*).

Genes associated with signaling. After the DC were exposed for 4 h to the encapsulated strain, the gene *Sema4b*, encoding semaphorine domain 4b, was down-regulated with a ratio of −1.2, while the interferon receptor alpha and beta gene (*Ifnar2*) was up-regulated with a ratio of 1.13. *Sema4* provides a costimulatory signal for T cells and is also involved in the regulation of Th1/Th2 responses (4).

The acapsular strain down-regulated the IL-17 receptor gene (*IL-17r*) in DC, a member of the novel inflammatory cytokine family, with a value of −1.01 at 4 h. This gene, however, was up-regulated at 18 h with a ratio of 1.09. In addition, the acapsular strain up-regulated the expression of interferon-related genes, such as *Igtp*, with a ratio of 2.18, and interferon induced with helicase C domain (*Ifih1*), with a ratio of 2.70. The suppressor of cytokine signaling 3 gene (*Socs3*) was also up-regulated with a ratio of 1.31 by the acapsular strain after 4 h, and the up-regulation decreased to a ratio of 1.09 at 18 h. *Socs3* is an inhibitor of cytokine receptor signaling mediated by the Janus kinase family kinases and may protect the host by limiting the excessive production of cytokines (1).

DISCUSSION

In this study, we analyzed changes in murine DC gene expression in relation to the presence of the polysaccharide capsule in *C. neoformans* during interaction with the fungus. We used two *C. neoformans* strains with isogenic backgrounds, one of which was acapsular by virtue of the disruption of the *CAP59* gene (6). These acapsular cells are completely devoid of capsular material as confirmed not only by India ink preparations but also by a negative reaction with anticapsular antibody from the Iatron Crypto kit, which is used for serotyping of *C. neoformans* cells. Our array study validates the previous findings by showing the profound differences between the encapsulated and acapsular strains in regulating maturation and activation of DC.

It is well known that mature DC express high levels of MHC and costimulatory molecules such as CD86 and CD83 (23) and the up-regulation of these genes observed after stimulation with acapsular strain reflects the capacity of the fungus to promote a series of events involved in DC maturation and activation.

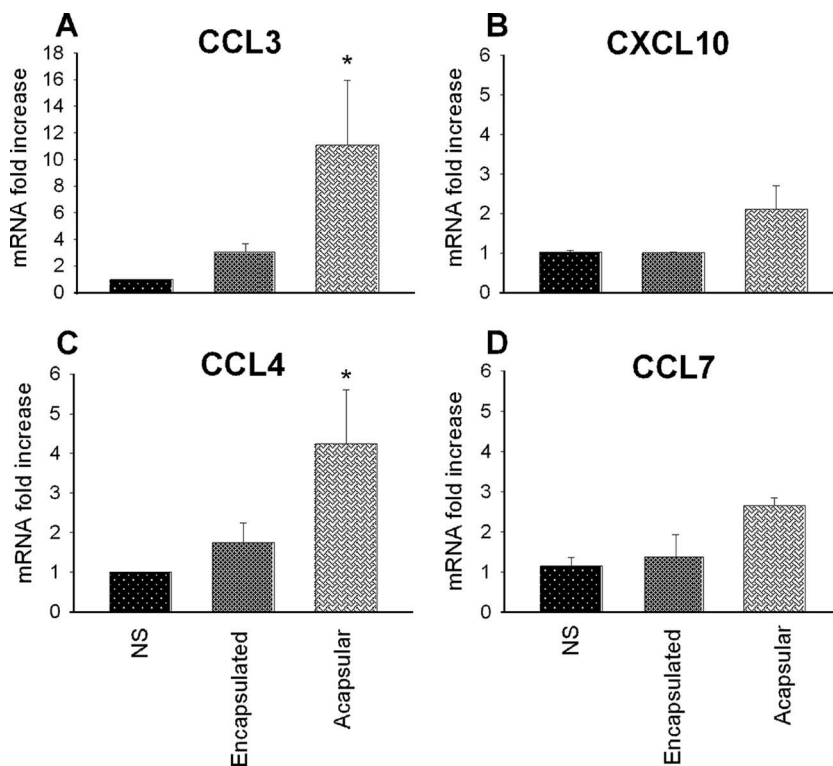These results are consistent with previous observations dem-

FIG. 3. Real-time PCR analysis of gene expression of chemokines from CD11c^{+} cells. RT-PCR of CD 11c^{+} DC isolated from the spleens of naive mice that were stimulated in vitro for 4 h at a ratio of 1:2 with *C. neoformans* encapsulated and acapsular strains. Expression levels of *CCL3* (A), *CXCL10* (B), *CCL4* (C), and *CCL7* (D) were compared to the expression levels of the β2-microglobulin gene. Each data point represents an average of three independent experiments. The induction levels were calculated by using the $2^{-\Delta\Delta CT}$ method. ∗, $P < 0.05$ (cells treated with encapsulated and acapsular yeasts versus untreated cells).

onstrating that the acapsular strain, differently from the encapsulated one, promotes the up-regulation of several costimulatory molecules (44); however, in this study, we provide evidence for the existence of other genes that participate in the final biological effect. As a matter of fact, besides the increase in CD86, CD83, and MHC-II-related genes, other genes, such as *Relb*, encoding a transcription factor associated most directly with differentiation and function of DC (5, 22, 46), as well as the *CD1d2* and *ICAM1* genes, which are involved in DC activation and maturation, are also activated. In both cases, up-regulation was exclusively ascribed to the acapsular strain, while the encapsulated strain failed to stimulate any activation.

There is compelling evidence that encapsulation of *C. neoformans* is a potent obstacle for the induction of proinflammatory cytokines such as IL-12 and TNF-α (19). Although our results validate previously reported data on the capacity of acapsular strain to induce IL-12 and TNF-α (45) and the inability of the encapsulated virulent strain to induce TNF-α (19), this is the first demonstration that an acapsular strain induces the chemokine genes in DC, while the encapsulated strain produced the opposite results either by down-regulating or not modulating the expression of some of these genes.

Exposure to the acapsular strain resulted in a prompt and intense response by DC, which resulted in the induction of a large set of cytokine genes, including those encoding TNF-α, IL-1, and interferon, as well as the genes for chemokines and cell adhesion molecules. Several cytokine-induced genes appeared to persist in an activated state even after 18 h of incubation. Thus, the DC were highly responsive to acapsular cells, and the nature of the response had the typical features of an immunostimulatory reaction. Conversely, the expression of only seven genes was modulated in 4 h of DC incubation with the encapsulated strain. Among these genes, *Tgfb3* and *Ifnra2* were up-regulated. The former is involved in inducing TGF-β and is considered an inhibitor of DC maturation (10), and the latter is involved in the production of alpha interferon and suppresses IL-12 production (7, 32), evidencing for the first time that not only was the encapsulated strain unable to induce activation but that it was capable of activating the gene that regulated the production of alpha interferon and could be involved in the suppression of IL-12 production (7). Thus, the inability of DC to produce IL-12 following stimulation with encapsulated yeast could be related to the induction level of this gene and the activation of other signaling pathways that suppress IL-12. These results reveal a complex network of gene expression that contributes to highlight the evasive maneuvers of the encapsulated strain. Another piece of evidence for this assertion is related to the capacity of the encapsulated fungus to induce down-regulation of five genes, including those encoding the E74-like factor 1 (*Elf1*) and sequestosome 1 (*Sqstm1*). The *Elf1* gene is a transcription factor that regulates the expression of cytokines such as granulocyte-macrophage colony-stimulating factor and IL-3 (27, 35), and the *Sqstm1* gene is involved in induction of NF-κB signaling (16, 48).

NF-κB is a ubiquitous transcription factor that is activated by a variety of stimuli and is a key regulator of the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 and chemokines such as CXCL8 (41). Therefore, it is conceivable that capsular material inhibits NF-κB activation and the consequent cytokine release via down-regulation of these genes.

The effect of capsular material as an immune inhibitor has been shown previously (42). Glucuronoxylomannan is able to induce an atypical response of peripheral blood mononuclear cells, allowing activation of NF-κB without stimulating TNF-α secretion (38, 42). Indeed, our data show that DC stimulated for 4 or 18 h with encapsulated strain are for the most part unresponsive and the nature of their minimal early response resembles an immune-inhibitory reaction. The only DC gene that was significantly up-regulated after 18 h of incubation with the encapsulated strain was *Ilf2*, which is involved in IL-2 mRNA induction (14, 25). This suggests that the induction of IL-2 expression by DC could be regulated by the presence of capsular material.

Different from the encapsulated strain, the acapsular strain activates genes that regulate the activation of NF-κB family genes and the endothelin-1 (*ET-1*) gene, which promotes TNF-α production through its action on the ET$_A$ receptor, thus demonstrating its proinflammatory function (49). Since ET-1 is almost ubiquitous in both lymphoid and nonlymphoid tissues, it is reasonable to hypothesize that it may participate in DC activation. In addition, up-regulation of the *ICAM1* gene suggests that the acapsular strain could favor the migration of DC through the endothelium, thereby promoting an inflammatory response that is considered essential for clearing *C. neoformans* from host tissues (9, 26, 29, 39). This is consistent with the observed rapid elimination of acapsular *C. neoformans* from infected animals (21).

The absence of DC stimulation observed with the encapsulated strain was not due to the lack of recognition by DC, because, in our experimental system, encapsulated *C. neoformans* cells interacted with DC and were even internalized. Previously, Kelly et al. reported that murine DC rarely phagocytized encapsulated *C. neoformans* (20). There were, however, several differences in the experimental systems that could account for the apparent discrepancy: (i) Kelly et al. used *C. neoformans* opsonized with 10% fetal bovine serum, whereas in our experiments, the fungus was opsonized with 20% of normal mouse serum; (ii) Kelly et al. used murine bone marrow-derived DC, while we used the D1 cell line; and (iii) Kelly et al. used the *C. neoformans* strain ATCC 62070, while we used the JEC21 strain. Several receptors present on our D1 cell line, such as CR3, FcγRII/FcγRIII (48), and CD205 (36), which is homologous to the mannose receptor (18), are known to be involved in *C. neoformans* phagocytosis (24, 40). These receptors could have been associated with the observed internalization of encapsulated yeast cells. Further evidence that supports the presence of interaction between encapsulated *C. neoformans* and D1 cells is that the encapsulated cells are able to modulate the expression of negative signals, such as TGF-β3 and Ifnra2.

The results of this study show for the first time the overall profile of gene expression in DC during their interaction with *C. neoformans* as well as the role of capsular material in neg-

atively influencing the pattern of gene expression and molecules involved in driving the T cells. Our data suggest that the cell wall antigens, such as α- and β-glucans and mannoproteins, that in encapsulated *C. neoformans* are confined to the inner layer play a fundamental role in inducing a protective response. In contrast, the outermost capsular part is involved in evasion or even silencing of the DC response. The cryptococcal capsule is reminiscent of the extracellular polysaccharides of bacteria, such as the capsules of gram-negative species, including *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Pseudomonas aeruginosa* (47). Both bacterial and *C. neoformans* capsules are antiphagocytic and primarily composed of carbohydrates which are not readily removed from the cell wall. Moreover, the encapsulated bacteria *S. pneumoniae*, *N. meningitidis*, *Haemophilus influenzae*, and *Klebsiella pneumoniae* share common pattern recognition receptors with *C. neoformans*, such as TLR2 and TLR4 (31, 34). Although no bacterial extracellular glycan is structurally similar to glucuronoxylomannan or galactoxylomannan of *C. neoformans* capsule, these polymers provide a context for investigation not only of the synthesis of the capsule but also of the nature of immune-silencing properties of encapsulated microorganisms. Collectively, these results could help elucidate the nature of immune-regulation that determines survival of encapsulated microorganisms in different host environments.

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Editor: A. Casadevall