Cryptococcus neoformans and *Candida albicans* Regulate CD4 Expression on Human Monocytes

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This study examined the capability of *Candida albicans* and *Cryptococcus neoformans* to modulate CD4 expression on human monocytes. *C. albicans* and an acapsular strain of *C. neoformans* induced higher levels of CD4 expression than encapsulated strains. Purified glucuronoxylomannan did not regulate CD4 expression on monocytes, but down-regulation of CD4 expression compared with stimulation by acapsular *C. neoformans* alone was observed when glucuronoxylomannan was used in combination with acapsular *C. neoformans*. The ability of opsonic factors to facilitate fungal-mediated CD4 overexpression suggests that binding or internalization (or both) of the yeast cells is a critical event. Protein synthesis was required, excluding redistribution of the intracellular pool of CD4 receptors to the cellular surface as the sole possible mechanism. Results demonstrate a new effect of fungi on professional phagocytic cells and raise the possibility that modulation of CD4 could influence gp120-mediated human immunodeficiency virus entry.

CD4, a transmembrane glycoprotein, is a member of the immunoglobulin-like superfamily that is expressed on thymus cells, T lymphocytes, hematopoietic progenitor cells, and monocytes/macrophages [1]. Monocytes and macrophages express CD4, but the number of molecules on the surface is lower than on T cells [2]. Moreover, the CD4 molecule is expressed in low density on the membrane surface, whereas the CD4 density within cytoplasm is high. In contrast to T cells, $CD4^+$ nonlymphocytic cells do not express $p56^{lck}$ protein kinase, but several other *src*-related protein tyrosine kinases could participate in CD4 signal transduction. However, kinase activity does not coimmunoprecipitate with CD4, and the lack of evidence for an association of tyrosine kinase with CD4 suggests that CD4 signal transduction may be kinase-independent [3]. The physiologic ligand and the function of CD4 on monocytes/macrophages remain unknown. One possible function for CD4 in nonlymphocytic cells is binding to major histocompatibility complex class II independently of T cell receptors [4]. This binding might allow attachment of circulating monocytes to tissue-located macrophages. Attachment to inflammatory sites could be achieved through regulation of cell surface expression of CD4 in monocytes. CD4 might be able to self-associate, this phenomenon being important for cell-to-cell interaction between monocytes and macrophages.

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The extracellular domain of CD4 is the primary surface receptor for human immunodeficiency virus (HIV) on monocytes/macrophages [5] that, in concert with chemokine receptors, permits HIV entry [6]. HIV-infected macrophages represent a "reservoir" for virus and an insidious vehicle for HIV dissemination to different organs [7]. During the advanced stage of AIDS, opportunistic fungi are often responsible for life-threatening infections; in particular, *Cryptococcus neoformans* affects 4%–8% of AIDS patients. Mucocutaneous candidiasis is another fungal infection that commonly occurs in AIDS patients.

Pettoello-Mantovani et al. [8] were the first to report that the capsular polysaccharide of *C. neoformans* enhances HIV replication in H-9 cells [8]. This report was followed by several studies that documented the ability of microorganisms such as *C. neoformans* [9, 10] and *Candida albicans* [10] to enhance HIV replication, but there are different views for the mechanism involved. Orendi et al. [9] suggested that *C. neoformans*–enhanced HIV replication is tumor necrosis factor (TNF)–independent. More recently, Harrison et al. [10] reported that *C. neoformans* or *C. albicans* enhances HIV expression in monocytic cells through a TNF-α– and NF-κB–dependent mechanism.

Our study examined the possibility that exposure to *C. neoformans* or *C. albicans* yeast cells could alter CD4 expression on human monocytes. Such modulation of CD4 could influence CD4/gp120 interactions and possibly modulate both syncytium formation and virus entry [11]. Our results demonstrate that *C. neoformans* and *C. albicans* induce up-regulation of CD4 on the monocyte surface.

Materials and Methods

Reagents and media. RPMI 1640 with glutamine and fetal calf serum (FCS) were obtained from Gibco BRL (Paisley, Scotland).

Type AB human serum (HS) from healthy donors was purchased from Sigma (St. Louis). Fluorescein isothiocyanate–conjugated (FITC) mouse anti–human CD4 monoclonal antibody (MAb type IgG1) was obtained from Calbiochem-Novabiochem (San Diego). Specific fluorescence was assessed by comparison with results from an irrelevant FITC-conjugated mouse IgG1 MAb (Sigma). Mouse anti–human CD14R MAb (IgG2a) and phycoerythrin conjugate were purchased from Ancell (Bayport, MN). Glucuronoxylomannan (GXM) from *C. neoformans* was isolated from culture supernatant fluid of a serotype A isolate (ATCC 24064) by differential precipitation with ethanol and cetyltrimethylammonium bromide [12]. A MAb reactive with GXM (MAb 2H1) was a gift of Arturo Casadevall (Albert Einstein College of Medicine, Bronx, NY). All media and buffers used in this study were tested for endotoxin contamination by limulus amebocyte lysate assay (Sigma), which has a sensitivity of ~0.05 to 0.1 ng/mL *Escherichia coli* lipopolysaccharide. All media and reagents tested negative. Monoiodoacetic acid (MIA) was purchased from Sigma. Human complement C3a, prepared from normal HS, was obtained from Cortex Biochem (San Leandro, CA). Human recombinant complement C5a from *E. coli* was obtained from Fluka Chemie (Buchs, Switzerland).

Preparation of peripheral blood monocytes (PBM). Heparinized venous blood was obtained from healthy volunteers and diluted with RPMI 1640. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation over Ficoll-Hypaque Plus (Pharmacia Biotech, Uppsala, Sweden) [13]. PBMC were recovered; washed twice in RPMI 1640 supplemented with 5% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL); plated into cell culture petri dishes (Nunc, Roskilde, Denmark); and incubated for 1 h at a concentration of 2×10^6 to 3×10^6/mL. Nonadherent cells were removed by washing the dishes three to five times with prewarmed RPMI 1640. Adherent cells (PBM) were recovered using a cell scraper (Falcon, Oxnard, CA), washed twice, and suspended with three different solutions comprising RPMI plus penicillin (100 U/mL), streptomycin (100 μg/mL), and one of the following: 10% HS, 10% heat-inactivated FCS, or 10% heat-inactivated HS.

Microorganisms. *C. albicans* (strain PCA-2) was supplied by D. Kerridge (Department of Biochemistry, University of Cambridge, UK). This is an agerminative strain that grows as a pure yeast form in vitro at 28 or 37°C in conventional media. The culture conditions have been previously described [14]. A virulent germinative strain of *C. albicans* (CA-6) isolated from a clinical specimen was also used in our studies. The origin, characteristics, and growth conditions of *C. albicans* CA-6 have been described [14].

Two encapsulated strains of serotype A *C. neoformans* were used; a thinly encapsulated strain (Central Bureau Schimmel [CBS], Delft, The Netherlands; no. 6995 = NIH 37) was obtained from CBS, and a large-capsule strain (NCPF 3168) was obtained from the National Collection of Pathogenic Fungi (London). An acapsular strain of *C. neoformans* var. *neoformans* (CBS no. 7698 = NIH B-4131) was also used.

The cultures were maintained by serial passage on Sabouraud agar (bioMérieux, Lyon, France). Log-phase yeasts were harvested by being suspended (a single colony) in RPMI 1640, washed twice with physiologic saline, counted on a hemocytometer, and adjusted to the desired concentration in RPMI [15].

Challenge of human monocytes with yeast cells and flow cytometry analysis. Suspensions of 1×10^6 monocytes in RPMI plus 10% HS in polypropylene tubes were mixed and vortexed with different strains of *C. neoformans* or *C. albicans* at an effector cell–to–target cell (E:T) ratio of 1:2 (data obtained with different E:T ratios are specified). The cell mixtures were incubated for different time periods in the presence of a fungistatic concentration of amphotericin B, as described by Herrmann et al. [16]. RPMI plus the above-mentioned amphotericin B was used in all experiments. After incubation at 37°C in the presence of 5% CO_2, the cells were collected by centrifugation, fixed in 3% paraformaldehyde in PBS, washed twice in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide, and mixed with FITC-conjugated mouse anti–human CD4. After 30 min of incubation on ice, the cells were washed twice and then stained for 30 min with phycoerythrin-conjugated anti-CD14. For each sample, CD4 expression was measured on the surface of $CD14^+$ cells by flow cytometry (FACScan; Becton Dickinson, San Jose, CA) [13]. Mean fluorescence of labeled cells was determined using logarithmic-scale histograms. Autofluorescence was assessed using untreated cells. An irrelevant FITC-conjugated isotype control was used as a negative control for each experiment.

Cycloheximide and actinomycin D treatment of monocytes. Cycloheximide from *Streptomyces griseu* and actinomycin D isolated from *Streptomyces* species were obtained from Sigma. Cycloheximide (2.5 μg/mL) was added to monocytes 30 min before challenge with yeasts as described previously [17]. Actinomycin D (500 ng/mL) was added to monocyte cultures at the time of challenge. Cell viability was >98% after treatment with cycloheximide or actinomycin D. Viability of cycloheximide- or actinomycin D–treated monocytes was measured with a colorimetric reaction that is based on the capacity of mitochondrial dehydrogenase of living cells to reduce MTT (Aldrich Chemical, Milan, Italy) into formazan. The quantity of formazan produced and measured at an optical density of 540 nm in a microplate reader (Sorin Biomedica, Saluggia, Italy) correlated with the number of living cells [18].

Statistical analysis. Statistical significance was calculated using Student's paired *t* test.

Results

Initial experiments examined expression of CD4 on monocytes after incubation with thinly encapsulated *C. neoformans* 6995, heavy encapsulated *C. neoformans* 3168, acapsular *C. neoformans* 7698, agerminative *C. albicans* PCA-2, or germinative *C. albicans* CA-6. Different E:T ratios were used, but only the E:T ratio of 1:2 is reported in figure 1. Acapsular *C. neoformans* 7698 and both agerminative (PCA-2) and germinative (CA-6) *C. albicans* were better stimulators of CD4 expression than the encapsulated strains of *C. neoformans* (6995 or 3168). Furthermore, CD4 up-regulation was dependent on the E:T ratio used. No modulation of CD4 expression was observed at an E:T ratio of 1:0.1. In contrast, a substantial up-regulation of CD4 expression was observed at an E:T ratio of 1:2 (figure 1).

We also determined whether incubation of monocytes with GXM (250 μg/mL) altered CD4 expression. The results show that GXM alone did not modulate CD4 expression (figure 1).

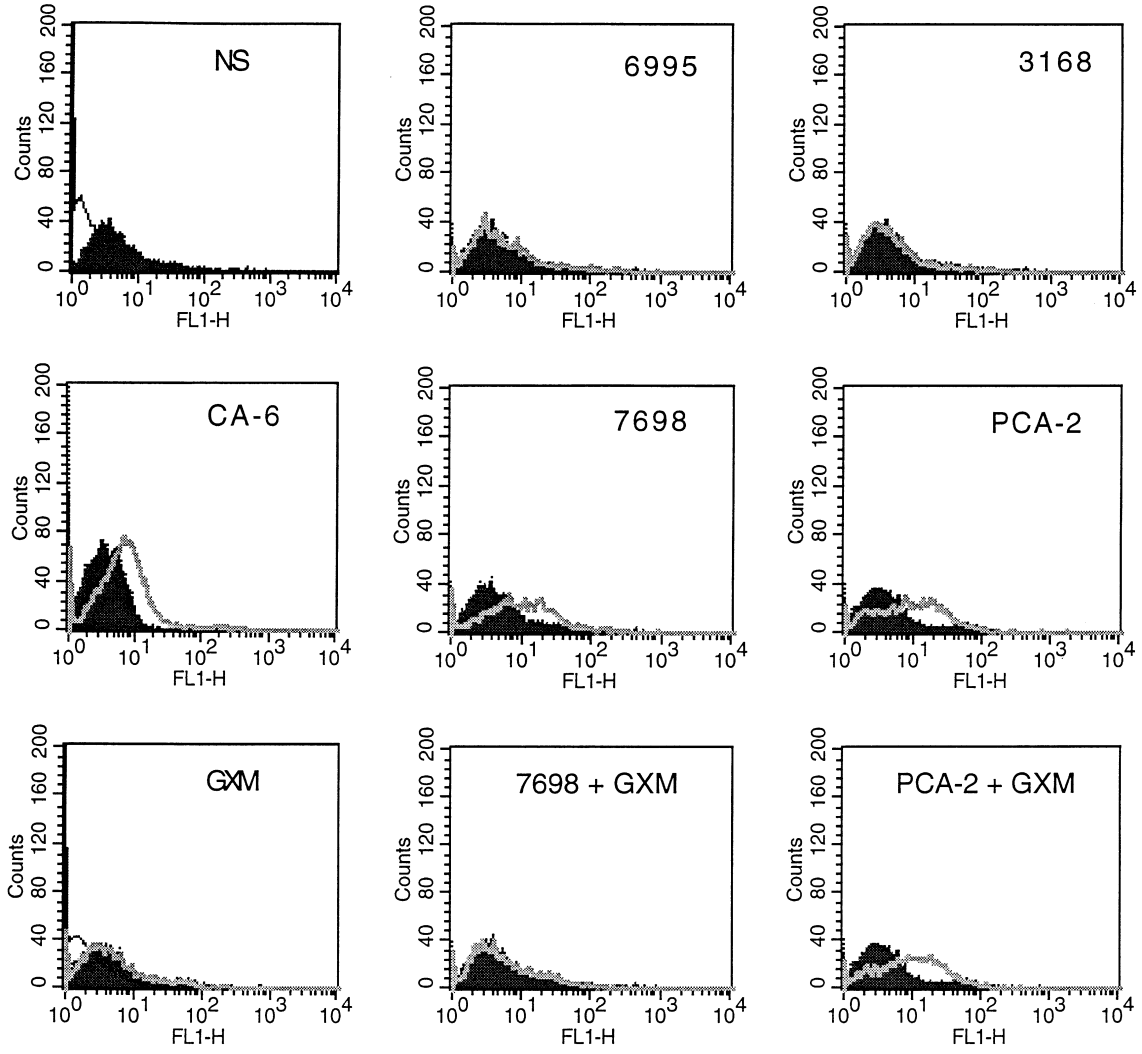

Figure 1. Effect of incubation of monocytes with yeast cells on expression of monocyte CD4. Monocytes were incubated for 18 h in absence of yeast cells (not stimulated, NS) or with encapsulated *C. neoformans* 6995, encapsulated *C. neoformans* 3168, acapsular *C. neoformans* 7698, agerminative *C. albicans* PCA-2, germinative *C. albicans* CA-6, glucuronoxylomannan (GXM, 250 mg/mL), acapsular *C. neoformans* strain plus GXM (250 μg/mL), or *C. albicans* PCA-2 plus GXM (250 μg/mL). Effector-to-target ratio was 1:2. Results are reported from 1 experiment of 3 performed with similar results. Dotted line represents staining profile with isotype-matched control antibody. Black lines represent CD4 expression on unstimulated monocytes. Gray lines represent CD4 expression on monocytes treated with indicated stimuli.

GXM binds to the acapsular strain and can confer an experimentally constructed capsule that protects the fungus from phagocytosis [19, 20]. To determine whether encapsulation of *C. neoformans* with GXM influenced the effect of the yeast on CD4 expression, monocytes were incubated with acapsular *C. neoformans* in the presence of GXM (250 μg/mL). The results showed that the presence of GXM suppressed the up-regulation of CD4 that would normally occur on monocytes exposed to acapsular *C. neoformans* alone. In contrast, GXM had no effect on *C. albicans*–induced CD4. This result is consistent with the observation that GXM has no effect on phagocytosis of *C. albicans* [20].

We evaluated the time course for yeast-induced expression of monocyte CD4. Monocytes were incubated for 3, 18, or 72 h with acapsular cryptococci, encapsulated cryptococci, or cells of *C. albicans* in the presence of 10% HS. The results showed significant enhancement of CD4 expression after incubation of monocytes with the yeast cells for 18 or 72 h (figure 2). However, the level of CD4 expression on monocytes incubated for 72 h in the absence of yeast stimuli was considerably increased over the level of expression after 3 or 18 h incubation. As a consequence, all subsequent experiments assessed CD4 levels on monocytes that were incubated with various stimuli for 18 h.

The results in figure 1 suggest that the presence of the *C.*

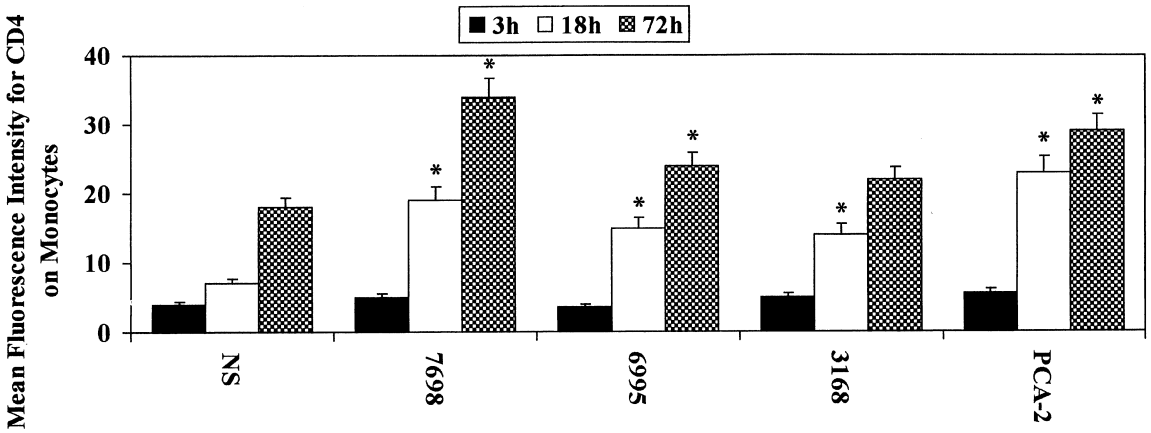

Figure 2. Time course for up-regulation of CD4 expression on monocytes incubated for various times in RPMI containing 10% human serum in absence of yeast cells (not stimulated, NS) or in presence of acapsular cryptococci (7698), thinly encapsulated cryptococci (6995), large capsule cryptococci (3168), or *C. albicans* PCA-2. Results represent mean of 4 separate experiments with monocytes from different donors. * $P < .05$ (yeast-treated vs. untreated cells).

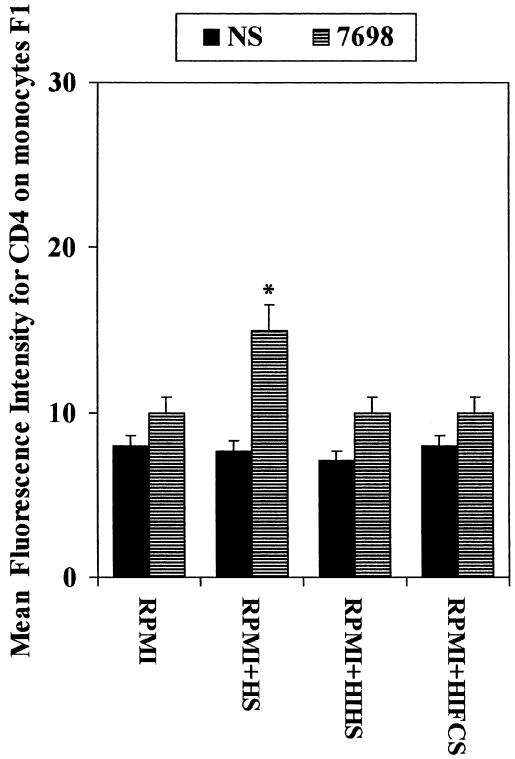

Figure 3. CD4 expression on monocytes that were incubated for 18 h with RPMI, RPMI containing 10% normal human serum (HS), RPMI containing 10% heat-inactivated human serum (HIHS), or RPMI containing 10% heat-inactivated fetal calf serum (HIFCS). Incubations were done in absence (solid bars; not stimulated, NS) or presence (striped bars) of acapsular cells of *C. neoformans* 7698. Results represent mean of 4 separate experiments done with monocytes from different donors. * $P < .05$ (7698-treated vs. nonstimulated cells).

neoformans capsule reduces the ability of the yeast to induce CD4 expression on monocytes. As the capsule is a potent inhibitor of phagocytosis [21], we hypothesized that one or more steps in the phagocytic process are involved in CD4 regulation. Consequently, we tested the contribution of serum opsonic factors to CD4 up-regulation. PBM were exposed to acapsular *C. neoformans* and cultured under various conditions, including RPMI in the absence or presence of HS, heat-inactivated HS, or heat-inactivated FCS. The results showed that strain 7698 could modulate CD4 expression only in the presence of HS (figure 3), suggesting that heat-labile serum factors are essential for stimulation of CD4 expression.

Encapsulated and acapsular *C. neoformans* are activators of the complement system, raising the possibility that the dependence of increased CD4 expression on heat-labile opsonins is due to generation of C3a and/or C5a, which are known to have regulatory effects on phagocytic cells [22]. As a consequence, we incubated monocytes (1×10^4) for 18 h with C3a (10 ng/mL) and C5a (10 ng/mL), individually or in combination, and CD4 expression was assessed by flow cytometry. The results showed that neither purified complement fragment alone or in combination had a significant effect on CD4 expression (data not shown). These results suggest that the phagocytic process itself is responsible for increased expression of CD4, rather than cleavage fragments of the complement system (C3a or C5a) that were generated during opsonization.

The contribution of opsonic factors was further studied using the anti-GXM IgG1 MAb 2H1 in combination with encapsulated *C. neoformans.* The results showed that anti-GXM MAb together with encapsulated *C. neoformans* stimulated increased CD4 expression on monocytes in a dose-dependent manner in the presence of heat-inactivated serum (figure 4).

Further proof for involvement of phagocytosis in stimulating

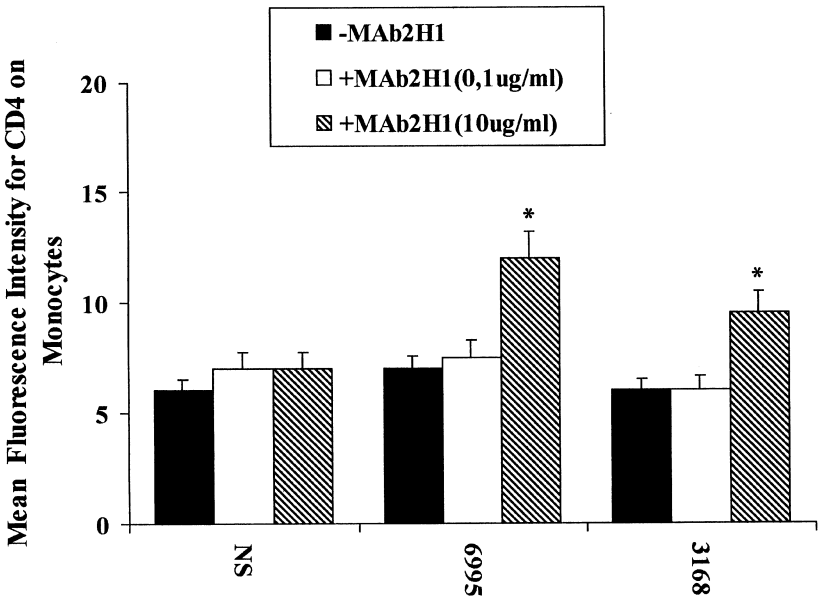

Figure 4. Effect of anticapsular antibodies on ability of encapsulated cryptococci to stimulate CD4 expression on monocytes. Monocytes were incubated for 18 h in RPMI containing 10% heat-inactivated human serum in absence of yeast cells (not stimulated, NS) or with cells of thinly encapsulated cryptococci (6995) or heavily encapsulated cryptococci (3168). Incubations were done in absence of anticapsular antibodies (solid bars) or in presence of 0.1 μg/mL (open bars) or 10 μg/mL (striped bars) of anti-glucuronoxylomannan monoclonal antibody (MAb) 2H1. Results are mean of 4 separate experiments with monocytes from different donors. * $P < .05$ (MAb 2H1–treated vs. MAb 2H1–untreated cells).

increased CD4 expression on monocytes was provided by the use of MIA, a potent inhibitor of phagocytosis. Incorporation of MIA (500 n*M*) into the incubation medium suppressed up-regulation of CD4 on monocytes exposed to acapsular *C. neoformans* or *C. albicans* (figure 5). An examination of the effect of MIA on the attachment of acapsular (7698) or capsulated (6995 or 3168) *C. neoformans* or *C. albicans* PCA-2 to monocytes showed an appreciable inhibition of binding in addition

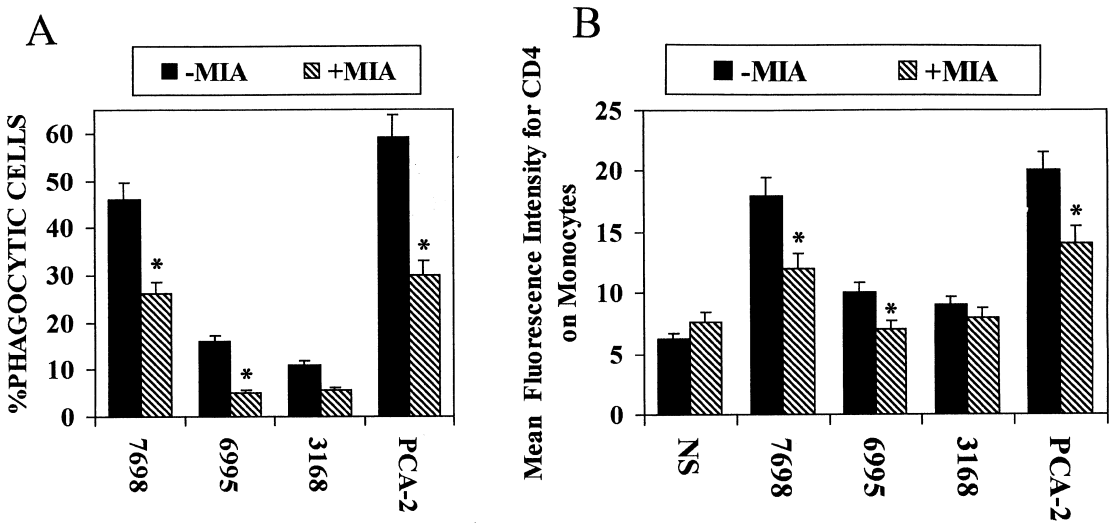

Figure 5. Effect of monoiodoacetic acid (MIA) on phagocytosis (**A**) or CD4 expression (**B**) on monocytes that were incubated for 18 h with RPMI containing 10% human serum in absence of yeast cells (not stimulated, NS) or in presence of acapsular cryptococci (7698), thinly encapsulated cryptococci (6995), heavily encapsulated cryptococci (3168), or *C. albicans* PCA-2. Results are mean of 4 separate experiments with monocytes from different donors. * $P < .05$ (MIA-treated vs. MIA-untreated cells).

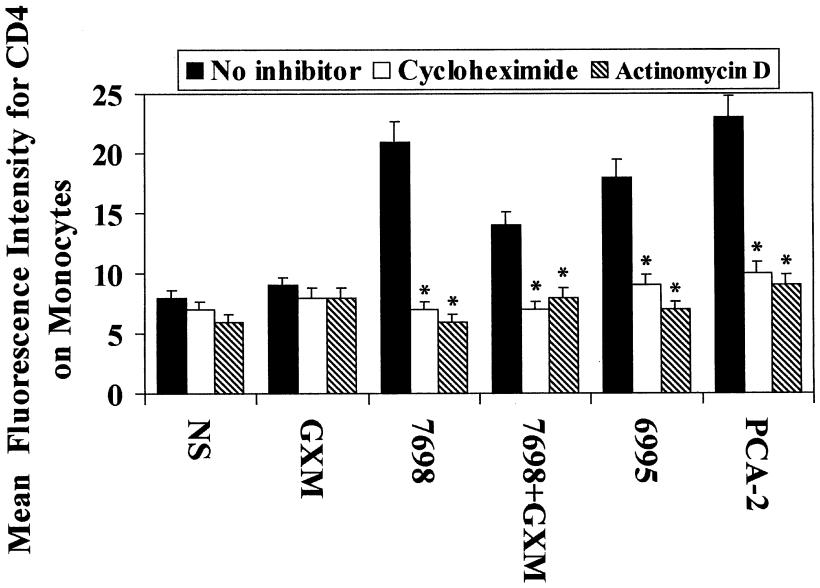

Figure 6. Effect of cycloheximide and actinomycin D on abilities of various yeast cells to stimulate CD4 expression on human monocytes. Monocytes were incubated for 18 h with 10% human serum in absence of yeast cells (not stimulated, NS) or with glucuronoxylomannan (GXM, 250 μg/mL), acapsular cryptococci (7698), acapsular cryptococci (7698) plus 250 μg/mL GXM, thinly encapsulated cryptococci (6995), or *C. albicans* PCA-2. Incubations were done in absence of inhibitor (solid bars) or in presence of 2.5 μg/mL cycloheximide (open bars) or 500 ng/mL actinomycin D (striped bars). Results represent mean of 4 separate experiments with monocytes from different donors. * $P < .05$ (cycloheximide- or actinomycin D–treated vs. noninhibited cells).

to the expected inhibition of phagocytic process (data not shown). Thus, ligation and the internalization processes (or both) appear to be key events in up-regulation of CD4 expression.

Experiments were performed to determine whether increased expression of CD4 required transcription and protein synthesis. Cycloheximide is a potent inhibitor of protein synthesis; actinomycin D binds to DNA via the deoxyguanosine residues to prevent DNA-dependent RNA synthesis [23]. Monocytes were incubated with acapsular cryptococci, thinly encapsulated cryptococci, *C. albicans,* or GXM (250 μg/mL) in the absence or in the presence of cycloheximide (2.5 μg/mL) or actinomycin D (500 ng/mL). Both cycloheximide and actinomycin D completely abrogated CD4 up-regulation by any of the stimuli that were examined (figure 6). The absence of increased CD4 expression in the presence of cycloheximide or actinomycin D could not be attributed to direct cytotoxicity by these compounds, because the monocytes retained cell viability at the concentrations and under the conditions used in our assay.

We previously demonstrated that *C. neoformans* or *C. albicans* yeast cells are inducers of cytokine secretion by monocytes/macrophages [15, 24], raising the possibility that endogenous cytokines play a role in the modulation of CD4 expression. Alternatively, products released by fungi during incubation (18–72 h) could influence CD4 expression. To determine whether soluble factors released by monocytes were involved in the up-regulation of surface CD4, supernatants from monocytes cocultured for 18 h with different fungi were recovered, filtered, added to autologous monocytes, and incubated for 18 h. Supernatants from stimulated monocytes could not modulate surface CD4 molecule expression on the autologous monocytes (data not shown). Experiments were also performed in which fungi were cultured in RPMI for 18 h; then supernatant fluids were harvested, filtered, and used as culture media for monocytes in the presence of 10% HS. After 18 h of incubation, CD4 expression was evaluated. No modulation of CD4 expression was observed (data not shown).

Discussion

Our data provide evidence that surface expression of CD4 on human monocytes is up-regulated after exposure to *C. neoformans* or *C. albicans.* The increased CD4 expression was not found after 3 h but was readily detected after exposure to the fungi for 18 or 72 h. Acapsular *C. neoformans* and *C. albicans* were better stimulators than encapsulated *C. neoformans.* Purified GXM did not directly affect CD4 expression on monocytes but selectively mitigated CD4 up-regulation induced by the acapsular strain. Ligation and internalization of the fungi were critical to fungal-induced CD4 expression on human monocytes. Such expression appeared to be independent of the presence of endogenous cytokines. CD4-induced expression required transcription and protein synthesis.

In agreement with previous observations [25], our results

show an increase of CD4 expression on human monocytes during in vitro culture (figure 2). However, CD4 expression was further stimulated by overnight culture with yeast cells, suggesting that expression of surface CD4 on nonlymphocytic cells is related to the stage of differentiation and activation. Previous reports demonstrated that CD4 expression on human monocytes is modulated by a variety of stimuli, including HIV envelope glycoprotein gp120 [26], lipopolysaccharide [27], and cytokines such as interleukin (IL)-1β, TNF-α, interferon-γ, and granulocyte-macrophage colony-stimulating factor [27]. Moreover, it has been reported that human herpesvirus 6 induces CD4 expression in CD4-negative cells [28]. Our results confirm that surface CD4 on nonlymphocytic cells is susceptible to regulation and suggest this regulation extends to include appropriately opsonized intact yeast cells.

C. neoformans and *C. albicans* are potent stimulators of cytokines from human monocytes, raising the possibility of cytokine involvement in CD4 up-regulation. Such a role for cytokines seems unlikely because our recent observations show that monocytes exposed to *C. neoformans* or *C. albicans* produce TNF-α, IL-1β [15], and IL-10 [24]. These cytokines are involved in down-regulation or nonregulation of CD4 expression [27]. Consistent with this premise, our results showed no modulation of CD4 molecules on fresh human monocytes treated for 18 h with supernatants from monocytes treated with *C. neoformans* or *C. albicans.* However, we cannot exclude the possibility that such stimulation could occur via priming with endogenous factors.

Fungal up-regulation of CD4 expression probably involves synthesis of new molecules, as demonstrated by complete inhibition of CD4 up-regulation by actinomycin D, an inhibitor of RNA synthesis, and cycloheximide, an inhibitor of protein synthesis. However, we cannot rule out the possibility that translocation of preformed CD4 to the cell surface requires protein synthesis.

The strongest up-regulation of CD4 was detected after stimulation with *C. albicans* or acapsular *C. neoformans;* encapsulated cryptococci were less effective stimulators. The major component of the *C. neoformans* capsule (GXM) did not directly modulate CD4 expression. However, the combined treatment of monocytes with GXM and acapsular *C. neoformans* reduced the level of enhanced CD4 expression compared with the level of stimulation produced by acapsular *C. neoformans* alone. This inhibitory effect appears to be specific because GXM had no effect on *C. albicans*–induced CD4 expression, suggesting that one or more steps in the phagocytic process is involved in the observed phenomenon. The importance of phagocytosis is further underlined by (1) the dependence of up-regulation by acapsular cryptococci on the presence of heat-labile opsonins (figure 3), (2) the ability of anti-capsular IgG to facilitate stimulation of CD4 expression on monocytes exposed to encapsulated cryptococci in the presence of heat-inactivated serum (figure 4), and (3) suppression of yeast-induced CD4 expression by MIA (figure 5). These results strongly suggest that either attachment or the phagocytic event itself is responsible for enhanced CD4 expression on monocytes. It remains to be established whether interaction with other microorganisms, such as bacteria and viruses, could produce a similar effect.

Taken together, our results demonstrate a novel consequence of the interaction between the organisms *C. albicans* and *C. neoformans* and human monocytes. The pathobiologic significance of these results for patients with fungal infections remains to be determined.

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