INFECTION AND IMMUNITY, Feb. 2005, p. 820–827 Vol. 73, No. 2
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.2.820–827.2005

Mannoproteins from *Cryptococcus neoformans* Promote Dendritic Cell Maturation and Activation

Donatella Pietrella,[1] Cristina Corbucci,[1] Stefano Perito,[1] Giovanni Bistoni,[2] and Anna Vecchiarelli[1]*

Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy,[1] and Biological Sciences, Imperial College London, London, United Kingdom[2]

Received 30 July 2004/Returned for modification 31 August 2004/Accepted 19 October 2004

Our previous data show that mannoproteins (MPs) from *Cryptococcus neoformans* are able to induce protective responses against both *C. neoformans* and *Candida albicans*. Here we provide evidence that MPs foster maturation and activation of human dendritic cells (DCs). Maturation was evaluated by the ability of MPs to facilitate expression of costimulatory molecules such as CD40, CD86, CD83, and major histocompatibility complex classes I and II and to inhibit receptors such as CD14, CD16, and CD32. Activation of DCs was measured by the capacity of MPs to promote interleukin-12 and tumor necrosis factor alpha secretion. DC-induced maturation and interleukin-12 induction are largely mediated by engagement of mannose receptors and presume MP internalization and degradation. DC activation leads to IκBα phosphorylation, which is necessary for nuclear factor κB transmigration into the nucleus. MP-loaded DCs are efficient stimulators of T cells and show a remarkable capacity to promote CD4 and CD8 proliferation. In conclusion, we have evidenced a novel regulatory role of MPs that promotes their candidacy as a vaccine against fungi.

Cryptococcus neoformans is a ubiquitous fungus that causes infection in immunocompromised hosts, particularly those with defective CD4 T-cell counts and functions, including patients with AIDS (22).

The advent of highly active antiretroviral therapy has drastically reduced the impact of cryptococcosis in AIDS; however, new groups of immunodepressed patients, such as ones with renal transplants, who are particularly susceptible to *C. neoformans* infection, are emerging (42). The fungus enters the body via inhalation and thereby reaches the lung, where it can cause pneumonia, although any organ system may be affected and meningoencephalitis is the most common clinical presentation.

Protective immunity has been attributed to a T-helper 1 (Th1)-type response (17) with consequent activation of macrophages via production of cytokines and direct antifungal activity (3).

Compelling evidence indicates that mannoproteins (MPs) from *C. neoformans* play a key role in inducing the T-cell-mediated immune response, which is critical for antifungal protection (32, 38). In particular, Murphy isolated a *C. neoformans* culture filtrate antigen characterized as an MP fraction that was able to induce a delayed-type hypersensitivity response (29); subsequently, MPs were also shown to stimulate lymphocyte proliferation (25, 27, 37). MP engagement of mannose receptors (MRs) on antigen-presenting cells is essential for optimal T-cell stimulation (27).

Previously we demonstrated that MPs from *C. neoformans* (CnMPs) stimulate an early and massive production of interleukin-12 (IL-12) by human monocytes (36). When monocytes were cocultured with autologous T cells, an intense proliferation of T cells with production of gamma interferon (IFN-γ) was observed (36, 37). The possibility that this type of immune response could be elicited in vivo after administration of MPs was corroborated in an experimental mouse model of systemic cryptococcosis (33). MP treatment conferred protection against *C. neoformans* lethal challenge by concomitant induction of a Th1-type response characterized by an early and massive presence of IL-12 (33). Indeed, MP-induced IL-12 is fundamental in promoting protection, because its deletion, at least in the early phase of the immune response, creates an imbalance that the immune system tries to circumvent by augmenting IL-18 release (34).

MPs are glycoconjugates usually containing 80 to 90% mannose expressed on the fungal surface and released during fungal growth (30). MPs are heterogeneous in *C. neoformans*; however, many of them share immunoregulatory effects (5, 9, 17, 27, 33). Two MPs (MP1 and MP2) extracted from *C. neoformans* are able to induce tumor necrosis factor alpha (TNF-α) and IL-12 production by human monocytes. However, MP2 is a better inducer of IL-12 and IFN-γ than MP1 (5, 36), and MP2 induces a protective Th1 response to *C. neoformans* infection in a mouse model (33, 34). In addition, MPs are expressed on a variety of fungi, including *Candida albicans* (4). One of them, the immunodominant 65-kDa MP of *C. albicans* (CaMP), has been extensively studied and characterized (15, 16, 21, 31). It possesses strong immunogenic properties, including induction of a T-cell response and partial protection against lethal infection (28). Recently we showed that CnMP induces cross-protection against systemic *C. albicans* challenge and that it is, at least in part, related to epitopes also common to CaMP (35). The protective T-cell response is governed by antigen-presenting cells, and dendritic cells (DCs) are the

* Corresponding author. Mailing address: Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto, 06122 Perugia, Italy. Phone and fax: 39-075-585-7407. E-mail: vecchiar@unipg.it.

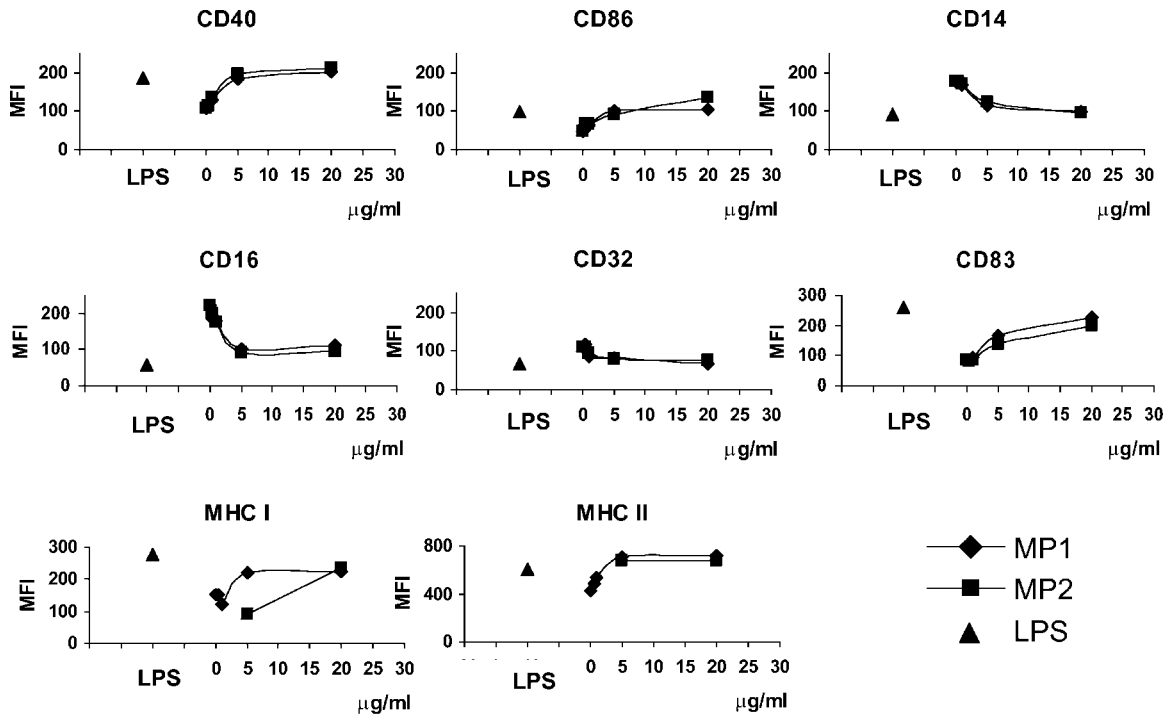

FIG. 1. Cell surface expression of MHC, CD16, CD32, and CS molecules on DCs upon addition of MPs. Immature DCs were treated with different concentrations of MP1, MP2, or LPS (1 μg/ml) for 48 h; stained with FITC-conjugated MAb to CD14, CD16, CD32, CD40, CD83, CD86, MHC class I, or MHC class II; and then examined by flow cytometry. Results are expressed as mean fluorescence intensities (MFIs). The data are representative of one of three independent experiments with similar results.

quintessential professional antigen-presenting cells. Human DCs are able to phagocytize *C. neoformans* yeast cells through CD32 and MRs. After phagocytosis, DCs process and present cryptococcal antigen to T cells, thereby inducing their proliferation and activation (43). It has been reported that various MPs from *C. neoformans* (6, 23, 27) share many biological effects; therefore, we were interested in testing the similarities or differences of two MPs isolated from culture supernatant of *C. neoformans*. Given that MPs are usually immunostimulating agents that favor protective T-cell responses (28, 35) and that DCs have a key role in this process, the purpose of our study was to evaluate the effects of MP1 and MP2 on DC maturation and activation.

MATERIALS AND METHODS

Reagents and media. RPMI 1640 with glutamine and fetal calf serum (FCS) was obtained from Gibco-BRL (Grand Island, N.Y.). Human serum type AB, cytochalasin D, and ammonium chloride were purchased from Sigma (Milan, Italy). All murine anti-human monoclonal antibodies, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin, were obtained from Ancell (Alexis Italia, Florence, Italy) and are specified for single experiments described below. Isotypic control antibodies were purchased from Sigma. Mouse monoclonal anti-human MRs were obtained from HyCult Biotechnology (Uden, The Netherlands).

Microorganisms. An acapsular mutant strain of *C. neoformans* var. *neoformans* CAP67 (NIH B-4131) was obtained from the American Type Culture Collection (Rockville, Md.). The CAP67 acapsular phenotype is a result of a mutation in a single gene which, when complemented, restores the capsule and the virulence of the strain (7). The cultures were maintained by serial passage on Sabouraud agar (Fluka, Buchs, Switzerland). Log-phase yeast cells were harvested with a hemocytometer and adjusted to the desired concentration in RPMI, 1640. The yeast was inactivated at 60°C for 30 min.

Preparation of MP extracts of *C. neoformans*. *C. neoformans*-secreted MP1 and MP2 were purified as previously described (8). Briefly, secreted MPs were purified from culture supernatants of *C. neoformans* NIH B-4131. Purification was performed by a combination of ultrafiltration affinity chromatography (concanavalin A), anion-exchange chromatography (DEAE), and gel permeation chromatography. Anion-exchange chromatography of the MPs yielded two fractions, MP1 (35.6 kDa) and MP2 (8.2 kDa) (5). MP1 and MP2 contained 7 and 13% protein together with 79 and 72% neutral sugars, respectively (44).

Preparations of the various cryptococcal components tested negative for endotoxin contamination by a *Limulus* assay (Coatest endotoxin; Kabi Diagnostica, Mölndal, Sweden) with a sensitivity of 25 pg of *Escherichia coli* lipopolysaccharide (LPS). Nevertheless, all in vitro experiments were carried out at least once in the presence of 10 μg of polymixin B (Sigma) per ml to neutralize any undetected contamination with bacterial LPS.

In vitro generation of human DCs. The generation of DCs from human peripheral blood monocytes was performed as described previously with minor modifications (40). Heparinized venous blood was obtained from healthy donors and diluted with RPMI 1640 (Gibco-BRL). Peripheral blood mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque PLUS (Pharmacia Biotech, Uppsala, Sweden); recovered; washed twice and suspended in RPMI 1640 supplemented with 5% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml); plated onto cell culture flasks (Corning Incorporated, Corning, N.Y.); and incubated for 1 h at a density of 2×10^6 to 3×10^6/ml. Adherent peripheral blood monocytes were recovered with a cell scraper (Falcon, Oxford, Calif.), washed twice, and purified by E rosetting to remove contaminating T cells. The cells recovered were >98% $CD14^+$ monocytes, as evaluated by flow cytometry. Isolated monocytes (2×10^6 to 3×10^6/ml) were incubated in RPMI 1640 plus 10% FCS containing 50 ng of human recombinant granulocyte macrophage–colony-stimulating factor (Biosource International, Camarillo, Calif.)/ml and 30 ng of human recombinant IL-4 (Biosource)/ml. After 6 days of culture, immature DCs were harvested, washed, and suspended in complete RPMI (cRPMI; RPMI 1640 plus 10% human serum type AB, penicillin [100 U/ml], and streptomycin [100 μg/ml]).

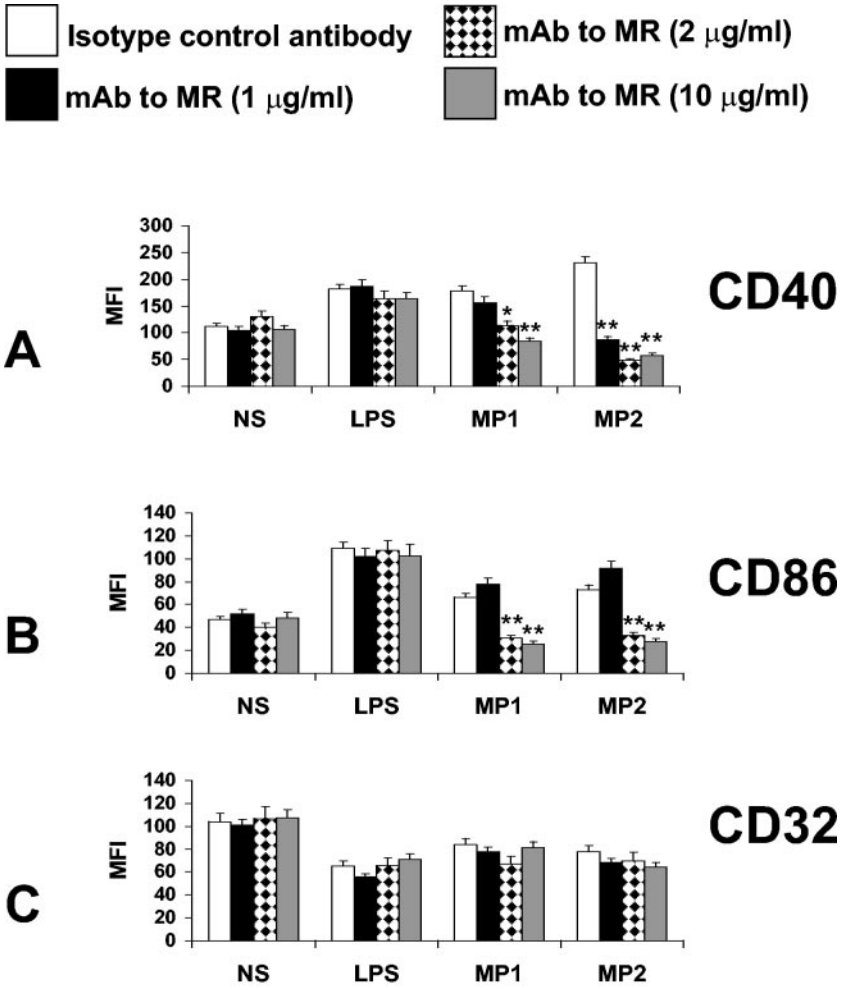

FIG. 2. Effects of MAb to MRs on CD40 (A), CD86 (B), and CD32 (C) expression induced by MPs. Immature DCs were incubated for 30 min with different concentrations of MAb to MR and then treated with MP1 or MP2 (5 μg/ml) or LPS (1 μg/ml) for 48 h. After incubation, the cells were stained with FITC-conjugated MAb to CD40, CD86, or CD32 and examined by flow cytometry. Results are expressed as mean fluorescence intensities (MFIs). Results represent the mean ± SEM for three separate experiments with cells from three different donors. Double asterisks indicate a P of <0.001, and single asterisks indicate a P of <0.05 (for results with DCs treated with MAb to MR versus results with DCs treated with isotypic control antibody).

Flow cytometry analysis of surface molecules. Surface molecule expression was quantified by flow cytometry after various culture incubation times. Suspensions of immature DCs (10^6/sample) in cRPMI were stimulated with MP1 and MP2 of *C. neoformans* in various concentrations or with LPS (1 μg/ml) as a positive control and were incubated for 48 h. After incubation at 37°C in the presence of 5% CO_2, the cells were collected by centrifugation, fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS), washed twice in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide, and mixed with mouse anti-human major histocompatibility complex (MHC) class I–FITC conjugate, mouse anti-human MHC class II–FITC conjugate, mouse anti-human CD14–FITC conjugate, mouse anti-human CD16–FITC conjugate, mouse anti-human CD32–FITC conjugate, mouse anti-human CD40–FITC conjugate, mouse anti-human CD83–FITC conjugate, or mouse anti-human CD86–FITC conjugate. After 45 min of incubation on ice, the cells were washed and analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, Calif.). In selected experiments, DCs were pretreated with 5 mg of glucan (Sigma)/ml for 30 min before MP addition.

Blocking of MP uptake by DCs. Immature DCs were pretreated with different doses of mouse anti-human MR for 30 min before MP or LPS addition. In separate experiments, internalization and processing of MPs were blocked by treatment of DCs with 2 μg of cytochalasin D/ml or 1 mM NH_4Cl for 30 min before the addition of the stimuli.

Determination of TNF-α and IL-12 production. A total of 2×10^6 of immature DCs/ml were incubated with 5 μg of MP1 or MP2/ml, heat-inactivated *C. neoformans* CAP67 (4×10^6/ml), or 1 μg of LPS/ml. In preliminary experiments, we tested cytokine induction and got the best production of TNF-α and IL-12 after 24 and 48 h, respectively. DCs were incubated for 24 h for TNF-α detection and 48 h for IL-12 detection. After stimulation, supernatant fluids were recovered and stored at −20°C. Cytokine presence in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) for human TNF-α (BD Biosciences Pharmingen, San Diego, Calif.) or for human IL-12 (Biosource).

Determination of lymphocyte proliferation, phenotypes of T cells, and cytokine production. DCs (2×10^4) were stimulated with MP1 or MP2 (5 μg/ml) for 2 days. After incubation, autologous lymphocytes (2×10^5) were added to the cultures. After 1, 2, and 5 days, proliferation was determined by [*methyl*-^{3}H]thymidine incorporation by proliferating cells. At the indicated time points, the cultures were pulsed overnight with 0.5 μCi of [*methyl*-^{3}H]thymidine (Amersham Life Science); thereafter, the cells were collected onto filter paper with a cell harvester (PBI International). The dried filters were counted directly in a beta-ray counter (Packard Instruments). Proliferation was expressed as means of results for the indicated replicates ± standard errors of the means (SEMs). In parallel experiments, at the same time that incubation was done, supernatants were recovered and the presence of IL-2, IFN-γ, and IL-10 was tested with an ELISA kit (Biosource).

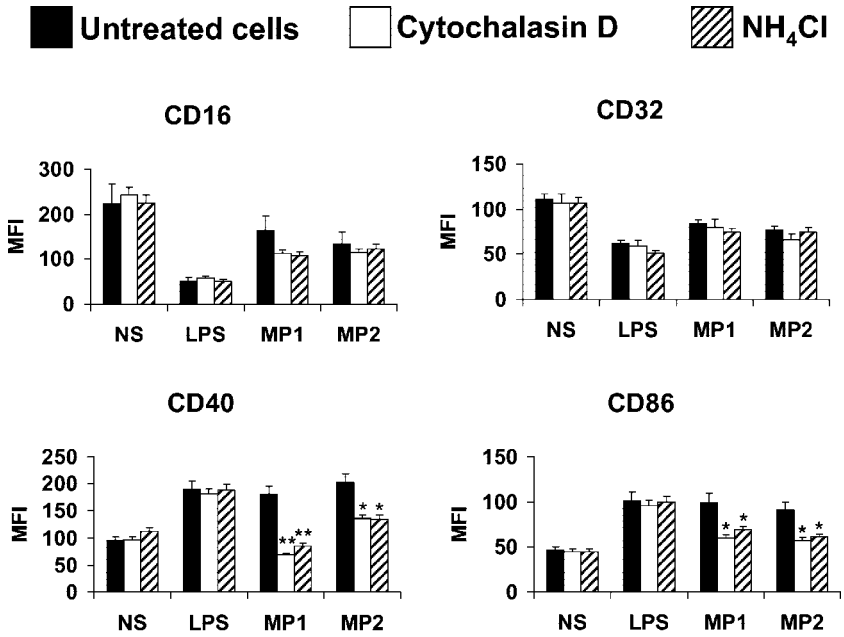

FIG. 3. Effect of cytochalasin D and NH_4Cl on CD16, CD32, CD40, and CD86 expression induced by MPs. Immature DCs were incubated for 30 min with cytochalasin D (2 μg/ml) or NH_4Cl (1 mM) and then challenged with MP1 or MP2 (5 μg/ml) or LPS (1 μg/ml) for 48 h; after incubation, the cells were stained with FITC-conjugated MAb to CD16, CD32, CD40, or CD86 and examined by flow cytometry. Results are expressed as mean fluorescence intensities (MFIs). Results represent the mean ± SEM for four separate experiments with cells from four different donors. Double asterisks indicate a P of <0.001, and single asterisks indicate a P of <0.05 (results with DCs treated with cytochalasin D or NH_4Cl versus results with untreated DCs).

In selected experiments, after 5 days, lymphocytes were recovered and collected by centrifugation, fixed in 1% paraformaldehyde in PBS, washed twice in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide, and mixed with mouse anti-human CD4–FITC conjugate (Sigma) and mouse anti-human CD8–phycoerythrin conjugate (Ancell). After 45 min of incubation on ice, the cells were washed and analyzed with a flow cytometer.

Determination of IκBα activation. DCs (10^6) were incubated in the presence of MP1 (5 and 20 μg/ml) or *C. neoformans* CAP67 (2×10^6) or with LPS (10 μg/ml) for 30 min at 37°C in cRPMI in the presence of 5% CO_2. After stimulation, the cells were washed with 1 ml of ice-cold PBS. Proteins from the cells were extracted with 200 μl of mammalian protein extraction reagent in the presence of Halt protease inhibitor cocktail (Pierce, Rockford, Ill.); lysates were collected by centrifugation for 10 min at 12,000 × *g*. The extracted proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Pierce) for 1 h at 100 V for Western blot analysis (Bio-Rad, Hercules, Calif.). Membranes were incubated in the blocking buffer containing Tris-buffered saline, 0.1% Tween 20, and 5% nonfat milk for 1 h at room temperature and then incubated overnight at 4°C with anti-IκBα or anti-phospho-IκBα (Cell Signaling, Beverly, Mass.) specific antibody. The membranes were washed several times with washing buffer (Tris-buffered saline–0.05% Tween 20) and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G in blocking buffer. After being washed, the membrane was incubated with an enhanced chemiluminescence detection system (SuperSignal chemiluminescent substrate; Pierce) and immunoreactive bands were visualized and quantified with a Chemidoc instrument (Bio-Rad).

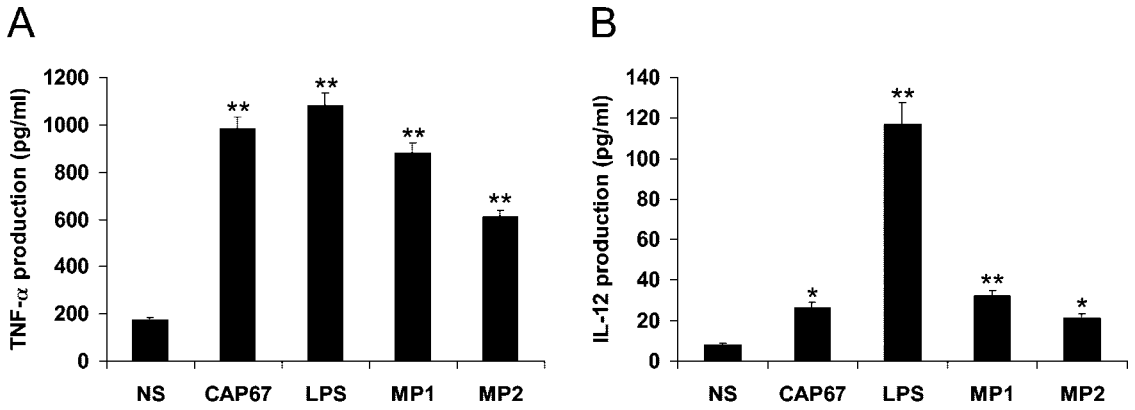

FIG. 4. TNF-α (A) and IL-12 (B) production by DCs in response to MPs. A total of 2×10^6 immature DCs/ml were stimulated for 24 h with acapsular *C. neoformans* CAP67 (4×10^6/ml), MP1 or MP2 (5 μg/ml), or LPS (1 μg/ml). After incubation, supernatants were recovered and then the presence of TNF-α or IL-12 was determined by ELISA. Results represent the mean ± SEM for three separate experiments with cells from three different donors. Double asterisks indicate a P of <0.001, and single asterisks indicate a P of <0.05 (for results with DCs treated with CAP67, LPS, MP1, or MP2 versus results with untreated DCs).

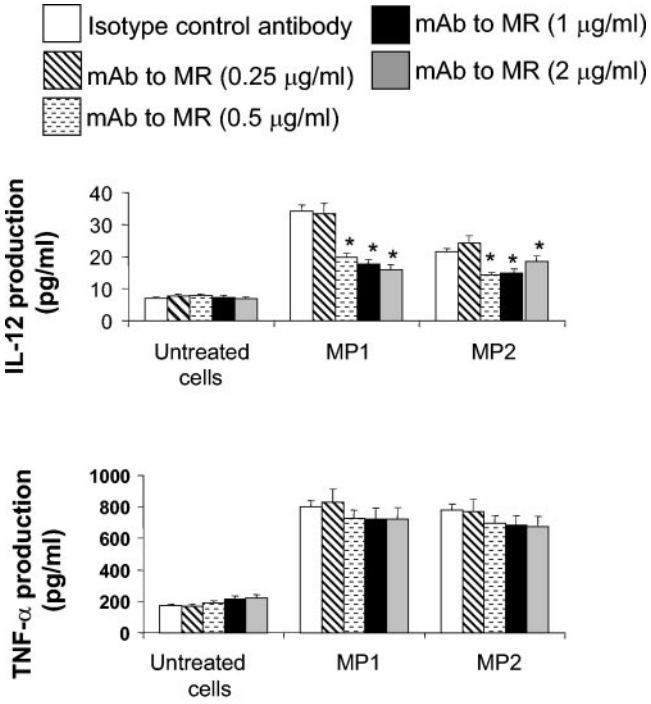

FIG. 5. Effects of MAb to MR on IL-12 and TNF-α production induced by MPs. A total of 2×10^6 immature DCs were incubated for 30 min with different concentrations of MAb to MR and then treated for 24 h with MP1 and MP2 (5 μg/ml). After incubation, supernatants were recovered and then the presence of IL-12 and TNF-α was tested by ELISA. Results, expressed in picograms per milliliter, represent the mean ± SEM for three separate experiments with cells from three different donors. An asterisk indicates a P of <0.05 (for results with DCs treated with MAb to MR versus results with DCs treated with isotype control antibody).

Statistical analysis. Statistical significance between groups was determined by an analysis of variance test. Results are presented as means ± SEMs. Each experiment was performed with different donors.

RESULTS

DC maturation induced by MP1 and MP2 of *C. neoformans*. The impact of MPs on human DC maturation was evaluated first. To this end, two MPs (MP1 and MP2) with different molecular weights were used. As shown in Fig. 1, both MPs were able to stimulate expression of CD40 at an optimal dose of 5 to 20 μg/ml. The effect was similar to that of a classical stimulator of DC maturation such as LPS (20). Under the same conditions, DCs also showed increased expression of CD86 when stimulated with MP1 or MP2, and, as for CD40 induction, 5 μg of MP/ml was sufficient to produce the highest level of stimulation, which was quantitatively similar to that of LPS.

CD14 and the receptors involved in the phagocytic process, such as CD16 and CD32, are strongly decreased in mature DCs, while CD83 and MHC classes I and II are highly expressed (13, 39). Indeed, the addition of MP1 and MP2 to DCs favored reduction of CD14, CD16, and CD32 expression; conversely, an increase of CD83 and MHC class I and II molecules was observed (Fig. 1).

MP1 and MP2 binding to MRs. It has been reported by Mansour et al. that the T-cell response to CnMP is largely dependent on recognition by MRs (27). Thus, the involvement of MRs in the observed MP-induced DC maturation was analyzed by blocking MRs with a specific monoclonal antibody (MAb) to human MR. The results illustrated in Fig. 2A show that both MP1 and MP2 need MR engagement to upregulate CD40 expression. Moreover, when the cells were stimulated with MP2, inhibition of CD40 was already evident with 1 μg of MAb to MR/ml, while for MP1, a dose of 2 μg/ml was required.

CD86 is considered an additional marker that increases during DC maturation; thus, we examined whether a MAb to MR inhibited CD86 overexpression induced by MP1 or MP2. The results show that by blocking the receptors with 2 or 10 μg of MAb to MR/ml, the upregulation of CD86 was inhibited (Fig. 2B). Conversely, upon analysis of CD32, which was downregulated after MP1 or MP2 addition, no modification was demonstrated in the presence of MAb to MR (Fig. 2C).

With parallel experiments, we tested the involvement of glucan in DC maturation. Indeed, glucan treatment (5 μg/ml for 30 min) was not able to regulate CD32, CD40, or CD86 (data not shown). Moreover, pretreatment of DCs with glucan (5 mg/ml for 30 min) before MP stimulation did not influence the MP-induced maturation (data not shown). These results suggest that the observed effects are ascribed exclusively to MP fractions.

Role of internalization and degradation of MP1 and MP2 in DC maturation. To verify whether the MP internalization and degradation process was involved in DC maturation, cells were treated with cytochalasin D, which inhibits antigen internalization by arresting microtubule polymerization, and NH_4Cl, which blocks antigen degradation by inducing intracellular alkalization (14, 24). The results illustrated in Fig. 3 show that cytochalasin D and NH_4Cl did not have any effect on the expression of CD16 and CD32, whereas upregulation of CD40 and CD86 was blocked.

DC cytokine production induced by MP1 and MP2. DCs initiate and drive the adaptive immune response via physical interaction with T cells and via production of soluble factors such as cytokines (19). Thus, cytokine levels were determined after stimulation with MP1 or MP2. The results show that appreciable levels of IL-12 (Fig. 4B) and high levels of TNF-α were detected soon after stimulation with MP1 and MP2 (Fig. 4A). Both MPs are able to induce TNF-α and IL-12, but the production of cytokines was lower than that triggered by LPS.

Next we performed experiments to verify whether the activation of DCs under our experimental conditions was through degradation of phospho-IκBα, which would account for the transmigration of nuclear factor κB (NFκB) into the nucleus (2). To this end, DCs (5×10^6) were treated with MP1 (20 μg/ml for 2 h), and the results show that there was a rapid degradation of phospho-IκBα (data not shown). These data suggest that DC maturation induced by MPs requires a cellular transduction pathway that involves phosphorylation of IκBα.

Role of uptake, internalization, and degradation of MPs in cytokine induction. The involvement of MR engagement in the induction of TNF-α and IL-12 was examined. The results illustrated in Fig. 5 show that production of IL-12, but not TNF-α, was mediated via MRs, as demonstrated by its inhibition in the presence of MAb to MR. Furthermore, inhibition of IL-12 but not TNF-α was observed in the presence of cytocha-

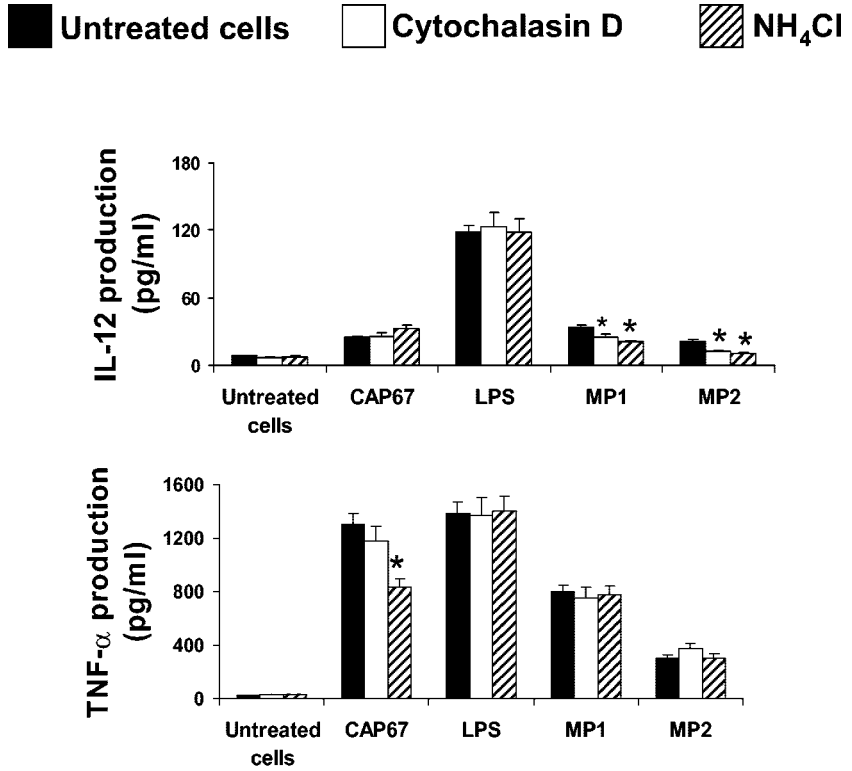

FIG. 6. Effects of cytochalasin D and NH_4Cl on IL-12 and TNF-α production induced by MPs. Immature DCs were incubated for 30 min with cytochalasin D (2 μg/ml) or NH_4Cl (1 mM) and then challenged with MP1 or MP2 (5 μg/ml), *C. neoformans* CAP67 (effector-to-target cell ratio, 1:2), or LPS (1 μg/ml) for 24 h. After incubation, supernatants were recovered and then the presence of IL-12 and TNF-α was determined by ELISA. Results, expressed in picograms per milliliter, represent the mean ± SEM for three separate experiments with cells from three different donors. An asterisk indicates a P of <0.05 (for results with DCs treated with cytochalasin D or NH_4Cl versus results with untreated DCs).

lasin D and NH_4Cl (Fig. 6). These results suggest that induction of IL-12 but not TNF-α necessitates the uptake of MPs via MRs and presumes MP internalization and degradation.

The secretion of LPS-induced IL-12 and TNF-α was unchanged in the presence of MAb to MR, cytochalasin D, or NH_4Cl.

MP-treated DCs induce T-cell activation. The functional activity of MP-treated DCs was tested by measuring its ability to induce T-cell activation. To this end, DCs were stimulated with both MP1 and MP2 and cocultured with autologous T cells for different days. As shown in Table 1, MP1 and MP2 induced a blastogenic response of T cells that reached a maximum after 5 days. The proliferating T cells were mainly $CD4^+$; moreover, the ratio of $CD4^+$ to $CD8^+$ cells did not change after stimulation (data not shown). Furthermore, we analyzed the cytokine production during T-cell activation. The results reported in Table 1 demonstrate increased production of IFN-γ and decreased production of IL-12 and IL-10 during incubation.

DISCUSSION

The results reported here show that MPs from *C. neoformans* promote DC maturation by favoring the expression of CD40, CD86, CD83, and MHC classes I and II and by inhibiting CD16 and CD32. The effect is dose dependent and occurs for MP1 and MP2. The engagement of MR triggers DC maturation through the induction of costimulatory (CS) molecules. In addition, internalization and degradation of MPs are necessary to induce CD40 and CD86 but not to regulate CD16 and CD32. DCs are able to secrete TNF-α and IL-12 when stimulated with MP1 and MP2; this activation involves IκBα phosphorylation and degradation. IL-12 induction, but not TNF-α

TABLE 1. Determination of lymphocyte proliferation and cytokine production in cocultures of MP-treated DCs plus T cells[a]

Day of incubation	Stimulus	T-cell proliferation (10^3 cpm)	Cytokine production (pg/ml)[b]		
			IL-2	IFN-γ	IL-10
1	None	2 ± 0.1	17 ± 1	3 ± 1	7 ± 1
	MP1	3 ± 0.1	96 ± 3**	30 ± 2**	37 ± 2**
	MP2	2 ± 0.2	54 ± 2**	52 ± 3**	13 ± 1*
2	None	2 ± 0.1	16 ± 0.9	4 ± 1	3 ± 1
	MP1	6 ± 2.0*	51 ± 2.1**	107 ± 5**	66 ± 6**
	MP2	8 ± 2.9*	38 ± 1.1*	130 ± 4**	45 ± 2**
5	None	3 ± 1.6	14 ± 1.1	13 ± 5	5 ± 1
	MP1	10 ± 2.5*	26 ± 0.4	118 ± 4*	20 ± 2**
	MP2	11 ± 3.4*	25 ± 0.6	185 ± 6**	16 ± 1*

[a] DCs (2×10^4) were stimulated with MP1 or MP2 (5 μg/ml) for 2 days. After incubation, autologous lymphocytes (2×10^5) were added to the culture. After 1, 2, and 5 days, proliferation levels were determined. Data shown are the mean ± SEM for results of three experiments with cells from three different donors.

[b] Asterisks indicate significant differences from results for untreated DCs plus T cells (= **, $P < 0.001$; *, $P < 0.05$).

induction, was mediated via MR uptake and requires internalization and degradation of MPs. MP-induced mature DCs are functionally active and promote T-cell proliferation and activation of a Th1 response.

In a previous paper, we demonstrated that MPs from *C. neoformans* are able to induce a Th1-type response in vitro by using monocytes and T cells separated from peripheral blood mononuclear cells from healthy donors (36), and in subsequent papers, we demonstrated that immunization with MPs induced protective responses to *C. neoformans* and *C. albicans* challenge (33, 35). DCs are critical in controlling early innate responses: they regulate long-lasting adaptive immunity and contribute to the maintenance of self-tolerance. DCs continuously monitor the environment through a multifaceted innate antigen receptor repertoire: their response to different stimuli results in conditioning of the activation of innate and then adaptive immune responses. MPs have a positive impact on DCs, thereby favoring their maturation. It is well known that mature DCs are efficient in presenting antigen and in inducing T-cell activation; in contrast, immature DCs may be tolerogenic (26). The maturation process involves enhanced expression of CS molecules and downregulation of FcγR. Indeed, both MP1 and MP2 stimulate CS molecules such as CD40, CD86, and MHC classes I and II and downregulate CD16 and CD32 in a dose-dependent manner. Upregulation of CS molecules occurs via MR engagement, which likely promotes an optimal antigen presentation process. Our results are consistent with the data published by Mansour et al. (27) showing that the blockage of MRs on antigen-presenting cells diminished MP-dependent stimulation of T cells.

MRs can be rapidly internalized, and their function involves ligand delivery into the intracellular compartment (10). Our observations that internalization and degradation of MPs are necessary to produce upregulation of CS molecules reinforce the role of MRs in transporting and delivering the cargo into late endosomes (10) that are characterized by the presence of active lysosomal hydrolases, which should effect the final degradation of MPs. Thus, carbohydrate residues could be necessary to promote MR engagement, and the protein portion may play a fundamental role in antigenic peptide presentation and in driving the final T-cell response.

Moreover, the observations that blocking of MP binding to MR and blocking of MP internalization and degradation inhibits the increase of CS molecules suggest that these processes are strictly correlated and specifically involve CS molecule induction but not expression of CD16 and CD32. This suggestion implies that the regulation of CD16 and CD32 occurs through other, undefined DC receptors. In our experimental system, cytokine induction by MPs, different from that by LPS, required MP internalization and degradation, suggesting that MPs and LPS activate DC via different signal pathways.

DCs produce cytokines, particularly IL-12, upon antigen encounter and can thus influence the ensuing adaptive immune response. Indeed, IL-12 and TNF-α are produced by DCs in response to MPs; however, only IL-12 release depends on the physical engagement of MR and MP internalization and degradation. Therefore, it is conceivable that IL-12 and TNF-α are induced by different cellular receptors and are probably secreted independently, at least in the first phase of MP-DC interaction. We speculate that the maturation and activation processes likely involving MR and other cellular receptors may be dependent on different MP-immunodominant epitopes recognized by DCs.

The production of proinflammatory cytokines is usually associated with activation of NFκB. In this study, we observed IκBα phosphorylation that has been implicated in the translocation of NFκB into the nucleus (18). Even though the signaling pathway transmitted by MR is not known (10), the MP pattern recognition receptors require a cellular transduction pathway that involves phosphorylation of IκBα.

DCs treated with MPs stimulate T-cell proliferation and production of IFN-γ. Kinetic analysis of cytokine production showed that there is a constant increase in IFN-γ levels; in contrast, there is a decrease in IL-2 and Il-10. This suggests that in the first phase of the immune response, both Th1 and Th2 responses are present, but in the second phase, the Th1 response dominates that of Th2.

The evidence that DCs stimulated with MP1 and MP2 induce T-cell proliferation and differentiation into Th1 promotes the role of these DCs in orchestrating a protective response against cryptococcal infection.

Various CnMPs that have been characterized (6, 23, 27) share many biological effects. In the present study, we found that MP1 and MP2 work in substantially similar manners to elicit protective responses.

Collectively, these data evidence two major points. First, they reveal a previously unknown role for CnMPs as inducers of DC maturation and activation. This role could be shared by other CnMPs and by MPs from other fungi such as *C. albicans* and *Aspergillus fumigatus* and may have important implications in the design of vaccines that induce protective immune responses to fungi. Second, aside from the physiological importance of MRs as mediators in binding a wide variety of microorganisms, including *C. albicans* (11), *Pneumocystis carinii* (12), *Leishmania donovani* (10), *Mycobacterium tuberculosis* (41), and *Klebsiella pneumoniae* (1), the proposed role of MRs as regulators of DC maturation and IL-12 secretion has potential implications for immunotherapy.

ACKNOWLEDGMENTS

This work was supported by grants to A.V. from MIUR, Rome, Italy (Prot. 2003068044_006), from the Italian Higher Institute of Health 1AF/F6 project, and from the EC Galar Fungail II project in the 6th Framework Programme on Research, Technological Development and Demonstration.

We are grateful to Jo-Anne Rowe for editorial assistance.

REFERENCES

1. **Athamna, A., I. Ofek, Y. Keisari, S. Markowitz, G. G. S. Dutton, and N. Sharon.** 1991. Lectinophagocytosis of encapsulated *Klebsiella pneumoniae* mediated by surface lectins of guinea pig alveolar macrophages and human monocyte-derived macrophages. Infect. Immun. **59:**1673–1682.
2. **Bowie, A., and L. A. O'Neill.** 2000. Oxidative stress and nuclear factor-κB activation: a reassessment of the evidence in the light of recent discoveries. Biochem. Pharmacol. **59:**13–23.
3. **Casadevall, A., and J. R. Perfect.** 1998. *Cryptococcus neoformans*, 1st ed. ASM Press, Washington, D.C.
4. **Chaffin, W. L., J. L. López-Ribot, M. Casanova, D. Gozalbo, and J. P. Martínez.** 1998. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol. Mol. Biol. Rev. **62:**130–180.
5. **Chaka, W., A. F. Verheul, V. V. Vaishnav, R. Cherniak, J. Scharringa, J. Verhoef, H. Snippe, and A. I. Hoepelman.** 1997. Induction of TNF-alpha in human peripheral blood mononuclear cells by the mannoprotein of *Cryptococcus neoformans* involves human mannose binding protein. J. Immunol. **159:**2979–2985.

6. **Chaka, W., A. F. M. Verheul, V. V. Vaishnav, R. Cherniak, J. Scharringa, J. Verhoef, H. Snippe, and I. M. Hoepelman.** 1997. *Cryptococcus neoformans* and cryptococcal glucuronoxylomannan, galactoxylomannan, and mannoprotein induce different levels of tumor necrosis factor alpha in human peripheral blood mononuclear cells. Infect. Immun. **65:**272–278.
7. **Chang, Y. C., and K. J. Kwon-Chung.** 1994. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. Mol. Cell. Biol. **14:**4912–4919.
8. **Cherniak, R. R. E., and S. H. Turner.** 1982. A galactoxylomannan antigen of *Cryptococcus neoformans* serotype A. Carbohydr. Res. **103:**239–250.
9. **Coenjaerts, F. E., A. M. Walenkamp, P. N. Mwinzi, J. Scharringa, H. A. Dekker, J. A. van Strijp, R. Cherniak, and A. I. Hoepelman.** 2001. Potent inhibition of neutrophil migration by cryptococcal mannoprotein-4-induced desensitization. J. Immunol. **167:**3988–3995.
10. **East, L., and C. M. Isacke.** 2002. The mannose receptor family. Biochim. Biophys. Acta **1572:**364–386.
11. **Ezekowitz, R. A., K. Sastry, P. Bailly, and A. Warner.** 1990. Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. J. Exp. Med. **172:**1785–1794.
12. **Ezekowitz, R. A., D. J. Williams, H. Koziel, M. Y. Armstrong, A. Warner, F. F. Richards, and R. M. Rose.** 1991. Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. Nature **351:**155–158.
13. **Fanger, N. A., K. Wardwell, L. Shen, T. F. Tedder, and P. M. Guyre.** 1996. Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. J. Immunol. **157:**541–548.
14. **Goldman, D., X. Song, R. Kitai, A. Casadevall, M. L. Zhao, and S. C. Lee.** 2001. *Cryptococcus neoformans* induces macrophage inflammatory protein 1α (MIP-1α) and MIP-1β in human microglia: role of specific antibody and soluble capsular polysaccharide. Infect. Immun. **69:**1808–1815.
15. **Gomez, M. J., B. Maras, A. Barca, R. La Valle, D. Barra, and A. Cassone.** 2000. Biochemical and immunological characterization of MP65, a major mannoprotein antigen of the opportunistic human pathogen *Candida albicans*. Infect. Immun. **68:**694–701.
16. **Gomez, M. J., A. Torosantucci, S. Arancia, B. Maras, L. Parisi, and A. Cassone.** 1996. Purification and biochemical characterization of a 65-kilodalton mannoprotein (MP65), a main target of anti-*Candida* cell-mediated immune responses in humans. Infect. Immun. **64:**2577–2584.
17. **Herring, A. C., J. Lee, R. A. McDonald, G. B. Toews, and G. B. Huffnagle.** 2002. Induction of interleukin-12 and gamma interferon requires tumor necrosis factor alpha for protective T1-cell-mediated immunity to pulmonary *Cryptococcus neoformans* infection. Infect. Immun. **70:**2959–2964.
18. **Karin, M.** 1999. How NF-κB is activated: the role of the IκB kinase (IKK) complex. Oncogene **18:**6867–6874.
19. **Langenkamp, A., M. Messi, A. Lanzavecchia, and F. Sallusto.** 2000. Kinetics of dendritic cell activation: impact on priming of T_H1, T_H2 and nonpolarized T cells. Nat. Immunol. **1:**311–316.
20. **Lanzavecchia, A., and F. Sallusto.** 2001. The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics. Curr. Opin. Immunol. **13:**291–298.
21. **La Valle, R., S. Sandini, M. J. Gomez, F. Mondello, G. Romagnoli, R. Nisini, and A. Cassone.** 2000. Generation of a recombinant 65-kilodalton mannoprotein, a major antigen target of cell-mediated immune response to *Candida albicans*. Infect. Immun. **68:**6777–6784.
22. **Levitz, S. M.** 1991. The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcosis. Rev. Infect. Dis. **13:**1163–1169.
23. **Levitz, S. M., S. Nong, M. K. Mansour, C. Huang, and C. A. Specht.** 2001. Molecular characterization of a mannoprotein with homology to chitin deacetylases that stimulates T cell responses to *Cryptococcus neoformans*. Proc. Natl. Acad. Sci. USA **98:**10422–10427.
24. **Levitz, S. M., S.-H. Nong, K. F. Seetoo, T. S. Harrison, R. A. Speizer, and E. R. Simons.** 1999. *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. Infect. Immun. **67:**885–890.
25. **Levitz, S. M., and E. A. North.** 1997. Lymphoproliferation and cytokine profiles in human peripheral blood mononuclear cells stimulated by *Cryptococcus neoformans*. J. Med. Vet. Mycol. **35:**229–236.
26. **Mahnke, K., J. Knop, and A. H. Enk.** 2003. Induction of tolerogenic DCs: 'you are what you eat. ' Trends Immunol. **24:**646–651.
27. **Mansour, M. K., L. S. Schlesinger, and S. M. Levitz.** 2002. Optimal T cell responses to *Cryptococcus neoformans* mannoprotein are dependent on recognition of conjugated carbohydrates by mannose receptors. J. Immunol. **168:**2872–2879.
28. **Mencacci, A., A. Torosantucci, R. Spaccapelo, L. Romani, F. Bistoni, and A. Cassone.** 1994. A mannoprotein constituent of *Candida albicans* that elicits different levels of delayed-type hypersensitivity, cytokine production, and anticandidal protection in mice. Infect. Immun. **62:**5353–5360.
29. **Murphy, J. W.** 1998. Protective cell-mediated immunity against *Cryptococcus neoformans*. Res. Immunol. **149:**373–386, 519–522.
30. **Nguyen, T. H., G. H. Fleet, and P. L. Rogers.** 1998. Composition of the cell walls of several yeast species. Appl. Microbiol. Biotechnol. **50:**206–212.
31. **Nisini, R., G. Romagnoli, M. J. Gomez, R. La Valle, A. Torosantucci, S. Mariotti, R. Teloni, and A. Cassone.** 2001. Antigenic properties and processing requirements of 65-kilodalton mannoprotein, a major antigen target of anti-*Candida* human T-cell response, as disclosed by specific human T-cell clones. Infect. Immun. **69:**3728–3736.
32. **Orendi, J. M., A. F. Verheul, N. M. De Vos, M. R. Visser, H. Snippe, R. Cherniak, V. V. Vaishnav, G. T. Rijkers, and J. Verhoef.** 1997. Mannoproteins of *Cryptococcus neoformans* induce proliferative response in human peripheral blood mononuclear cells (PBMC) and enhance HIV-1 replication. Clin. Exp. Immunol. **107:**293–299.
33. **Pietrella, D., R. Cherniak, C. Strappini, S. Perito, P. Mosci, F. Bistoni, and A. Vecchiarelli.** 2001. Role of mannoprotein in induction and regulation of immunity to *Cryptococcus neoformans*. Infect. Immun. **69:**2808–2814.
34. **Pietrella, D., P. Lupo, F. Bistoni, and A. Vecchiarelli.** 2004. An early imbalance of interleukin 12 influences the adjuvant effect of mannoproteins of *Cryptococcus neoformans*. Cell. Microbiol. **6:**883–891.
35. **Pietrella, D., R. Mazzolla, P. Lupo, L. Pitzurra, M. J. Gomez, R. Cherniak, and A. Vecchiarelli.** 2002. Mannoprotein from *Cryptococcus neoformans* promotes T-helper type 1 anticandidal responses in mice. Infect. Immun. **70:**6621–6627.
36. **Pitzurra, L., R. Cherniak, M. Giammarioli, S. Perito, F. Bistoni, and A. Vecchiarelli.** 2000. Early induction of interleukin-12 by human monocytes exposed to *Cryptococcus neoformans* mannoproteins. Infect. Immun. **68:**558–563.
37. **Pitzurra, L., A. Vecchiarelli, R. Peducci, A. Cardinali, and F. Bistoni.** 1997. Identification of a 105 kilodalton *Cryptococcus neoformans* mannoprotein involved in human cell-mediated immune response. J. Med. Vet. Mycol. **35:**299–303.
38. **Quinti, I., C. Palma, E. C. Guerra, M. J. Gomez, I. Mezzaroma, F. Aiuti, and A. Cassone.** 1991. Proliferative and cytotoxic responses to mannoproteins of *Candida albicans* by peripheral blood lymphocytes of HIV-infected subjects. Clin. Exp. Immunol. **85:**485–492.
39. **Reis e Sousa, C., A. Sher, and P. Kaye.** 1999. The role of dendritic cells in the induction and regulation of immunity to microbial infection. Curr. Opin. Immunol. **11:**392–399.
40. **Sallusto, F., and A. Lanzavecchia.** 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. **179:**1109–1118.
41. **Schlesinger, L. S.** 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J. Immunol. **150:**2920–2930.
42. **Stephen, C., S. Lester, W. Black, M. Fyfe, and S. Raverty.** 2002. Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. Can. Vet. J. **43:**792–794.
43. **Syme, R. M., J. C. Spurrell, E. K. Amankwah, F. H. Green, and C. H. Mody.** 2002. Primary dendritic cells phagocytose *Cryptococcus neoformans* via mannose receptors and Fcγ receptor II for presentation to T lymphocytes. Infect. Immun. **70:**5972–5981.
44. **Turner, S. H., R. Cherniak, and E. Reiss.** 1984. Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans*. Carbohydr. Res. **125:**343–349.

Editor: T. R. Kozel