Cellular Microbiology (2006) **8**(4), 602–612
doi:10.1111/j.1462-5822.2005.00651.x
First published online 9 November 2005

Candida albicans mannoprotein influences the biological function of dendritic cells

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Summary

Cell wall components of fungi involved in induction of host immune response are predominantly proteins and glycoproteins, the latter being mainly mannoproteins (MP). In this study we analyse the interaction of the MP from *Candida albicans* (MP65) with dendritic cells (DC) and demonstrate that MP65 stimulates DC and induces the release of TNF-α, IL-6 and the activation of IL-12 gene, with maximal value 6 h post treatment. MP65 induces DC maturation by increasing costimulatory molecules and decreasing CD14 and FcγR molecule expression. The latter effect is partly mediated by toll-like receptor 2 (TLR2) and TLR4, and the MyD88-dependent pathway is involved in the process. MP65 enables DC to activate T cell response, its protein core is essential for induction of T cell activation, while its glycosylated portion primarily promotes cytokine production. The mechanisms involved in induction of protective response against *C. albicans* could be mediated by the MP65 antigen, suggesting that MP65 may be a suitable candidate vaccine.

Introduction

Candida albicans, a dimorphic fungus, is a component of the normal microflora of skin, mucosa and alimentary tract of the healthy host (Newman and Holly, 2001). Commensalism can easily turn into mucosal candidiasis in immunocompromised subjects, such as AIDS patients (Coleman *et al*., 1993; Brawner and Hovan, 1995); moreover, deep-seated candidiasis predominantly occurs in neutropenic bone-marrow transplant patients (Viviani *et al*., 1992; Hoppe *et al*., 1997; Grossi *et al*., 2000). In healthy women the most common *C. albicans* infection is recorded at vaginal level (Fidel and Sobel, 1994).

Compelling data show that both natural and adaptive immune responses are essential to induce protection against the fungus. In particular, several studies have underlined the critical roles of polymorphonuclear leukocytes and generation of Th1-type response (Ashman, 1998).

Cell wall components involved in elicitation of host immune response are predominantly proteins and glycoproteins, the latter being mostly mannoproteins (MP). The 65 KDa MP (MP65) from the cell wall of *C. albicans* has been purified and biochemically characterized, and some of its immunological properties have been previously elucidated (Gomez *et al*., 1996; 2000). It emerges from *in vitro* studies that MP65 represents the major target of T cell response in humans (Torosantucci *et al*., 1993; Nisini *et al*., 2001) and is involved in induction of predominant Th1 response (La Sala *et al*., 1996), considered protective against *C. albicans* (Ashman, 1998). An *in vivo* study in a mouse experimental model showed that MP65 is able to induce a delayed-type hypersensitivity reaction, Th1 profile cytokines and partial protection against lethal *Candida* infection (Mencacci *et al*., 1994).

Dendritic cells (DC), which orchestrate Th-cell-dependent immunity, sense *C. albicans* yeast. Recent evidence suggests that the use of distinct recognition receptors contributes to the disparate patterns of reactivity observed in response to challenge with *C. albicans* (Roeder *et al*., 2004). In this regard it has been reported that DC recognize *Candida* cells through mannose-fucose receptor (Newman and Holly, 2001), toll-like receptor 2 (TLR2) and TLR4 (Roeder *et al*., 2004). Moreover, *C. albicans* induces immunostimulation through cell surface molecules, such as mannan, recognized by the immune system through pathogen-associated molecular patterns that are located in the cell wall of fungi (Roeder *et al*., 2004).

In previous studies we demonstrated that MP from *Cryptococcus neoformans* are able to induce protective Th1 response against both *C. neoformans* and *C. albicans* and that MP65 confers partial protection against *C. neoformans* (Pietrella *et al*., 2002). Thus, we hypothesized that MP from *C. neoformans* and MP from *C. albicans* share some common antigenic determinants (Pietrella *et al*., 2002).

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These observations prompted us to analyse the interaction of MP65 with DC that govern the subsequent T cell activation and differentiation. We therefore investigated: (i) the capacity of MP65 to bind human DC and the potential pattern recognition receptors (PRR) involved, (ii) whether a particular receptor influences DC maturation, activation and function and (iii) involvement of the protein core and glycosylated portion in Ag presentation and subsequent T cell activation.

Results

In a first series of experiments we investigated whether MP65 was able to promote proinflammatory cytokine secretion and in particular IL-12, which is considered a key cytokine for inducing Th1-type response. To this end myeloid CD11c$^+$ DC were treated with MP65 for 18 h, after which a robust production of TNF-α and IL-6 was observed (Fig. 1). IL-12 was also induced after MP stimulation as shown in Fig. 1. A kinetic profile of transcripts for IL-12, evaluated by real-time polymerase chain reaction (PCR), showed appreciable levels of expression 6 h after treatment that rapidly declined after 24 and 48 h. Maximum levels of IL-12 were observed after stimulation with avirulent *C. albicans* (CA-2). Moreover, the gene expression profile kinetics were similar for both MP65 and CA-2. The possibility that the MP activity was due to LPS contamination was ruled out by Polymyxin B addition. As shown in Fig. 1, Polymyxin B blocked the LPS effect on DC activation, but did not modify the effect of MP.

Given that mature DC are able to stimulate an efficient T cell response while immature DC and plasmacytoid CD11c$^-$ DC are involved in tolerance induction (Rutella and Lemoli, 2004), we evaluated whether MP65 is able to favour the expression of several maturation-dependent surface molecules such as CD40, CD80, CD86, MHC class II, and to inhibit FcγRs such as CD16, CD32 and CD64, that are strongly reduced in mature DC. In our experimental system immature DC were treated with MP65 for 48 h and LPS was used as a positive stimulator. The results show that CD32 expression was inhibited (Fig. 2) with a rate of inhibition similar to that achieved with LPS. CD16 expression decreased after MP65 stimulation; the reduction was less than that obtained with LPS

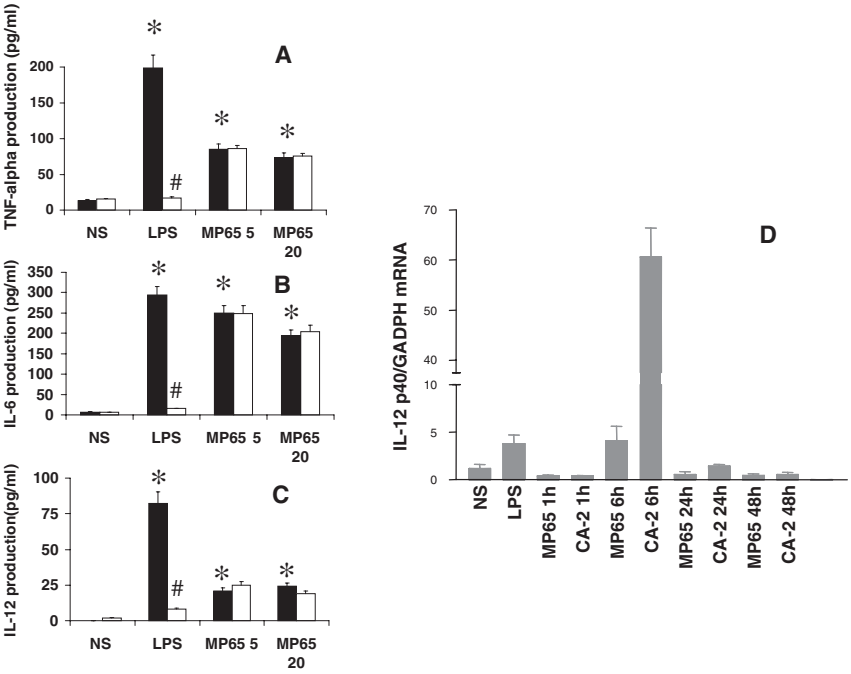

Fig. 1. TNF-α (A), IL-6 (B) and IL-12 (C) production by DC in response to MP65 and analysis of IL-12p40 transcripts (D). For cytokine production, 2×10^6 immature DC were not stimulated (NS) or stimulated with MP65 (5 or 20 μg ml^{-1}) or LPS (1 μg ml^{-1}) in presence (empty bars) or absence (solid bars) of Polymyxin B (10 μg ml^{-1}). After 24 h of incubation supernatant fluids were recovered and tested for TNF-α, IL-6 and IL-12 by ELISA. Results represent the mean of three separate experiments with cells from three different donors. For RT-PCR analysis of IL-12p40 transcripts (D), $2–5 \times 10^6$ DC were not stimulated (NS) or stimulated with 5 μg ml^{-1} of MP65 or *C. albicans* (CA-2) or LPS for different times. RNA isolation was performed with Triazol reagent and cDNA was synthesized from RNA using random primers and reverse transcriptase. Quantification of gene expression was performed by RT-PCR. All reactions were performed in triplicate and the thermal cycling conditions were: 2 min at 95°C, followed by 50 10 s cycles at 95°C, and 30 s at 60°C. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T: value where each PCR cycle reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the IL-12p40 gene and the mean C_T value of that sample for the endogenous control (GAPDH). *$P < 0.05$ (DC treated with LPS or MP65 vs. not stimulated DC), #$P < 0.001$ (DC treated with Polymyxin B and LPS or MP65 vs. DC treated with LPS or MP65).

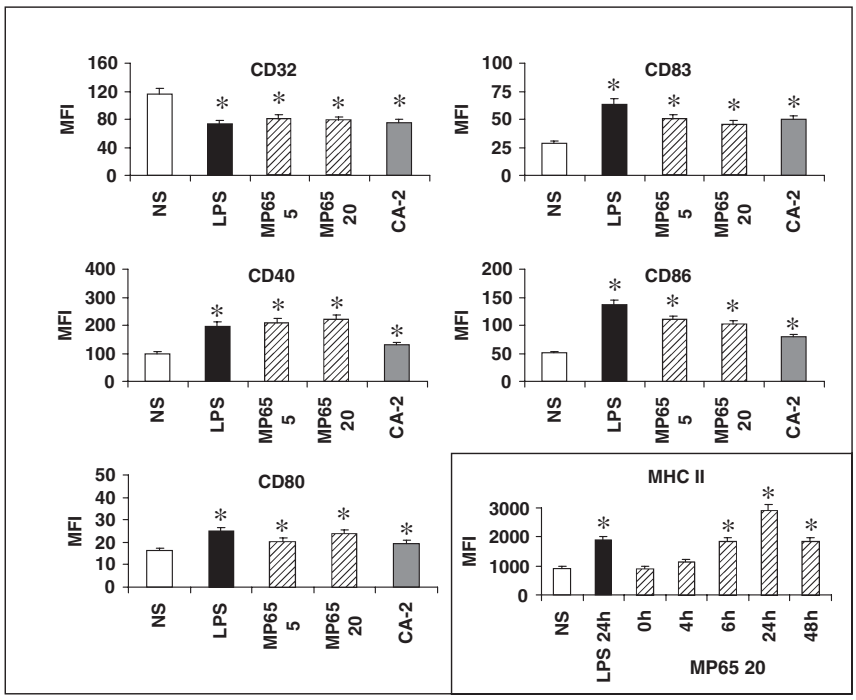

Fig. 2. Cell-surface expression of CD32, CD40, CD80, CD83, CD86 and MHC II molecules on DC treated with MP65. Immature DC (1×10^6) were not stimulated (NS) or stimulated for 48 h with *C. albicans* (CA-2, 2×10^6 ml^{-1}), MP65 (5 or 20 μg ml^{-1}) or LPS (1 μg ml^{-1}), stained with FITC-conjugated mAb to CD32, CD40, CD80, CD83, CD86, MHC II and then examined by flow cytometry. Results are expressed as mean fluorescence intensity (MFI). Results represent the mean ± SEM of three separate experiments with cells from three different donors. *$P < 0.05$ (DC treated with CA-2, MP65 or LPS vs. untreated DC).

stimulation (data not shown). Conversely, an increase in costimulatory molecules was observed after stimulation with MP65 or LPS. In particular, upregulation of CD40, CD80, CD86 and CD83 was observed (Fig. 2). Given that DC maturation is also associated to an increase in MHC molecules, we performed experiments to analyse the effect of MP65 on MHC class II expression. A significant upregulation of MHC class II expression was observed after 6 h of incubation, reaching a maximum within 24 h (Fig. 2).

To verify whether these mature DC were indeed efficient in inducing T cell activation, MP65-treated DC were co-cultured with CD4 T cells and T cell proliferation was measured. A strong induction of T cell response was manifested after 3–5 days of incubation, correlated with an increased production of IL-2 and IFN-γ (Fig. 3). Recent evidence points out that *C. albicans* exploits PRR such as mannose receptor (MR), TLR2 and TLR4. In particular, engagement of TLR4 is considered critical for induction of activation signals (Roeder *et al.*, 2004). As immature myeloid DC express TLR2 and TLR4, and MP65 is part of an external MP component of *C. albicans,* we verified whether MP65 induced myeloid DC maturation occurs via TLR2, TLR4 or via MR. The results show that upregulation of CD40 and downregulation of CD32 were abrogated when TLR4 was blocked. Conversely, no effect was manifested for CD83 and CD86 expression (Fig. 4A). Moreover the blockage of TLR2 resulted in inhibition of CD40 similar to that observed by using zymosan, a well known TLR2 ligand. Conversely no modulation was observed for CD32, CD83 and CD86 (Fig. 4A). Furthermore, by blocking TLR4 and TLR2, a drastic reduction of TNF-α was observed (Fig. 4B), internal control LPS and zymosan were used for stimulating the cells. MR involvement in DC maturation was tested in parallel experiments and the results showed that the blockage of MR did not influence either modulation of costimulatory molecules or expression of FcγRs, such as CD16 and CD32 (not shown).

Toll-like receptor signalling has been shown to involve members of the myeloid differentiation protein MyD88 family of adaptor proteins (Imler and Zheng, 2004). The post-receptor pathway that leads to NF-κB activation begins with the assembly of a membrane-proximal complex between TLR and adaptor molecules, such as MyD88 and IL-1-receptor-associated kinases (IRAK1). Eventually, phosphorylation of IkBα, the NF-κB inhibitor, affected by the IkBα kinases, releases NF-κB, which translocates to the nucleus and modulates gene expression (Janssens and Beyaert, 2002). Thus, we analysed whether MP65 treatment involves MyD88-dependent pathways. The results obtained after MP65 stimulation show a clear involvement of MyD88, activation starts after 30 min of incubation, reaching a maximum after 24 h. Concomitantly, IRAK1 expression following MP65 stimulation was observed and phosphorylation of IkBα was evidenced after 30 and 120 min with a subsequent degradation after

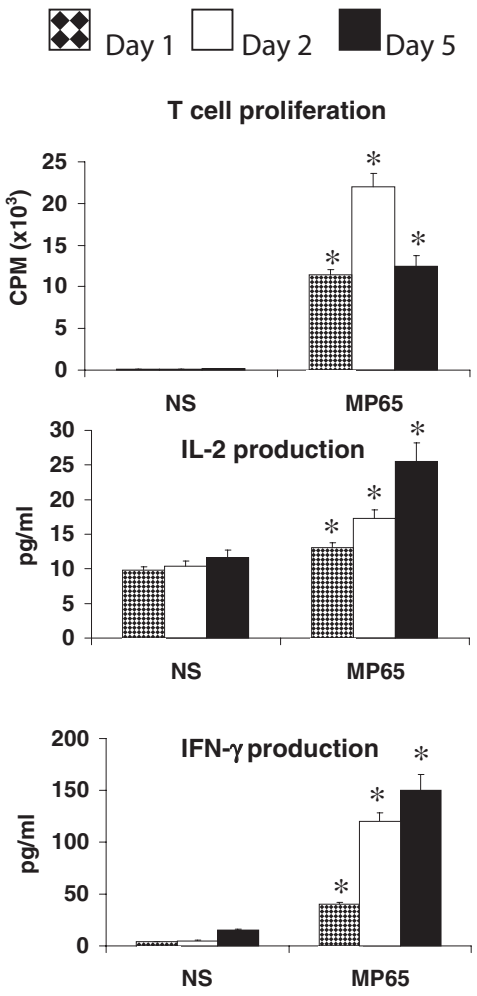

Fig. 3. Determination of T cell proliferation and IL-2 production induced by DC stimulated with MP65. DC (2×10^4) were stimulated with MP65 (5 μg ml^{-1}) for 2 days. After incubation, autologous lymphocytes (2×10^5) were added to the culture. After 1, 2 and 5 days proliferation was determined by [methyl-^{3}H]-thymidine incorporation by proliferating cells. Proliferation results are reported as cpm ($\times 10^3$), IL-2 and IFN-γ production were reported as pg ml^{-1}. Results are the mean of three experiments with cells from three different donors. *$P < 0.05$ (DC treated with MP65 plus T cells vs. DC untreated plus T cells).

24 h. IkBα generated after LPS stimulus appeared to be degraded later (after 24 h) (Fig. 5).

Dendritic cells were treated with MP65 in the presence or absence of mAb to MR, to determine possible involvement of MR in induction of proinflammatory cytokines, and TNF-α, IL-12, and IL-6 were tested in supernatant fluids after 24 h of incubation. The results reported in Fig. 6A show that blockage of MR results in inhibition of IL-12, while TNF-α or IL-6 appears unaffected. Given that MR is involved in IL-12 induction, we evaluated whether it is also involved in promoting T cell activation and Th1 response. To this end, MP65-treated DC were co-cultured for 1, 2, 5 days with autologous T cells and T cell proliferation and IFN-γ production were evaluated in the presence or absence of mAb to MR. The results reported in Fig. 6B show that both T cell proliferation and IFN-γ production were strongly inhibited by blockage of MR. This phenomenon was probably influenced by both inhibition of antigen uptake and reduction of IL-12.

Subsequently, we analysed whether the core protein or the glycosyl moiety of MP65 was involved in DC activation and/or T cell response. DC were treated with deglycosylated or deproteinized MP65, after which cytokine production (Fig. 7A), T cell proliferation, and T cell polarization were determined (Fig. 7B). The protein core and glycosyl moiety participate in TNF-α induction, while IL-12 induction does not seem to be influenced by proteic portion. Conversely the deproteinization strongly influenced T cell proliferation, IL-2 and IFN-γ production. Deglycosylation did not influence the proliferative response and the polarization of T cells. Even though the deglycosylation of MP65 reduced IL-12 production, the IL12 produced was still sufficient to drive TH1 polarization of lymphocytes. Furthermore CD40 expression was evaluated after DC stimulation with MP65 deglycosylated. The results showed that CD40 expression resulted unmodulated 3 and 24 h post treatment (data not shown).

Discussion

Candida albicans induces immunostimulation via causative molecules including MP, which are recognized by immune cell PRR. In particular, MP65 has been shown to promote T cell proliferation (Nisini *et al*., 2001) and differentiation into Th1 subsets (La Sala *et al*., 1996). We recently demonstrated that MP65 shares antigenic properties with MP from *C. neoformans* and is able to promote survival of mice treated with *C. albicans* or *C. neoformans* (Pietrella *et al*., 2002).

In this study we analysed the interaction of MP65 with human immature myeloid DC and demonstrated that MP65: (i) facilitates DC maturation by increasing CS molecule expression and by decreasing CD14 and FcγR expression, (ii) promotes stimulation of proinflammatory cytokines, such as TNF-α and IL-6, (iii) induces appreciable expression levels of transcripts for IL-12 6 h post treatment, (iv) induces DC maturation via TLR2 and TLR4 and the TLR-MyD88 pathway is involved in this process, (v) enables DC to activate T cell response and lastly (vi) promotes cytokine production through its glycosylated portion while its protein core is essentially involved in induction of T cell response.

It has been reported that MP65 is able to induce proliferation of PBMC and production of IL-1β, IL-6 and IFN-γ, but not of IL-4 (Torosantucci *et al*., 1993). A subsequent study demonstrated that PBMC from healthy donors responding to MP65 resulted in memory T cells being able

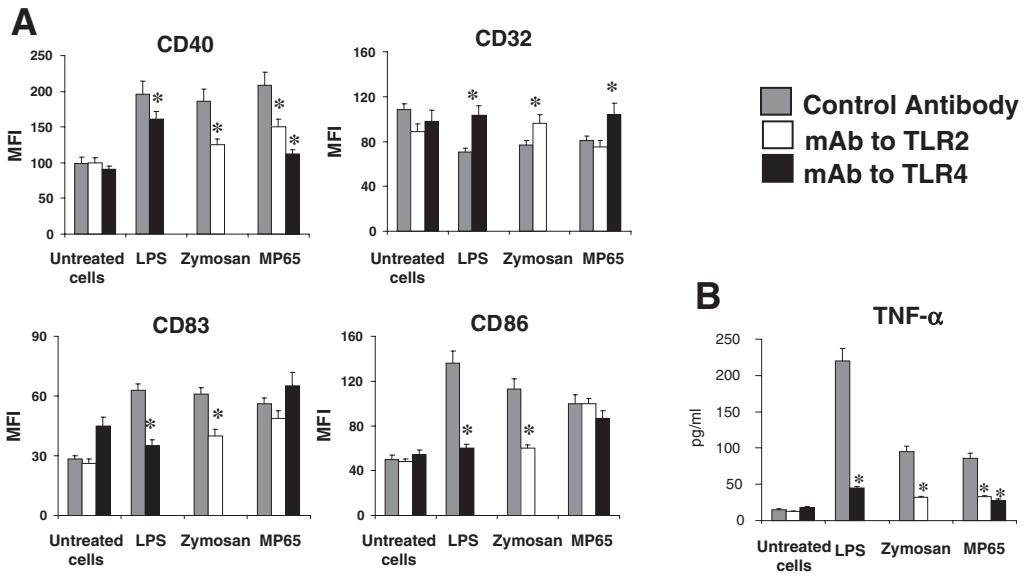

Fig. 4. Effect of mAb to TLR2 and TLR4 on CD40, CD32, CD83 and CD86 expression and TNF-α production induced by MP65. Immature DC were incubated for 30 min with mAb to TLR2, mAb to TLR4 or control antibody (2 μg ml^{-1}) and then treated with MP65, LPS (1 μg ml^{-1}) or Zymosan (500 μg ml^{-1}) for 48 h. After incubation, cells were stained with FITC-conjugated mAb to CD40, CD32, CD83 and CD86 and examined by flow cytometry. Supernatant fluids were recovered and tested for TNF-α production after 24 h of incubation. Results are expressed as mean fluorescence intensity (MFI), and represent the mean ± SEM of three separate experiments with cells from three different donors. *$P < 0.05$ (DC treated with mAb to TLR2 or mAb to TLR4 vs. DC treated with isotype control antibody).

to produce IFN-γ (La Sala *et al*., 1996). In addition, stimulation of delayed-type hypersensitivity response and induction of Th1 cytokines has been reported in a mouse experimental model (Mencacci *et al*., 1994). Finally, recent evidence qualifies MP65 as an important adhesin involved in the virulence expression of the fungus, therefore MP65 neutralization by antibodies is protective against experimental candidiasis (Antonio Cassone, unpubl. results). The present report clarifies some aspects of MP65 interaction with DC, which are quintessential APC, and describes cellular and molecular events that lead to DC activation and maturation.

Dendritic cell activation was evaluated by induction of proinflammatory cytokines TNF-α, IL-6 and IL-12; the latter being a critical cytokine that drives Th1 protective response characterized by IFN-γ production. The kinetic profile of mRNA encoding IL-12 was comparable for both MP65 and the avirulent strain of *C. albicans* (CA-2), although stimulation by CA-2 was quantitatively more powerful than stimulation by MP65. This suggests that the immunostimulating properties of the purified MP65 antigen could be comparable to those of intact avirulent live yeasts, which multiply in organs and induce protection against a lethal challenge with a highly virulent strain. DC maturation was inferred by an increase in costimulatory molecules, such as CD40, CD80, CD86, CD83 and MHC class II molecules as well as by inhibition of FcγRs, such as CD16 and CD32. Immature myeloid DC CD11c$^+$ express TLR2 and TLR4 in contrast with plasmacytoid DC, that express TLR7 and TLR9 (Kadowaki *et al*., 2001). As *C. albicans* interacts with TLR2 and TLR4, and the latter is involved in the activation of the cells (Roeder *et al*.,

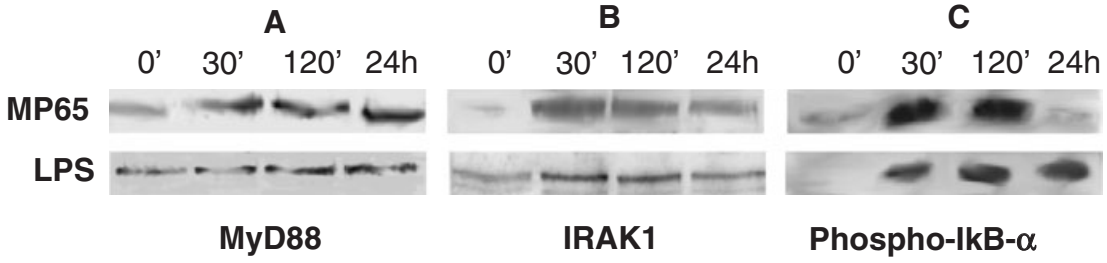

Fig. 5. Effect of MP65 on NF-κB activation dependent on the MyD88 pathway. Immature DC (5×10^6) were treated for 2 h with MP65 (5 μg ml^{-1}) or LPS (1 μg ml^{-1}). Then incubation cells were lysed and Western blotting was performed with Abs that recognize MyD88, IRAK-1 and phospho-IκBα. Data are representative of three independent experiments.

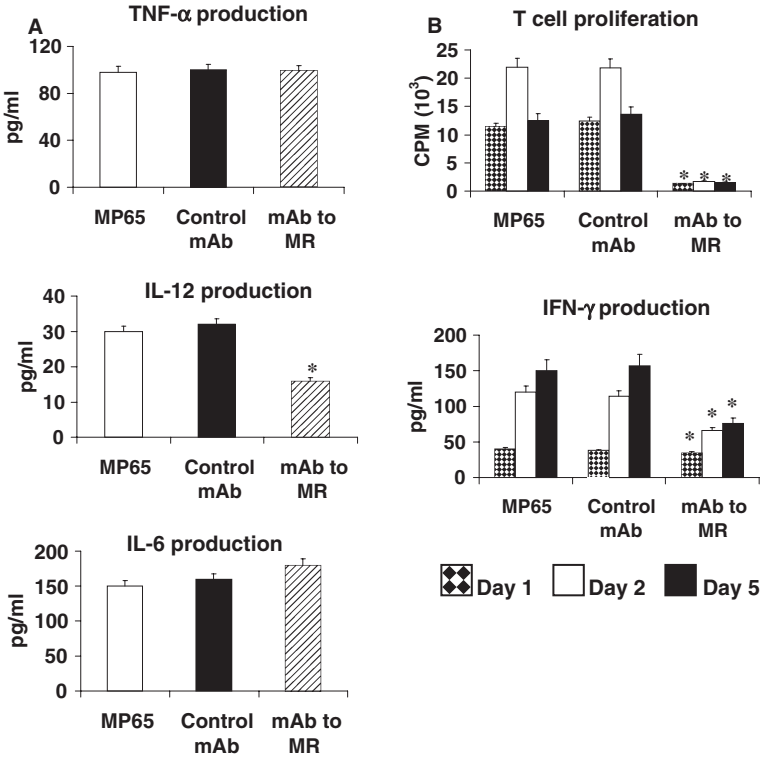

Fig. 6. Effect of mAb to MR on T cell proliferation and cytokine production. Immature DC were incubated for 30 min with control mAb or mAb to MR (2 μg ml^{-1}) and then treated for 24 or 48 h with MP65 (5 μg ml^{-1}). After 24 h of incubation, supernatants were recovered, then TNF-α, IL-12 and IL-6 presence was tested by ELISA. To determine T cell proliferation and IFN-γ production, DC were treated with MP65 and after 48 h of incubation, autologous lymphocytes were added to the culture. Proliferation was measured by [methyl-^{3}H]-thymidine incorporation after 1, 2 or 5 days of incubation. In parallel experiments supernatants were harvested and IFN-γ was tested by ELISA. Proliferation results are reported as cpm (× 10^3) and cytokine production were reported as pg ml^{-1}. Results are expressed as pg ml^{-1}, and represent the mean of three separate experiments with cells from three different donors. *$P < 0.05$ (DC treated with mAb to MR vs. DC treated with isotype control antibody).

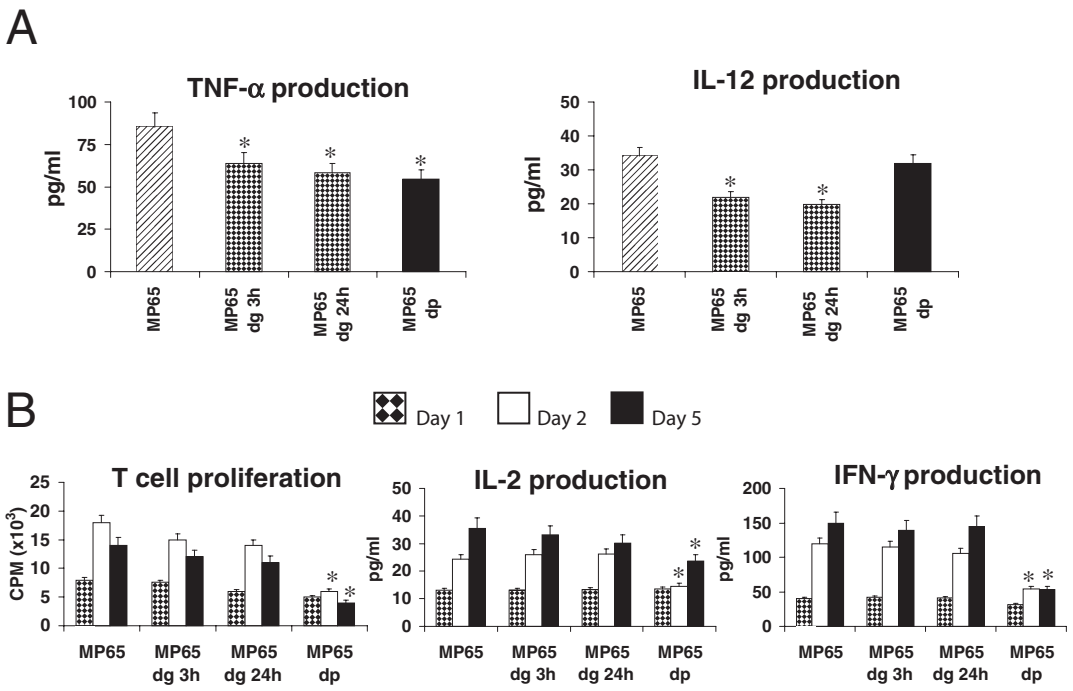

Fig. 7. Effect of deglycosylation and deproteinization of MP65 on TNF-α and IL-12 production by DC (A) and determination of lymphocyte proliferation, IFN-γ and IL-2 production (B) induced by DC stimulated with MP65. For deglycosylation, MP65 was treated with 0.1 M NaOH for 3 and 24 h. After incubation the pH was neutralized by adding acetic acid and the deglycosylated protein (MP65 dg 3 h or MP65 dg 24 h) was used in the experiments. Digestion of the MP was performed by enzymatic treatment with proteinase K. The digested MP65 (MP65 dp) was recovered and used in the experiments. *$P < 0.05$ (DC treated with MP65 deglycosylated or deproteinized vs. DC treated with MP65).

2004), the possibility that MP65 could affect these receptors was considered in this study. Partial involvement of TLR2 and TLR4 in MP65-induced maturation was demonstrated by blockage of TLR4 and TLR2 that resulted in complete inhibition of CD40 and CD32 regulation. CD83 and CD86 expression induced by MP65 was not altered by blockage of TLR2 and TLR4, suggesting that regulation of these molecules is independent from TLR2 and TLR4 and possibly involves different receptors.

It is well known that TLR activation usually occurs via MyD88-dependent signalling, through IRAK1 and IkBα phosphorylation, and that the latter promotes the translocation of NFκB into the nucleus (Janssens and Beyaert, 2002). A pathogen can activate different members of the MAPK family, the NF-κB cascade and the phosphoinositide 3-kinase (PI3-K) cascade. The first pathway is associated with inflammatory response, the second induces tolerance. Recent evidence suggests that Langherans cells stimulated with a human papillomavirus protein, show the activation of PI3-K which in turn reduces Akt. Akt is involved in downregulation of MAPK pathway and inhibits IkB phosphorylation (Fausch *et al*., 2005). Indeed we observed that activation of MyD88 takes place via MP65 stimulation and that the signal transduction pathway involves IRAK1 and IKBα. Given the evidence that activation and maturation of DC requires the signal transduction molecule, MyD88, with consequent NFκB activation (Re and Strominger, 2001), it is conceivable that MP65 promotes cytokine secretion and DC maturation, at least in part, through a TLR-induced pathway.

Recently it has been reported that *C. albicans* binds to TLR2 and TLR4 and that protection involves MyD88-signalling (Roeder *et al*., 2004). In this study, we identify for the first time, a *C. albicans* antigen responsible for TLR2 and TLR4 engagement that, through a signal transduction pathway, which involves a complex of MyD88 and IRAK1, leads to DC maturation and activation. This strongly suggests that the molecular mechanisms involved in induction of protective response triggered by intact *C. albicans* could be ascribed in part to its external antigen, MP65.

The existence of multiple receptors for MP65 was confirmed by MR involvement in MP65-mediated IL-12 induction. The partial reduction of IL-12 production in presence of antibody to MR suggests that part of the cytokine is produced through the linkage of the mannose portion with the receptor, this hypothesis is supported by the decreased production of IL-12 by DC in response to deglycosylated MP65. It is noteworthy that nevertheless, IL-12 results similarly inhibited under both conditions: deglycosylation of MP65 and MR blockage, Th1 response resulted unaffected or abrogated respectively. This suggests that in these experimental conditions, the presence or absence of other cytokines such as IL-18, IL-23, that sustain IFN-γ production, could play a role in maintaining or suppressing, respectively, Th1 differentiation. Moreover MR did not show any involvement in TNF-α and IL-6 induction or in promoting DC maturation. There are controversial data about the role of MR in promoting protective or non-protective immune responses against microorganisms (Lee *et al*., 2003). Anyhow, it is clear that MR are involved in endocytosis of antigens (East and Isacke, 2002). Thus, MP65 could stimulate maturation and activation of DC via TLR, while the engagement of MP65 with MR is necessary for antigen internalization and presentation for the subsequent T cell activation. This is supported by previous data showing that antigen uptake mediated by MR enhances antigen presentation by DC (Tan *et al*., 1997), although multiple mechanisms are probably operative.

The analysis of the functional status of MP65-treated DC indicated that these latter cells appeared to be efficient APC, as evidenced by induction of T cell proliferation and production of IL-2, a cytokine involved in the proliferation process. Moreover, the core protein of MP65 is strongly involved in induction of T cell proliferation, as demonstrated by inhibition of T cell response using deproteinized MP65. Furthermore, deglycosylation of MP65 inhibits TNF and IL-12 release. The ability of the glycosylated MP to induce proinflammatory cytokines supports the notion that TLR2 and TLR4 bind the glycosylated moiety and transmit activation and maturation signals via MyD88. Conversely, the core protein of the MP plays a critical role in antigen presentation process and as a consequence, the proteic portion plays a fundamental role in inducing T cell response. This is consistent with previous data from other research groups working with a variety of experimental systems and approaches (Nisini *et al*., 2001). In addition, given that the protein core affects IL-12 and IFN-γ production, it is conceivable that it promotes protective TH1 response, and as a consequence, protective immunity. Moreover IL-12 is a cytokine important in TH1 polarization driven by myeloid DC (Kalinski *et al*., 1999) suggesting that both proteic and mannose portions of MP65 are essential for T cell differentiation.

Collectively our results provide new information about the role of the MP65 antigen in the induction of *C. albicans* resistance and show that (i) MP65 could be a useful tool to promote DC maturation and activation and, as a consequence, antigen presentation process, (ii) DC are potential vehicles to induce or potentiate MP65-induced resistance, (iii) the MP65 core protein and glycosylated parts are necessary for induction of protective immune response, and are primarily involved in non-overlapping effects and (iv) the employment of TLR and the MyD88-signalling pathway implicated in protective response could be achieved with MP treatment. This suggests that a suitable preparation of MP65 could be exploited as a candidate vaccine for humans.

Experimental procedures

Reagents and media

RPMI 1640 with glutamine and FCS were obtained from Gibco-BRL (Grand Island, NY). Human serum type AB was purchased from Sigma (Milan, Italy). All murine anti-human mAbs, conjugated with FITC or PE, were obtained from Ancell (Alexis Italia, Florence, Italy), and are specified in single experiments below. Isotype control Abs were purchased from Sigma. Mouse monoclonal anti-human MR, anti-human TLR2 and anti-human TLR4 were obtained from HyCult biotechnology (Uden, the Netherlands).

MP65 purification

MP65 was provided by Antonio Cassone (Istituto Superiore Sanità, Rome, Italy). The MP65 was affinity purified from the material spontaneously released from *C. albicans* cultures grown to the mycelial form as described elsewhere (Gomez *et al*., 1996). Briefly, the fungus was grown in Lee's medium with 1 pg of tucamycin ml^{-1} for 24 h at 37°C. The culture supernatant was concentrated and dialysed by ultrafiltration (Diaflow Ultrafilter YM10; Amicon, Danvers, MA) and passed through two sequential affinity columns which were prepared by covalently coupling mAb7H6 or mAb 4H12 to protein A-Sepharose CL4B (Pharmacia) with dimethylpimelimidate (Sigma). The first column (mAb 4H12) was used to minimize non-specific binding of material from the mycelial secretion to the MP65 specific mAb 7H6 column. MP65 was eluted from the second column (mAb 7H6) with 100 mM triethylamine, pH 11.5, neutralized with 2 M Tris, dialysed against double distilled water and kept at –20°C. The purified antigen was substantially free from other MP or proteins as assessed by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), silver staining, ConA detection, and immunoblotting with mAbs of polyclonal antibodies directed against other components of the mycelial secretion (Gomez *et al*., 2000). Total polysaccharide and protein composition of MP65 were determined by phenol-sulphuric acid method and Bio-Rad (Hercules, CA) protein assay respectively (Gomez *et al*., 2000).

Preparations of the various candidal components tested negative for endotoxin contamination using a *Limulus* assay (Coatest endotoxin, Kabi Diagnostica, MölIndal, Sweden) with a sensitivity of 25 pg of *Escherichia coli* LPS. Nevertheless, all *in vitro* experiments were carried out at least once in the presence of 10 μg ml^{-1} of polymixin B (Sigma), a polycationic antibiotic, to neutralize any undetected contamination with bacterial LPS (Kurt-Jones *et al*., 2000).

Microorganisms

In selected experiments the avirulent strain of *C. albicans* (CA-2) was used, the characteristics and culture conditions have been previously described (Pietrella *et al*., 1998). The yeast was inactivated at 60°C for 30 min.

In vitro *generation of human DC*

The generation of DC from human peripheral blood monocytes (PBM) was performed as previously described with minor modifications (Sallusto and Lanzavecchia, 1994). Heparinized venous blood was obtained from healthy donors and diluted with RPMI 1640 (Gibco-BRL). PBMC were separated by density-gradient centrifugation over Ficoll-Hypaque PLUS (Pharmacia Biotech, Uppsala, Sweden), recovered, washed twice and suspended in cRPMI 1640, plated onto cell-culture flasks (Corning Incorporated, Corning, NY) and incubated for 1 h at a density of 2×10^6–3×10^6 ml^{-1}. Adherent PBM were recovered using a cell scraper (Falcon, Oxford, CA), washed twice, and purified by E-rosetting to remove contaminating T cells. The recovered cells were > 98% $CD14^+$ monocytes, as evaluated by flow cytometry. Isolated monocytes ($2–3 \times 10^6$ ml^{-1}) gave rise to immature myeloid DC after culture in RPMI 1640 plus 10% FCS containing 50 ng ml^{-1} human recombinant GM-CSF (Biosource International, Camarillo, CA) and 30 ng ml^{-1} of human recombinant IL-4 (Biosource). After 6–7 days of culture, immature myeloid DC resulted positive for CD11c but negative for CD14. These immature DC become mature myeloid DC after stimulation with endotoxin (Rissoan *et al*., 1999). Immature DC were harvested, washed, and suspended in RPMI 1640 plus human serum type AB (Sigma), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), referred to as cRPMI and used for subsequent experiments.

Flow cytometry analysis of surface molecules

Surface molecule expression was quantified by flow cytometry after various culture incubation times. Suspensions of immature DC (1×10^6 $sample^{-1}$) in cRPMI were stimulated with *C. albicans* MP65 (5 and 20 μg ml^{-1}), or LPS (1 μg ml^{-1}) as a positive control for TLR4 and 500 μg ml^{-1} of Zymosan (Sigma) as a positive control for TLR2 (Kazemi *et al*., 2005), and were incubated for the indicated time. After incubation at 37°C in the presence of 5% CO_2, cells were collected by centrifugation, fixed in 1% paraformaldehyde in PBS, washed twice in PBS containing 0.5% BSA and 0.1% sodium azide, and mixed with, mouse anti-human MHC class II 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, cells were washed and analysed using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA).

Blocking of PRR uptake by DC

Immature DC were pretreated with different doses of mouse anti-human MR, anti-human TLR2 or anti-human TLR4 for 30 min before MP65, LPS or Zymosan addition.

Determination of TNF-α, IL-6 and IL-12 production

Immature DC (2×10^6 ml^{-1}) were incubated with 5–20 μg ml^{-1} of MP65, heat-inactivated *C. albicans* CA-6 (4×10^6 ml^{-1}) or 1 μg ml^{-1} of LPS in presence or absence of Polymyxin B. In preliminary experiments we tested cytokine induction in a time course and found the best production of IL-6 and TNF-α after 24 h and IL-12 after 48 h. DC were incubated with MP65 for 24 h for TNF-α and IL-6 detection, and 48 h for IL-12. After stimulation supernatant fluids were recovered and stored at –20°C. Cytokine presence in culture supernatant fluid was measured by ELISA

for human TNF-α (BD Biosciences Pharmingen, San Diego, CA) or for human IL-12 (Biosource, Camarillo, CA).

Determination of lymphocyte proliferation, phenotype of T cells and cytokine production

Dendritic cells (2×10^4) were stimulated with MP65 (5 μg ml^{-1}) for 2 days. After incubation, autologous lymphocytes (2×10^5) were added to the culture. Phytohemoagglutinin (PHA, 5 μg ml^{-1}) was used as a positive control. (After 1, 2 and 5 days, proliferation was measured by [methyl-^{3}H]-thymidine incorporation. At the indicated time points, cultures were pulsed overnight with 0.5 μCi of [methyl-^{3}H]-thymidine (Amhersham Life Science), then cells were collected onto filter paper using a cell harvester (PBI International, Milan, Italy). The dried filters were counted directly in a β-counter (Packard Instruments, Boston, MA). Proliferation was expressed as mean values of indicated replicates ± SEM. In parallel experiments, at the time of incubation, supernatants were recovered and the presence of IL-2 and IFN-γ was tested by ELISA kit (Biosource).

Determination of intracellular signals

Dendritic cells (5×10^6) were incubated in the presence of MP65 (5 μg ml^{-1}) or with LPS (10 μg ml^{-1}) for 30 min at 37°C in RPMI plus 10% HS in the presence of 5% CO_2. After stimulation cells were washed with 1 ml of ice-cold PBS. Proteins from cells were extracted with 200 μl of Mammalian Protein Extraction Reagent (M-PER) in the presence of Halt™ Protease Inhibitor Cocktail Kit (Pierce, Rockford, IL), and lysates were collected by centrifugation for 10 min at 12 000 *g*. Extracted proteins were separated by SDS-10% PAGE and then transferred to a nitrocellulose membrane (Pierce) for 1 h at 100 volt for Western blot analysis (Bio-Rad, Hercules, CA). Membranes were incubated in the blocking buffer containing TBS, 0.1% of Tween 20 and 5% non-fat milk for 1 h at room temperature, then incubated overnight at 4°C with specific antibody anti-MyD88 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IRAK1 (Santa Cruz Biotechnology) or antiphospho IκBα (Cell Signaling, Beverly, MA). The membranes were washed several times with washing buffer (TBS-0.05% Tween 20) and incubated for 1 h at room temperature with a horseradish peroxidase-conjugate anti-rabbit IgG in blocking buffer. After washing, the membrane was incubated with an enhanced chemiluminescence detection system (SuperSignal Chemilunescent Substrate, Pierce), and immunoreactive bands were visualized and quantified by Chemidoc Instrument (Bio-Rad).

RNA extraction

Dendritic cells ($2–5 \times 10^6$) were stimulated with 5 μg ml^{-1} of MP65 for different time periods. Then incubation cells were recovered and RNA isolation was performed with Trizol reagent (Life Technologies, Grand Island, NY). Extracts were made from 2 to 5×10^6 DC and samples were homogenized with 0.5 ml of Trizol reagent by passing the cell lysate several times through a pipette, then they were incubated for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes by addition of 0.1 ml of chloroform. Tubes were vigorously shaken by hand for 15 s and incubated for 2–3 min at room temperature. Samples were centrifuged for 15 min at 12 000 *g* at 2–8°C. RNA in the aqueous phase was recovered in an RNase-free tube, and 0.25 ml of isopropyl alcohol was added. Samples were incubated at room temperature for 10 min and centrifuged for 15 min at 12 000 *g* at 2–8°C. The RNA pellet was washed with 75% ethanol (1 ml), vortexed, centrifuged at 10 000 *g* for 5 min at 2–8°C, and air dried. The residue was dissolved in RNase-free water and stored at −80°C. RNA concentration was determined spectrophotometrically (optical density of 260 nm), and integrity was verified by running samples on a denaturating formaldehyde agarose gel. To prevent genomic contamination, DNase I (Invitrogen) was used for 15 min at room temperature. The DNase I was inactivated at 95°C for 5 min in presence of 2.5 mM of EDTA according to the manufacturer's directions (Amplification Grade; Life Technologies).

cDNA synthesis

cDNA was synthesized from RNA (1 μg) using random primers and reverse transcriptase (RT) (Superscript III; Invitrogen) according to the manufacturer's instructions. Genomic DNA contamination was checked by PCR of the sample, without adding RT.

Reverse transcriptase polymerase chain reaction

Quantification of gene expression was performed by RT-PCR. A 100 ng template was used in 50 μl of final volume reaction containing the following reagents: 0.3 μM of each primer and 25 μl of 2 × SYBR Green PCR Master MIX (FINNzyme, Finland). All reactions were performed in triplicate and the thermal cycling conditions were: 2 min at 95°C, followed by 50 cycles of 95°C for 10 s, and 60°C for 30 s in iCycler iQ instrument (Bio-Rad). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T: value at which each PCR cycle reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta C_T$) between the ΔC_T values of the test sample and of the control sample for each target gene. The relative quantization value was expressed and shown as $2^{-\Delta\Delta C_T}$.

All PCR primers were designed with the PRIMER3-OUTPUT software, using published sequence data from the NCBI database. Primers:

hIL-12 p40: 5′-agcagtgaggtcttaggctct-3′ and 5′-ccagtacacctgtca caaagga-3′

hGAPDH: 5′-gacaacagcctcaagatcatcagc-3′ and 5′-gtagaggcag ggatgatgttctgg-3′

Statistical analysis

Statistical significance between groups was performed using ANOVA test. Results are presented as mean ± SEM. Each experiment was performed with cells taken from three different donors.

Acknowledgements

We thank Gabriella F. Mansi for expert editorial assistance and Maria Testa and Giovanni Rizzo for their excellent technical assis-

tance. This work was supported by a grant from the Italian Ministry of Education, Universities and Research, COFIN 2003068044-006 and Marie Curie Network Grant, Galar Fungail II Project for A.V.

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