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Article Type: RESEARCH ARTICLE

The *Iranian Journal of Immunology* is a quarterly Peer-Reviewed Journal Published by Shiraz Institute for Cancer Research and the Iranian Society of Immunology & Allergy, Indexed by Several World Indexing Systems Including: Index Medicus and Pubmed

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The Effects of *Candida Albicans* Cell Wall Protein Fraction on Dendritic Cell Maturation

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ABSTRACT

Back ground: *Candida albicans* is a member of the normal human microflora. *C. albicans* cell wall is composed of several protein and carbohydrate components which have been shown to play a crucial role in *C. albicans* interaction with the host immune system. Major components of *C. albicans* cell wall are carbohydrates such as mannans, β glucans and chitins, and proteins that partially modulate the host immune responses. Dendritic cells (DC), as the most important antigen-presenting cells of the immune system, play a critical role in inducing immune responses against different pathogens. **Objective:** We investigated the effect of the cell wall protein fraction (CPF) of *C. albicans* on DC maturation. **Methods:** The CPF of *C. albicans* cells was extracted by a lysis buffer containing sodium dodecyl sulphate, 2-mercaptoethanol and phosphate-buffered saline. The extract was dialyzed and its protein pattern was evaluated by electrophoresis. Dendritic cells were purified from Balb/c mice spleens through a three-step method including mononuclear cell separation, as well as 2-h and overnight cultures. The purified CPF was added at different concentrations to DC. The purity and maturation status of DC were determined by flow cytometry using monoclonal antibodies against CD11c, MHC-II, CD40 and CD86. **Results:** Treatment of DC with 10 μ g/ml of CPF increased the expression of maturation markers including MHC-II, CD86 and CD40 on DC compared to the control group. **Conclusion:** In this study we used *C. albicans* CPF with the molecular weight of 40-45 kDa for pulsing and maturation of dendritic cells. Since according to our results CPF significantly increased the expression of maturation markers on DC, we suggest that CPF may act as an efficient immunomodulator, or may be used as a potential adjuvant to boost the host immune system against infections.

Keywords: *Candida albicans*, Dendritic cells, T Cell Proliferation, Immunomodulator

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INTRODUCTION

Candida albicans is a member of the normal microflora of the alimentary tract and the mucocutaneous membranes of a healthy host (1). It is the main cause of a wide range of human diseases and since the early diagnosis of an invasive infection is difficult, strategies for infection prevention seem to be extremely appealing (2).

The major components (80%-90%) of the cell wall of *C. albicans* include carbohydrates, mannan or polymers of mannose covalently associated with proteins to form glycoproteins, β glucan, and chitin. Proteins (6%-25%) and lipids (0.07%-1%) are present as minor wall constituents (3). The protein component of *C. albicans*, including both mannoprotein and nonmannoproteins, is composed of some 40 or more moieties. *Candida albicans* cell wall proteins have been shown to adhere to host tissues and ligands. These protein components have the capacity to stimulate and modulate the host immune responses (4).

T helper 1 (Th1) cell-mediated immunity plays a key role in protection against opportunistic pathogens such as *C. albicans* (5,6). The establishment of specific T cell responses is related to the interaction of the fungus with antigen-presenting cells (APC). Among APC, dendritic cells (DC) are particularly capable of priming and expanding *Candida*-specific T lymphocytes (7,8). Dendritic cells are the most potent APC, and play a central role in the initiation and control of immune responses (9). Moreover, depending on their subsets and maturation state, DC may also be important for the induction of immunological tolerance and regulation of T cell-mediated cytokine profiles (10).

Dendritic cell maturation is characterized by the expression of molecules such as MHC-II, CD80, CD86 and CD40 (11,12). At least two distinct pathways of DC development, including the myeloid and lymphoid ones, have been identified in mice. In this species, myeloid DC play an immune-stimulatory role and shift T cell responses to Th2, whereas the latter subset suppresses immune responses and induces Th1 immunity (10). Overall, regarding their subsets, microenvironment and maturation status, DC play an essential role in immunomodulation (13).

It has recently been reported that the *C. albicans* cell wall has some antigenic constituents with important features such as modulation of antifungal host immune responses. Fungal antigens may stimulate immune responses including DC maturation and specific cell mediated immunity (4). Therefore such components may be used as an adjuvant that can affect host immune responses. The aim of this study was to investigate whether the protein fraction of the *C. albicans* cell wall, as an immunomodulator, can induce DC maturation.

MATERIALS AND METHODS

Animals. Balb/c mice (8–12 weeks) were purchased from the Pasteur Institute (Tehran, Iran) and were kept under optimal conditions of hygiene, temperature and humidity with 12-h light/dark intervals. All experimental procedures with the animals were approved by the Ethics Committee of Tarbiat Modares University (Tehran, Iran).

Candida albicans

Strain and Culture. *C. albicans* standard strain (ATCC 10321) was grown on glucose-yeast extract peptone medium (2% glucose, 0.1% peptone, 0.3% yeast extract) contain-

ing 100 µg/ml streptomycin and 50 mg/ml chloramphenicol. The yeast cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS).

Preparation of Cell Wall Protein Fraction (CPF) from *C. albicans*. The CPF of *C. albicans* was prepared according to the Casanova method (14) with minor modifications. In brief, 0.45 mm glass beads and a lysis buffer containing sodium dodecyl sulfate (SDS, 2.3 g), 2-mercaptoethanol (0.3 M), phenylmethylsulfonyl fluoride (0.001 M) as an antiprotease and PBS (10 mM) were used for mechanical and chemical disruption of the yeast. Breakage of yeast cells in this solution was monitored by light microscopic observation. The supernatant was heated in hot water (80 °C) for 5 min, and was then put on ice. Broken yeast cells were centrifuged at 14 000 × g for 10 min at 4 °C, and the supernatant was dialyzed.

Protein concentration was determined by the Bradford assay. The protein pattern of CPF was determined by SDS-polyacryl-amide gel electrophoresis. Ultrafiltration was done to eliminate low-molecular-weight CPF, (molecular weight 40 kD). With this method the proteins with molecular weights lower than 40 kD were pelleted and the supernatant was used. The limulus amebocyte lysate test was performed to verify the absence of glucan in CPF.

Dendritic Cell Separation. Dendritic cells were prepared from Balb/c mice spleens according to the method reported elsewhere (15,16) with minor modifications. Briefly, spleens were taken from Balb/c mice under sterile conditions and injected with an enzymatic cocktail comprised of 0.5 mg/ml collagenase D (Roche, Germany) and 20 µg/ml DNase (Roche). The resultant cell suspension was kept at 4°C. Afterwards, the injected spleens were minced into very small pieces and suspended in 5–10 ml RPMI-1640 (Gibco, UK) containing collagenase D (1 mg/ml) and DNase (0.02 mg/ml). Then they were digested for 30-45 min at 4°C and 5% CO₂. To avoid cell aggregations, EDTA (5 mM, pH 7.2) was added at the end of the incubation period and the cell suspension was pipetted several times. The dissociated tissues were passed through a metal mesh and the resulting cell suspension was mixed with the suspension obtained from the enzyme cocktail injection.

The whole cell suspension was washed twice with PBS containing 5mM EDTA at 4°C, followed by centrifugation at 340 × g for 10 min. The pellet was resuspended in 2–4 ml RPMI, loaded slowly on 2-4ml 12% (wt/vol) Nycodenz (Axis-Shield, Norway) and centrifuged at 4°C and 620 × g for 16 min. Low-density cells were collected from the interface, washed twice with RPMI and cultured in complete RPMI medium containing 5% FBS, 2 mM L-glutamine, penicillin 50 IU/ml and streptomycin 50 µg (Gibco) for 120 min (16). The non-adherent cells were detached by gently washing the plates with 37°C RPMI and adherent cells were cultured for another 14-16 h in complete RPMI medium. Afterwards, the non-adherent cells were harvested and washed twice with cold PBS containing 5 mM EDTA.

Flow Cytometric Analysis. To evaluate the purity of isolated DC and the effect of CPF on DC maturation, cells were stained for their phenotypic markers by a standard direct method using monoclonal antibodies. Briefly, DC were treated on ice with 0.5% normal hamster serum for 15 min. The cells were then incubated with PE-conjugated hamster anti-mouse CD11c (1µg/10⁶ cells) in PBS containing 2% FBS for 30 min at 4°C and then with one of the FITC-conjugated rat antimouse monoclonal antibodies (BD Pharmingen, USA) including anti-MHC-II, anti-CD86 and anti-CD40 for the next 30 min. Then the cells were washed twice and resuspended in 0.5 ml of cold PBS–FCS and

kept on ice until analyzed by flow cytometry (Partec, Germany). Suitable isotype controls were used for all stainings.

Treatment of Dendritic Cells with *C. albicans* Protein Fraction. Dendritic cells were divided into three groups. The test group was pulsed with the CPF of *C. albicans*, and the other two groups were considered as the negative and positive controls. To optimize the concentration of antigen needed for DC treatment in the test group, after removing the non-adherent cells, the enriched DC were treated with different CPF concentrations (10, 50, 100 and then 5, 10, and 15 µg/ml) during an overnight culture. Dendritic cells cultured in the absence of antigen were considered as the negative control group, and the positive control group contained TNF-α-treated DC cultured in the absence of CPF. To determine DC maturation, after a 14-16 h incubation period, the nonadherent cells (enriched DC) were collected and analyzed for the surface expression of MHC-II, CD86 and CD40 using FACS flow cytometry (Partec). To verify the results, all the above experiments were repeated five times (15,17,18).

Statistical Analysis. All data are presented as the mean ± SD, and the Mann-Whitney test was used to verify that the differences between the control and test groups. P-Values ≤ 0.05 were regarded as statistically significant

RESULTS

Figure 1 shows the electrophoretic pattern of CPF of *C. albicans* on 10% agarose gel, and Figure 2 shows the electrophoretic pattern of the fraction after ultrafiltration. The concentration of CPF was determined to be 2 mg/ml with the Bradford assay.

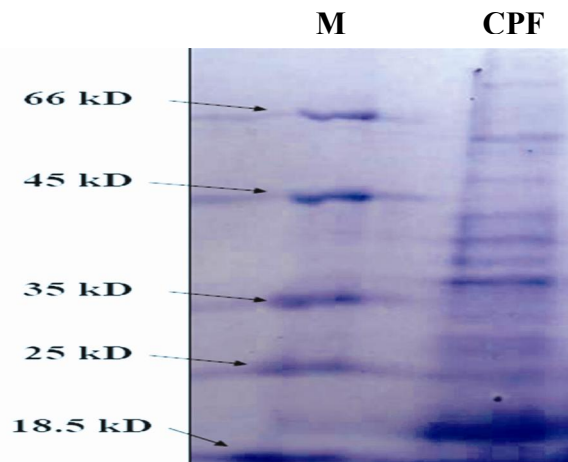


Figure 1. Electrophoretic pattern of CPF on 10% agarose gel (SDS-PAGE) before ultrafiltration. In this pattern a wide spectrum of proteins with different molecular weights is shown. M: Marker, CPF: cell wall protein fraction

Preparation of Enriched Dendritic Cells. We used Nycodenz density gradient media and a short-term culture to enrich splenic DC. Having used 12 % (wt/vol) Nycodenz, the yield of low-density cells in the first step of purification was 6%-10% of the starting cell population. After 2 h of culture and removal of the nonadherent cells, only 0.5% of spleen cells were attached to the plate. After overnight culture, DC were mature and floated. Purified DC were more than 90% viable. The yield of DC was about $3-5 \times 10^5$

cells per spleen, and they were more than 90% pure as determined by flow cytometry using staining with an anti-CD11c antibody (Figure 3).

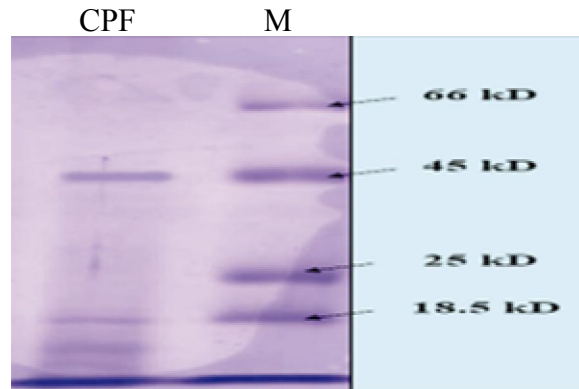


Figure 2. Supernatant electrophoretic pattern of CPF on 10% agarose gel (SDS-PAGE) after using ultrafiltration. This method eliminated many proteins with molecular weights lower than 40 KDa. M: Marker, CPF: cell wall protein fraction

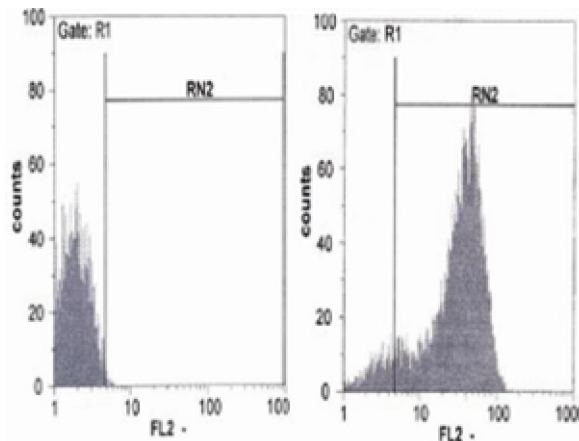


Figure 3. Analysis of mouse DC specific surface marker (CD11c) by flow cytometry. DCs were more than 90% pure (right plan). As the control, cells were treated with isotype matched anti-body to exclude any non-specific binding (left plan).

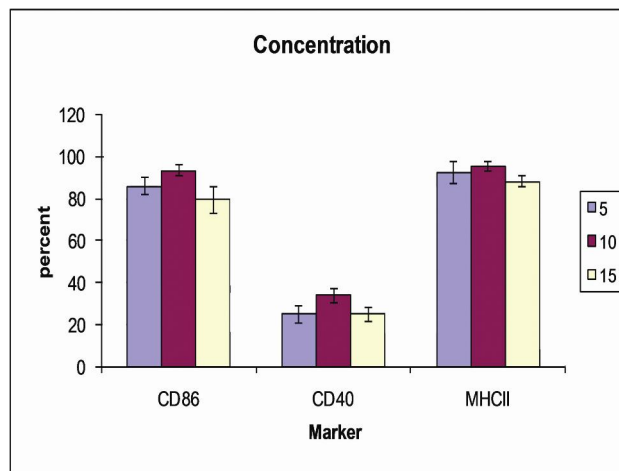


Figure 4. Comparison of surface marker expression on DC treated with different concentrations of CPF (5, 10 and 15 µg/ml) during overnight culture to optimize CPF concentration (CD40 $p=0.016$, CD86 $p=0.009$).

Dendritic Cell Maturation in Presence of Cell Wall Protein Fraction of *C. albicans*. Dendritic cells were treated with varying concentrations (5, 10 or 15 $\mu\text{g/ml}$) of *C. albicans* CPF to optimize the concentration. Analysis of the expression of CD86 and CD40 on the DC surface led to the selection of 10 $\mu\text{g/ml}$ as the optimal CPF concentration for DC treatment (Figure 4). Accordingly, DC were treated with 10 $\mu\text{g/ml}$ CPF to test the effect of this fungal fraction on DC maturation. The data from flow cytometric analysis indicated that CPF treatment significantly upregulated MHC-II, CD86 and CD40 expression on the DC surface in comparison to the negative control group (Figure 5).

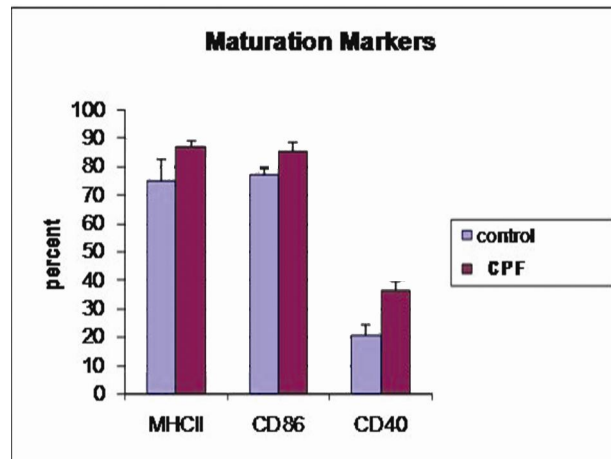


Figure 5. Comparison of maturation marker expression on the surface of DC cultured in presence (CPF group) or absence of CPF (Control group), using flow cytometry ($p=0.021$).

DISCUSSION

Upon evaluating the expression of cell surface markers including MHC-II, CD86 and CD40, we found that *C. albicans* CPF enhanced the maturation of DC as the most potent APC of the immune system.

DC are APC that are responsible for activation of undifferentiated T cells and the generation of primary T cell responses (19). In the present study we used *C. albicans* CPF with a molecular weight of about 40-45 kDa to pulse DC. The results showed that the Nycodenz density gradient media used for DC enrichment did not have any side effects on their morphologic characteristics. Using 12% (wt/vol) Nycodenz, 4%-5% of spleen cells remained in the interface. Thus DC were partially enriched in this stage. As a result of maturation overnight, DC became light, suspended and aggregated. We obtained $3-5 \times 10^5$ highly pure DC from each spleen after overnight culture.

Several studies have shown the effect of different components of *C. albicans* on DC or monocyte maturation and function. Kikuchi et al. indicated that Candida β -D-glucan enhanced the maturation of DC and their cytokine synthesis. They also suggested that Candida β -D-glucan can be used as a novel DC stimulator (20). In another study, it was shown that cell wall components of *C. albicans* such as glucan and mannoprotein efficiently stimulated chemokine production by monocytes (21). Also, a distinctive feature of DC is their ability to internalize different fungal morphotypes, such as both Candida yeast and hyphae (22).

Accordingly, we investigated the expression of common maturation markers including MHC-II, CD86 and CD40 on CPF-treated DC, and found out that treatment with 10

$\mu\text{g/ml}$ of *C. albicans* CPF enhanced the maturation of DC. Our data indicated that this protein fraction may have unique effector molecules with regulatory effects on DC. It has been shown that DC surface receptors such as TLR-2 and TLR-4 are involved in the interaction of these cells with the *C. albicans* cell wall during candidiasis (23). Therefore, the candidal fraction may have induced DC maturation via interaction with the receptors mentioned above. However, to define the exact receptors and the subsequent intracellular pathways resulting in DC maturation, further investigations is needed. Additionally, it would be tempting to investigate whether CPF-matured DC can induce Th1 cell activation and cytokine production.

In conclusion, we demonstrated in this study that the protein fraction of the cell wall of *C. albicans* can significantly induce DC maturation, providing a basis for further research on the use of *C. albicans* cell wall protein fraction as a potent adjuvant.

ACKNOWLEDGEMENTS

We wish to thank Tarbiat Modares University (Tehran, Iran) for financial support and the Immunology Department of Tarbiat Modares University, where this work was done.

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