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# *In Vitro* Effects of *Candida albicans* and *Aspergillus fumigatus* on Dendritic Cells and the Role of Beta Glucan in this Effect

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**A** – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

## Abstract

**Background.** Dendritic cells (DCs) are able to initiate and regulate the immune response to fungal infections. β-glucan stimulates the immune system, modulating cellular and humoral immunity. It has a beneficial effect in fighting fungal infections.
**Objectives.** We investigated the *in vitro* effect of *C.albicans* and *A.fumigatus* infection on human DCs. The cytokine levels were determined by ELISA.
**Material and Methods.** Human PBMCs isolation was performed by Ficoll-hypaque density gradient centrifugation method. DCs maturation was analysed by using flow cytometry. The cytokine levels were determined by ELISA.
**Results.** DCs stimulated by *C. albicans* and *A. fumigatus* induced DC maturation by increasing CD80 and CD86 co-stimulatory molecules. DCs stimulated by fungi produced IL-8 and IL-12p70. Whereas IL-10 production from the stimulated DCs did not differ from uninfected DCs. Also, the addition of β-glucan to the DCs stimulated by fungi promoted the activation and maturation of DCs.
**Conclusions.** Our results suggest that DCs are capable of initiating an innate and adaptive immune response against fungal infections. In addition, β-glucan can be used as a novel stimulator to DC-based vaccination against fungal infections (**Adv Clin Exp Med 2014, 23, 1, 17–24**).

**Key words:** dendritic cells, fungal infection, β-glucan.

Dendritic cells (DCs) are antigen presenting cells that reside in the peripheral regions such as skin, nose mucosa, the respiratory system and the digestive system and in all tissues apart from the brain, testis and eyes, and they play key roles in the initiation of adaptive immunity. These cells, recognized as professional antigen presenting cells, induce the immune response by stimulating the undifferentiated *T* cells [1]. When activated, dendritic cells mature and migrate to regional lymph nodes in order to activate antigen specific *T* cells [2]. Moreover, DCs play an effective role in the interaction between innate and adaptive immunity [3]. DCs also exist in nonlymphoid tissues consisting of antigen entrance sites such as the skin while they are widespread in lymphoid tissues [4].

DCs express great amounts of CD11c and MHC class II molecules. During their migration, DCs have some phenotypic and functional changes. When they mature, MHC molecules, co-stimulatory molecules such as CD86, CD80 and CD40 are activated. These molecules are essential for the activation of *T* cells. During maturation, *Th* cells (*T*-helper cells) are differentiated as Th1 and Th2 according to the cytokine type secreted. IL-12 secreted from DCs causes Th1 differentiation and it is important in developing the immune response against bacterial infections. At the same time, IL-10 secreted from DCs causes Th2 differentiation and this carries some importance for the immune response against extracellular parasites [5].

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DCs are the immune cells that have been the focus of many studies recently. There have been some findings that many pathogens attempt to affect the host immune response by interacting with DC functions [6]. They have crucial roles in infections, host-microbe interactions and the balance between protective immunity and immunopathology [6].

Opportunistic fungal infections such as *Candida albicans (C.albicans)* and *Aspergillus fumigatus (A.fumigatus)* cause some life threatening infections, especially during immunosuppressive conditions such as AIDS or organ transplants, and they have an increasing incidence. Some different host defense mechanisms play effective roles during infections against fungi. The innate immune response is basically effective during the early stages of the infection [6].

DCs have important roles in the initiation of cellular response against fungi. It has been discovered that DCs have direct effects on both *in vitro* and *in vivo* anti-fungal immunity. DCs phagocytose both yeast and hyphal forms of the fungus [6]. Phagocytosis of *A.fumigatus conidia* by DCs leads to the secretion of some cytokines. So, it is considered that DCs initiate the adaptive immune responses to *Aspergillus* species and directly influence the outcome of an *Aspergillus* infection [3, 7].

The changes occurring in dendritic cell structures and functions because of fungal infections play important roles in the development of antifungal immunity [3]. Therefore, the use of therapeutic approaches in fungal infections targeting DCs carry a great importance in the prevention of the agents that cause mortality especially in immunosuppressive patients and the treatment of these infections.

β-glucan (*beta-glucan*), a polysaccharides, is a component of the cell wall structure in various microorganisms such as pathogen bacteria and fungus. β-glucan is one of the strongest immune system stimulators known so far and plays an effective role in the protection against several infection agents. β-glucan has some strong effects in the immunotherapy of some diseases such as cancer. Recently, β-glucan has been accepted as an important pharmacological agent in human beings since its several immunomodulatory effects such as anti-cytotoxic, anti-mutagenic and anti-tumorigenic effects have been identified [8].

In our study, we aimed to identify the changes in the structure and function of DCs due to some fungi and the effect of β-glucan administered externally on those changes.

# Experiment

## Isolation of Human Peripheral Blood Mononuclear Cells (PBMC)

Heparinized blood was collected from 20 healthy human donors. PBMCs were separated from the blood by Ficoll-hypaque density gradient centrifugation (Sigma, UK). After centrifugation, buffy coats were collected and washed in Phosphate Buffered Saline (PBS, Gibco, Germany) 3 times and resuspended at a concentration of $2 \times 10^6$ cells/mL in a complete RPMI 1640 medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.05 mM 2ME and supplemented with 10% fetal calf serum (Gibco, Germany). Cell viability was 95% by the trypan blue exclusion test [2]. PBMCs were allowed to adhere to 6-well plates. After 2 h at 37°C the nonadherent cells were removed and the adherent cells were separated by incubated with $Mg^{2+}$ and $Ca^{2+}$ free PBS containing 0.5 mM EDTA at 37°C [2].

## Preparation of Dendritic Cell Culture

The cell culture medium (Complet RPMI) was RPMI-1640 (Sigma, Germany) supplemented with Penicillin (100 U/mL, Biochrom AG, Germany), Streptomycin (100 μg/mL, Biochrom AG, Germany), L-glutamin (2 mM, Biochrom AG, Germany), 2-mercaptoethanol (50 μM, Fluka, Switzerland), and 10% heat-inactivated FCS (Biochrom AG, Germany). At day 0, the cells were seeded at $2 \times 10^6$ per 100 mm dish in 10 mL complete RP-MI containing 50 ng/mL rhGM-CSF (Biosource, USA) and 1.000 U/mL IL-4. The culture medium was partially replaced every 3 days and fresh cytokines (rmGM-CSF and IL-4) were added. For complete maturation, on day 10, non-adherent cells were collected by gentle pipetting, centrifuged at 300 × g for 5 min at RT, and resuspended with 10 mL fresh complete RPMI in a 100 mm tissue culture plastic dish containing 100 U/mL rmGM-CSF and lipopolysaccharide (LPS, Sigma, Germany) at 1 μg/mL. The cells were then cultured for 1 or 2 more days. The cultured cells were washed once and an aliquot volume mixed 1 : 1 in Trypan Blue solution (Sigma, Germany). DCs were counted as viable under the microscope in a Neubauer chamber and the number was adjusted to $5 \times 10^6$ cells/mL.

## Cell Separation and Flow Cytometry

CD11c$^+$ DCs were purified by magnetic cell sorting (MACS) by using positive selection, according to the manufacturer's protocol (Miltenyi, USA). Briefly, the cells were incubated with magnetic microbeads conjugated with monoclonal anti-human CD11c antibodies in MACS buffer for 15 min at 4°C. After this, the cells were run through a MACS column (Miltenyi) in a magnetic field. The column was then removed from the magnet, and the positive cells were flushed out. Then CD11c$^+$ DCs were analyzed further by flow cytometer (Coulter, USA).

## Introduction of Fungi and DCs

One mL of BM-DCs ($10^6$) were delivered in per well. One mL of *A. fumigatus* ($10^6$ conidia/mL) and *C. albicans* ($10^6$ yeast/mL) were added to the wells, respectively. Thusly, the DCs were stimulated by the fungi. Amphotericin *B* was used to prevent fungal overgrowth in the wells. Control culture flasks did not contain fungi, only the medium and DCs. Each sample was studied in triplicate. β-glucan (Immunex) was added to the control culture plates and culture plates containing fungi. All plates were incubated for 24 h in 5% $CO_2$ at 37°C. At the end of the time period, the contents of the culture plates were transferred into tubes and centrifuged. The culture supernatants were removed and stored at –70°C until used in ELISA.

## Flow Cytometric Analysis of PBMCs

The pelleted cells were washed in PBS and stained with antibodies for 30 min at 4°C. The DCs ($2 \times 10^6$ cells/mL) were incubated with monoclonal antibodies specific to human CD antigens. The antibodies used were anti-CD80, and anti-CD68 (e-bioscience, USA). IgG1-FITC/IgG2-PE were used as isotypic control. Flow cytometric analyses were done by using a Coulter FC500 flow cytometer (Coulter, USA).

## Cytokine Secretion

The levels of cytokines such as IL-8 (İnterleukin-8), IL-10 and IL-12p70 were determined by specific enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer's instructions (Biosource, USA). The concentration of cytokines was determined spectrophotometrically. The absorbance was read at 450 nm (BioTek, USA). We constructed a standard curve using cytokine standards. The cytokine concentrations for unknown samples were calculated according to the standard curve.

# Statistical Analysis

The data was analyzed with the SPSS statistical package (15.0 Version). Results are expressed as mean + standard deviation (SD). The Kolmogorov-Smirnov test was used to analyze the normal distribution of the variables. The Levene's test was used to assess variance homogeneity. Data with a normal distribution was analyzed with one-way analysis of variance (ANOVA). The Bonferroni test was used as *post hoc* analysis. $P < 0.05$ was considered to be significant.

# Results

## Analysis of DCs

The analysis of cells by flow cytometry on a plot of CD11c vs side scatter showed that more than 94% of the purified cells expressed CD11c$^+$ (Fig. 1).

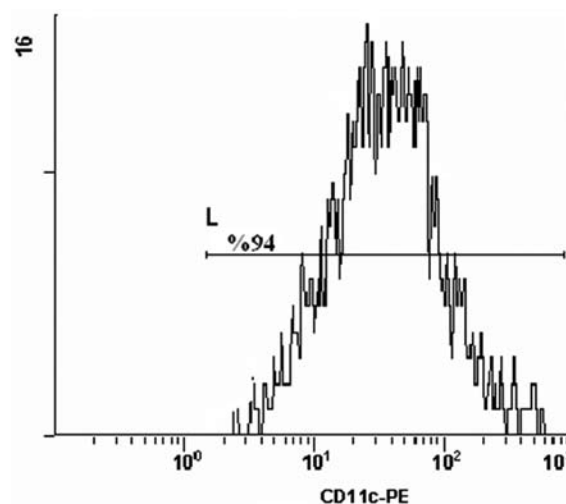


**Fig. 1.** Flow cytometric analysis of CD11c (+) DCs

## DCs Maturation

In the analysis of the effectiveness of *C. albicans* and *A. fumigatus* on DC maturation, it was found that the expression of DC co-stimulatory molecules such as CD80 and CD86 remarkably increased after the stimulation of DCs with both of the fungi. In addition, the expression of these co-stimulatory molecules increased in the wells containing DCs infected by fungi with the addition of

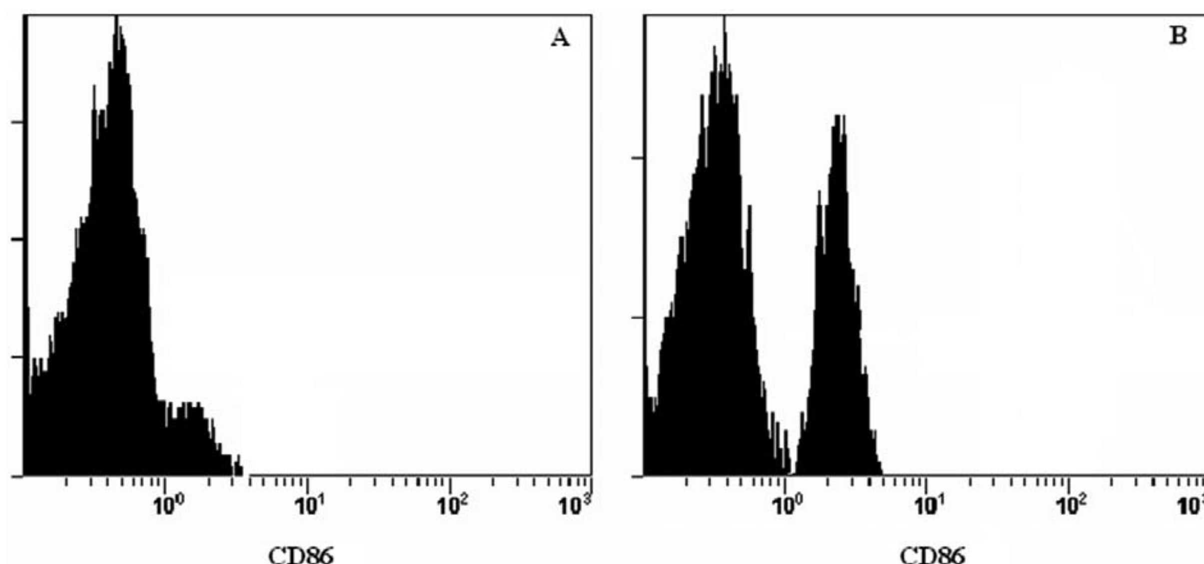


**Fig. 2.** The expression of CD86 of DCs (A. Dendritic cell, B. Dendritic cell infected with *C. albicans*)

β-glucan after fungal infection; however, this increase was not significantly different from the increase in the wells infected by fungus without β-glucan ($p > 0.05$).

The increase in the expression of these co-stimulatory molecules after the addition of β-glucan to the DCs stimulated by fungus shows that DCs have effective antigen-presenting cell function after fungal infection and in the presence of β-glucan.

## The Levels of Cytokines

In our study, we observed that the IL-8 and IL-12p70 cytokine levels increased in DCs stimulated by *C. albicans* and *A. fumigatus* compared to the wells without any infection. However, these increases were not statistically significant ($p > 0.05$). The stimulation of DCs with fungus did not lead to any significant changes in IL-10 levels compared to the wells with only DCs. Nevertheless, the addition of β-glucan to DCs stimulated with fungus caused statistically significant changes in the levels of IL-8, IL-10 and IL-12p70 ($p < 0.05$).

The changes observed in IL-8 cytokine levels after 24, 48 and 72 h are shown in Fig. 3. According to the figure, IL-8 cytokine levels after 24, 48 and 72 h in DCs stimulated with fungus increased compared to the wells with only DCs, but the changes were not statistically significant ($p = 0.291, 0.445, 1$, respectively). The addition of β-glucan to the DCs stimulated with *C. albicans* led to statistically significant changes after 24, 48 and 72 h ($p < 0.05$) (Fig. 3).

The changes observed in IL-10 cytokine levels in the wells containing DCs stimulated by *C. albicans* after 24, 48 and 72 h are shown in Fig. 4. The stimulation of DCs by fungus did not lead to significant changes in IL-10 secretion after 24, 48

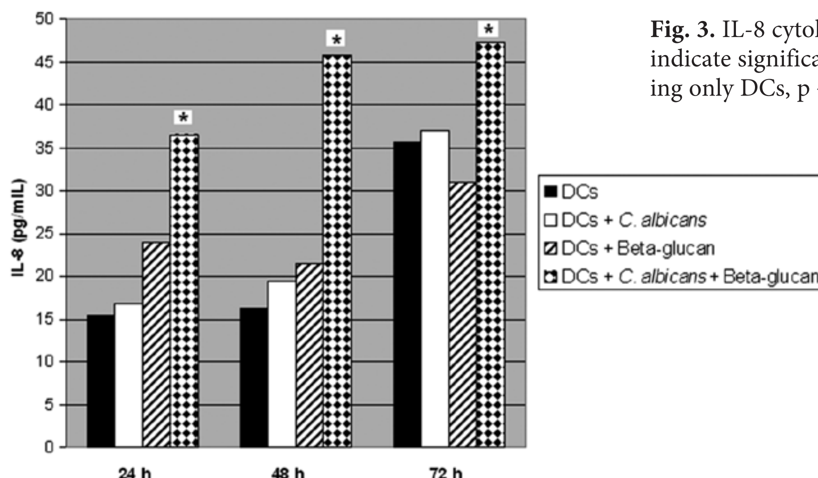


**Fig. 3.** IL-8 cytokine levels in dendritic cells (Asterisks indicate significant differences from samples containing only DCs, $p < 0.05$)

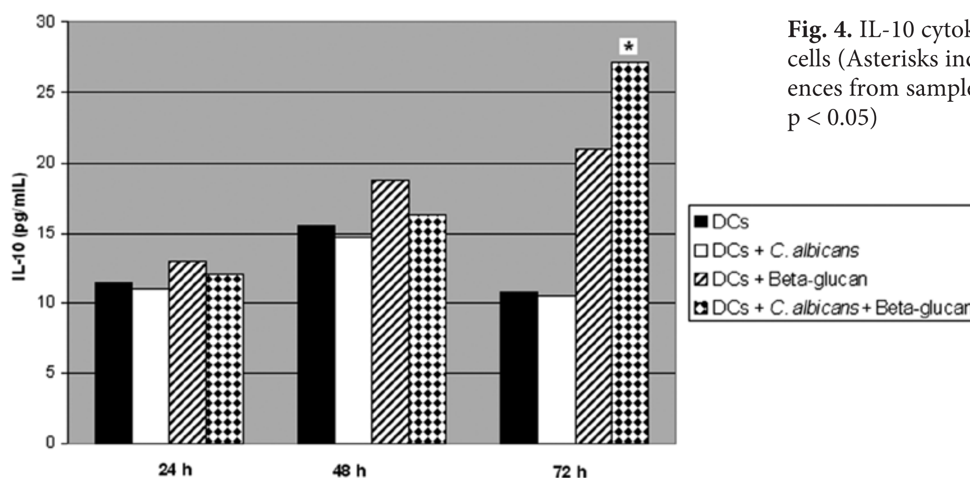


**Fig. 4.** IL-10 cytokine levels in dendritic cells (Asterisks indicate significant differences from samples containing only DCs, $p < 0.05$)

and 72 h in the wells containing only DCs ($p = 1$). While the addition of β-glucan to DCs stimulated by *C. albicans* did not cause any statistically significant increases in IL-10 levels after 24 and 48 h compared to the wells without β-glucan, there was a statistically significant increase in IL-10 levels after 72 h ($p < 0.05$) (Fig. 4).

The changes in IL-12p70 cytokine levels in the wells containing DCs stimulated with *C. albicans* after 24, 48 and 72 h are shown in Fig. 5. When DCs were stimulated with fungus, there was an increase in the secretion of IL-12p70 compared to the wells containing DCs non-stimulated by fungus. However, the changes after 24, 48 and 72 h were not found statistically significant ($p = 0.292$, 0.089, 0.477, respectively). Still, the addition of β-glucan to DCs stimulated with *C.albicans* led to statistically significant changes after 24, 48 and 72 h ($p < 0.05$) (Fig. 5).

The same results were obtained with DCs stimulated by *A. fumigatus* (data not shown).

# Discussion

DCs, professional antigen presenting cells, play important roles in the initiation of the cellular response against foreign antigens. They are also effective in providing innate defense against microbial antigens. DCs are activated by different microorganisms or their products [9]. Having been activated when the infecting agent is recognized by the DC, DCs play some roles in the activation and control of the adaptive immunity through the cytokines they secrete [10].

DCs are also important in the defense against fungal infections. DCs are effective against *C. albicans* infections as antigen presenting cells. They also phagocytose both yeast and hyphal forms of the fungus. It has been found that DCs not only phagocytose, process and present fungal to T cells, but also kill fungi as efficiently as macrophages do [11]. DCs also have an important role in the initiation of the cellular immune response against fungi. Fungi

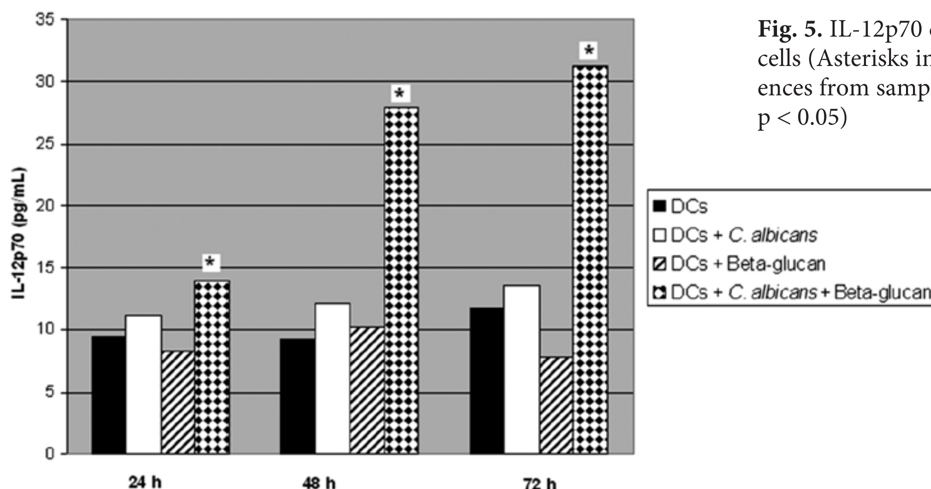


**Fig. 5.** IL-12p70 cytokine levels in dendritic cells (Asterisks indicate significant differences from samples containing only DCs, $p < 0.05$)

and its products cause a strong activation stimulus in DCs, leading to DC maturation with upregulation of co-stimulatory molecules and production of cytokines leading to different *T* cell responses. DCs are important for further treatment approaches since they are effective in enhancing the anti-fungal immune response [12]. Perruccio et al. reported that the adoptive transfer of DCs activate Th cells and effect the outcome of fungal infections in hematopoietic stem cell transplantation [6].

In our study, aimed to determine DC activation signals and the release of cytokines such as IL-8, IL-10 and IL-12p70 following DCs stimulated with *A. fumigatus* and *C. albicans* strains, we observed some changes in the cell surface co-stimulatory molecules and DC functions after DCs were infected by fungi such as *A. fumigatus* and *C. albicans*. Furthermore, the presence of β-glucan affected the activation of DCs infected by fungi.

There have been a lot of studies focusing on the immunomodulatory effects of β-glucan. However, there have been few dealing with the effects of β-glucan on the maturation and activation of DCs after fungal infections. For this reason, we aimed to determine the effects of β-glucan administration on DCs during fungal infections.

The DC maturation process is one of the important phases in the initiation of the adaptive immune response. The maturation signals for DCs may derive from various sources. This process is regulated by extracellular stimulators such as microbial products or membrane associated ligands. DC maturation occurs due to the changes in morphological, phenotypical and functional features of DCs. DC activation is characterized by cytokine secretion and the expression of certain surface markers. These are essential for DCs to stimulate effective *T* cell response. DCs affect immune response by increasing co-stimulatory molecule expression. CD80 and CD86 are the co-stimulatory molecules crucial for both *T* cell response and Th differentiation.

In our study, DCs stimulated by *A. fumigatus* and *C. albicans* led to DC maturation. DC maturation is characterized by an increase in the expression of co-stimulatory molecules on the dendritic cell surface and by the secretion of cytokines such as IL-12 that provide strong stimulators for *T* cell development and differentiation [13]. Our study showed that co-stimulatory molecules (CD80 and CD86) and proinflammatory cytokines (IL-8, IL-12p70) increased after DC was infected by *A. fumigatus* and *C. albicans.*

The increase in the expression of these co-stimulatory molecules and cytokine secretion in the DCs stimulated by *A. fumigatus* and *C. albicans* indicate the importance of DC activation during such fungal infections. However, the infection of DCs by fungus did not cause a remarkable change in IL-10 levels, an anti-inflammatory cytokine eliciting Th2 type response, compared to the DCs non-stimulated by fungus. The insignificant change in IL-10 levels after fungal infections may be explained by the fact that Th1 cell-mediated response plays an effective role in the protection against fungal infections [14].

Romagnoll et al. have reported that different forms of *C. albicans* enhance DC maturation and stimulate Th1 type response [15].

Gafa et al. have also reported that *in vitro* stimulation of DC by *A. fumigatus* increases the surface expression of CD11b and CD18 activation signals and they have added that DC plays an important role in the regulation of the innate and adaptive immune response against *A. fumigatus* by enabling the production of various chemokines [3].

Netea et al. have indicated that although DC stimulated by *C. albicans* produced a great amount of TNF-α and IL-8, TNF and IL-8 release by DC was only 5–10% of that released by monocytes or macrophages [11].

IL-12 is a cytokine that plays an important role in the interaction between innate and adaptive immunity. The expression of IL-12 is one of the most specific markers for DCs functionally activated [15]. IL-12 stimulates natural killer cells and *T* cells to secrete cytokines and to initiate lytic activity. Moreover, IL-12p70 directs the immune system towards Th1 response [16]. DCs stimulated by *C. albicans* and *A. fumigatus* play important roles in Th1 cell differentiation by generating IL-12 secretion. Clinical and experimental studies have shown that Th1 cell reactivity plays an important role in the control of fungal infections such as invasive aspergillosis [3].

β-glucan has several immunopharmacological effects such as IL-6, TNF and nitrogen oxide secretion from macrophages, the adjuvant effect in the antibody production and antitumor effect [17]. Since it enhances DC maturation, it can be used in DC-based vaccination and in combination therapies to boost DC maturation [15].

In our study, the addition of β-glucan, an important immunomodulator, to DCs stimulated by fungus caused an increase in the expression of co-stimulatory molecules and also in the proinflammatory and antiinflammatory cytokine response.

Depending on the results of our study that IL-8 and IL-12p70 secretion increased after stimulation by fungus and there was even a further increase with the addition of β-glucan, we may conclude that these cytokines may play some roles in the fungal infections and the immunomodulatory effects associated with β-glucan. The increase in

IL-8 and IL-12p70 cytokines in DCs stimulated by fungus indicates the importance of Th1 response during fungal infections. Moreover, the infection of DCs by fungus did not lead to significant changes in the secretion of IL-10, which is the indicator of Th2 response, and emphasizes again the importance of Th1 response during fungal infections.

Avasti et al. have reported that *in vitro* infection of bone-marrow derived DC in mice with dimorphic fungi such as *Coccidioides posadasii* lead to a significant increase in the levels of IL-12. They also suggest that the differences in the activation status of DC in mice may be responsible for the discrepancy in their susceptibility to *C. posadasii* [18].

The findings derived from our study show that β-glucan has strong effects on DC maturation and activation during fungal infections. It is considered that by enabling DC maturation and activation, β-glucan activates cytokine response, and so it is considered to effect the regulation of immune response [19]. Kikuchi et al. have also reported that *Candida* β-glucan may augment DC maturation [15].

Consequently, DC-based therapeutic approaches have already been considered to be important in the prevention of fungal infections. As was observed in our study, the changes in the maturation and activation of DC during fungal infections becomes more remarkable in the presence of β-glucan and so this agent may significantly enhance DC maturation and activation. The effect of β-glucan on DC endows it with an important role during the immune response. Since β-glucan is an important alternative for the therapeutic approaches regulating host immune response, DC based immunotherapeutic approaches in the presence of β-glucan for immunosuppressed patients with fungal infections are expected to be very effective in future treatments.

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