Original Article

Expression of the surface antigens of lymphocytes and the levels of cytokines in mice infected with *Aspergillus fumigatus*

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Abstract

Background: *Aspergillus fumigatus* (*A. fumigatus*) is an opportunistic fungus that causes invasive aspergillosis. Determining the immune changes during *A. fumigatus* infection and the factors leading to such changes clearly will make it possible to prevent the spread of the infection and to provide new strategies in the treatment of infection. Thus, the present study aims at determining the changes of lymphocyte surface antigens which develop during *A. fumigatus* infection and the role of cytokines in immune response.

Methodology: The expression of the surface antigens of lymphocytes was analysed by flow cytometry and the cytokine levels were determined by ELISA in a mouse model of aspergillosis.

Results: It was observed that in mice infected by *A. fumigatus* the percentage of CD19+ B cells and the levels of IL-4 and IL-10 increased when compared to those in noninfected mice cells.

Conclusions: These results suggest that Th2 type cytokines are important in the pathogenesis of *A. fumigatus* infection. Humoral immunity is considered to be effective during *A. fumigatus* infection because of the increase in Th2 type response.

Key Words: *Aspergillus fumigatus*, flow cytometry, immune response.

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Introduction

Aspergillus fumigatus (*A. fumigatus*), the most common cause of invasive aspergillosis, is an opportunistic fungus. *A. fumigatus* infections have also been associated with mortality in immunocompromised patients [1]. The immune system is important for host defense, and host defense reactions against microorganisms are numerous and varied. Cell-mediated immunity and humoral immunity are the principle protective immune responses against fungal infections. Acquired immunity also plays significant roles in the fungus-host interaction. Nevertheless, nonspecific cellular immunity mediated by macrophages, neutrophils and natural killer (NK) cells may provide the main defences against fungi [2]. Specific antibodies occur during fungal infections but the role of antibodies in protective immunity is unclear [3].

The susceptibility to *Aspergillus* infections has been associated with host immunosupression [4]. It is suggested that in experimental mouse models, T cells play an effective role in the protection of invasive fungal infections. Neutrophils, macrophages and monocytes are primarily important as effector cells. Effector cells function to destroy the pathogen during the fungal infection. Also, they are recruited to sites of infection by the action of inflammatory signals, such as those expressed by chemokines and cytokines [5].

Cytokines, which are polypeptides produced by various cells such as mast cells, T and B cells, macrophages, and endothelial cells, regulate inflammatory and immune reactions [6]. The T helper 1 (Th1) subset of CD4+ T cells secretes IFN-γ (Interferon), IL-2 (Interleukin-2), and TNF-β (Tumor necrosis factor) cytokines and is responsible for the activation of cell-mediated immunity and cytotoxic CD8+ T cells. The Th2 subset of CD4+ T cells secretes IL-4, IL-6, and IL-10 cytokines and is responsible for the stimulation of humoral immune response, through which it helps B cell activation [7]. It is considered that Th1 type cytokine responses are associated with strong immune responses and resistance to lethal fungal infections, while Th2 type cytokine

responses correlate with minimum cell-mediated immune responses and the increase of antibody production [8].

Even though *aspergillus* specific cellular and humoral response occurring during *aspergillus* infection is considered not to play an important role in the host response, antigen specific antibody and T cell responses have been detected during pulmonary aspergillosis syndromes [9].

The aim of this study was to determine the changes caused by *A. fumigatus* infection on leukocyte surface markers and Th1 and Th2 cytokine profiles in experimental mouse models in order to understand the immunological mechanisms formed in *A. fumigatus* infection.

Materials and Methods

Mice

Twelve female BALB/c mice were used in this study. All mice were 6-8 weeks old. Animals were kept under standard laboratory conditions.

Infection of mice

The animal experiments were conducted according to the "Guide for the Care and Use of Laboratory Animals" of the Gazi University School of Medicine. Local Ethical Committee, from which permissions were also obtained. For the infections, six mice were injected with a suspension containing *A. fumigatus* (DSM 1386) conidia at 10^6 CFU/ml concentration from the lateral tail vein. Three mice were used as a control group. The control group was injected with buffer only. *Aspergillus* infection in mice was confirmed by blood culture.

Isolation of mononuclear cells from mouse spleen

The first group mice infected with *A. fumigatus* (n=3) were killed by cervical dislocation following ether anesthesia at the 24th hour of infection. The second group of mice infected with *A. fumigatus* (n=3) were killed by cervical dislocation following anesthesia at the 72nd hour of infection. Their spleens were removed and mononuclear cells were isolated. After the spleens were mashed, the suspension was centrifuged and the supernatant discarded. Erythrocytes were removed by using Tris/ammonium sulfate solution. After centrifugation, buffy coats were collected and washed in Phosphate Buffered Saline (PBS) three times and resuspended at a concentration of $2x10^6$ cells/ml in complete RPMI 1640 medium containing 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2ME and supplemented with 10% fetal calf serum (Gibco). Cell viability was 95% by the trypan blue dye exclusion assay. The same procedure was performed in the control group without *A. fumigatus* infection (n=6). Six healthy mice were used as a control group. Three mice from the control group were killed after 24 hours and the remaining three mice were killed after 72 hours through cervical dislocation.

Flow Cytometric Analysis of mononuclear cells

Samples stained in two-color immunofluorescence were analyzed using a Coulter FC500 flow cytometer (Coulter, USA). Monoclonal antibodies used for staining were anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD19, anti-mouse CD69 and anti-mouse CD25 (e-bioscience, USA). Mononuclear cells were washed in PBS and then 10^6 cells/ml were incubated with monoclonal antibodies specific for mouse CD antigens for 30 minutes at 4^oC. After centrifugation at 500×g for 5 min and subsequent washing with 2 ml PBS containing 0.1% sodium azide, the cell pellets were resuspended in 0.5 ml of 1% paraformaldehyde in PBS. The stained cells were analyzed by a flow cytometer (FC500 flow cytometer, Coulter). FITC-conjugated anti-rat IgG2b antibody and PE-anti-rat IgG2b antibody were used as isotype controls. Lymphocytes were gated based on forward scatter/side scatter profiles. Each assay was studied in duplicate.

Levels of cytokines

Cardiac blood samples were collected for the cytokine assay. After centrifugation, the plasma was separated and the samples stored at -80^oC until they were analyzed. Levels of cytokines such as IL-2, IL-4, IL-10, IFN- γ, TNF-α (Tumor necrozis factor) were determined by specific Enzyme-linked immunosorbent assay (ELISA) techniques according to the manifacturer's instructions (Biosource, California, USA). The concentration of cytokines was determined spectrophotometrically. The absorbance was read at 450 nm. We constructed a standard curve using cytokine standards. The cytokine concentrations for

unknown samples were calculated according to the standard curve.

Statistical analysis

In the control group and infected mice group, expression of cell surface markers on mononuclear cells and cytokine levels were analysed using the one-way analysis of variance (ANOVA). The Bonferroni test was used as *post hoc* analysis. $p<0.05$ was considered to be significant.

Results

Expression of cell surface markers on mononuclear cells

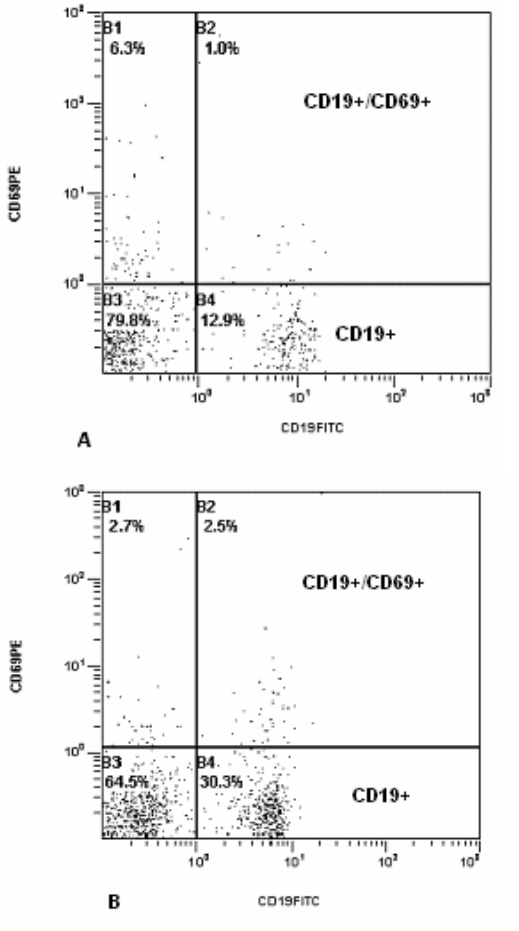
In this study, we did not find a significant difference between the CD4+ lymphocyte and CD8+ T lymphocyte percentages of the mononuclear cells in mice infected with *A. fumigatus* and those of the control groups without *A. fumigatus* infection ($p>0.05$).

Also, surface expression of CD25 (IL-2R), normally increased on activated T lymphocytes, was not found to have increased in the specimens with *A. fumigatus* infection in 24 hours compared to the control groups. No significant differences could be observed after 72 hours. Similarly, the expression of CD69, the marker found on activated lymphocyte surfaces, did not show any significant difference between the mononuclear cells infected with *A. fumigatus* and the control cells non-infected with *A. fumigatus* for ($p>0.05$).

In our study, CD19 expression found on B lymphocyte surfaces increased significantly in mononuclear cells of the mice infected with *A. fumigatus* in both 24 and 72 hours compared to those of the controls not infected with *A. fumigatus* ($p<0.05$). CD69 surface antigen of which expressions increased on the surface of activated B lymphocytes increased in the infected mice compared to the control groups not infected, but the rise was not statistically significant ($p=0.05$) (Figure 1).

In our study, no significant difference was observed in T lymphocyte percentages and T cell activation between the mice infected with *A. fumigatus* and the the control group non-infected by *A. fumigatus*. However, there was a remarkable increase in B lymphocyte percentages in the infected mice. Also, there was an increase in B lymphocyte activation compared to the control group, but it was not statistically significant. Thus, an increase was detected in B lymphocytes during *A. fumigatus* infection.

Figure 1. CD19 and CD69 expression on lymphocytes. (A) CD19+CD69+ expression in control group. (B) CD19+CD69+ expression in infected group.

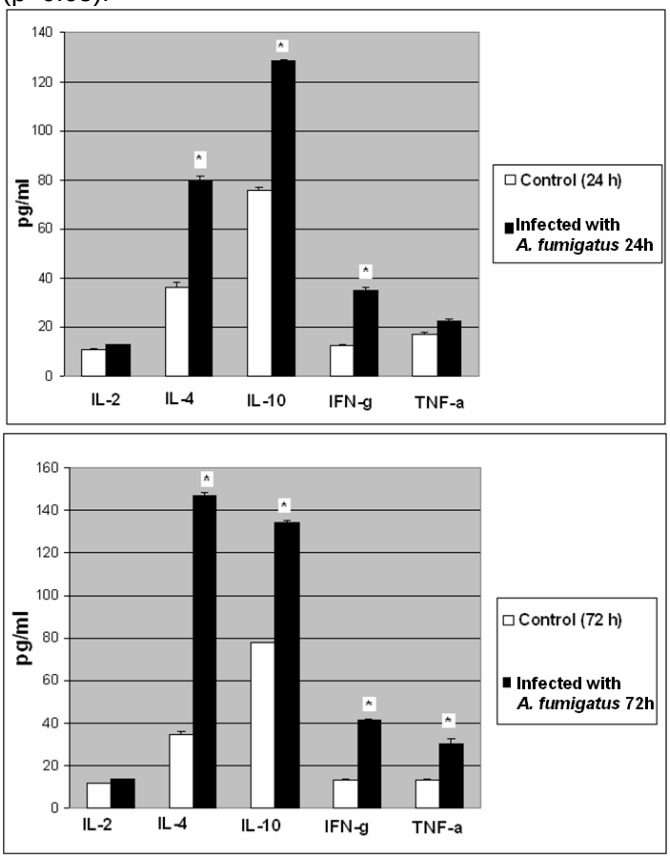


Cytokine levels

In this study we determined that the concentrations of IL-4, IL-10, and IFN-γ in the cardiac blood sample of the infected mice were significantly higher than those in uninfected controls in both 24 and 72 hours ($p<0.05$). But the increases were more marked in the levels of IL-4 and IL-10 ($p<0.001$) (Figure 2). Whereas the levels of TNF-α did not show any significant differences with the presence of *A. fumigatus* infection compared to the control group without infection in 24 hours, there was a remarkable increase after 72 hours ($p=0,125$, $p= 0.002$, respectively). We found no difference in the levels of the IL-2 in infected groups compared to those of the controls ($p>0.05$).

Therefore, it was observed that there was a statistically significant increase in all of the cytokines other than IL-2 in the mice infected with *A. fumigatus* compared to the control group.

Figure 2. The levels of cytokines in controls and in infected groups (Upper: 24 h post-infection; Below: 72 h post-infection). Values represented are mean ±SD. Asterisks indicate significant differences from controls ($p<0.05$).



Discussion

Antigen-specific immune response to *Aspergillus* infection results in the stimulation of CD4+Th-cell subsets [4].The difference in susceptibility to *A. fumigatus* infection appears to be determined primarily by the balance between CD4+, Th1 and Th2 responses [10]. T-helper (Th) cytokines contribute to phagocytic cell-mediated host defense against *A. fumigatus* [11].

In our study, we investigated the changes of CD surface markers and cytokine profiles during the infection of *A. fumigatus* in mice. Our study revealed that there was no difference between infected groups and control groups in relation to the percentage of CD4+, CD8+ and total T-lymphocytes. Also, CD69 and CD25 (IL-2R) surface antigens, which normally increase in expression on activated T lymphocytes, did not increase in the specimens with *A. fumigatus* compared to the control groups.

Stanzani *et al*. reported in their study that *A. fumigatus* filtrates led to a relatively weak activation of T cells [1]. Scanlon *et al.* indicated that *A. fumigatus* induced CD4+ T cell proliferation, whereas there was no significant increase the numbers of CD8+ T cells. Also, they informed that the pulmonary surfactant protein-A directly suppressed *A. fumigatus*-stimulated CD4+ T cell function [12].

In this study, our failure to detect a difference in the percentage of T cells might be related to the killing of the mice at the 24th hour and 72nd hour of *A. fumigatus* infection. Thus, it is suggested that elongation of killing time of mice may cause significant differences. Indeed, Rivera *et al.* reported that CD4 T cells were not in high levels in the bronchoalveloar fluid of the mice infected with live and heat activated *A. fumigatus* conidia before the post infection day 7 and CD4 T cells were first detected only on the third day. Therefore, in our study, it is considered that there would be more CD4 cells if the mice were analyzed seven days after postinfection [13].

In this study, we observed a marked increase in the percentage of CD19+ B lymphocytes in the mice infected with *A. fumigatus*. Since the expression of CD69, the marker found on the surface of activated B lymphocytes, also increased in infected groups, it is considered that humoral immunity may play an important role during *A. fumigatus* infection. Rivera *et al*. also informed that humoral immune responses to *A. fumigatus* antigens mounted in mice infected with live but not heat-inactivated fungus [13].

In *A. fumigatus* infection, Th1 cell responses are associated with resistance and onset of protective immunity, whereas Th2 responses are associated with progressive disease, more tissue damage, and poor survival [10].

In our study, we observed no significant change in the level of IL-2 in the infected mice compared to control group. The levels of TNF-α, pro-inflammatory cytokine, were observed to be higher in the infected group than they were in the control group at 24 and 72 hours after the infection. Similarly, Centeno-Lima *et al.* reported that TNF-α expression increased 2 days after the

infection with *A. fumigatus* [14]. TNF-α is important to the host response to infectious challenge, including fungal pathogens. It is a critical component of innate immunity in both immunocompromised and immunocompetent hosts [15]. TNF-α is secreted very early during infection [10]. Then, in our study, the increase in the TNF-α secretion on the first three days was expected. So, TNF-α enhances specific phagocytic activity against *Aspergillus* conidia by macrophages as an early event [10].

In addition, in our study, the levels of other cytokines such as IL-4, IL-10, and IFN-γ were significantly higher in the infected group compared to the control groups. The increases were more marked in the levels of IL-4 and IL-10 in infected mice, indicating that Th2 type cytokines can play an essential role in *A. fumigatus* infection. Both the increase of the percentages and the activations of B cells and the increase of Th2 type cytokines such as IL-4 and IL-10 suggest that there is a marked Th2 response during *A. fumigatus* infection. Consequently, the immune response to *A. fumigatus* infection may be characterized by minimal cellular response and increased antibody production. Actually, the studies have reported that Type 2 CD4+ T cell responses increase in allergic bronchopulmonary aspergillosis (ABPA) [16].

Kurup *et al.* informed that there was an enhanced Th2 response in the mice exposed to crude *A. fumigatus* extract and *A. fumigatus* spores [17]. In some other studies, it was observed that Th2-phenotype cytokines such as IL-4 and IL-10 increase susceptibility to *A. fumigatus* infection and contribute to the progression of infection [18, 19]. IL-10 is an important regulator of the T-cell-mediated immune response to *A. fumigatus*. Studies in murine models of mice deficient in IL-10 revealed increased resistance to *A. fumigatus* infection; accordingly, mice expressing high serum IL-10 levels showed decreased resistance to aspergillosis [10]. The fact that Th2 responses increased in our study suggests that the susceptibility to *A. fumigatus* infection is also increased.

Rivera *et al.* informed that Th 1 type cytokines were formed predominantly during the exposure to live conidia while the production of Th2 type cytokine was prominent following the immunization with heat inactivated conidia [13]. We observed that Th2 response was more prominent following the infection with live conidia. This difference presumably results from the fact that the levels of cytokines were analyzed in the earlier period of infection (at 24 and 48 postinfection hours) in our study and in a later period (on the seventh postinfection day) in the former study. Therefore, our further studies will focus on the immune response observed in the late period of *A. fumigatus* infection.

In the light of the findings we obtained in our study it is considered that Th2 type cytokine response plays an important regulatory role during *A. fumigatus* infection. Depending on the predominant Th2 response, humoral immune response leads to production of antibodies. A full study of the elements and mechanisms of humoral and cell-mediated responses taking place after *A. fumigatus* infection will be very important for understanding its pathogenesis and may provide new strategies for prevention and treatment of invasive aspergillosis as well as new strategies for vaccination in immunocompromised patients.

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Conflict of interest: No conflict of interest is declared.