Effect of Invasive Aspergillosis Infection on the Immune Responses of Cancer Mice

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Abstract

Objective(s)
Using a cancer murine model of invasive aspergillosis (IA), we investigated the expression of TLR-2, Dectin-1 and the level of cytokine production by CD4+ T helper cells in different groups of mice (with or without cancer), also, the effect of invasive aspergillosis on the immune response pattern of cancer mice.

Materials and Methods
Patterns of susceptibility and resistance to infection obtained with different groups of mice injected with Aspergillus fumigatus conidia. TLR-2 and Dectin-1 analyzed applying flowcytometry and cytokine production of cultured splenocytes by ELISA method.

Results
Cancer mice that challenged with A. fumigatus conidia showed significant increase in TLR-2 and Dectin-1 levels compared with the two other control groups (normal mice challenged with A. fumigatus and non-infected cancer mice). Moreover, it showed insignificant decrease in IFN-γ and IL-10 levels and insignificant increase in TNF-α level. The data demonstrated remarkable rise in IL-4 level and the mortality of cancer mice that intravenously infected with A. fumigatus.

Conclusion
Probably IA causes stimulation in innate immunity and Th2 cells, also some disorganization in cytokine production in CD4+ T helper cells. We hypothesize that concomitance of IA and cancer may change the microenvironment for local or systemic immune responses. Other complementary studies could help supporting our hypothesis.

Keywords: Aspergillosis, Cancer, Cytokines, Dectin 1, Toll-like receptor 2
Invasive Aspergillosis and Cancer

**Introduction**

*Aspergillus fumigatus*, the major causative agent of aspergillosis, is a ubiquitous and opportunistic fungus that elicits respiratory infections, such as sinusitis, allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (IA) (1, 2). In the immunosuppressed or neutropenic host, invasive pulmonary aspergillosis, characterized by hyphal invasion and destruction of pulmonary tissue, is the most common manifestation of *Aspergillus* infection, although local infections may also occur (1).

Although *Candida* species are still considered the predominant fungal pathogens among immunocompromised individuals (3), the increasing importance of aspergillosis is widely recognized (4). A recent analysis of invasive fungal infection in patients with hematological malignancies has reported an increase in infections caused by *A. fumigatus* from 0.9% to 2.9% between 1989 and 2003 (5).

In mice, both innate (6, 7) and acquired immunity (8, 9) contribute to resistance to IA. It has been shown that neutropenia alone is not sufficient to render mice susceptible to *A. fumigatus* infection, unless the macrophage line of defense is overcome by high challenge doses, activated conidia, or cortisone suppression of macrophage conidiacidal activity (7, 10).

The macrophages ingest conidia (spores) through pathogen recognition receptors (PRRs) such as TLRs and Dectin-1 that recognizes specific fungal cell wall compartments (11). Therefore, could coordinate the inflammatory response to *Aspergillus* species (12). Recent study has demonstrated the key role of PRRs in regulating innate and antigen dependent immunity in response to fungal infection (13).

Dectin-1 and TLRs are synergistic in mediating production of cytokines (14). However, only a few studies have dealt with acquired immunity to *Aspergillus* infection. It has become clear in recent years that the antigen-specific immune response results in selective or preferential stimulation of CD4+ T helper (Th) cell subsets. The activation of Th cell subsets leads to patterns of cytokine secretion and unique T cell functions. For humans, antigen-specific T cells from patients with allergic bronchopulmonary aspergillosis were characterized as being CD4+ Th2 like in their cytokine synthesis pattern (15).

It has been demonstrated that Balb/c mice had high levels of circulating immunoglobulin E (IgE), eosinophils and produced interleukin-4 (IL-4), IL-5 in response to particulate *Aspergillus* antigens (16-18), indicating the occurrence of a Th2 response in the experimental allergic aspergillosis. Recently, the role of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) in protection of mice with IA has been reported (19).

In the present study, we examined the susceptibility of tumor-bearing mice to aspergillosis and studied the effects of infection upon expression of TLR2/Dectin-1, as well as cytokine profiles of ex vivo cells from infected mice.

**Materials and Methods**

**Animal**

A total of forty eight 8-10 weeks female inbred Balb/c mice (Institute Pasteur, Tehran, Iran) kept in the animal house of Faculty of Veterinary Medicine, on the basis of standard condition.

The mice divided into 4 groups, with 12 mice in each group as bellows:

- Group A (mice engrafted with tumor and infected with *A. fumigatus* conidia intravenously)
- Group B (mice engrafted with tumor and intravenously administered with normal saline)
- Group C (mice infected with *A. fumigatus* conidia, intravenously)
- Group D (mice administered with normal saline).

**Tumor induction in mice**

Sterile and small (3-5 mm) lumps of tumor from a mouse with spontaneous breast cancer (Institute Pasteur, Tehran, Iran) engrafted subcutaneously to anesthetized mice, received intradermal injection of ketamin 1%.

When the size of tumor grew up to 6-8 mm (approximately 6 day), a histopathologic
specimen was taken and the next process accomplished.

Microorganism, culture and infection
A. fumigatus isolate 36607 (ATCC, Manassas, Virginia, United States) was grown up on Sabouroud glucose agar (Difco, Detroit, MI) containing chloramphenicol, for 5 days at room temperature. Conidia harvested by washing the slant culture with 5 ml of sterile, phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20. The suspension vortexed for 1 min to break up the chains of conidia and then was filtered through 40 µm nylon filters to remove hyphal fragments. Finally, the suspensions centrifuged (1200 g, 3 min) and resuspended in normal saline, the absorbance at 620 nm adjusted to 0.6 and then enumerated on a hemacytometer.

The viability of the conidia was approximately 95%, as determined by serially diluting and plating out the inoculums on Sabouroud glucose agar.

For IV infection, animals injected via the lateral tail vein with $5 \times 10^6$ conidia in 0.5 ml of sterile PBS. Mice succumbing to fungal challenge routinely necropsied for histopathologic confirmation of IA. For histology, tissues excised and immediately fixed in formalin. Sections of paraffin (3-4 mm) embedded tissues stained with the periodic acid Schiff.

After injection of normal saline or A. fumigatus conidia, the animals followed up for 10 days for the mortality rate.

Isolation of peritoneal macrophage
Mice anesthetized and scarified. The peritoneal cavity exposed and approximately 10 ml of sterile 0.05 M EDTA solution injected into the peritoneal cavity. A second needle then inserted along a lateral side of the peritoneal cavity and the majority of the EDTA solution was withdrawn.

On removal from the peritoneal cavity, the cells containing EDTA solution immediately placed on ice. They pelleted by centrifugation at 1500 rpm, after which the red blood cells lysed, using an ammonium chloride lysing buffer. They washed twice in RPMI, then resuspended in 15% Dulbecco’s modified Eagle’s medium (DMEM).

Isolation and culture of spleen cells
Spleens aseptically removed and placed in 60 mm tissue culture dishes containing 5 ml cold PBS. Cells dispersed by teasing and gentle aspiration through a 21-gauge needle. The suspensions filtered through fine-gauge stainless mesh, washed twice and counted with a hemacytometer. The cells resuspended at $10^7$/ml in RPMI 1640 culture medium (Gibco, Grand Island, USA) supplemented with hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer, 2 mM L-glutamine, 0.6 mg/ml gentamicin and 5% fetal bovine serum (FBS) (HyClone Labs, Logan, UT).

Flowcytometery
The expression of TLR-2 and Dectin-1 on the surface of macrophages analyzed by flowcytometery on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, USA) using monoclonal anti mouse Dectin-1-Phycoerythrin (PE) and anti mouse TLR-2-FITC (R&D Systems, Abingdon, UK). Sample preparation and staining procedure were according to the company instruction.

Cytokine determination
Spleen mononuclear cells cultured in 96-well, round-bottomed plates. After 3 days of culture, the supernatant removed from each well. Supernatants tested for IL-4, IL-10, IFN-γ and TNF-α by the use of commercial antigen-capture enzyme-linked immunosorbent assay (ELISA) kits (R&D systems).

Statistical analysis
Data analyzed using SPSS ver.15 software. Comparisons between groups made with analyses of variance and appropriate ad hoc testing. The two-tailed unpaired t-test and the two-tailed nonparametric Mann-Whitney test employed. Significance accepted at $P<0.05$. 
Invasive Aspergillosis and Cancer

Results

Mortality following intravenous administration of *A. fumigatus* conidia in cancer mice

We analyzed the survival of healthy and cancer Balb/c mice upon intravenous administration of conidia from *A. fumigatus*.

As shown in Figure 1, after administration of $5 \times 10^6$ conidia, around 50% of cancer animals with invasive aspergillosis died by day 6 and only around 30% survived by the end of the experiment (day 10). In contrast, only around 10% of cancer mice died on day 6 and almost 70% of them survived up to day 10. These results for mice challenged IV with *A. fumigatus* were 30% and 50%, respectively. However, all of the healthy mice survived during the test period.

Comparison of the survival curves indicates significant decrease in the survival of cancer mice when they challenged with *A. fumigatus* intravenously.

![Figure 1](image1)

Figure 1. Survival rate of study population after 10 days of *A. fumigatus* conidia or normal saline injection. A: tumor bearing mice challenged with *A. fumigatus* conidia, B: tumor bearing mice injected normal saline, C: mice infected with *A. fumigatus* conidia and D: healthy mice received normal saline.

TLR-2 and Dectin-1

Flowcytometry used for staining and analysis of surface expression of TLR-2 and Dectin-1 on peritoneal macrophages.

After induction of aspergillosis infection in cancer mice, Dectin-1 and TLR-2 expression on peritoneal macrophages, significantly increased in compare to IA or cancer animals ($P=0.005$ and $P=0.001$, respectively) (Figures 2 and 3).

Cytokine Production

Supernatants of cultured splenocytes from different study groups analyzed for cytokine production (Table 1).

![Figure 2](image2)

Figure 2. Dectin-1 expression on freshly isolated peritoneal macrophages. Expression was markedly increased with invasive aspergillosis infection in tumor bearing and normal mice (A and C respectively) compared with tumor bearing and normal animal without *Aspergillus* infection (B and D respectively). Unshaded histograms: Control Ab staining; shaded histograms, Dectin-1 specific staining.

Unshaded histograms: Control Ab staining; shaded histograms, Dectin-1 specific staining.

![Figure 3](image3)

Figure 3. Distribution and surface expression of TLR-2 on freshly isolated peritoneal macrophages. Highest surface expression was observed after *Aspergillus* infection in tumor bearing mice and normal mice (A and C respectively) in comparison with tumor bearing mice and normal mice without invasive aspergillosis (B and D respectively). Unshaded histograms: Control Ab staining; shaded histograms, Dectin-1 specific staining.
Table 1. Cytokine production of study groups from activated splenocytes was analyzed in supernatants of 72 hr. All of data were analyzed with normal control group (D) by ANOVA test ($P<0.05$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-4 (Mean±SD)</th>
<th>IFN-γ (Mean±SD)</th>
<th>IL-10 (Mean±SD)</th>
<th>TNF-α (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>127.85±32.18</td>
<td>59.69±1.98</td>
<td>433.05±26.61</td>
<td>457.97±183.01</td>
</tr>
<tr>
<td>B</td>
<td>78.29±0.91</td>
<td>72.34±16.37</td>
<td>601.07±25.29</td>
<td>380.42±87.43</td>
</tr>
<tr>
<td>C</td>
<td>92.44±10.42</td>
<td>60.75±2.48</td>
<td>708.06±120.52</td>
<td>342.44±120.39</td>
</tr>
<tr>
<td>D</td>
<td>65.45±4.27</td>
<td>71.32±14.21</td>
<td>311.78±87.45</td>
<td>227.71±47.26</td>
</tr>
</tbody>
</table>

**Production of TNF-α**

TNF-α production from spleen cells in tumor bearing mice, with or without *Aspergillus* infection, increased but not significantly in compared to the control healthy mice ($P=0.28$ and 0.33 respectively).

**Production of IFN-γ**

When the tumor bearing mice, with *Aspergillus* infection compared to the other groups (tumor, *Aspergillus* infected and healthy mice), no significant decrease in IFN-γ secretion observed ($P=0.9$ and 0.8 and 0.9, respectively). However, IFN-γ levels in *Aspergillus* infected tumor bearing mice were lower than those in the other groups.

**Production of IL-10**

Cancer mice that challenged with *A. fumigatus* conidia showed insignificant decrease in IL-10 levels compared to the normal healthy control groups. However, in comparison with the normal mice, IL-10 was significantly higher in the normal mice challenged with *A. fumigatus* and non infected tumor bearing mice ($P=0.001$ and $P=0.01$, respectively).

**Production of IL-4**

Significant increase in IL-4 levels detected in *Aspergillus* infected tumor bearing mice, compared to the PBS treated normal mice ($P=0.031$). Interestingly, this rise in IL-4 levels was not significant in the other groups.

**Discussion**

Invasive aspergillosis is a disease of immunocompromised host and is a rapidly progressive often fatal infection. This infectious process is characterized by invasion of blood vessels and lymphatics resulting in multifocal infiltrate (20). Several previous studies have highlighted the importance of natural and adaptive immunity in the defense against aspergillosis by recognition and clearance of the organism (6-8).

In this study, we examined the possibility that IA could decrease the survival of cancer animal model and change the immune responses. Experimental IA established in mice, which implanted with tumor cells, in order to test the survival index, Dectin-1, TLR-2 and cytokine production in comparison with the control groups. This model of IA established by inoculating viable *A. fumigatus* conidia intravenously.

The previous study demonstrated that *Aspergillus* infection could increase the mortality rate of infected animal (21). For the first time, we have reported that the mortality rate of tumor bearing mice infected with *Aspergillus* significantly increased.

As it was noted in the previous reports, levels of Dectin-1 and TLR-2 in the peritoneal macrophage increased during IA (11, 22-24). This indicated the role of macrophages and phagocytic cells in the early innate immune responses to the fungal infection.

The present study confirms that expression of Dectin-1 and TLR-2 on the peritoneal macrophages of cancer mice which challenged with *A. fumigatus* dramatically increased. This is a good evidence for concomitance of these pattern recognition receptors (PRRs) for the induction of inflammatory responses in selected group (25-27).

Different reports demonstrated the importance of both TLR2 and TLR4 at least in vitro, using murine peritoneal macrophages, bone-marrow-derived dendritic cells, TLR-transfected cells, and human blood
mononuclear cells (23, 24, 28). Bellocchio et al reported a significantly higher infection of the lungs of TLR2−/− mice than of the lungs of wild-type mice and an enhanced susceptibility of TLR2−/− mice to a secondary challenge with *A. fumigatus* (22).

Dectin-1 collaborates with TLR2, as well as acting independently, to initiate a range of innate immune responses and orchestrating immune system machinery for suitable responses in fungal infections or some other infections or diseases such as cancer (22).

TNF-α is one of the main cytokines produced by cells of the macrophage lineage, after TLR2 activation (29, 30) and is a critical primary mediator in the initiation of pulmonary innate immunity in the experimental aspergillosis (31, 32).

In the previous study observed that the lack of TLR2 apparently renders macrophages less responsive to *A. fumigatus*. Indeed, TNF-α production reduced in TLR2−/− compared to TLR2+/+, both in vivo and in vitro (22).

In our study the level of TNF-α in tumor bearing mice challenged with IA insignificantly increased. It is not fully understood, but it seems that the detection of TNF-α in peritoneal macrophages culture could help for resolving this puzzle.

Cytokines are differentially regulated in mice that are resistant or susceptible to Aspergillus infection. It was emphasized that Th1 cells are effective for the protection responses against IA infection (33). For human antigen-specific T cells from patients with allergic bronchopulmonary aspergillosis, characterized as being CD4+ Th2 like in their cytokine synthesis pattern (15).

IL-10 is a regulatory cytokine which is secreted mainly by the regulatory lymphocytes. This cytokine could change the infection course and outcome as it supports the progression of invasive diseases (34). As noted in animal models that treated with IL-4 or IL-10 antagonists, infection would not tend to be aggressive. In contrast, Th2 responses are associated with the disease progression and the onset of no protective responses, as observed in mice treated with IFN-γ or IL-12 antagonists (41). It was strictly emphasized that the production of IL-4 correlated with infection progression, as its depletion early in infection resulted in decreased fungal loads in the organs and the cure of mice from infection. Conversely, it has been found that IFN-γ has a protective effect on mice with IA and neutralization of this cytokine resulted in an increased pathology and a concomitant increased expression of the IL-10 (35).

The results of this study demonstrated the occurrence and non differential expansion of Th2-cell activation in *Aspergillus* infected tumor bearing mice by showing that culture of spleen lymphocytes produces IL-4 and IFN-γ, in addition to IL-10. Here we show that development of protective acquired immunity in cancer mice that are infected with *A. fumigatus* conidia is disturbed and Th2 responses increased. However, only significantly increase in IL-4 production observed in tumor bearing mice that infected with *Aspergillus* conidia.

The data of the present study are compatible with a scenario in which a disarray of microenvironment, as a result of second systemic infection such as IA, could be altered the immune responses in tumor animal model who are challenged by IA. Probably systemic aspergillus infection causes stimulation in innate immunity and Th2 cells and some disorganization in the cytokine production from CD4+ T helper cells and acquired immunity. Also, IA causes decrease in surveillance of cancer mice. We hypothesize that concomitance of IA and cancer may changes the microenvironment for local or systemic immune responses.

In this condition immune responses may go wrong and have efficiency for defending against the tumor or fungal infections host. Moreover, this irregulation of immune responses could be result for a decreased survival rate of this animal in compare to mice that administrated with *Aspergillus* conidia.

**Conclusion**

These results while indicating the role of innate immune system receptors and show
better understanding of reciprocal regulation between the different Th subsets and cytokine production may be useful in the proper management of IA. These could be better established by additional studies about the regulatory T cells and matrix proteins that are responsible for the cancer metastasis.

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References