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Differential mechanisms of resistance to sublethal systemic *Aspergillus fumigatus* infection in immunocompetent BALB/c and C57BL/6 mice

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ABSTRACT

Studies of systemic and pulmonary *Aspergillus fumigatus* infection demonstrated differential susceptibility of inbred mice of various genetic background to lethal outcome, with an opposite pattern of Th1 cytokine interferon-γ (IFN-γ) and Th2 cytokine interleukin-4 (IL-4) in susceptible *vs* resistant mice. We have shown recently reciprocal IFN-γ and IL-4 expression in spleens of Th1-prone C57BL/6 mice in sublethal systemic aspergillosis. In this study, resistance to systemic (*i.v.*) *A. fumigatus* infection was investigated in Th2-prone BALB/c mice by survival rate at different fungal inocula, efficiency of reduction of visceral organ and spleen fungal burden at sublethal conidia dose and splenic immune response to this dose and compared to C57BL/6 mice. No strain differences in survival were noted at three *A. fumigatus* doses, with similar extent and dynamics of fungal eradication from all organs following sublethal conidia dose injection. Progressive decrease in spleen fungal burden was associated with different dynamics and quality of changes in spleen activity of BALB/c and C57BL/6 mice. Increased spleen mass and cellularity was noted in both strains, with higher values in BALB/c mice at some time points what might be ascribed to peripheral blood cell recruitment, as well as hematopoietic activity and red pulp upgrowth. Infection tipped the balance towards pro-inflammatory antifungal splenic response by a highly increasing IFN-γ and without changing the IL-4 expression in BALB/c mice, in contrast to down-regulating anti-inflammatory (IL-4) and a moderately increasing IFN-γ response in C57BL/6 mice. Jointly, stimulation of IL-17 expression noted in both strains provided an optimal inflammatory *milieu* in the spleen of infected mice that might have contributed to efficient removal of conidia.

Introduction

Aspergillus fumigatus is an ubiquitous saprophytic mould species with airborne conidia (Latge 1999; Mullins et al. 1984; Nolard 1994). Owing to their small diameter, as well as environmental factors, conidia easily gain access to the lungs (Goodley et al. 1994; Hospenthal et al. 1998). Infections occur rarely in healthy, immunocompetent individuals due to efficient fungal elimination by host immune defense (Latge 1999). Increase in numbers of immunocompromised individuals has, however, placed *A. fumigatus* as the second major cause of invasive fungal infections in humans contributing to morbidity/mortality in these individuals (Latge 1999).

The use of animal models has been indispensable for current understanding of various aspects of *Aspergillus* infection (Clemons and Stevens 2005). Four murine models of aspergillosis that are most often used to explore the mechanisms of resistance to this microbe are invasive pulmonary aspergillosis, bronchopulmonary aspergillosis, systemic (disseminated) infection and central nervous system infection (Clemons and Stevens 2005). By using intranasal or intratracheal inoculation of *A. fumigatus* conidia, that mimic the natural route of infection, the role of innate host defense and the generation of Th1 cytokines in the lungs were shown to play a critical role in the resistance of mice to invasive pulmonary aspergillosis (Cenci et al. 1998; Centeno-Lima et al. 2002). To

Abbreviations: *A. fumigatus*, *Aspergillus fumigatus*; CFUs, colony forming units; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IFN-γ, interferon-γ; IL, interleukin; *i.v.*, intravenous; mRNA, messenger RNA; NK cells, natural killer cells; NO, nitric oxide; p.i., post infection; RT-PCR, real-time polymerase chain reaction; SMA, Sabouraud's maltose agar; Th, T helper.

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increase the susceptibility to infection in these models (Polak 1998; Schmidt 2002), immunosuppressive treatments (glucocorticoids and cyclophosphamide) are required, which were shown to modulate the host immune response both before as well as during *A. fumigatus* infection (Armstrong-James et al. 2009). Administration of *A. fumigatus* by a systemic route (by *i.v.* inoculation) establishes infection in immunocompetent mice with a relatively uniform pattern of disease. This model of infection is used for infection dissemination studies and infection to mortality ratio investigation and is particularly useful in the determination of mechanisms involved in host resistance or susceptibility to lethal outcome of *A. fumigatus* infection (Cenci et al. 1997; Clemons et al. 2000; Mirkov et al. 2008; Nagai et al. 1995; Polak 1998).

Studies of both systemic and pulmonary *A. fumigatus* infection demonstrated that inbred mice of various genetic background differ significantly in their degree of susceptibility to lethal outcome of infection (Cenci et al. 1997; Zaas et al. 2008). An opposite pattern of IFN-γ and IL-4 in susceptible *vs* resistant strains was noted, with higher IFN-γ production and lower IL-4 levels in individuals which survived infection compared to non-survivors (Cenci et al. 1997, 1998). Interestingly, no differences were noted in susceptibility to lethal systemic *A. fumigatus* infection (Cenci et al. 1997) between C57BL/6 and BALB/c mice, identified as prototypic Th1 and Th2 strains, respectively, according to their predisposition towards IFN-γ or IL-4 cytokine response to microbial infections (Mossman and Coffman 1989). Similarly, in invasive pulmonary aspergillosis studies, immunosuppressed C57BL/6 and BALB/c mice exhibited similar pattern of survival following conidia administration (Svirshchevskaya et al. 2009; Zaas et al. 2008), although the survival differed somewhat depending on immunosuppression regimen (Stephens-Romero et al. 2005). Consequently, these both strains were considered more "resistant" to *A. fumigatus* infection as opposed to "susceptible" mice such as A/J, DBA/2J, C3H/HeJ, CBA and A/Sn mice (Svirshchevskaya et al. 2009; Zaas et al. 2008). In the light of Th1/Th2 paradigm and knowing that C57BL/6 and BALB/c were shown to express disparate cytokine (IFN-γ or IL-4) responses in a variety of parasitic and bacterial infections (Gervais et al. 1984; Guler et al. 1996; Tam et al. 1999; Thach et al. 2000; Wakeham et al. 2000) it seems worthwhile to compare IFN-γ and IL-4 expression in response to *A. fumigatus* infection in these mice. There are some data concerning Th1 and Th2 cytokine production in resistant BALB/c mice (Cenci et al. 1998) which showed increase in IFN-γ and low levels of splenic IL-4 production in lethal infection, but with similar levels of production of both cytokines in sublethal infection with this fungus. In addition, we have shown recently reciprocal expression (increased IFN-γ *vs* decreased IL-4) of these two cytokines in Th1-prone C57BL/6 mice in response to disseminated sublethal *A. fumigatus* infection (Mirkov et al., 2010). Beside Th1 and Th2 cytokines, the importance of activation of Th17 cells and expression of IL-17, a main cytokine produced by these cells in immune response to fungi was stressed (Curtis and Way 2009). The role for IL-17 in pulmonary aspergillosis is demonstrated recently in C57BL/6 mice (Zelante et al. 2009) and our recent investigations showed the involvement of IL-17 in systemic *A. fumigatus* infection in this strain (Mirkov et al., 2010). To our knowledge, there are no data which explore influences of genetic predisposition on IL-17 in aspergillosis.

In this study, resistance of Th2-prone BALB/c mice to systemic *A. fumigatus* infection was explored by measuring survival rate at different conidia doses and by efficiency of conidia elimination from visceral organs and spleen at sublethal conidia dose and compared to Th1-prone C57BL/6 mice. Basic indices of splenic response (spleen mass/cellularity and proliferation) to sublethal *A. fumigatus* infection were compared in these two mouse strains, as well as quantitative and temporal expression of IFN-γ, IL-4, and IL-17, main cytokine representatives of Th subsets. Data were presented which demonstrated roughly similar survival in both strains at high conidia inocula, with progressive and similar decrease in liver, lung, kidney and spleen fungal burden at sublethal infection. Conidia elimination in spleens was associated with changes in activity and inflammatory cytokine *milieu* created differently in the two strains. Animals of both strains responded to infection by increase in relative mass, cellularity and proliferative capacity, but with more pronounced red pulp proportion and hematopoiesis in spleens of BALB/c mice. While highly pronounced increase in IFN-γ and IL-17 expression, but unchanged IL-4 expression characterized antifungal spleen cytokine response in BALB/c mice, increase in the IFN-γ and IL-17 cytokine expression was accompanied by down-regulation of anti-inflammatory IL-4 response in C57BL/6 mice. Obtained data confirmed the importance of IFN-γ in immunity to *A. fumigatus* and demonstrated the involvement of IL-17 in resistance to systemic aspergillosis in both strains.

Materials and methods

Mice

Eight to twelve week old female BALB/c and C57BL/6 mice, four to six per group, were used for the study. The animals were bred and conventionally housed at the Institute for Biological Research "Sinisa Stankovic" (Belgrade, Serbia) in a controlled environment and provided with standard rodent chow and water *ad libitum*. All experiments were approved by the Ethical Committee of our Institute. Fungal burden assessment was conducted at days 1, 3, 7 and 15 post infection (p.i.) and at days 3, 7 and 15 p.i. for the evaluation of splenic response.

Fungal culture conditions

The human isolate of *A. fumigatus* was obtained from the Institute of Public Health of Serbia "Dr Milan Jovanovic-Batut". The microorganisms were grown on Sabouraud's maltose agar (SMA, Torlak, Belgrade, Serbia) for 7 days (Booth 1971). Conidia were harvested by flooding the surface of agar slants with non-pyrogenic sterile physiological saline.

Animal infection, determination of survival rate and organ fungal burden

Anesthetized mice (Ketamidor, Richter Pharma, Austria) were inoculated with 1×10^6, 1×10^7 or 5×10^7 conidia of *A. fumigatus* in 0.1 ml of pyrogen-free saline *via* the lateral tail vein. Control mice received saline solely. Survival, clinical appearance and body weight were monitored daily for 15 days and survival percentage was calculated for each group. Fungal burden in the spleen, liver, kidneys and lungs was determined by quantitative colony forming units (CFUs) assay (Sheppard et al. 2006). At selected time points (1, 3, 7 and 15 days) p.i. organs were aseptically removed and their wet masses weighed using a precision balance (±0.01 g). Tissue specimens were homogenized with IKA T18 basic homogenizer (IKA Works Inc., Wilmington, NC) in 5 ml of sterile saline, on ice. Primary homogenate dilutions were quantitatively cultured by serial dilution, plated on SMA plates supplemented with streptomycin sulfate (ICN-Galenika, Belgrade, Serbia), incubated at 37 °C for 24–48 h, and the number of CFUs per gram of tissue was determined.

Peripheral blood total leukocyte and differential counts

Total leukocyte counts were determined at days 3 and 7 following conidia administration by Türck staining and by using an improved Neubauer hemocytometer. Leukocyte differential counts were performed by differentiating at least 200 cells from