International Journal of Medical Microbiology 306 (2016) 187–195

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

The absence of microbiota delays the inflammatory response to *Cryptococcus gattii*

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ARTICLE INFO

Article history:
Received 4 November 2015
Received in revised form 14 March 2016
Accepted 28 March 2016

Keywords:
Cryptococcus gattii
Cryptococcosis
Germ-free mice
Microbiota

ABSTRACT

The inflammatory response plays a crucial role in infectious diseases, and the intestinal microbiota is linked to maturation of the immune system. However, the association between microbiota and the response against fungal infections has not been elucidated. Our aim was to evaluate the influence of microbiota on *Cryptococcus gattii* infection. Germ-free (GF), conventional (CV), conventionalized (CVN—mice that received feces from conventional animals), and LPS-stimulated mice were infected with *C. gattii*. GF mice were more susceptible to infection, showing lower survival, higher fungal burden in the lungs and brain, increased behavioral changes, reduced levels of IFN-γ, IL-1β and IL-17, and lower NFκBp65 phosphorylation compared to CV mice. Low expression of inflammatory cytokines was associated with smaller yeast cells and polysaccharide capsules (the main virulence factor of *C. gattii*) in the lungs, and less tissue damage. Furthermore, macrophages from GF mice showed reduced ability to engulf, produce ROS, and kill *C. gattii.* Restoration of microbiota (CVN mice) or LPS administration made GF mice more responsive to infection, which was associated with increased survival and higher levels of inflammatory mediators. This study is the first to demonstrate the influence of microbiota in the host response against *C. gattii.*

1. Introduction

Cryptococcus gattii causes cryptococcosis, affecting the lungs and central nervous system (CNS) of immunocompetent individuals, and cryptococcal meningoencephalitis is the most severe clinical manifestation (Perfect et al., 2010; Franco-Paredes et al., 2015). Severe *C. gattii* infection is due to defective induction of the host immune response, resulting in low levels of proinflammatory cytokines (Brouwer et al., 2007). During infection, important changes occur in the capsular polysaccharides and cell morphology, affecting pathogenicity (Okagaki et al., 2010a,b).

Axenic animals, created in sterile conditions without microbiota contact and colonization (Germfree—GF), have been used as a model to study the effect of the absence of microorganism on the host physiology and anatomy (Yi and Li, 2012). The gut microbiota regulates the development and maturation of the immune system (Kamada et al., 2013). Compared to conventionally raised mice, GF mice show reduced Peyer's patches (Sommer and Bäckhed, 2013), mesenteric lymph nodes (Round and Mazmanian, 2009), cytokine production, serum immunoglobulin levels (Kamada et al., 2013), and hematopoiesis (Khosravi et al., 2014). These features make GF animals more susceptible to infection by bacteria (Khosravi et al., 2014), e.g., *Listeria monocytogenes* (Inagaki et al., 1996; Mittrücker et al., 2014), *Salmonella enterica* Typhimurium (Nardi et al., 1989), and *Klebsiella pneumoniae* (Fagundes et al., 2012). The absence

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http://dx.doi.org/10.1016/j.ijmm.2016.03.010
1438-4221/

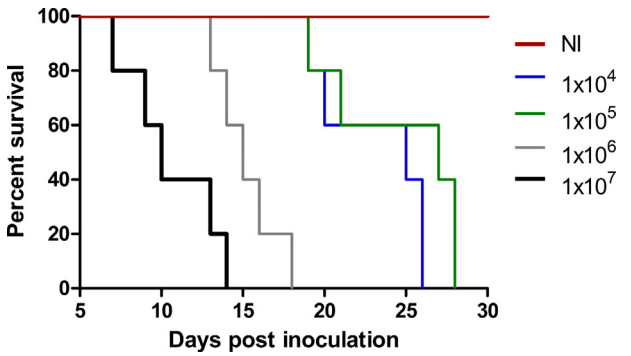

Fig. 1. *Cryptococcus gattii* induces lethality in germfree (GF) mice in an inoculum-dependent manner.
GF mice (n = 6) were inoculated by the intratracheal route with 1×10^4, 1×10^5, 1×10^6, or 1×10^7 *C. gattii* cells. Mice in the uninfected group (NI) were administered PBS. The results shown are percent survival postinfection.

of microorganisms can have either a negative (Ichinohe et al., 2011) or positive (Kuss et al., 2011) effect on viral infection (Wilks et al., 2013). Although these studies demonstrated the role of host microbiota on viral and bacterial infections, the effects on fungal infections are not yet known.

The aim of this study was to investigate the influence of gut microbiota on murine cryptococcosis caused by *C. gattii*. Our data demonstrate that the absence of microbiota leads to a delayed inflammatory response and morphological alterations in the yeasts during cryptococcosis.

2. Material and methods

2.1. Animals and ethics

GF *Swiss*/NIH mice (6–8-weeks old) from the GF nucleus (Taconic, Germantown, NY, USA) and *Swiss* conventional (CV) mice were used in this study. GF mice were maintained in flexible plastic isolators (Standard Safety Equipment) using classical gnotobiology techniques. As a microbiological control, fecal samples were plated on thioglycollate broth and brain heart infusion (BHI) broth and incubating at 37 °C (Pedroso et al., 2015). Groups of GF mice were subjected to conventionalization (CVN mice; i.e., fecal samples removed from the large intestine of CV mice were homogenized in saline [10%] and administered by oral gavage to GF mice, which were colonized for 21 days prior to intratracheal [i.t.] inoculation of *C. gattii*) (Souza et al., 2004). CVN mice were i.t. infected with *C. gattii* in the next day after the conventionalization was completed. Other groups received LPS (*Escherichia coli* 0111: B4, 4 mg/kg; Sigma-Aldrich) i.p. 48 h prior to *C. gattii* inoculation. The animal protocol was approved by the Comitê de Ética no Uso de Animais (CEUA) of Universidade Federal de Minas Gerais (Protocol 287/2012). Mice were sacrificed under anesthesia (i.p. ketamine [60 mg/kg] and xylazine [10 mg/kg]) by cervical dislocation.

2.2. Infection of mice with C. gattii

The L27/01 strain of *C. gattii* was cultured on Sabouraud dextrose agar (SDA) for 48 h at 35 °C and colonies were suspended in PBS to generate inoculum at 1×10^4, 1×10^5, 1×10^6, or 1×10^7 CFU/animal. Mice (n = 6/group) were anesthetized by i.p. injection of ketamine hydrochloride (80 mg/kg) and xylazine (15 mg/kg) in sterile saline and inoculated (i.t.) with 30 μL of cryptococcal cells and were monitored daily to determine the inoculums to use in subsequent experiments (Santos et al., 2014).

2.3. Fungal burden, myeloperoxidase (MPO) and β-N-acetylglucosaminidase (NAG) activities, cytokine and chemokine levels, NFκBp65 phosphorylation, and histopathology

GF, CV, CVN, and LPS-stimulated mice were inoculated (i.t.) with 1×10^7 CFU/animal (standardized inoculum) or PBS only (control). Lungs and brain were obtained at 1 or 10 days post inoculation, homogenized in PBS and plated onto SDA for measurement of fungal burden (Maxeiner et al., 2007).

The MPO assay and cytokine analysis was performed according to Elian et al. (2015) and NAG assay according to Aires et al. (2013), both with modifications. MPO and NAG assay were used as an indirect measurement of neutrophil and macrophages accumulation in lungs, respectively. Briefly, 100 mg of the lungs were used for cytokines analysis and MPO/NAG assays. The tissue was homogenized with 1 mL of PBS (pH 7.0) containing anti-proteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 Kallikrein inhibitor units of aprotinin A, all purchased from Sigma-Aldrich and 0.05% tween 20). The samples were centrifuged for 10 min at 3000*g*, at 4 °C and the supernatant was frozen at −20 °C and utilized for cytokines analysis. The levels of cytokines TNF-α, IFN-γ, IL-1β, IL-10 and IL-17 was determined by ELISA with commercially available antibodies according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN, USA).

The pellet obtained as described above was resuspended in 1.9 mL of buffer, pH 4.7 (0.1 M NaCl, 0.02 M $NaH_2PO_4 \cdot 1H_20$, 0.015 M Na_2-EDTA) and centrifuged again (12,000 *g* for 10 min). The supernatant was discarded and to the precipitate was added 0.9 mL of 0.2% NaCl solution followed by addition of an equal volume of solution containing 1.6% NaCl and 5% glucose. After homogenization, the content was equally divided into two new tubes, one for MPO assay and another for NAG assay. The tubes were centrifuged (12,000*g*, at 4 °C, for 10 min), the supernatant discarded and the pellet resuspended in 0,6 mL of 0.05 M $NaPO_4$buffer (pH 5.4) containing 0.05% hexadecytrimethyl-amonium bromide (HTAB) (Sigma-Aldrich) for MPO assay and in 0.6 mL 0.9% saline solution containing 0.1% (v/v) Triton X-100 for NAG assay. After frozen three times in liquid nitrogen, the content was centrifuged at 4 °C at 12,000*g* for 10 min. The supernatant was collected in a new tube and kept in −80 °C.

For MPO assay, 25 μL of the supernatant was added to microplates of 96 wells, in triplicate, following addition of same volume of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich) and incubation at 37 °C for 5 min. After that, 100 μL of 0.002% (v/v) H_2O_2 was added and the samples were again incubated at 37 °C for 5 min. After the incubation period, the reaction was stopped by adding 100 μL1 M H_2SO_4. The absorbance reading was taken at 450 nm. The result was expressed in MPO/100 mg of tissue.

For NAG assay, the supernatant was diluted in citrate-phosphate buffer, pH 4.5 (0.1 M citric acid and 0.1 M Na_2HPO_4), in proportion of 1:3, and100 μL of each diluted sample was added in microplate of 96 wells, in triplicate. Then, 100 μL 2,24mMp-nitrophenyl-*N*-acetyl-β-D-glicosaminide (Sigma-Aldrich) substrate diluted in a citrate-phosphate buffer was added in each well and the microplate was incubated at 37 °C for 5 min. After reaction, 100 μL of 0.2 M glycine buffer, pH 10.6 (0.8 M glycine, 0.8 M NaCl and 0.8 M NaOH) were added to stop the reaction. The absorbance was read at 400 nm. The result was expressed in NAG/100 mg of tissue.

NFκBp65 phosphorylation levels in the lungs of GF and CV mice was determined by western blotting (Lv et al., 2014). Briefly, samples containing 30 μg of protein were resolved on an SDS-PAGE gel and transferred to a PVDF membrane. The membrane was incubated with specific primary antibodies (1:2000; Sigma, St. Louis, MO, USA) and then with a secondary anti-mouse HRP-conjugated antibody (1:5000; Amersham), and the signal was quantified using