Journal of
Medical Microbiology & Diagnosis

Silva et al., J Med Microb Diagn 2016, 5:4
DOI: 10.4172/2161-0703.1000244

Research Article Open Access

Effect of GXM (Glucuronoxylomannan) on the Inflammatory Response in Lung Infection Caused by *Cryptococcus neoformans* (Serotype A) in Immunodeficient Murine Model (BALB/c-SCID)

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Abstract

GXM (glucuronoxylomannan) is the major component surrounding the capsule of *Cryptococcus neoformans* having multiple biological functions, one of them and most important, the reduction in production of inflammatory cytokines. They were evaluated in this study the correlation of the production GXM with TNF, IL-6, IL-10, as well, as survival in murine model (BALB-c) and severe combined immunodeficiency (SCID). The animals were infected intravenously with 0.1 ml of a suspension containing 3.0 × 10^6 model, BALB-c compared to the model. The high production GXM as well as the induction of viable cells of *C. neoformans*. There was an increase in the production of GXM, as well as a decrease in survival in (SCID) a severe inflammatory response in this model may be due to a compromised immune system.

Keywords: GXM; *C. neoformans*; BALB/c; SCID; Pulmonary cryptococcosis; TNF-α; IL-6; IL-10; Inflammatory response

Introduction

Cryptococcosis is a subacute or chronic systemic mycosis with a cosmopolitan nature [1], showing tropism for the central nervous system (CNS) [1,2]. This disease is caused by yeast of the genus *Cryptococcus* [3]. Two variety are known: *C. gattii* (Serotypes B and C) and *C. neoformans* (Serotypes A, D and AD); [4], Franzot, et al. proposed subdividing the *C. neoformans* variety into var. *neoformans* (Serotype D) and var. *grubii* (Serotype A). Serotype A is the most prevalent [1].

Infectious particles of the yeast can be spread through the air and infect susceptible hosts, caused pulmonary cryptococcal [5]. Life-threatening diseases caused by *C. neoformans* in immunosuppressed hosts such as AIDS patients occur at a global rate of approximately 1 million cases per year 1. Life-threatening diseases caused by *C. neoformans* in immunosuppressed hosts such as AIDS patients occur at a global rate of approximately 1 million cases per year [6]. The prominent anti-phagocytic polysaccharide capsule is unique to *Cryptococcus* spp. and is considered to be an essential virulence factor that has multiple effects on host immunity [3]. This capsule is composed primarily of glucuronoxylomannan (GXM), which comprises more than 90% of the capsule's polysaccharide mass [7].

The model of systemic cryptococcosis in mice with severe combined immunodeficiency (SCID) is useful for immunological and therapeutic study of the disease in immunodeficient hosts [8], and is a valuable tool that contributes to understanding how these infections occur [9].

The greater susceptibility to the disease in these models is justified by the important role of B and T cells contributing to the animal's protection against severe infection [10]. In experimental infections, the yeast *primarily* affects the *lungs* and then reaches other organs, preferentially the brain [11].

Tumor necrosis factor (TNF-α) is an important marker in cryptococcosis, both in humans and in mice, several *in vitro* studies showed that the capsule components are capable of stimulating the production of TNF-α by various cell types [12-14]. Pro-inflammatory cytokines with various biological functions (among them, increasing the power of phagocytosis), modulate the expression of other cytokines such as IL-1 and IL-6 being secreted by macrophages, neutrophils and T cells [15]. Decreased production of IL-6 may be an important additional mechanism whereby the cryptococcal capsule, destroys the protective immune response [16]. The survival of patients with cryptococcal meningitis may be associated with increased levels of Il-6, TNF-α and IF-γ, [17,18]. Interleukin 10 (IL-10) is a multifunctional cytokine produced by many different cell types, including alternatively and classically activated macrophages, dendritic cells, B cells and CD4 T regulatory cells, and is an important regulator of innate immunity and interferes with the production of inflammatory mediators by polymorphonuclear neutrophils, monocytes and macrophage [17].

The aim of this study was to correlate the production of GXM in the inflammatory response in the immunocompetent murine model (BALB/c) and severe combined immunodeficiency (BALB/c-SCID).

Material and Methods

Cryptococcus neoformans strain

The standard strain ATCC 90112 (*Cryptococcus neoformans* var. *grubii*-Serotype A), maintained in tubes containing Sabouraud dextrose agar (Difco Laboratories, Detroit, MI, USA) and glycerol at -20°C, in the laboratory of pathogenic yeasts of the Department of Microbiology,

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Received August 12, 2016; **Accepted** November 25, 2016; **Published** December 01, 2016

Citation: Silva EG, Silva SM, Paula CR, Ruiz L (2016) Effect of GXM (Glucuronoxylomannan) on the Inflammatory Response in Lung Infection Caused by *Cryptococcus neoformans* (Serotype A) in Immunodeficient Murine Model (BALB/c-SCID). J Med Microb Diagn 5: 244. doi:10.4172/2161-0703.1000244

Institute of Biomedical Sciences of São Paulo University, São Paulo, Brazil was evaluated in this research.

Animals and experimental cryptococcosis

A total of 65 mice with BALB/c-SCID, with a mean weight of 20 g to 25 g and 65 immunocompetent mice (BALB/c) with a mean weight of 25 g to 27 g, obtained from the animal center, responsible for breeding isogenic animals, at the Institute for energy and nuclear research, São Paulo, Brazil, were used in the study. These mice were housed in microisolator cages, provided with sterile feed and water and randomly distributed into ten groups. The strain ATCC 90112 was cultivated in YPD medium [1% yeast extract (Difco), 1% Bacto Peptone (Difco) and 2% dextrose (Sigma-Aldrich, Milwaukee, WI, USA)] for 18 h at 30°C; the cells were collected after centrifugation, washed twice in phosphate buffer solution (PBS) and resuspended at the inoculation concentration. Nine groups of each murine model with 5 mice each were inoculated with 100 µL of the suspension containing 3×10^5 yeast cells via the lateral tail vein and were euthanized in the various moments (1, 2, 3, 5, 7, 9 and 11 days) after infection to be evaluated. Among these nine groups one group (n=10) was not euthanized, serving as the positive control; and the other group (n=10) was inoculated with PBS, serving as the negative control. The groups were maintained during the study period (40 days). Animal handling and treatment observed the ethical principles of the Brazilian college of animal experimentation (COBEA).

Removal of lungs

At the end of each moment the study, all mice were euthanized in a CO_2 chamber and the lungs were aseptically removed and weighty to be used in the measurement of GXM, and cytokines.

Homogenizing of lungs

The organs were homogenized in a solution containing 1.0 mL of phosphate buffer solution (PBS) supplemented with 0.05% Tween 20, 1% protease inhibitor (Sigma-Aldrich) and 1% phenyl methyl sulfonyl fluoride (PMSF-1 mM) (Sigma-Aldrich). They were centrifuged for 5 minutes at 14000 rpm and homogenates of the organ that were not used immediately were stored at -80°C for later use.

Measurement of GXM in lungs

GXM was measured in the lung as by the method of capture ELISA as described in the literature [19,20]. The tests were performed in microtiter plates with 96 wells. Microplates were briefly coated with 1 µg/ml goat Ab to mouse IgG1 (Southern Biotechnology, Birmingham, AL). To capture the antigen, the antibody MAb 18B7 (5 µg/mL) was used [21,22]. The supernatant of the homogenate digested with proteinase K (Sigma-Aldrich) before being added to the plate was incubated at 100°C for 5 minutes to inactivate the enzyme. GXM was detected by MAb 12A1 [21,22], followed by the addition of anti-IgM conjugated with alkaline phosphatase. As a substrate for alkaline, phosphatase p-nitrophenyl phosphate (PNPP)-(Sigma-Aldrich) was used. Absorbance was measured at 405 nm and the amount of GXM in the solution was calculated from standard curves [23].

Levels of TNF-α, IL-6 and IL-10 in lungs homogenates

The inflammatory mediators TNF-α, IL-6 and IL-10 were measured by the ELISA (Enzyme-linked Immunosorbent assay) using the kits (Mouse IL-6 (Interleukin-6) ELISA Ready-SET-Go), (Mouse IL-10 (Interleukin-10 ELISA Ready-SET-Go) and mouse TNF-α ELISA Ready-SET-Go) were purchased from eBioscience and followed the manufacturer's guidelines.

Statistical Analysis

The mean survival times were estimated by the Kaplan-Meier method and compared among groups by using the log-rank test. The data obtained in relation to the levels of TNF-α (cell necrosis factor), IL6 (interleukin-6) and IL-10 in the lungs underwent logarithmic transformation to achieve an approximation of a normal distribution, prior to statistical analysis using the Mann-Whitney test. The correlation between the levels of cytokines production was performed using the Pearson correlation test. All the statistical tests were performed using the software GraphPad Prisma 5 (GraphPad Prism™, Version 5.0, and GraphPad Software Incorporated). Differences were considered significant when $p<0.05$.

Results

Survival

The average survival of immunocompetent animals (BALB/c) was 15 days and the animals with BALB/c-SCID 12 days after the initial infection. There were not significant decreases ($p<0.05$) in survival of groups of (BALB/c-SCID) animals compared to the group of (BALB/c) animals.

Measurement of GXM in lung homogenates

There was no detectable GXM in the immunocompetent model (BALB/c) on days 2, 3, 7 and 9 after initial infection. In the model with Severe Combined Immunodeficiency (BALB/c-SCID), GXM was not detected on day 2 after the initial infection. There was a significant production of GXM ($p<0.05$) in this model on day 1 (8.26 ± 0.16) and day 11 (4.27 ± 0.07), compared to immunocompetent model (4.5 ± 0.13), (2.6 ± 0.22), respectively (Figure 1).

Production of TNF-α (Tumor necrosis factor), IL-6 (Interleukin 6) and IL-10 (Interleukin 10)

TNF-α

The level of production at cytokine pro-inflammatory TNF-α in the lung homogenate in the murine model immunocompetent were significantly higher ($p<0.05$) on day 5 (33.25 ± 0.2) of infection, compared with murine model Immunodeficiency, (21.97 ± 0.2), (Figure 2).

No detected level of production this cytokine on day 7 of infection, this murine mode, the levels of production this is cytokine were significantly higher ($p<0.05$), compared with murine model immunocompetent on day 1 (79.0 ± 0.6), (44.9 ± 0.7) on day 2 (18.3 ± 0.06), (16.7 ± 0.2) on day 3 (36.3 ± 0.3), (21.5 ± 0.3) on day 9 (35.9 ± 0.4), (17.1 ± 0.1) and on day 11 (25.3 ± 0.2), (15.8 ± 0.4), (Figure 2).

IL-6 (Interleukin-6)

Detectable levels of IL-6 were not observed on days 9, and 11 of infection in the murine model immunocompetent. The level was significantly higher ($p<0.05$), when compared with murine model with Severe Combinated Immunodeficiency on day 2 (19.0 ± 0.3), (14.8 ± 1.6), on day 5 (38.2 ± 0.7), (22.1 ± 0.3) and day 7 (17.8 ± 0.1), (14.6 ± 0.2) of infection (Figure 3).

IL-10 (Interleukin-10)

Detectable levels of IL-10 do not was observed on days 7, 9 and 11; after initial infection in the murine model immunocompetent, and in day 11 in the murine model with Severe Combinated Immunodeficiency. The level of production this cytosine was

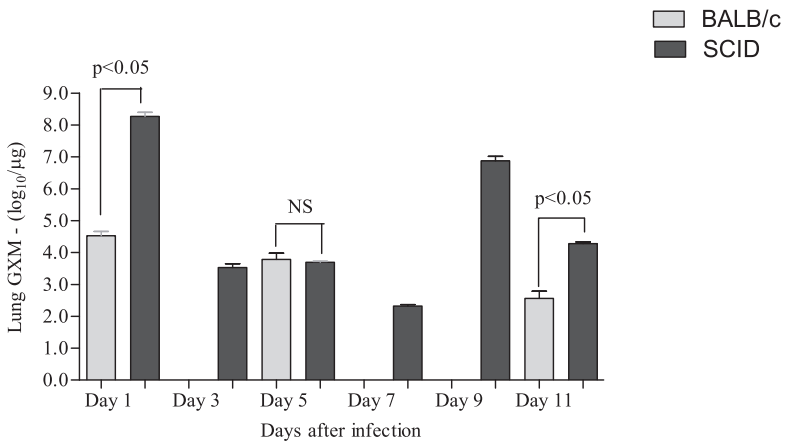

Figure 1: GXM production in the lung homogenate (1:100) evaluated after 1, 2, 3, 5, 7, 9 and 11 days after intravenous inoculation of 3.0×10^5 viable cells of *C. neoformans* (ATCC 90112. Serotype A) in murine model immunocompetent murine model (BALB/c) was detected only in days 1, 5 and 11 and murine model with severe combined immunodeficient (BALB/c-SCID) no detected on day 2. NS, Difference not significant . Differences wer e considered Statistical significant for $p<0.05$ (Mann-Whitney test).

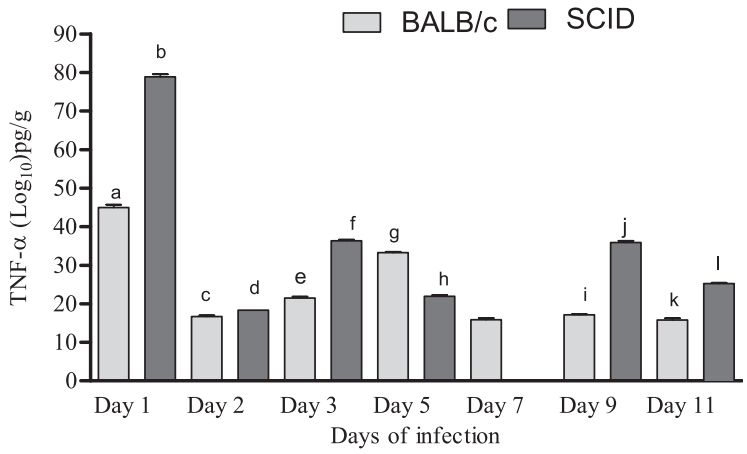

Figure 2: TNF-α level the lung homogenate in murine model immunocompetent (BALB/c) and with severe combined immunodeficiency (SCID), inoculated intravenously with 3.0×10^5 viable cells of *C. neoformans* (ATCC 90112. Serotype A). Evaluated on days: 1, 2, 3, 5, 7, 9 and 11 of infection. No detected-on day 7 of infection in murine model (SCID). Different letters indicate statistical significant difference for $p<0.05$ (Mann-Whitney test).

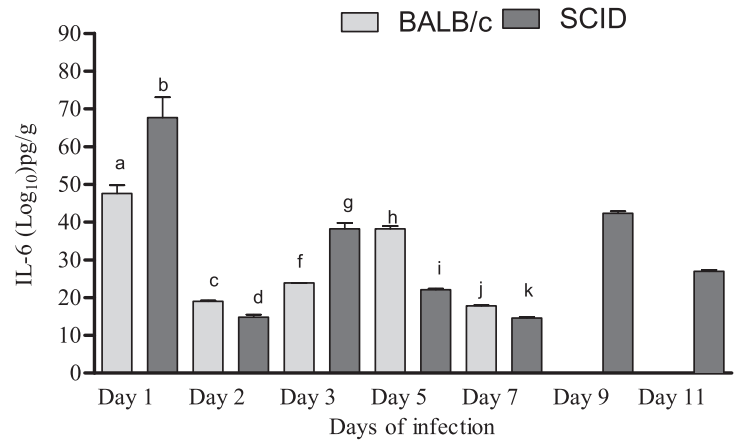

Figure 3: IL-6 level the lung homogenate in murine model immunocompetent (BALB/c) and with severe combined immunodeficiency (SCID), inoculated intravenously with 3.0×10^5 viable cells of *C. neoformans* (ATCC 90112. Serotype A). Evaluated on days: 1, 2, 3, 5, 7, 9 and 11 of infection. No detected on days 9 and 11 of infection in murine model (BALB/c). Different letters indicate statistical significant difference for $p<0.05$ (Mann-Whitney test).

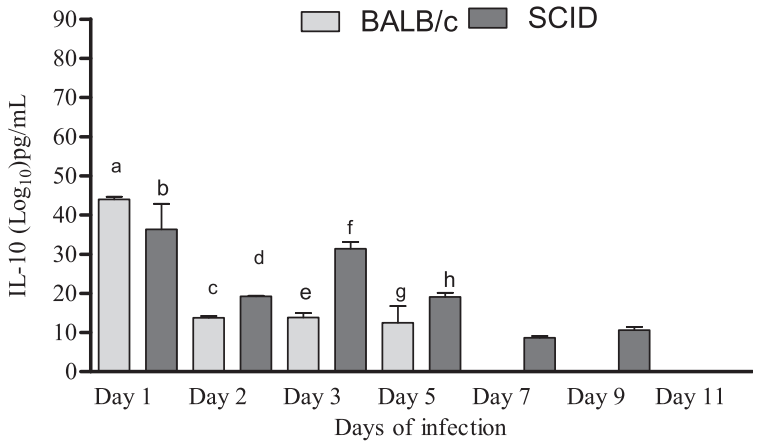

Figure 4: IL-10 level the lung homogenate in murine model immunocompetent (BALB/c) and with severe combined immunodeficiency (SCID), inoculated intravenously with 3.0 × 10^5 viable cells of *C. neoformans* (ATCC 90112. Serotype A). Evaluated on days: 1, 2, 3, 5, 7, 9 and 11 of infection. No detected on days 7, 9 and 11 of infection in murine model (BALB/c), and no detected on day 11 of infection in murine model with Severe Combinated Immunodeficiency. Different letters indicate statistical significant difference for p<0.05 (Mann-Whitney test).

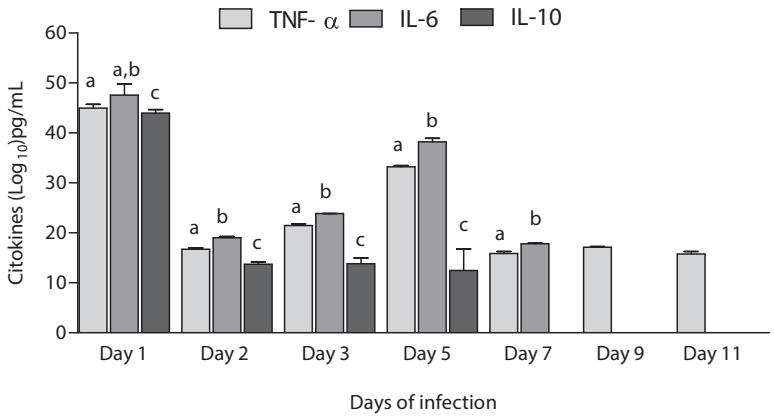

Figure 5: Comparative analysis between the levels of production of cytokines, TNF, I-6 and IL-10 in immunocompetent murine model (BALB/c) inoculated intravenously with 3.0 × 10^5 viable cells of *C. neoformans* (ATCC 90112-Serotype A). Evaluated on days 1, 2, 3, 5, 7, 9 and 11 of infection. IL-10 is not detected on days 7, 9 and 11 of infection, IL-6 not detected on 9 and 11. Different letters indicate statistically significant differences for p<0.05 (Mann-Whitney test).

significantly on day 1 of infection in the murine model with Severe Combinated immunodeficiency, (44.0 ± 0.7), compared with murine model immunocompetent (36.3 ± 6.5), (Figure 4).

Comparative analyses into TNF, IL-6 and IL-10

There was no significant difference in levels of production of TNF-α and IL-6 in the immunocompetent model on day 1 of infection. The production of IL-6 was significantly higher on days 2, 3, 5 and 7 of infection in this model. The production of TNF-α was significantly higher on days 9 and 11 of infection, (Figure 5). The correlation between the production of IL-6 and IL-10 was strongly negative (r= -0.93) on day 2 of infection, and strongly negative on day 3 of infection between the levels of production of TNF and IL-6.

In the model with SCID no significant difference in the production of TNF and IL-6 on 3 and 5 of infection. On days 1 and 2 infection of TNF production was significantly higher. The production of IL-6 was significantly higher on days 9 and 11 of infection. The levels of IL-10 was significantly higher on days 2 and 7 of infection. There was a strong positive correlation (r=0.96) between the production of IL-6 and IL-10 in 3 days of infection and on day 9 of infection between TNF and IL-6 (r=0.99).

Discussion

TNF-α is a proinflammatory cytokine which is an important protection against lung infections caused by *C. neoformans* [24]. In this work, we quantified the levels of production of proinflammatory cytokines (TNF-α and IL-6), as well as the cytokine which mediates IL-10 in lung homogenates.

The induction from an intense inflammatory process at the beginning of an infection was found in both models studied, since the levels of TNF-α were significantly higher in this during this period. The presence of an inflammatory process represents a fundamental role in restricting *C. neoformans* at the site of infection [25]. The intense inflammatory process that happened early in the infection was not able to restrict the presence of *C. neoformans* in the model with SCID, because through microbiological analysis of lung tissue a significant colonization of the yeast was found, as well as high levels of GXM still on the 9th and the 11th day of infection in this model. A statistical

study of the correlation between the production of TNF-α and GXM was strongly positive, suggesting that GXM was responsible for the induction of a severe inflammatory process in this model. GXM can induce the production of proinflammatory cytokines, such as TNF-α and IL-6 in neutrophils [26].

A vigorous early inflammatory response is associated with the development of a strong protective immunity and, consequently, the resolution of the infection [27]. The intense inflammatory response observed in the model with SCID was not enough to resolve the infection; this may have been due to the absence of an inflammatory response mediated by T and B cells. The murine mutation SCID is on chromosome 16 and causes these animals not to have immunity mediated by T and B cells [28,29].

The production of TNF-α before the 14th day of the lung infection by *C. neoformans* is essential for the development of protective immunity by T cells in the lungs [30]. IL-6 has a protective role in immune response against *C. neoformans* [31]. High levels of IL-6 were observed at the beginning of the infection in both models; however, the level of IL-6 on the model of SCID was significantly higher also on the 9th and 11th days of infection. High levels of IL-10 were observed in both models studied at the beginning of the infection; however, in the immunocompetent model that just occurred within the first 3 days. IL-10 was responsible for the regulation of inflammation in the immunocompetent model because we found a strong positive correlation between the levels of production of proinflammatory cytokines and IL-10 [32]. Interleukin 10 (IL-10) is a multifunctional cytokine produced by many different cell types, including alternatively and classically activated macrophages, dendritic cells, B cells and CD4 T regulatory cells. It is an important regulator of innate immunity and interferes with the production of inflammatory mediators by polymorphonuclear neutrophils, monocytes and macrophages [33]. The levels of IL-10 in the immunodeficient model were high in all periods of infection; however, this high production was not able to regulate inflammation in this model, as verified by analysis of levels of TNF-α and IL-6. The induction of severe inflammation observed may have been responsible for the reduction of survival of these animals, compared with the survival observed in the immunocompetent model. The deficiency modulating immune response mediated by T and B lymphocytes, in the model of combined immunodeficiency, leads us to infer that the inflammatory response observed in this model was derived from the innate immunity mediated by macrophages and mainly of neutrophils. In a murine model of immune system cells and CD4 T and CD8 T has been important in clearing *C. neoformans* pulmonary infection [34]. The humoral response, mainly immunoglobulin G, also increases the host defense against cryptococcosis [35], however, T cells have a modulatory effect on the response of B cells against infection with *C. neoformans* in mice [31].

C. neoformans is an intracellular parasite of phagocytic cells [36]. After phagocytosis the yeast can survive inside the macrophage, proliferating and eventually promoting cell lysis [37,38]. Neutrophils can contribute greatly to an innate immune response in cryptococcosis [39]. The migration of neutrophils in lung tissue is important in early protection of mice against progressive cryptococcosis [40,41]. The course of lung infection is characterized by a rapid response of neutrophils, with phagocytosis of Cryptococcus observed on the 7th day after the initial infection [39], since phagocytosis and the killing of *C. neoformans* by neutrophils are reduced due to the presence of a large polysaccharide capsule [42]. In this study we verified that the innate immune response was not effective in protecting animals against progressive cryptococcosis in the immunodeficient model by comparing it to the high levels of production of GXM, as well as high isolation of yeast in the lungs of these animals during the entire period of infection. GXM can suppress various functions of macrophages [43]. GXM is also able to influence cells such as neutrophils, however, in a different way. While in neutrophils. GXM is expelled from the cell, in macrophage an accumulation of GXM occurs an accumulation intracellular [44]. Therefore, our results lead us to suggest that the murine model of SCID is more susceptible to pulmonary infection caused by *C. neoformans*, as previously reported by other authors [29].

The severe inflammatory response caused by the innate immune system in this model may have been an important factor in reducing survival of these animals. Deficiency of adaptive immune response in mice of severe combined deficiency, the abnormal increase in the innate immune response may be an important cause of systemic immune response syndrome (SIRS) and reduced survival in this model [45]. GXM was responsible for greater survival of yeast in the lungs of these animals and was the main modulator of the severe inflammation observed in this model. There was an increase in the production of GXM, in (SCID) a severe inflammatory response in this model may be due to a compromised immune system.

The BALB-c/SCID model used in this research is especially important for the analysis of the pathophysiology of cryptococcal disease due to most human cases of disseminated cryptococcosis are associated with the CD4 + T cell deficiency in AIDS patients. In summary, GXM contribute to the pathogenesis of *C. neoformans* infections due to a variety of immunomodulatory effects between them inhibits the production of proinflammatory cytokines, induces inhibitory factors such as IL-10 and induces apoptosis in splenic mononuclear cells from normal rats [46-48].

Future studies should be carried out to examine the influence of GXM in apoptosis, as well, as their interaction with macrophages in this model.

Acknowledgements

The authors are grateful to FAPESP and CNPq, for financial support, Dr. Arturo Casadevall for providing the antibodies: Mab 18B7 and Mab 12A1. Conflict of interest statement: No conflict of interest with the present study.

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