UNIVERSIDADE DE UBERABA

PRÓ-REITORIA DE PESQUISA, PÓS-GRADUAÇÃO E EXTENSÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

1ª TURMA DE MESTRADO

Expression of CD23 and CD11b molecules by macrophage populations in the course of pulmonary infection with *Paracoccidioides brasiliensis* conidia in susceptible and resistant mice.

ALUNO: ROBERT BOAVENTURA DE SOUZA

ORIENTADOR: PROF. DR. MARCELO FERNANDES DA SILVA

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Dissertação de Mestrado, no formato de artigo submetido à publicação, como um dos quesitos indispensáveis a obtenção do título de Mestre, conforme regimento do Programa de Mestrado em Odontologia

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UBERABA

NOVEMBRO DE 2009

Ata da Sessão Pública de defesa de dissertação para obtenção do título de Mestre em Odontologia, área de concentração em Biopatologia, a que se submeteu o aluno Robert Boaventura de Souza – matrícula 6100210-1, orientado pelo Prof. Dr. Marcelo Fernandes da Silva.

Aos vinte e sete dias do mês de novembro do ano de dois mil e nove, às 13H30min, na sala 2C05 da Universidade de Uberaba, reuniu-se a Comissão Julgadora da defesa em epígrafe indicada pelo o Colegiado do Programa de Mestrado em Odontologia da Universidade de Uberaba, composta pelos Professores Doutores: Marcelo Fernandes da Silva - **Presidente**, Tony de Paiva Paulino e Denise Bertulucci Rocha Rodrigues, para julgar o trabalho do candidato Robert Boaventura de Souza, apresentado sob o título: "Expression of CD23 and CD11 b molecules by macrophages populations in the course of pulmonary infection with Paracoccidioides brasiliensis conidia in susceptible and resistant mice". O Presidente declarou abertos os trabalhos e agradeceu a presença de todos os Membros da Comissão Julgadora. A seguir o candidato dissertou sobre o seu trabalho e foi argüido pela Comissão Julgadora, tendo a todos respondido às respectivas argüições. Terminada a exposição, a Comissão reuniu-se e deliberou pelo seguinte resultado:

APROVADO X
REPROVADO (anexar parecer circunstanciado elaborado pela Comissão Julgadora)

Para fazer jus ao título de MESTRE EM ODONTOLOGIA ÁREA DE CONCENTRAÇÃO BIOPATOLOGIA, a versão final da tese, considerada Aprovada devidamente conferida pela Secretaria do Mestrado em Odontologia, deverá ser entregue à Secretaria dentro do prazo de 30 dias, a partir da data da defesa. O aluno Aprovado que não atender a esse prazo será considerado Reprovado. Após a entrega do exemplar definitivo, o resultado será homologado pela Universidade de Uberaba, conferindo título de validade nacional aos aprovados. Nada mais havendo a tratar, O Senhor Presidente declara a sessão encerrada, cujos trabalhos são objeto desta ata, lavrada por mim, que segue assinada pelos Senhores Membros da Comissão Julgadora, pelo Coordenador do Programa de Mestrado em Odontologia da UNIUBE, com ciência do aluno. Uberaba, aos 27 dias do mês de novembro de dois mil e nove.

Prof. Dr. Marcelo Fernandes da Silva _____

Prof. Dr. Tony de Paiva Paulino _____

Profª. Drª. Denise Bertulucci Rocha Rodrigues _____

Prof. Dr. José Bento Alves _____
Coordenador do Programa de Mestrado em Odontologia da UNIUBE

Poliana Gomes da Silva Alves _____
Secretária de Pós-Graduação

Ciência do Aluno: _____

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Corresponding Author: Dr Marcelo Fernandes da Silva, PhD

Corresponding Author's Institution: University of Uberaba

First Author: Robert B Souza, MS

Order of Authors: Robert B Souza, MS; Marden E Mattos-Jr, Undergraduated; Luiz P Queiróz-Jr, Undergraduated; Marcelo S Araújo, MS; Marcelo Fernandes da Silva, PhD; Célio L Silva, PhD

Abstract: We have been studying the expression of CD11b, CD23 and other cellular markers of macrophage populations in the inflammatory events in the lung of mice inflamed with β-glucan enriched-Paracoccidioides brasiliensis cell wall fraction. In this paper, we used flow cytometry and RT-PCR to analyse the expression of those molecules in the macrophages obtained from mice with different degrees of resistance or susceptibility against P. brasiliensis. When β-glucan enriched-cell wall fraction, or viable conidia, was introduced by intranasal or intraperitoneal routes, the expression of CD23 was significant higher in macrophages obtained from susceptible mice. In the other hand, the expression of CD11b/CD18 was enhanced in macrophages obtained from both resistant and susceptible mice. The expression of CD23 or CD11b/CD18 was up or down-regulated in vitro by the addition of recombinant IL-4 or IFN-γ. Despite of these findings, proinflammatory macrophage subpopulations from both mice strains had no significant differences concerning CD11b or CD23 mRNA expression when infected with conidia by intranasal route. Our results indicated that CD23+ macrophages could be related to the initial inflammatory events in the lung of susceptible mice, which seemed to be not involved with alterations in the level of mRNA expression

Suggested Reviewers: Terezinha S Peracoli PhD
Professor, Microbiology and Immunology, Biological Institute - University of São Paulo State
peracoli@ibb.unesp.br
Dr. Peracoli is one of the most experienced researchers on experimental paracoccidioidomycosis in Latin America, with several contributions to better understading of resistance or suscetibility against P. brasiliensis

Angel González PhD
Researcher, Medical and Experimental Mycology Unit, , Corporación para Investigaciones Biológi
agonzalezm@cib.org.co
Dr González has been publishing serveral papers regarding the role of pulmonary inflammatory cells in the course of fungal infections.

Denise Rodrigues PhD

Professor, Immunology, University of Triangulo Mineiro
denise.rodrigues@uniube.br
Dr Rodrigues has important contributions in inflammation processes associated to infectious diseases with consequences on Th1 or Th2 and Treg activation.

Opposed Reviewers:

Uberaba-MG, dec 12, 2009

To Editorial Office of Immunity

We are submitting an original manuscript entitled "Expression of CD23 and CD11b molecules by macrophage populations in the course of pulmonary infection with *Paracoccidioides brasiliensis* conidia in susceptible and resistant mice".

It is focusing original data obtained throughout the experiments on the cellular basis of inflammatory reaction against *Paracoccidioides brasiliensis*, which is one of the major interests of the corresponding author's research group. The cellular and molecular mechanisms behind the host x *Paracoccidioides brasiliensis* relationships are leading subjects of several paper published abroad.

Immunity is one of the most important journal devoted to the study of vary aspects of human immunity and experimental animal biology applied to several infectious diseases.

All the authors agreed with the submission to this journal and none of them has any potential financial conflict of interests related to this manuscript.

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3. Credit to authorships is only to those who have participated substantially in the preparation of this manuscript;

4. This paper is not currently under consideration for publication elsewhere.

We look forward to hearing from you,

The authors.

Corresponding author: Dr Marcelo Fernandes da Silva

Laboratory of Microbiology and Immunology

University of Uberaba

Minas Gerais - Brazil

Expression of CD23 and CD11b molecules by macrophage populations in the course of pulmonary infection with *Paracoccidioides brasiliensis* conidia in susceptible and resistant mice.

Robert Boaventura de Souza[1,2]; Marden E. Mattos-Jr[3]; Luiz de Pádua Queiroz-Jr[3]; Marcelo Fernandes da Silva[1,2] Marcelo Sivieri de Araújo[2] and Célio L. Silva[4].

[1]Program of Post-Graduation in Odontology of Pro-Rectory of Research, Post-Graduation and Extension – University of Uberaba.

[2]Laboratory of Biopathology, University of Uberaba

[3]Laboratory of Biomedical Science - University of Uberaba

[4]Department of Biochemistry and Immunology - School of Medicine of Ribeirão Preto - University of São Paulo - Brazil.

Running Title: $CD23^{+}$ macrophages in the susceptibility and resistance against *Paracoccidioides brasiliensis*

[1] To whom correspondence should be addressed: Dr Marcelo Fernandes da Silva - Pro-Rectory of Research, Post-Graduation and Extension – University of Uberaba- Uberaba - 38050-500. Minas Gerais. Brazil. Phone: (55) 34-3319-8839 E-mail: marcelo.fernandes@uniube.br

List of abbreviations: CW: cell wall components;; BAL: bronchoalveolar lavage fluid; FCM: flow citometry; FCS: fetal calf serum; i.n.: intranasal route; i.p.: intraperitoneal route; MoAb: monoclonal antibody; PCMEx: experimental paracoccidioidomycosis, PEC : peritoneal exsudate macrophages.

SUMMARY

We have been studying the expression of CD11b, CD23 and other cellular markers of macrophage populations in the inflammatory events in the lung of mice inflamed with β-glucan enriched-*Paracoccidioides brasiliensis* cell wall fraction. In this paper, we used flow cytometry and RT-PCR to analyse the expression of those molecules in the macrophages obtained from mice with different degrees of resistance or susceptibility against *P. brasiliensis*. When β-glucan enriched-cell wall fraction, or viable conidia, was introduced by intranasal or intraperitoneal routes, the expression of CD23 was significant higher in macrophages obtained from susceptible mice. In the other hand, the expression of CD11b/CD18 was enhanced in macrophages obtained from both resistant and susceptible mice. The expression of CD23 or CD11b/CD18 was up or down-regulated *in vitro* by the addition of recombinant IL-4 or IFN-γ. Despite of these findings, proinflammatory macrophage subpopulations from both mice strains had no significant differences concerning CD11b or CD23 mRNA expression when infected with conidia by intranasal route. Our results indicated that $CD23^{+}$ macrophages could be related to the initial inflammatory events in the lung of susceptible mice, which seemed to be not involved with alterations in the level of mRNA expression.

INTRODUCTION

Resident macrophage populations could be found at different organs being capable to adapt to their local microenvironment, especially in the lungs (Waldorf et al., 1989; Tournier et al., 2008). Several contributions have been studying macrophage´s subpopulations with respect to phenotype and cellular activation patterns (Dorger et al., 2001; Geissmann et al., 2003). The heterogeneity of macrophages has been currently focused and may be important for the diversity, flexibility and validity of innate and adaptive immune responses (Laskin et al., 2001) against fungal infections (Gonzalez et al., 2008; Guillot et al., 2008). Paracoccidioidomycosis is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis (P. brasiliensis*) characterized by a large broad of clinical manifestations associated with unbalanced Th2 response and disseminated infection (Mello et al., 2002). In addition, the pulmonary involvement in the course of natural infection supports the lungs as primary target in human paracoccidioidomycosis (Severo et al., 1979). Experimental model of resistance and susceptibility against *P. brasiliensis* has been established (Calich et al, 1985) using the pulmonary route of infection with conidia (Gonzalez et al., 2008) regarding the natural infection rather than the inoculation of yeasts by intravenous (i.v.) route. Thus, experimental paracoccidiodomycosis (PCMEx) represents a viable model to study macrophage subpopulations and their role in acute inflammatory events in the lung with consequences to host versus parasite's relationships. It has been shown that phagocytes have an important defense role in the natural resistance against *P. brasiliensis*. Recently, a number of cell surface molecules involved in cell adhesion, co-stimulation, motility and migration have been recognized (Moreira et al., 2006; David et al., 2007). Moreover, the possible roles of CD23 and CD11b as receptors for polysaccharides (Thorton et al., 1996) in the pulmonary inflammatory events against cell wall fractions from *P. brasiliensis* have been currently described (Queiroz-Jr et al., 2009). In the present study, we

investigated the differences on macrophage subpopulations and level of molecule expression of CD11b/CD18 and CD23 among peritoneal exsudate macrophages (PEC) and bronchoalveolar lavage macrophages (BAL) obtained from resistant and susceptible mice after challenge with *P. brasiliensis* derivatives. The data reported here suggest that $CD23^+$ macrophages predominated in the BAL of susceptible mice, instead of $CD11b^+$. The differences on CD23 and CD11b expressions were also affected *in vitro* by IFN-γ and IL-4. Therefore, CD23 mRNA expression was not significant between susceptible and resistant mice strains. Thus, the involvement of non-specific events found in PCMEx could be associated to a role of $CD23^+$ macrophage populations in the lungs, but not to an increased level of mRNA expression.

RESULTS

Selective recruitment of macrophage populations under inflammation caused by β-glucan from P. brasiliensis cell walls.

We started to study the expression of CD23 and CD11b/CD18 in multiple flow cytometry acquisitions of BAL from different strains of mice under experimental inflammatory events as described elsewhere (Queiroz-Jr et al., 2009) that are summarized in Figure 1. The acute inflammatory cells in the BAL showed a prevalence of $CD23^+$ macrophages in BALB/c, B6 and B10.A ($p<0.001$) instead of A/Sn which showed a significant difference ($p<0.01$) in $CD11b^+$ macrophages.It can be observed that BALB/c and A/Sn showed a cellular inflammatory pattern with higher levels of CD11b ($p<0.001$) in PEC when compared with CD23. On contrary, B6 and B10.A seemed to exhibit a higher level of CD23 ($p<0.001$) than CD11b mainly at 2 days post-treatment by intranasal (i.n.) route with β-glucan from *P. brasiliensis*. It was also possible to note that intraperitoneal (i.p) inoculation of β-glucan from

P. brasiliensis caused a persistent expression of CD11b by PEC in all mouse strains, except in B10.A.

Expression of CD23 and CD11b/CD18 in the course of pulmonary infection with conidia from P. brasiliensis

Since CD23 and CD11b levels varied among resistant and susceptible mice inflamed with β-glucan from *P. brasiliensis*, the expression of these molecules was monitored up to 60 days post-infection with viable conidia. A typical FCM (flow citometry) acquisition of BAL can be observed in Figure 2. The macrophages present in BAL from mice inflamed with cell wall derivatives or infected with conidia from *P. brasiliensis* were characterized (Queiroz-Jr et al., 2009) as $F4/80^{+}$, Ia^{+}, TCR^{-} and $CD4/CD8^{-}$ (not shown). The BAL macrophages from B10.A strain showed a persistent expression of CD23 throughout the infection, ranging from 18 to 46 % at 30 days post-infection ($p<0.01$ Figure 3; $p<0.001$ Figure 4). The BAL macrophages from A/Sn were highly and persistently positive to CD11b/CD18, while the expression of CD23 decreased after 4 days of infection. In the Figure 4 is possible to verify the comparison between CD23 and CD11b/CD18 at 30 days post-infection. It can be noted that susceptible mice exhibited the highest ($p<0.001$) levels of CD23 whereas resistant mice showed significant ($p<0.001$) expression of CD11b/CD18.

Expression of CD23 and CD11b/CD18 are modulated by IFN-γ and IL-4.

Since experimental model of *P. brasiliensis* infection with conidia seemed to be established on the basis of IFN-γ and/or IL-4 production (Gonzalez et al., 2000) we decided to verify the interference of those interleukins in the expression of CD23 and CD11b/CD18 by mononuclear adherent cells recovered from lungs from susceptible/resistant mice infected with conidia. In the Table 1 is possible to note that IFN-γ up-regulated ($p<0.001$) the expression of CD11b whilst CD23 seemed to be unaltered by the addition of the same

interleukin in vitro. Besides, the addition of IL-4 to macrophages cultures caused a significant increase ($p<0.001$) in the expression of CD23 mainly in susceptible mice.

Expression of CD11b and CD23 mRNA in the acute phase of pulmonary infection

According to the degree of resistance or susceptibility, each strain of mice infected with conidia from *P. brasiliensis* showed different pattern of mRNA expression to CD11b or CD23 when compared to its controls (Figure 5 and 6). Two days post-infection, both strains of mice produced similar amount of CD11b and CD23 mRNA (Figure 6). In the peak of acute inflammatory reaction caused by the inoculation of viable conidia, the B10.A mice presented an influx of inflammatory cells characterized as mix of neutrophils and macrophages (Figure 5 A;D) which exhibited low CD11b mRNA and high CD23 mRNA expressions (not significant) when compared to its non-infected controls (Figure 6A). On contrary, the A/Sn mice exhibited an inflammatory pattern (Figure 5 B;E) with highest level ($p<0.01$) of CD11b mRNA and the lowest level (not significant) of CD23 mRNA expression (Figure 6A). Nonetheless, after 4 days of pulmonary infection, the inflammatory reaction associated to the conidial infection was better noted in the B10.A mice (not shown).The expression of CD11b and CD23 mRNA varied among mouse strains, and it were higher, but not significant in infected B10.A when compared to its controls (Figure 5H; 6B). The A/Sn-infected mice produced the lowest levels of mRNA of both CD11b and CD23. It is possible to note that B10.A mice seemed to produce higher levels of mRNA to CD11b and CD23 when compared to A/Sn mice. Similar results were obtained after 15 days post-infection (not shown).

DISCUSSION

The results presented herein enlarge our previous observation on the involvement of the CD11b/CD18$^+$ and CD23$^+$ macrophage subpopulations in the initial inflammatory events in the course of PCMEx (Queiroz-Jr et al., 2009). The expression of mRNA to CD23 and CD18 by pulmonary cells from susceptible and resistant mice strains was compatible with phenotyping of inflammatory cells in the lungs. Susceptible mice strains exhibited higher counts of CD23$^+$ alveolar macrophages in the BAL and they failed to express higher level of mRNA to CD23 after conidial infection. In fact, the susceptible B10.A mice when infected with conidia form *P. brasiliensis* presented an acute influx of cells in the BAL, which was detected in both cytometry acquisition and histological analysis. RT-PCR from those cells showed an increase of mRNA expression to CD11b/CD18, an important integrin, which is compatible to a constant cellular migration from intravascular compartment to the alveoli. Intermediary/resistant mice strains exhibited lower counts of CD23$^+$ macrophages and more preserved architecture of alveoli and controlled influx of CD11b/CD18$^+$ cells in beginning of the infection. The cells from resistant mice express mRNA of both CD23 and CD11b molecules. These results corroborate recent findings on the different macrophages abilities of resistant and susceptible mice in the pulmonary infections (Rambert et al., 2009; Loures et al., 2009).

The cell wall components extracted from the yeast are representative of the main cell wall polysaccharide (β-glucan and chitin) of the fungi-derived wild infectious conidia (Garcia et al., 2009) and are of value to investigate the initial steps of inflammatory event in the host-fungi relationships. The notion that macrophage subpopulations present selective receptors for glucans (Cain et al., 1987; Thorton et al., 1996) and that they would be able to generate different inflammatory responses in the initial events of paracoccidioidomycosis (Gonzalez et al, 2005) give further evidences to our hypothesis. As the conidia from *P. brasiliensis* are

carbohydrate-rich structures, there is a possibility of the involvement of pattern recognition receptors (PRRs) having consequences in host's innate immunity to this fungus. The cell wall derivatives from *P. brasiliensis*, which is β-glucan enriched, could be triggering Toll-like receptors-mediated innate mechanisms as observed in *Candida albicans* (Van de Veerdonk et al., 2009) infection, with preferential secretion of TGF-β and IL-6 (Calich et al., 2008). Both interleukins were also detected in $CD23^+$ pulmonary macrophages cultures from C57.Bl/6 mice inflamed with *P. brasiliensis* derivatives (Queiroz-Jr et al., 2009).

We conclude that an effort to address macrophage functional patterns towards the inflammatory process could reveal a distinct phenotype based on the CD11b/CD18 and CD23 might alter macrophage responsiveness and phenotype in the course of natural infection, since $CD23^+$ cells induced by *P. brasiliensis* cell wall components and conidia could be involved in the acute immunological disturbances at lungs. All the micro environmental influences on pulmonary macrophages are likely to be contributing to their heterogeneity in tissues after the exposure of *P. brasiliensis* cell wall derivatives.

EXPERIMENTAL PROCEDURES

Animals

Male specific pathogen-free isogenic BALB/c and C57/Bl.6 (B6) mice ageing 6-8 weeks were obtained from the University of Campinas (UNICAMP - Brazil) and A/Sn and B10.A mice ageing 6-8 weeks were purchased from the Biological Science Institute from University of São Paulo - Brazil (ICB-USP) central stocks and kept at animal facility at the University of Uberaba under conventional conditions with food and water *ad libitum.* Animal manipulations were done in agreement to the institutional guidelines for animal welfare.

General Reagents

All reagents, unless indicated, were purchased from Sigma® Chemical Co. (St. Louis, MO, USA). All water used to prepare solutions was obtained from Milli-Q Plus device. LPS-free media were used throughout the experiments. RT-PCR protocols were done with RNAse free consumables and solutions.

Fungal strain, culture conditions, conidia prepararation and β-glucan purification

Virulent *P. brasiliensis* Pb18 was kindly gifted by Dr. Maria Terezinha Serrão Peraçoli from Bioscience Institute from University of São Paulo State (IBB –UNESP- Botucatu) cultured at 37° C for 20 days in PDA (Potato Dextrose Agar) medium. The cells were then harvested, formallin killed and washed several times with distilled water. Conidia were obtained by cultivation of virulent *P. brasiliensis* Pb18 at 25° C for 45 days in PDA medium and then propagules were recovered by glass fiber filtration as described elsewhere (Restrepo et al., 1986). Fractionation of *P. brasiliensis* cell walls resulted in a preparation enriched in β-glucan as described previously (Alves et al., 1987; Oliveira et al., 1993).

Inflammatory treatments and intranasal route of infection

In order to cause a pulmonary inflammation, animals were randomly separated in different groups that were inoculated by intranasal route, using a G26 gauge (BD), with a final volume

of 0.1 mL of one the following treatments: sterile PBS; 0.1 μg of LPS; 15 μg of F1 fraction from *P. brasiliensis*. After 1, 6, 12, 24, 48, 96 and 198 hours the BAL was recovered. Thus, mice were submitted to euthanasia under profound anesthesia. Then the trachea was surgically exposed and cut to introduce a thin plastic cannula connected to 1 mL syringe. A volume of 2 mL of cold PBS-heparin was introduced into the lungs and the lavage performed with several flushes. The entire volume infused to each mouse was recovered, and kept at 4° C. Control groups received PBS only. Similar protocol was used with the intention to cause pulmonary infection with 2x 10^6 viable cells.ml^{-1}. Viability of inocula was confirmed by sowing conidia in PDA medium plate and let to stand at 37°C during 21 days to observe percentage of yeast growth. To cause a peritoneal inflammation, a final volume of 0.5 mL of PBS containing 100 μg of Fraction F1 from *P. brasiliensis* was injected by i.p. route. Control group received PBS only (without the injection of thioglycolate as irritating agent). PEC were harvested 1, 6, 12, 24, 48, 96 and 198 hours after treatments using 5 mL of cold PBS-heparin and flushing the cavity several times.

Flow cytometry and cell culture of mononuclear cell populations

The suspension of 1 x 10^6 mL^{-1} of BAL or PEC cells was incubated at 4° C for 30 min with 1 μg of rat monoclonal anti-CD11b (CD11b/CD18, Pharmingen) anti-CD23 (Mac-2, Boehringer Mannheim) diluted 1:100 in FACS-PBS containing 5 % BSA, 8% FCS, 1% mouse control, 2.5% of normal goat or rat serum and 0.02% sodium azide. To develop the reaction, 10 μl of anti-rat IgG, raised in goat and labelled with FITC (Dako) and diluted 1:2000 in the FACS-PBS was added and the preparations were incubated at 4° C for 30 min. All cell suspensions were analysed in FACScan Cell Sorter (Becton Dickinson®) using gate analysis for lymphocytes or macrophages comparing the intensity of fluorescence of cells from treated groups to that of control cells incubated with an unrelated and isotype matched antibody. The FCM acquisitions were performed after a standartisation in FSC and SSC

detectors and data were analysed using LYSIS II® software. Coefficient of variation among all controls in each experiment was less than 10%. The cell cultures were done always in the absence or in the presence of biological active substances such as LPS of *Escherichia coli* used in the dose of 10ηg per well (50ηg.mL^{-1}); recombinant IFN-γ (Genetech) used in the dose of 12.5 U per well respectively (60 U.mL^{-1}) or IL-4 in the concentration of 50 ηg per well (100 ηg.mL^{-1}). The macrophages from BAL or PEC were adjusted to 5x10^5 cells placed into 96 wells flat bottom plates and incubated in a humid incubator at 37°C at 5% of CO_2 during 24 hours. After incubation, the cells were carefully harvested and submitted to flow cytometry protocol to investigate the expression of CD23 and CD11b as described.

Histological processing of the lungs

After 2 and 4 days of initial infection, the control and experimental mice were sacrificed and the lungs surgically removed. A piece from each right and left lobes from lungs were taken and kept in plastic cassettes and fixed in 10% buffered formalin. Then, the samples were dehydrated in alcohol and submitted to diaphanization in xylol. The samples were immersed in paraffin by 2 hours at room temperature until solidification. After, the blocks were submitted to microtome (LEICA model RM2145) to yield several sections of 6 µm from tissues. The sections were put onto glass slides and then it were hematoxilin-eosin stained, examined histologically and photographed by using a Nikon Eclipse E200 microscope and software Cybelink Power VCR II®.

RNA extraction, RT-PCR and PCR to CD11b and CD23 of BAL cells.

After recovering the BAL from each experimental group, the cells suspensions were centrifuged at 5000 g during 10 min and the cell counts adjusted to 2.5 x 10^6.mL^{-1} following the addition of 700 µL of Trizol® (Invitrogen) in a polypropilene tube and kept at –86°C until use. The cell preparations were thaw under ice bath and filled with 200 µL of chloroform and then mix by 15 seg in the vortex, and let to stand at room temperature by 3

min. Following centrifugation at 10.000 g during 15 min at 4°C, the aqueous phase were transferred to a new polypropilene tubes which were filled with 500 µL of chilled isopropanol. Then the last preparation was incubated for 2 hr at –86°C. At the end of last incubation, the preparations were centrifuged again and the pellets were dissolved with 8 µL of ultrapure DEPC treated water.

The quantity/quality of total RNA was analysed by agarose gel electrophoresis under denaturing conditions by using MOPS (3- N morpholinopropanosuphfonic acid) and formaldehyde. Briefly, to each 0.75 g de agarose, it was added 5 mL de 10X MOPS and 36 mL of autoclaved water. After heating, a volume of 9 mL of formaldehyde was added to preparation under chapel of exhaustion. Each 5 uL sample of RNA was mix to 10 µL of loading buffer and it was resolved after running by 1:30 min under 100 V and 55 mA. The RNA bands were visible after exposition to 0.1 % ethidium bromide in 1X TBE solution and analysed under UV transilluminator digital apparatus.

In order to generate cDNA, to each 5 µL of mRNA from experimental and controls groups, it was added 1 µL de oligo(dT) primer, 1 µL de 10mM dNTP mix and 3 µL of DEPC-treated water. A volume of 1µL of mRNA from HeLa cells (Invitrogen) was used as an amplification control. All mRNA preparations were submitted to a brief agitation and centrifugation, followed by incubation at 70° C during 10 min and in ice bath for 1 min.

All preparations were added with 7 µL of RT-PCR mix composed of 2.5 µL 10X RT buffer, 2 µL de 0.1 M DTT, 0.5 µL 25 mM $MgCl_2$ and 2 µL of DEPC-treated water. All samples were mixed, and then submitted to brief centrifugation. The samples were transferred to thermocycler (XP Thermal Cycler – Bioer - China) let to stand 2 min at 4° C, 2 min at 42° C and chill; after a brief centrifugation, all samples received 2 µL of 200 $U.\mu L^{-1}$ RT SuperScript III (Invitrogen) following by a new incubation of 1 hr at 37°C. After the last incubation, all cDNA samples were kept under –20° C until use.

In order to run PCR from cDNA, each sample was thaw under ice bath and then added with 12.5 uL of PCR master mix (Promega) containing dNTPs, Taq DNA Polimerase and $MgCl_2$, 3 μL containing a mix of each primer pairs (IDT Technologies) for CD11b (5'- CAG ATC AAC AAT GTG AAC GTA TGG G -3' / 3'-G TTC GCC GTC ATG TTC CTG TAC TAC -5'), CD23(5'- GCA CGC CTC ATC ACT GAA AGG -3'/ 3'- TGG GGT TTT TCA CTT GGG -5') or β-actin (5'- ATG GAT GAC GAT ATC GCT -3'/ 3'- T GGA CTG TCT GAT GGA GTA -5'), 5 μL of cDNA template and 4.5 μL of DEPC-treated water. After vortex, all samples were transferred to a thermocycler (XP Thermal Cycler – Bioer - China) running 2 min at 94° C following 38 cycles of 15 seg at 94°C, 30 seg at 55°C and 1 min at 72°C. After a final extension at 72° C, the samples were chilled.

The PCR products were resolved in 0.8% agarose gel in 1X TBE buffer after 100 V and 55 mA during 1:30 h. Bands were revealed after exposition to 0.1 % ethidium bromide in 1X TBE solution under constant oscilatory movement during 20 min and the analysed under UV-transilluminator. The images were acquired by the UV transilluminator (UVP M-26 Benchtop® - USA) and band intensity and volume measured by the Life Science Software (USA) by three independent subjects.

Statistical analysis

The One-way ANOVA was used to test the variance of data. The unpaired two-tailed Student's t test was used to determine the significance of differences between means from control versus experimental groups. The data from flow cytometry were generated by the software WinMDI 2.8 and analysed by non-parametric Kruskall-Wallis test. The ratio of mRNA expression from each control and experimental groups was analysed by non-parametric Mann-Whitney test. Unless otherwise indicated, all relevant comparisons were significantly higher than $p < 0.05$. Some results of flow cytometry are presented having the abbreviations: Gm: Geometrical median; Cv: Coefficient of variation; Md: Median.

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385 Figure Legends

386

387 **Figure 1**: Differences on cellular expression of CD11b and CD23 in the inflammatory
388 reaction caused by β-glucan from *P. brasiliensis* cell walls by i.p or i.n. routes in the mouse
389 strains with different degrees of resistance (A/Sn), intermediary (BALB/c) or susceptibility
390 (B6; B10.A) against *P. brasiliensis* infection. The intensity of fluorescence in each group is
391 presented by median of three independent acquisitions. Non-parametric Kruskall-Wallis's test
392 with (*) $p < 0.001$ or (**) $p < 0.01$.

393 **Figure 2:** Pattern of typical acquisition of inflammatory cells from BAL of A/Sn and B10/A
394 mouse strains infected with conidia from *P. brasiliensis* by intranasal route (**A**). Note the
395 prevalence of CD11b and the slightly augmentation in expression of CD23 in A/Sn mice in
396 the onset of inflammatory events. The expression of CD23 was sustained by macrophages
397 from B10.A mice. $CD11b^{DIM}$ represents an intermediary fluorescence higher than isotype
398 matched controls; $CD11b^{BRIGHT}$ represents the highest fluorescence when compared to
399 isotype matched controls. Comparison of CD11b expression (**B**). Comparison of CD23
400 expression in gate R2 (**C**)

401 **Figure 3**: Expression of CD11b and CD23 in the course of *P. brasiliensis* conidial infection
402 of A/Sn or B10.A mouse strains 4 days post intranasal infection. $CD11b^{DIM}$ (M2) represents
403 an intermediary fluorescence higher than isotype matched controls; $CD11b^{BRIGHT}$ (M1)
404 represents the highest fluorescence when compared to isotype matched controls. Comparison
405 of CD11b expression (**A**). Comparison of CD23 expression in gate R2 (**B**)

406 **Figure 4**: Expression of CD23 and CD11b in the course of *P. brasiliensis* conidial infection
407 of A/Sn or B10.A mouse strains 30 days post intranasal infection. $CD11b^{DIM}$ (M2) represents
408 an intermediary fluorescence higher than isotype matched controls; $CD11b^{BRIGHT}$ (M1)
409 represents the highest fluorescence when compared to isotype matched controls. A)
410 Comparison of CD11b expression. B) Comparision of CD23 expression in gate R2

411 **Figure 5**. Histological photomicrographs of lungs stained with haematoxilin and eosin (HE)
412 after inoculation of 2×10^4 viable conidia of fungus *P. brasiliensis* in A/Sn and B10.A mice.
413 Lung sections of B10.A mouse 2 days post infection showing many alveolar neutrophils and
414 macrophages scattered in the acute inflammatory infiltrate (**A;D**). Lung sections of A/Sn
415 mouse after 2 days of infection showing a better control of infection with hyperemic vessels
416 and cleaned alveoli (**B;E**). Lung sections of B10.A and A/Sn control mice after 2 days of
417 inhalation with 0,9% saline solution, showing absence of inflammatory process (**C;F**).
418 Photograph of 1% agarose gel electrophoresis showing products from RT-PCR to ß-actin
419 (600pb) CD11b (500pb) and CD23 (900 bp) in B10.A control (lanes 1, 3, 4 and 14, 15,16
420 respectively) and infected mice (5, 6, 7 and 17,18,19 respectively); A/Sn control (8, 9 and
421 20,21,22) and infected mice (11, 12, 13 and 24, 25, 26 respectively). La: Ladder; RT-PCR
422 positive (1) and negative (2) controls. (**G;H**)
423

424 **Figure 6:** CD23 or CD11b mRNA expressions in the acute phase of pulmonary infection
425 with *P. brasiliensis* conidia at 2 (**A**) and 4 (**B**) days post-infection. The bars indicate the ratio
426 of the β actin/CD11b or β actin/CD23 mRNA expressions. * $p<0.01$ Mann-Whitney.
427

428 Table Heading

429

430 Table I: IFN-γ e IL-4 up-regulate the expression of CD11b and CD23 by mononuclear
431 adherent cells from BAL after i.n. conidia infection. Mononuclear cells were recovered from
432 lungs at 15 days post-infection. Data are representative from experiments within two
433 independent replicates. Bold values indicate alterations (* increase with $p<0.001$) († decrease
434 with $p<0.001$) in the specific fluorescence intensity analised by Student's t test.

Table I

Macrophage Sources	*In vitro* Treatments	*Medians of Specific Fluorescence Intensity*			
		Susceptible		*Intermediary*	*Resistant*
		CD23 (B10.A)	**CD23 (B6)**	**CD11b (BALB/c)**	**CD11b (A/Sn)**
BAL	**RPMI**	40	55	25	33
	LPS	55	45	**65***	**80***
	IFN-γ	**17†**	**23†**	**75***	**88***
	IL-4	**80***	**78***	30	32
PEC	**RPMI**	22	17	70	59
	LPS	**32***	**34***	**99***	**99***
	IFN-γ	15	15	**99***	**99***
	IL-4	**55***	**35***	75	60

Figure 1: Prism converted to MS Office 2000

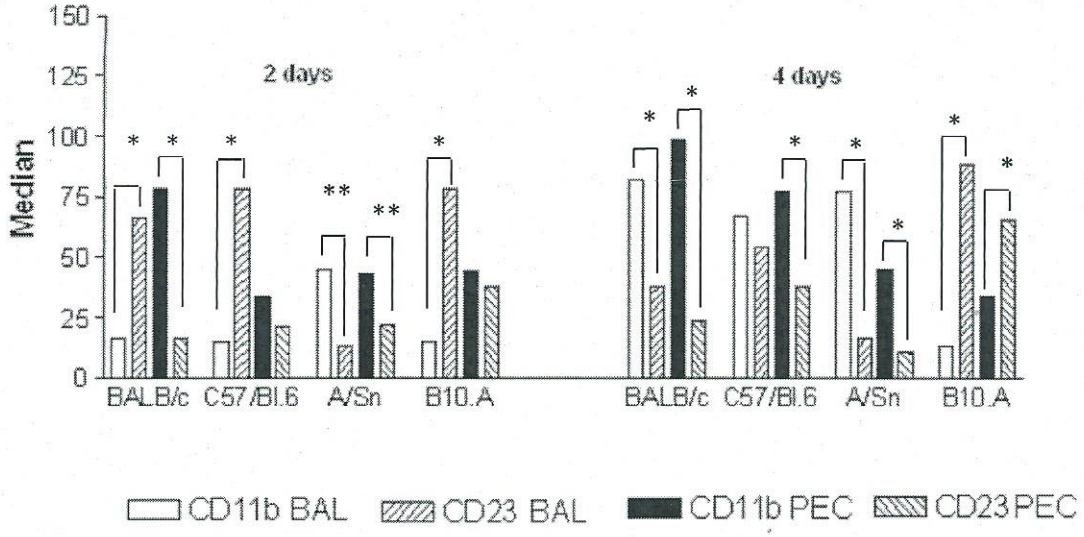

Figure 2: *WinMidi converted to MS Office 2000*

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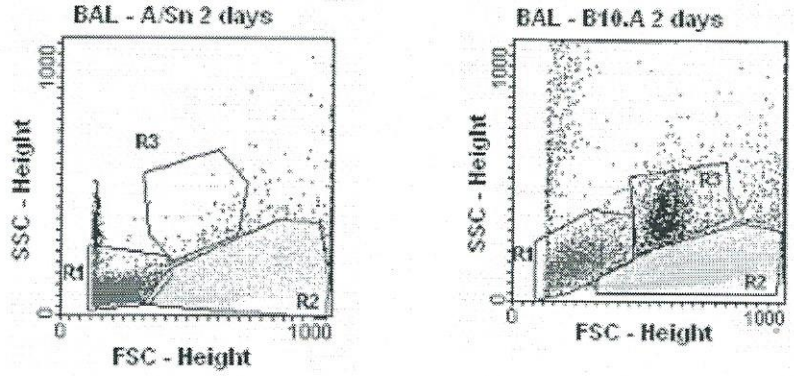

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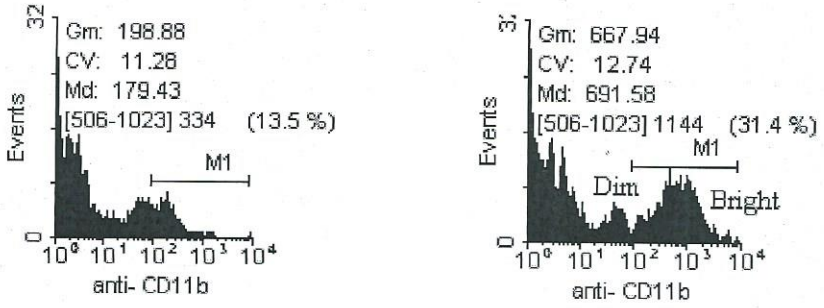

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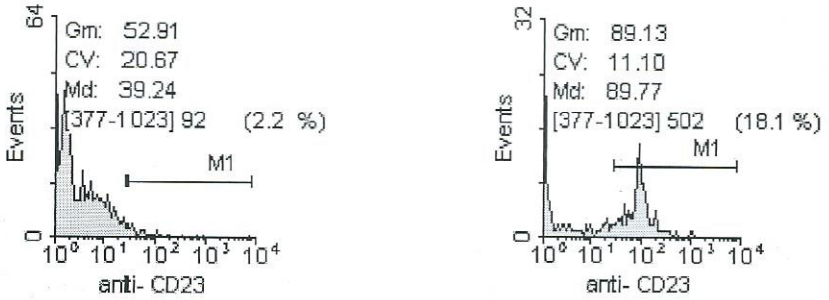

Figure 3: WinMidi converted to MS Office 2000

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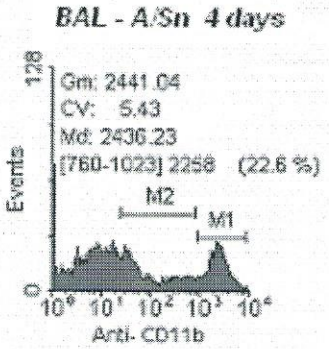

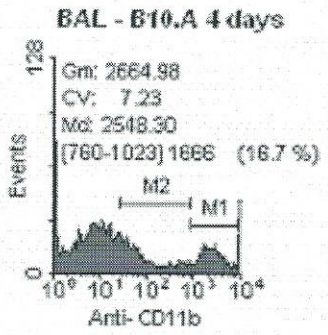

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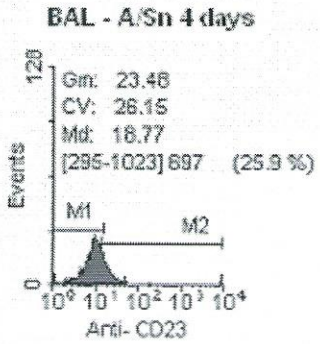

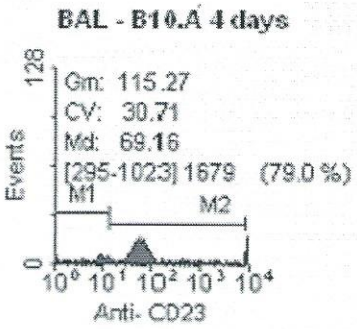

Figure 4: WinMidi converted to MS Office 2000

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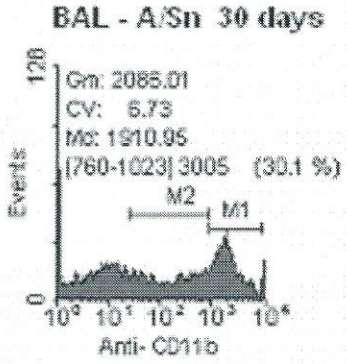

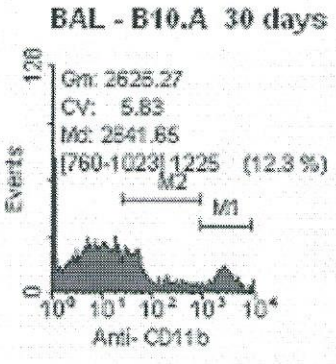

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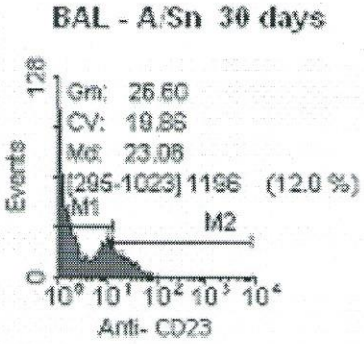

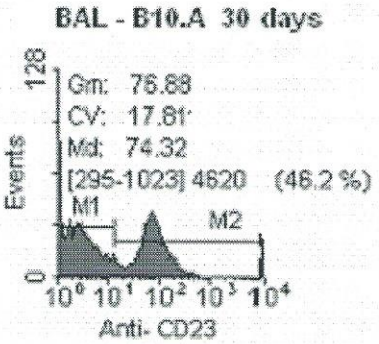

Figure 5

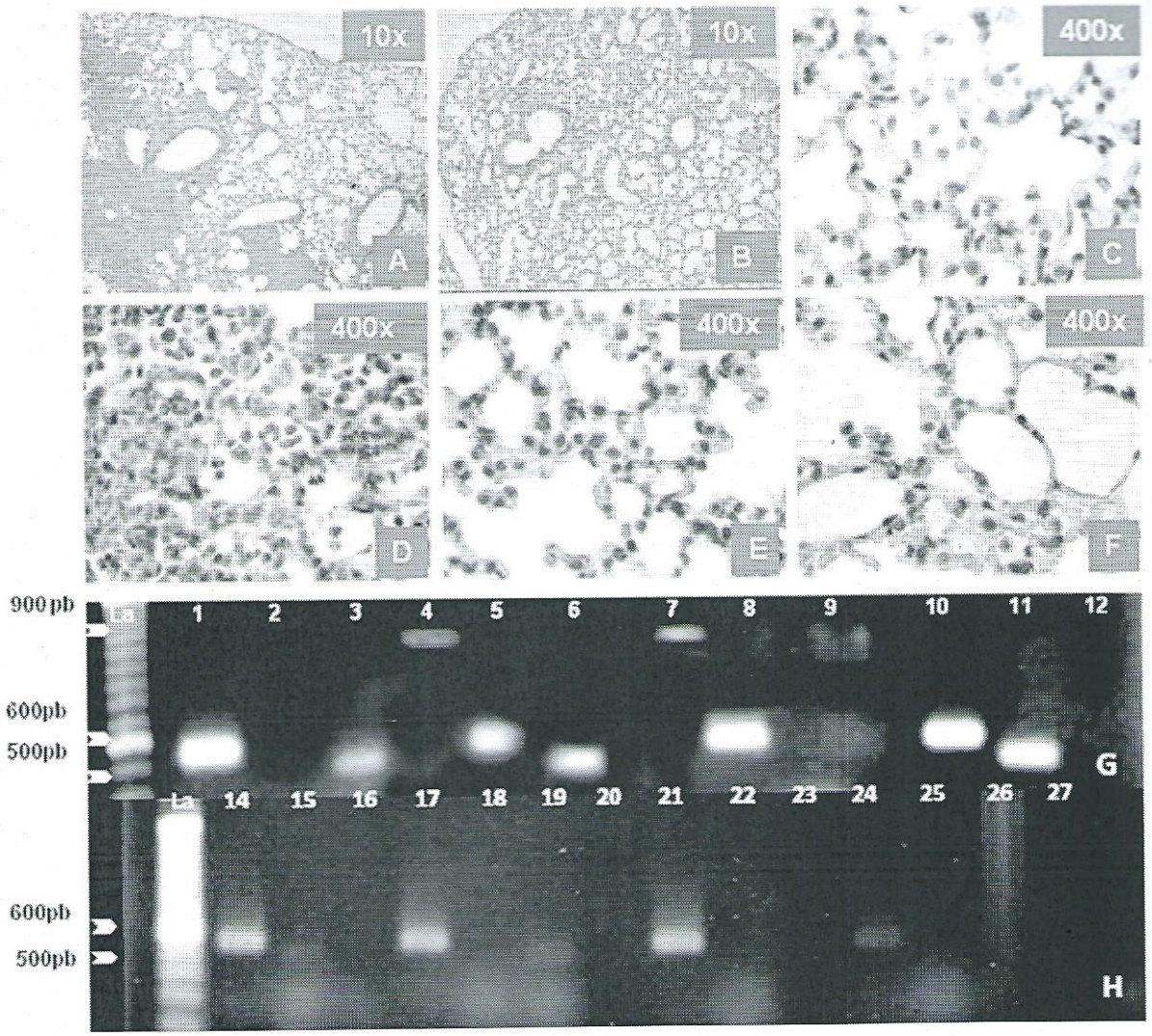
10x
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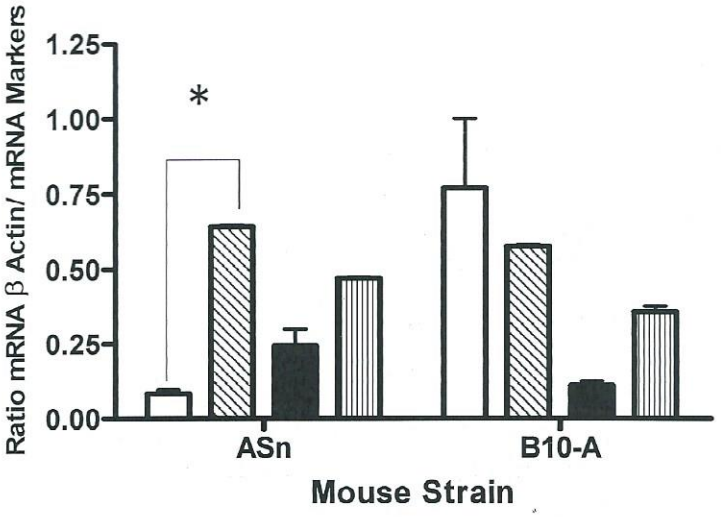
Figure 6: Prism converted to MSOffice 2000

A



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