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The low efficiency of dendritic cells and macrophages from mice susceptible to *Paracoccidioides brasiliensis* in inducing a Th1 response

S.R. Almeida and J.D. Lopes

Disciplina de Imunologia, Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil

Abstract

In the present study we evaluated T cell proliferation and Th lymphokine patterns in response to gp43 from *Paracoccidioides brasiliensis* presented by isolated dendritic cells from susceptible and resistant mice. T cell proliferation assays showed that dendritic cells from susceptible mice were less efficient than those from resistant mice. The pattern of T cell lymphokines stimulated by dendritic cells was always Th1, although the levels of IL-2 and IFN-γ were lower in T cell cultures from susceptible mice. To determie whether different antigen-presenting cells such as macrophages and dendritic cells stimulated different concentrations of Th1 lymphokines, the production of IFN-γ and IL-2 was measured. It was observed that dendritic cells were more efficient than macrophages in stimulating lymphoproliferation in resistant mice. However, no significant difference was observed for IFN-γ or IL-2 production. When cells from susceptible mice were used, macrophages were more efficient in stimulating lymphoproliferation than dendritic cells, but no difference was observed in the production of Th1 cytokine. Taken together, these results suggest the lower efficiency of dendritic cells and macrophages from B10.A mice in stimulating T cells that secrete Th1 lymphokines *in vitro*, an effect that may be involved in the progression of the disease *in vivo*.

Correspondence
J.D. Lopes
Disciplina de Imunologia
EPM, UNIFESP
Rua Botucatú, 862, 4º andar
04023-900 São Paulo, SP
Brasil
Fax: +55-11-5572-3328
E-mail: daniel@ecb.epm.br

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- Th1/Th2

Introduction

Paracoccidioidomycosis (PCM) is a human systemic mycosis caused by the termally dimorphic fungus *Paracoccidioides brasiliensis*. It is endemic in Latin America and can lead to high mortality rates in the absence of specific therapy or in immunodepressed individuals (1-4). The infection can be acquired by the respiratory route (5), by inhalation of propagules produced by the fungal mycelial forms that, once in the lungs, undergo differentiation into yeast cells, the infective form of *P. brasiliensis*. The infection induces the formation of granulomatous lesions primarily in the lungs and then may disseminate to other organs and systems.

It has been reported that a high level of humoral immune response is associated with increased disease dissemination and the se-

verity of its manifestations. On the other hand, the cell-mediated immune response seems to play an important role in resistance to *P. brasiliensis*.

T helper (Th) lymphocytes can be classified into at least two subsets based on the array of secreted lymphokines. Th1 cells produce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13. For instance, Th1, but not Th2 cells, have been reported to mediate delayed-type hypersensitivity and Th2 cells have been shown to cause B cell proliferation and differentiation (6). The selective activation of the proper Th1 or Th2 populations for each infectious pathogen can be critical for the development of protective immunity and is regulated by complex mechanisms. Manipulation of the Th phenotype by altering the level of IFN-γ, IL-12, or IL-4 at the time of infection has demonstrated the critical role of these cytokines in the priming of the CD4+ phenotype and, consequently, the outcome of the disease (7-9).

In the murine model of PCM, resistant mice (A/Sn) are assumed to direct the immune response to Th1 activation with predominant secretion of IFN-γ and IL-2, leading to resolution of the disease. On the other hand, susceptible mice (B10.A) activate Th2 with low levels of IFN-γ and early secretion of high levels of IL-5 and IL-10, thus leading to progressive disease (10,11).

As shown for other diseases (12), many factors may regulate resistance or susceptibility. The dose of the antigen, the antigen-presenting cells (APC), and the co-stimulatory microenvironment are some of those postulated as polarizing factors in determining the preferential immune response pathway to be activated (13,14). Studies with fully differentiated cloned T cell populations have suggested that APC may determine the phenotype of responding T cells (15). Disease resulting from infection with certain organisms (e.g., *Leishmania major*, *Mycobacterium leprae*, and *Schistosoma mansoni*) may be resolved according to the type of Th cell response elicited (16). Many studies have indicated that the selective induction of CD4+ T cells with distinct lymphokine profiles (6) may be dictated by the dose and type of antigen as well as by the route of immunization (17). These factors may favor the involvement of a particular APC type as well as the production of particular cytokines by either accessory cells/APC or T cells in the microenvironment. APC such as macrophages and dendritic cells have been shown to direct Th1 development by secretion of IL-12.

The macrophage was the first APC to be clearly identified (18). In the past few years, a wide variety of other cell types have also been shown to function as APC, like B cells (19), vascular endothelial cells, activated T cells (20), L cell fibroblasts transfected with MHC class II genes (21) and dendritic cells (22,23). Thus, a wide variety of cell types which constitutively express, or can be induced to express, MHC class II molecules on their surface can potentially function as APC.

The most extensively studied APC are macrophages, B cells and dendritic cells. These cells are likely to be the most important APC *in vivo* because they are located in secondary lymphoid tissues where T lymphocyte activation takes place. However, these cells differ in the relative quantity of expression of class II MHC antigens, in binding, uptake and processing of any given antigen and in the secretion of co-stimulatory factors (24). These differences could influence the efficiency of these cells in the activation of CD4+ T cell subsets.

Dendritic cells have attracted increasing interest on the part of immunologists since they are specialized in the capture, processing, and transport of the antigen to lymphoid organs, where they probably sensitize antigen-specific naive T lymphocytes (25). Recently, it has been shown that immature dendritic cells sequester intact antigens in lysosomes, processing and converting them

into peptide-MHC II complexes upon induction of dendritic cell maturation (26). The increased ability of maturing dendritic cells to load MHC class II molecules with antigenic cargo contributes to the >100-fold enhancement of the subsequent primary immune response observed when immature and mature dendritic cells are compared as adjuvants in culture and in mice (27).

In the mouse, two subclasses of dendritic cells have been described that differ in CD8α expression and localization in lymphoid organs. *In vitro* studies have suggested that CD8α+ dendritic cells may play a role in the regulation of the immune response, whereas conventional CD8α- dendritic cells may be more stimulatory (28). Maldonado-Lopez and colleagues (29) showed that both dendritic cell subclasses efficiently prime antigen-specific T cells *in vivo*, and direct the development of distinct Th populations. Administration of CD8α- dendritic cells induced a Th2-type response, whereas injection of CD8α+ dendritic cells led to Th1 differentiation.

The main antigenic component of *P. brasiliensis* is a 43-kDa secreted glycoprotein (gp43) which is recognized by 100% of patient sera (30) and also presents T cell epitopes (31). Indeed, the delayed-type hypersensitivity response is not observed in responder individuals when the *P. brasiliensis* extract is depleted of gp43 (32). We recently compared the T cell populations stimulated in resistant (A/Sn) and susceptible (B10.A) mice when gp43 was presented by macrophages or B cells. The results showed that, in resistant mice, purified gp43 seemed to have been preferentially presented by macrophages and to have stimulated Th1 lymphokine production. On the other hand, in susceptible mice, gp43 was distinguishably presented by B cells, which led to stronger activation of Th2 subsets (33).

In the present study, we compared the T cell population stimulated in resistant and susceptible mice when gp43 was presented by macrophages or dendritic cells. Both APC stimulated the same Th1 cell subset. However, dendritic cells were more efficient in stimulating T cells than macrophages in resistant mice. On the other hand, macrophages were more efficient than dendritic cells in stimulating T cells in susceptible mice.

Material and Methods

Animals

A/Sn and B10.A female mice, 8 to 12 weeks old, were used. Mice were obtained from the São Paulo University animal facilities.

Preparation of fungal antigens

For gp43 purification, exoantigen of *P. brasiliensis* B-339 was prepared as previously described (34) and passed through an adsorbent column consisting of murine anti-gp43 monoclonal antibody (mAb) 17c (35) coupled to an Affi-Gel 10 column (Bio-Rad Laboratories, Hercules, CA, USA). Gp43 was eluted with 0.1 M citric acid buffer, pH 2.8, neutralized with 1 M Tris, pH 9.0, and further concentrated in a 10-K Amicon apparatus (Amicon Division, Beverly, MA, USA). Protein contents were determined by the Bradford method (36) and every gp43 purification step was monitored by SDS-PAGE (37).

Immunization of mice

Purified gp43 was emulsified in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA). Fifty microliters of the emulsion containing 50 µg of the antigen was subcutaneously injected into the hind foot pads and the base of tail. One week later, the animals were sacrificed and inguinal, para-aortic, and popliteal lymph nodes were excised for T cell isolation.

Purification of APC

Macrophages. Macrophages from peritoneal exudates of naive mice were isolated by selective adherence to glass Petri dishes for 1 h at 37°C. The adherent cells were detached by scraping with a silicone stopper and then harvested, washed, and resuspended in complete medium, RPMI 1640 (Sigma) containing 10% heat-inactivated FCS (Gibco BRL, Grand Island, NY, USA) and 100 nM 2-mercaptoethanol (Sigma). The resulting population contained at least 90% macrophages, as assessed by morphology and specific staining using Mac-1 mAb (CD11b-FITC, Pharmingen, San Diego, CA, USA). Macrophages were irradiated with 1500 rad in a Nordion Gammacell 3000 apparatus, washed three times, plated onto 96-well plates (Costar, Cambridge, MA, USA) at increasing concentrations of 0.25, 0.5, 2.0, and 5.0 x 10^4 cells per well, and incubated overnight at 37°C in an atmosphere of 5% CO_2 before T cell addition.

Dendritic cells. Splenic dendritic cells were obtained by teasing spleens from naive mice disrupted on Petri dishes, using a sterile 20-ml syringe pestle and allowed to adhere for 2 h at 37°C in an atmosphere of 5% CO_2 in air, after which non-adherent cells were removed by thorough washing with RPMI medium. Adherent cells containing dendritic cells were incubated in complete medium. After 16 h at 37°C, non-adherent or loosely adherent cells containing dendritic cells were harvested by gentle pipetting. These cells were then treated with anti-Thy1.2 plus complement to remove remaining T cells and rosetted with sheep red blood cells (SRBC) coupled with anti-SRBC to remove FcR+ cells. Dead cells and rosetted cells were removed by separation on Isopac-Ficoll. The resulting population contained at least 90% of dendritic cells, as assessed by morphology and specific staining using anti-CD11c-FITC mAb (Pharmingen). Dendritic cells were irradiated with 1500 rad in a Nordion Gammacell 3000 apparatus, washed three times and plated onto 96-well plates (Costar) as described above.

T cell isolation

Lymph nodes were removed from immunized mice and the cell suspensions obtained were depleted of adherent cells by 1-h incubation on glass Petri dishes at 37°C and then passed through nylon wool columns for B cell removal.

Proliferative response assays

T cell suspensions in complete medium were added at a concentration of 2 x 10^5 cells per well to 96-well plates coated with increasing concentrations of irradiated APC in the presence of an optimal concentration of gp43 (10 µg/ml). Cells were co-cultured for 72 h and then pulsed for 16 h with ^{3}H-labeled thymidine (1 Ci/ml). Cells were collected with an automated cell harvester and incorporated radioactivity was measured by liquid scintillation spectrometry. Data are reported as means ± SD counts per minute of [^{3}H]-thymidine incorporation.

T cell lymphokine assays

T cell supernatants were obtained by culturing 2 x 10^6 T cells with 1 x 10^5 APC on 24-well plates (Costar), in the presence or absence of gp43 (10 µg/ml), in a final volume of 1 ml. IFN-γ, IL-2, IL-4, and IL-10 were quantified in the supernatants by ELISA (Pharmingen), according to manufacturer instructions. Interleukin activities were determined using curves with serial dilutions of mouse recombinant IFN-γ, IL-2, IL-4, and IL-10. The specific antibodies used for ELISA were: anti-IL-2 (JES6-1A12), anti-IL-4 (11B11), anti-IL-10 (JES5-2A5) and anti-IFN-γ (R4-6A2). Results are reported as the mean obtained from three experiments ± SD.

Statistical analysis

Statistical comparisons were made by analysis of variance (ANOVA) and by the Tukey-Kramer test. All values were reported as the mean ± SD of the mean.

Results

Proliferative response to gp43 presented by dendritic cells

Purified immune T lymphocytes from resistant and susceptible strains of mice were stimulated *in vitro* with optimal concentrations of gp43 (10 µg/ml) presented by increased concentrations of syngeneic dendritic cells. Figure 1 shows that dendritic cells from the A/Sn strain were more efficient in stimulating T cells than those from the B10.A strain ($P<0.01$).

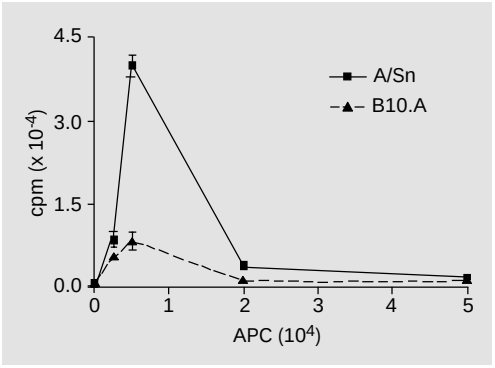

Figure 1 - Proliferation of immune T cells from A/Sn and B10.A mice stimulated with gp43 (10 µg/ml) in the presence of increasing concentrations of syngeneic dendritic cells. The [^{3}H]-thymidine incorporation by T cells plus different antigen-presenting cells (APC) in the absence of gp43 was as follows: A/Sn T cells, 1,500 ± 95, and B10.A, 1,300 ± 110. Results are representative of three independent experiments and are reported as arithmetic means ± SD.

Production of cytokines by lymphocytes in the presence of gp43 from dendritic cells

Figure 2 shows that when dendritic cells were employed as APC only IL-2 and IFN-γ were detected in the supernatant of 36-h T cell cultures from both strains of mice, although the lymphokine concentrations were higher in those from resistant mice ($P<0.001$ for IL-2 and IFN-γ). As controls, T cells and APC were cultured separately in the presence of gp43. IL-2, IFN-γ, IL-4 and IL-10 were not detected in the supernatant of these cultures (data not shown).

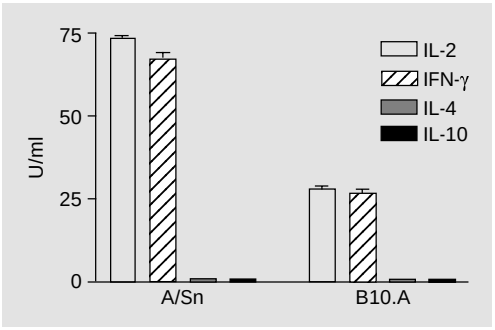

Figure 2 - Lymphokine profiles of T cells from A/Sn or B10.A mice stimulated with gp43 (10 µg/ml) in the presence of syngeneic dendritic cells. Culture supernatants were assayed by ELISA for the presence of IL-2, IFN-γ, IL-4 and IL-10. The levels of cytokines in the absence of gp43 were: A/Sn (IL-2: 5 U/ml, IFN-γ: 6.5 U/ml) and B10.A (IL-2: 4 U/ml, IFN-γ: 5.5 U/ml). Results are representative of three independent experiments.

Proliferative response to gp43 presented by different APC

Figure 3A shows that both macrophages and dendritic cells from A/Sn mice were efficient in stimulating T cells, whereas the concentration of dendritic cells required for optimal proliferation was lower than that used for macrophages. On the other hand, Figure 3B shows that macrophages from

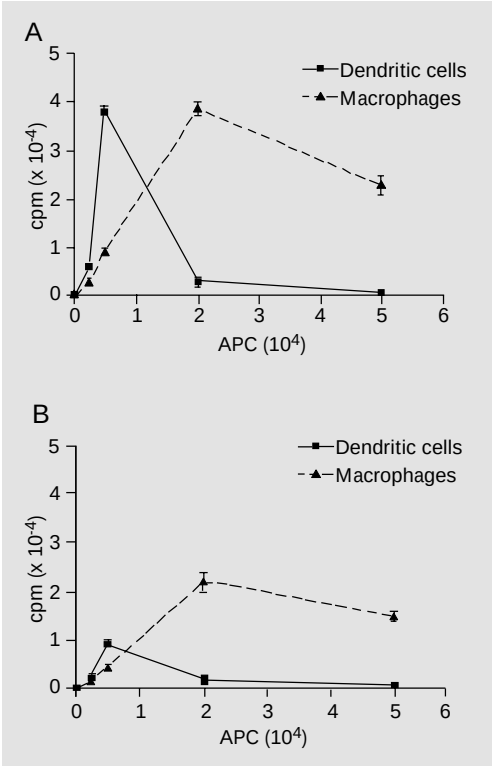

Figure 3 - Proliferation of immune T cells from A/Sn (A) and B10.A (B) mice stimulated with gp43 (10 µg/ml) in the presence of increasing concentrations of syngeneic dendritic cells (squares) or macrophages (triangles). Results are representative of three independent experiments and are reported as arithmetic means ± SD. APC, Antigen-presenting cells.

B10.A mice were more efficient in stimulating T cells.

Comparison of T cell subsets stimulated with different APC

T cell subsets stimulated with gp43 presented by syngeneic APC, macrophages or dendritic cells were characterized. As shown in Figure 4, macrophages and dendritic cells stimulated the production of IL-2 and IFN-γ in both strains of mice, although Th1 lymphokine concentration was higher in resistant than in susceptible mice ($P<0.001$).

Discussion

A recent report showed that a sustained secretion of Th1 cytokines (IL-2 and IFN-γ) plays a dominant role in the mechanism of resistance to *P. brasiliensis* infection. In contrast, the secretion of low levels of IFN-γ associated with the production of Th2 cytokines characterizes the progressive disease of susceptible animals (10).

Accepting the paradigm that the effector immune response is determined by activation of either Th1 or Th2 subsets, the identification of T cell subsets secreting different profiles of lymphokines might contribute to the understanding of the regulation of effector immune responses to *P. brasiliensis*.

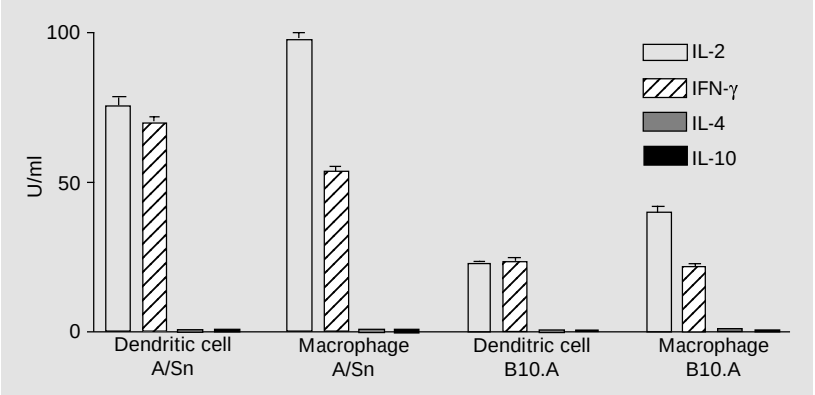

Figure 4 - Lymphokine profiles of T cells from A/Sn or B10.A mice stimulated with gp43 (10 µg/ml) in the presence of syngeneic dendritic cells or macrophages. Supernatants were assayed by ELISA for the presence of IL-2, IFN-γ, IL-4 and IL-10. The levels of cytokines in the absence of gp43 were: A/Sn (dendritic cell, IL-2: 6 U/ml, IFN-γ: 6.5 U/ml; macrophage, IL-2: 3 U/ml, IFN-γ: 4.5 U/ml) and B10.A (dendritic cell, IL-2: 3 U/ml, IFN-γ: 4 U/ml; macrophage, IL-2: 3 U/ml, IFN-γ: 4.5 U/ml). Results are representative of three independent experiments.

Based on these assumptions, we first evaluated T cell proliferation in response to gp43 presented by isolated dendritic cells from both A/Sn and B10.A mice. It was observed that the number of APC required to reach optimal proliferation was always comparable for both strains of mice. However, dendritic cells from susceptible mice were less efficient than dendritic cells from resistant mice.

In order to verify the pattern of T cell lymphokines stimulated by dendritic cells, T cells from resistant and susceptible mice were incubated with syngeneic APC and the cytokines quantified. It was observed that APC from both mouse strains stimulated IL-2- and IFN-γ-secreting T cells. However, both IL-2 and IFN-γ levels were lower in T cell cultures from B10.A mice. These results demonstrated the lower capacity of dendritic cells from susceptible mice to stimulate lymphoproliferation and Th1 subsets. These data suggest that the lower capacity of dendritic cells from susceptible mice could contribute to the susceptibility of B10.A mice to *P. brasiliensis* infection. This assumption is based on well-established demonstrations that dendritic cells have a highly developed function in the immune system as specialized APC which initiate T cell-dependent immune responses (38). Their strategic positioning in nonlymphoid tissues and their ability to circulate via blood and lymph to lymphoid organs suggest their important role in the initiation of responses against invading pathogens.

Nevertheless, a recent report showed that *P. brasiliensis*-infected or gp43-immunized BALB/c mice, which exhibit an intermediary pattern of PCM susceptibility, when challenged with a virulent strain of *P. brasiliensis*, produced high IgG1, IgG2a, IgG3, and IgE levels, which were considered compat-

ible with a mixed Th1/Th2 response. These findings reinforced the assumption that gp43 is the main antigen involved in the immune response to *P. brasiliensis* and suggested that this molecule may be presented by different APC (39).

The T cell response to gp43 presented by dendritic cells or macrophages was characterized and compared in resistant and susceptible mice in order to verify whether the APC could influence the outcome of *P. brasiliensis* infection. We first evaluated T cell proliferation in response to gp43 presented by isolated macrophages or dendritic cells from A/Sn and B10.A mice. In resistant mice, macrophages and dendritic cells were similarly able to induce proliferation of sensitized T cells, whereas in susceptible mice, dendritic cells were less efficient than macrophages. However, in resistant mice it was observed that the number of dendritic cells necessary to induce proliferation was lower when compared with macrophages. The high efficiency of dendritic cells in stimulating T cells could be correlated with internalization of antigen (gp43). Engering et al. (40) recently reported that dendritic cells use micropinocytosis for continuous uptake of large amounts of soluble antigens and express high levels of mannose receptors that can mediate internalization of glycosylated ligands. Dendritic cells can present mannosylated antigens 100- to 1000-fold more efficiently than non-mannosylated antigens (40). These findings are relevant considering that gp43 is a high mannose glycoprotein (31).

In order to verify the pattern of cytokines secreted by T cells stimulated with macrophages or dendritic cells, the levels of IFN-γ, IL-2, IL-10 and IL-4 were measured in culture supernatants. It was observed that both macrophages and dendritic cells stimulated IL-2- and IFN-γ-secreting T cells (Th1), whereas the concentrations of Th1 cytokines were higher in T cell cultures from resistant mice. It is worth mentioning that IL-4 and IL-10 were not detected in the same supernatants.

Two reports have shown that the cell population containing the highest proportion of CD8α+ dendritic cells was consistently less efficient in stimulating the proliferation of antigen-specific cells *in vitro* (41). A ligand for Fas was demonstrated on the surface of CD8α+ dendritic cells but not CD8α- dendritic cells, and the suboptimal activation of T cells by CD8α+ dendritic cells was associated with marked T cell apoptosis via Fas engagement (42). However, Maldonado-Lopez et al. (29) showed that injection of pulsed CD8α+ and CD8α- dendritic cells induced equally strong T cell proliferative responses upon *in vitro* restimulation. These findings suggest that the nature of the dendritic cells that present the antigen to naive T cells may dictate the class selection of the adaptive immune response. Therefore, it is tempting to speculate that B10.A and A/Sn could have a predominance of dendritic cell subsets which could determine the different proliferation and production of cytokines by T cells.

It is reasonable to consider that a number of circumstances, such as the amount of antigen injected, the immunization route used and the cytokine microenvironment, could contribute to determining the resistance or susceptibility to PCM. Nevertheless, the present study demonstrated a lower efficiency of dendritic cells and macrophage from B10.A mice in stimulating T cells that secrete Th1 lymphokines *in vitro*. Considering the central role of these cells in the initiation as well as the effector function of the immune response, the low efficiency in stimulating production of Th1 cytokines such as IFN-γ could be implicated in the determination of a susceptible pattern *in vivo*. Several reports have shown that resistance to *P. brasiliensis* infection is linked to IFN-γ production (43,44). Based on the present data, it is tempting to speculate that distinct antigen-processing mechanisms of APC in resistant and susceptible mice, such as the expression of genetic differences in the ability to take up antigens, qualitative and

quantitative expression of MHC class II antigens and differences in the proteolytic machinery for antigen processing, may be involved in determining a susceptibility pattern to *P. brasiliensis in vivo*.

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