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SUSCEPTIBILITY OF PERITONEAL MACROPHAGE FROM DIFFERENT SPECIES OF NEOTROPICAL PRIMATES TO *EX VIVO Leishmania (L.) infantum chagasi*-INFECTION

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SUMMARY

This study examined the susceptibility of peritoneal macrophage (PM) from the Neotropical primates: *Callithrix jacchus*, *Callithrix penicillata*, *Saimiri sciureus*, *Aotus azarae infulatus* and *Callimico goeldii* to *ex vivo Leishmania (L.) infantum chagasi*-infection, the etiological agent of American visceral leishmaniasis (AVL), as a screening assay for evaluating the potential of these non-human primates as experimental models for studying AVL. The PM-susceptibility to infection was accessed by the PM-infection index (PMI) at 24, 72 h and by the mean of these rates (FPMI), as well as by the TNF-α, IL-12 (Capture ELISA) and Nitric oxide (NO) responses (Griess method). At 24h, the PMI of *A. azarae infulatus* (128) was higher than those of *C. penicillata* (83), *C. goeldii* (78), *S. sciureus* (77) and *C. jacchus* (55). At 72h, there was a significant PMI decrease in four monkeys: *A. azarae infulatus* (128/37), *C. penicillata* (83/38), *S. sciureus* (77/38) and *C. jacchus* (55/12), with exception of *C. goeldii* (78/54). The FPMI of *A. azarae infulatus* (82.5) and *C. goeldii* (66) were higher than *C. jacchus* (33.5), but not higher than those of *C. penicillata* (60.5) and *S. sciureus* (57.5). The TNF-α response was more regular in those four primates which decreased their PMI at 24/72 h: *C. jacchus* (145/122 pg/μL), *C. penicillata* (154/130 pg/μL), *S. sciureus* (164/104 pg/μL) and *A. azarae infulatus* (154/104 pg/μL), with exception of *C. goeldii* (38/83 pg/μL). The IL-12 response was mainly prominent in *A. infulatus* and *C. goeldii* which presented the highest FPMI and, the NO response was higher in *C. goeldii*, mainly at 72 h. These findings strongly suggest that these New World primates have developed a resistant innate immune response mechanism capable of controlling the macrophage intracellular growth of *L. (L.) i. chagasi*-infection, which do not encourage their use as animal model for studying AVL.

KEYWORDS: Peritoneal macrophage susceptibility; Neotropical primates; *Leishmania (L.) infantum chagasi*-infection; TNF-α; IL-12; Nitric oxide response.

INTRODUCTION

Leishmaniasis consists in a group of diseases caused by protozoan parasites belonging to the genus *Leishmania* Ross 1903. They are digenetic and heteroxenous parasites, with an obligated intracellular amastigote stage living and multiplying into the macrophages and, in a few extension, into the dendritic cells of their vertebrate hosts[16,27].

Clinically, there are two different forms of leishmaniasis which exist depending on the *Leishmania* species involved and the immune-genetic profile of man; the cutaneous or mucocutaneous form and the visceral one. They comprise important anthropozoonotic diseases widely spread in four continents, presenting an endemic or epidemic character in many regions and being considered as one of the six tropical diseases of major interest by the World Health Organization[42].

Actually, one of the most discussed subjects at leishmaniasis scientific meetings is concerning the need for new experimental models for studying these diseases, mainly considering the possibility of using non-human primates due to their physiologic and phylogenetic proximity with humans[2]. Thus, the real reasons for using non-human primates as experimental models for studying leishmaniasis are not due to the ethical order but also because these animals may give rise to the clinical-immunological features similar to those found in human diseases[29].

In this way, it is interesting to record that some Neotropical primates have been tested before with relative success as experimental models for American visceral leishmaniasis (AVL), as follows: *Callithrix jacchus*, *Aotus trivirgatus* (*Aotus infulatus*) and *Saimiri sciureus*[7,11,21]. In a similar way, the African green monkey *Cercopithecus aethiops*[13] and the *Presbytis entellus*[24,25,26] have also been used for studying the Old World's visceral leishmaniasis. In this context, the successful results by using the *Cebus apella* as experimental model for studying cutaneous leishmaniasis due to *Leishmania (V.) braziliensis*, *L. (L.) amazonensis*

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and *L. (V.) lainsoni* in the north of Brazil[34,35] should also be emphasized. Based on these findings, it was also investigated *in vivo* the susceptibility of *C. apella* to experimental infection due to *L. (L.) infantum chagasi*, the etiological agent of AVL[32]. In contrast, however, to the experimental infection with the cutaneous disease agents, the *C. apella* showed to be resistant against the inoculation with either amastigote (intravenous via) or promastigote (intradermal via) forms of *L. (L.) i. chagasi*[10].

Thus, regarding the above comments, we have studied the susceptibility of peritoneal macrophage from some species of Neotropical primates to *L. (L.) i. chagasi*-infection, aiming to evaluate the potential of these non-human primates as experimental model for studying AVL. The susceptibility of peritoneal macrophage from each monkey species to *L. (L.) i. chagasi*-infection was accessed using a screening assay which allowed to evaluate the peritoneal macrophage-infection index (PMI), as well as some pro-inflammatory cytokines (TNF-α and IL-12) and the nitric oxide (NO) responses. The reproducibility of this screening assay has been prior demonstrated through a pilot study using not only *L. (L.) i. chagasi*, but also *L. (V.) braziliensis, L. (V.) guyanensis* and *L. (L.) amazonensis*[9], the principal etiological agents of American cutaneous leishmaniasis (ACL) in the north of Brazil[33].

MATERIAL AND METHODS

***1. Experimental animals*:** This study was carried out with Neotropical primates from the National Center of Primates (Ministry of Health), in Ananindeua municipality, Pará State, Brazil, where the peritoneal macrophage (PM) cells were collected from the five different species of primates: *Callithrix jacchus*, *Callithrix penicillata*, *Saimiri sciureus*, *Aotus azarae infulatus* and *Callimico goeldii*. All of them were young males, born and bred in captivity in the National Center of Primates in good environmental and dietetic conditions (Fig. 1).

This study had IBAMA license (029-2006) to collect and transport biological material of primates from captivity and it was approved by the Animal Research Ethics Committee of Evandro Chagas Institute (Health Ministry, Brazil), protocol number 004/2006, and the Ethics Committee of research programs, Medicine School of São Paulo University, São Paulo State, Brazil, protocol number 0255/2007.

2. Parasite: *L. (L.) i. chagasi*-strain (MCAO/BR/2003/M22697/Pará State) isolated from a dog viscera naturally infected was identified by monoclonal antibodies and multi-locus enzyme electrophoresis at the Leishmaniasis laboratory, Parasitology Department, Evandro Chagas Institute, Belém (PA) where the parasite was maintained in the criobank at $^{-}$196 ^{o}C.

In order to guarantee the continued virulence of *L. (L.) i. chagasi*-strain, the stationary phase promastigotes from Difco B45 blood-agar culture medium[39] were re-inoculated through an intra-peritoneal route in laboratory hamsters. Three months later, the amastigote forms from the hamsters' viscera were transferred to the liquid culture medium RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% bovine fetal serum (Cultilab); 100 U/mL of penicillin-G; 100 µg/mL gentamicin; 2 mM L-glutamine and 10 mM HEPES. The parasites were then cultivated at 25 ºC and the stationary phase promastigote forms, established by growth-curve, showed to be best used after seven days of culture. The final concentration of stationary phase promastigotes in the inoculum was 2 x 10^6/mL.

3. Isolation of peritoneal macrophage from primates: In order to obtain the peritoneal macrophages of the different primate species, the animals were anesthetized with a solution of ketamine (10 mg/kg) plus xylazine (1 mg/kg) (2:1 ratio) and the peritoneal cavities were washed with 80 mL for small size primates such as *C. jacchus* and *C. penicillata*, and 120 mL for middle size primates such as *S. sciureus*, *A. infulatus* and *C. goeldii*, of RPMI-1640 medium supplemented with 1.0% FBS, 10 mg/mL gentamicin and 100 Ul/mL penicillin. The peritoneal macrophage (PM) was obtained from a pool of three specimens of each

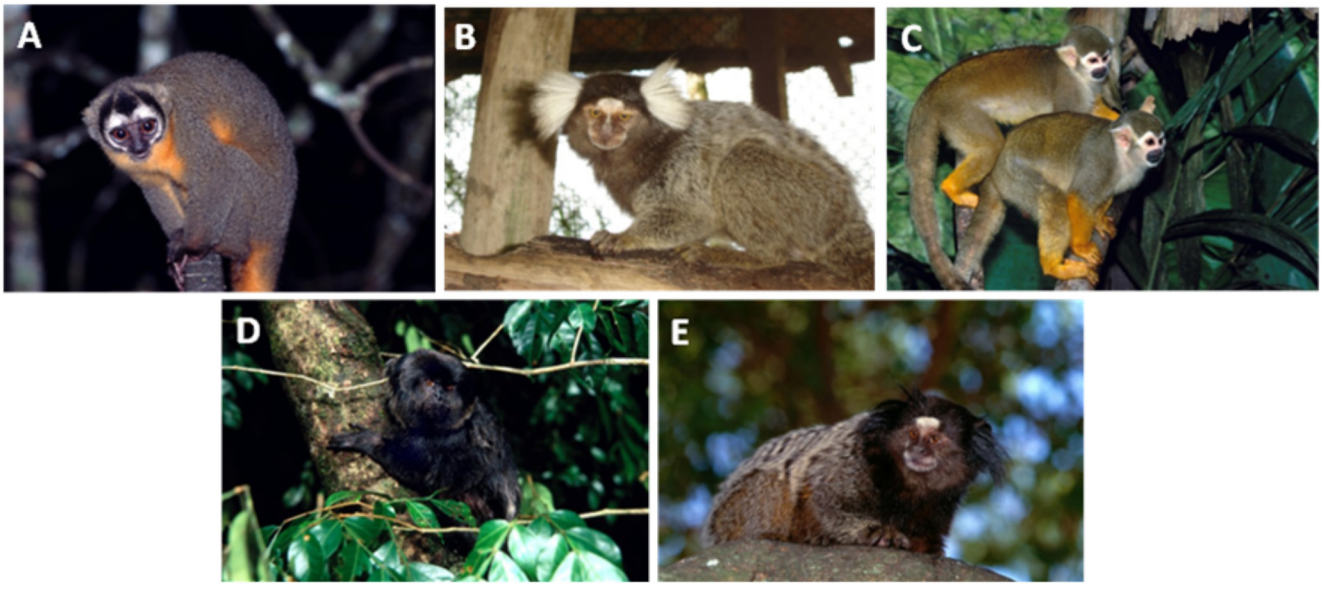

Fig. 1 - Neotropical primate species born and bred in captivity at the National Center of Primates in Ananindeua municipality, Pará State, Brazil, that were used for studying the *ex vivo* peritoneal macrophage *L. (L.) i. chagasi*-infection. A- *Aotus infulatus*; B- *Callithrix jacchus*; C- *Saimiri sciureus*; D- *Callimico goeldii* and E- *Callithrix penicillata*.

primate species. Following aspiration of the fluid, a suspension of cells was examined in a Neubauer chamber and its concentration adjusted to approximately 2 x 10^6 macrophages /mL in RPMI-1640 medium without serum. A volume of 50 µL (about 10^5 cells) of this suspension was added to cell-culture plates (Nunc) containing sterile cover-slips which were incubated at 35 ^{0}C with 5.0% CO_2 for two hours to the adhesion of the macrophages. After this time the cover-slips were washed with RPMI-1640 medium to remove unattached cells and an addition of 600 µL of RPMI-1640 was done for incubation during a further 24 hours.

4. Macrophage-*Leishmania *interaction: *L. (L.) i. chagasi*-stationary phase promastigotes were washed twice by centrifugation (1,600 x g for 10 minutes) in sterile PBS (pH 7.2), and the concentration was adjusted to 8 x 10^6 parasites/mL. The PM-infection was based on the ratio of four parasites per macrophage in 50 µL suspension of the cell-culture and this procedure was used in triplicate for each experiment. The mean of the triplicate values at 24 and 72 hours was the result of each experiment. This experiment was repeated three times for each primate species, and the PM-infection index (PMI) of each monkey species was based in the mean of these three independent experiments. Following these two time-points (24 and 72 hours) of cultured peritoneal macrophage *L. (L.) i. chagasi*-interaction, two more procedures were also carried out : i) the supernatants of these three experiments were collected in pool to evaluate the TNF-α, IL-12 and nitric oxide (NO) levels, thus, these results were expressed as unique value and not as a mean of the three experiments as was done for PMI; ii) the cover-slips were washed in PBS, fixed in absolute methyl alcohol, and stained by Giemsa to determine the PMI. These selected times (24 and 72 hours) for determining the PMI were used with the aim of observing the dynamic evolution of infection. In addition, an average value obtained from these two time-points of infection was also analyzed in attempting to represent the degree of macrophage-infection susceptibility to each primate species; this infection-index was regarded as the final PMI (FPMI).

The number of infected macrophages and parasites per macrophage was determined in 200 cells of cover-slip from each primate species examined. The PMI was expressed as the percentage of infected macrophage multiplied by the average number of amastigotes per macrophage[19].

5. Determination of nitric oxide (NO) response: The NO response was estimated by determining the nitrite concentration present in the supernatant of macrophage cultures infected by stationary phase culture promastigotes, using the Griess method[15]. This method consists of a calorimetric reaction in which the supernatant of the culture (300 µL) is incubated with an equal volume of recently prepared Greiss reagent (1.0% sulfanilamide, 0.1% naphazoline hydrochloride, and 2.5% orthophosforic acid) for 10 minutes at room temperature. The absorbance was determined in a Zeiss spectrophotometer, with an optic density of 540 nm against to a white formed by culture medium (RPMI-1640) and Griess reagent in equal proportions. The results were expressed as µM of NO based on a standard curve formed with known concentrations of sodium nitrites ($NaNO_2$) dissolved in culture medium. In all assays, the control was the supernatant from an uninfected macrophage culture.

6. *Determination of TNF-α and IL-12 response:* An ELISA anti-monkey assay was used for evaluating the TNF-α and IL-12 response in the supernatants from the peritoneal macrophage cultures infected with *L. (L.) i. chagasi*, using available commercial kits (Biosource Laboratory®). All assays were performed in accordance with the manufacturer instructions. The assays sensitivities were < 2 pg/mL for TNF-α and < 4 pg/mL for IL-12.

7. Statistics: The statistically significant differences of the infection rates (PMI and FPMI), cytokines (TNF-α and IL-12) and NO levels amongst the different species of primates were obtained using the Bio-Estat 4.0 software[4]. The binomial and χ^2 tests for independent variables were used to compare these differences with $p < 0.05$ considered to be significant.

RESULTS

1. PMI and FPMI of neotropical monkeys by L. (L.) i. chagasi: At 24 hours of infection, it was observed that PMI of *A. azarae infulatus* (128) was higher ($p < 0.05$) than those other primates: *C. penicillata* (83), *C. goeldii* (78), *S. sciureus* (77) and *C. jacchus* (55), amongst which there were no differences ($p > 0.05$) (Fig. 2).

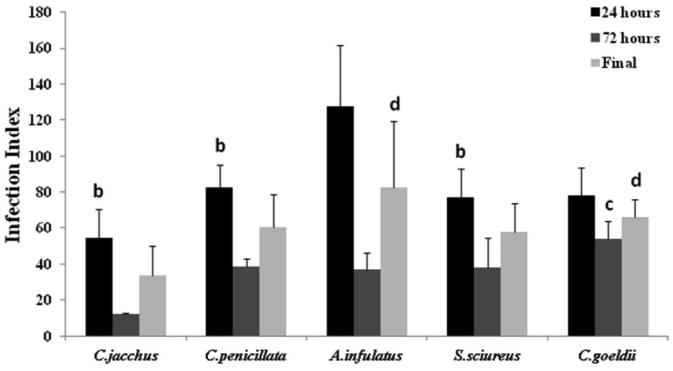

Fig. 2 - Average and standard deviation of the infection index of peritoneal macrophage of Neotropical primates infected with promastigotes of *L. (L.) i. chagasi*, where: (a) Infection Index of *A. infulatus* > *C. penicillata*, *S. sciureus*, *C. goeldii* and *C.jacchus* at 24 hours; (b) Infection index of *A. infulatus, C. penicillata*, *S. sciureus*, and *C.jacchus* at 24 hours > 72 hours; (c) Infection index of *C. goeldii* > *C. jacchus* at 72 hours; (d) Final infection index of *A. infulatus* and *C. goeldii* > *C. jacchus* ($p < 0.05$).

At 72 hours of infection, there was a significant ($p < 0.05$) decrease in the PMI of four primates, as follows: *A. azarae infulatus* 128/37, *C. penicillata* 83/38, *S. sciureus* 77/38 and *C. jacchus* 55/12, with exception of *C. goeldii* 78/54 ($p > 0.05$) which was also the only primate with PMI (54) higher ($p < 0.05$) than that of *C. jacchus* (12). Thus, it should be emphasized that the PMI(s) of *C. jacchus*, at 24 (55) and 72 (12) hours of infection were the lowest amongst all primates examined (Fig. 2).

With regards to the FPMI, it was noted that *A. azarae infulatus* (82.5) and *C. goeldii* (66) showed the highest rates of infection, although they were only higher ($p < 0.05$) than that of *C. jacchus* (33.5), but not ($p > 0.05$) than *C. penicillata* (60.5) and *S. sciureus* (57.5) (Fig. 2).

2. ***TNF-α, IL-12 and NO responses of ex vivo peritoneal macrophage-infection***

2.1. *TNF-α response:* At 24 hours of infection, there were not found differences ($p > 0.05$) of TNF-α levels in the supernatants of infected PM-cultures of the primates *S. sciureus* (164 pg/µL), *C. jacchus* (145 pg/mL),

C. penicillata (154 pg/mL) and *A. azarae infulatus* (154 pg/mL), although these levels were higher ($p < 0.05$) than *C. goeldii* (39 pg/mL) (Fig. 3).

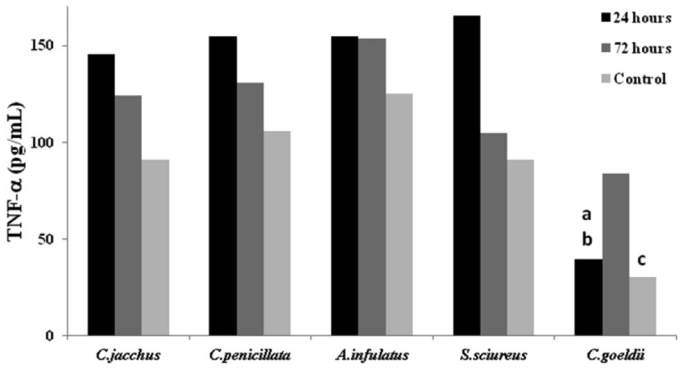

Fig. 3 - Concentration of TNF-α (pg/mL) in the supernatants of peritoneal macrophage cultures of Neotropical primates infected with promastigotes of *L. (L.) i. chagasi,* where: (a) *C. goeldii* < *S. sciureus*, *A. infulatus*, *C. penicillata* and *C. jacchus* at 24 hours*;* (b) *C. goeldii* at 24 hours < 72 hours; (c).Non infected cultures of *C. goeldii* < *S. sciureus*, *A. infulatus*, *C. penicillata* and *C. jacchus* ($p < 0.05$).

At 72 hours of infection, there were two notable findings; first, it was observed that the TNF-α levels found in four primates have shown similar profiles ($p > 0.05$) to those at 24 hours of infection: *S. sciureus* (104 pg/mL), *C. jacchus* (122 pg/mL), *C. penicillata* (130 pg/mL) and *A. azarae infulatus* (153 pg/mL); second, it was noted that although the TNF-α level of *C. goeldii* (83 pg/mL) had increased ($p < 0.05$) concerning the 24 hours of infection (39 pg/mL), this increase was not able to avoid the condition of the lowest TNF-α-produced primate species. In addition, it was also noted that the TNF-α levels found in the supernatants of non-infected PM-cultures (controls) had similar profiles to those of infected PM-cultures (24 and 72 hours), such as: *S. sciureus* 90 pg/mL, *C. jacchus* 90 pg/mL, *C. penicillata* 105 pg/mL and, *A. azarae infulatus* 124 pg/mL. Thus, they were also higher ($p < 0.05$) than *C. goeldii* (29 pg/mL), which were demonstrated in the infected PM-cultures (39 and 83 pg/mL) and in the PM controls (29 pg/mL), the TNF-α levels of *C. goeldii* were lower ($p < 0.05$) than those other primates (Fig. 3).

2.2. *IL-12 response:* At 24 hours of infection, there were not found differences ($p > 0.05$) of IL-12 levels in the supernatants of infected PM-cultures of the monkeys *S. sciureus* (153 pg/mL) and *C. jacchus* (117 pg/mL), just the two primate species with the lowest FPMI (57.5 and 33.5). However, it was also observed that the IL-12 levels of *A. azarae infulatus* (419 pg/mL) and *C. goeldii* (456 pg/mL), the two primates with the highest FPMI (82.5 and 66), were higher ($p < 0.05$) than those of *C. jacchus* (117 pg/mL) and *S. sciureus* (153 pg/mL), but not than the *C. penicillata* (283 pg/mL) which occupied an intermediary (60.5) FPMI level (Fig. 4).

Following 72 hours of infection, it was detected a significant increase ($p < 0.05$) of IL-12 levels in the supernatants of infected PM-cultures of *A. azarae infulatus* (419/24h→690/72h pg/mL) and *C. goeldii* (456/24h→666/72h pg/mL), but not ($p > 0.05$) in *C. penicillata* (283/24h→333/72h pg/mL). In contrast, there was a relative decrease ($p > 0.05$) of IL-12 levels in the supernatants of infected PM-cultures of *C. jacchus* (117/24h→74/72h pg/mL) and *S. sciureus* (153/24h→98/72h pg/mL) (Fig. 4). Thus, the impression regarding the IL-12 response at 72 hours of experiment was similar to what happened at 24 hours.

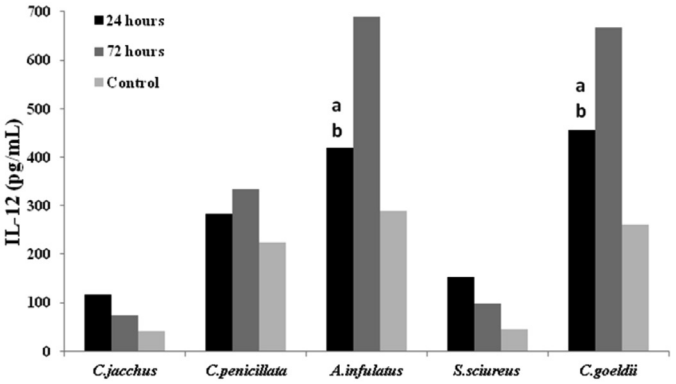

Fig. 4 - Concentration of IL-12 (pg/mL) in the supernatants of peritoneal macrophage cultures of Neotropical primates infected with promastigotes of *L. (L.) i. chagasi,* where: (a) *C. goeldii* and *A. infulatus* > *S. sciureus* and *C. jacchus* at 24 hours; (b) *C. goeldii* and *A. infulatus* at 24 hours < 72 hours ($p < 0.05$).

2.3. Nitric oxide (NO) response: At 24 hours of infection, it was demonstrated that the NO levels detected in the supernatants of infected PM-cultures of *C. jacchus* (3.2 μM) were higher ($p < 0.05$) than those other primate species; *C. penicillata* (0.3 μM), *A. azarae infulatus* (0.7 μM), *S. sciureus* (0.9 μM) and *C. goeldii* (0.5 μM), amongst which there were not found ($p > 0.05$) differences (Fig. 5).

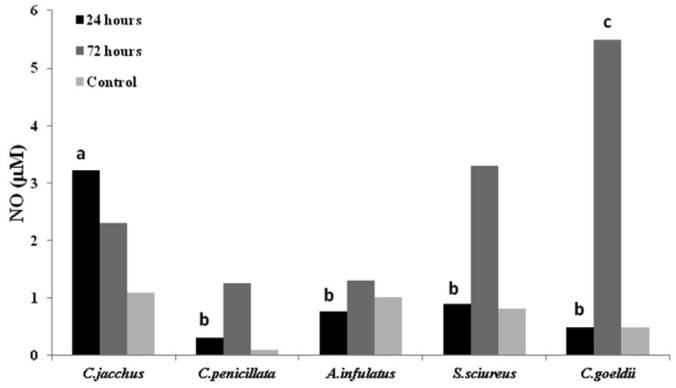

Fig. 5 - Concentration of nitric oxide (NO) (μM) in the supernatants of peritoneal macrophage cultures of Neotropical primates infected with promastigotes of *L. (L.) i. chagasi*, where: (a) *C. jacchus* > *C. penicillata*, *A. infulatus*, *S. sciureus* and *C. goeldii*; (b) *A. infulatus*, *C. penicillata, S. sciureus* and *C. goeldii* at 24 hours < at 72 hours; (c) *C. goeldii* > *C. jacchus* ($p < 0.05$).

At 72 hours of infection, it was noted an important ($p < 0.05$) NO response in the supernatants of infected PM-cultures of those primates which have showed low NO levels at 24 hours of infection, as follows: *C. penicillata* (0.3/24h→1.25/72h μM), *A. azarae infulatus* (0.7/24h→1.3/72h μM), *S. sciureus* (0.9/24h→3.3/72h μM) and, surprising, *C. goeldii* (0.5/24h→5.5/72h μM). In contrast, there was a relative ($p > 0.05$) decrease in the NO response of *C. jacchus* (3.2/24h→2.3/72h μM), which was only lower ($p < 0.05$) than the *C. goeldii* (5.5 μM) (Fig. 5).

DISCUSSION

Firstly, it should be emphasized that the most interesting feature of

this work was the use of the peritoneal macrophage from five different species of Neotropical primates: *C. jacchus*, *C. penicillata*, *S. sciureus*, *C. goeldii* and *A. azarae infulatus* for studying its susceptibility to *ex vivo L. (L.) i. chagasi*-infection, the etiological agent of AVL. Thus, it was tried to identify which monkey species would have the potential of susceptibility to *in vivo L. (L.) i. chagasi* infection, aiming to establish an experimental model which might be useful for reproducing the parasitological, clinical and immunological features found in human AVL. Besides, it was also desired to demonstrate in the supernatants of *L. (L.) i. chagasi*-infected peritoneal macrophage cultures the production of some pro-inflammatory cytokines, such as TNF-α and IL-12, as well as the NO, which are strongly associated to the development of macrophage resistance against *Leishmania*-infection[5,22,31].

In this way, two rates were used for evaluating the susceptibility of peritoneal macrophage from Neotropical primates: i) the peritoneal macrophage-infection index (PMI) at intervals of 24 and 72 hours of infection and, ii) the final peritoneal macrophage-infection index (FPMI) accessed by the average of these two time-points rates, which aimed to express the potential susceptibility of each primate species to *ex vivo L. (L.) i. chagasi*-infection. Considering that TNF-α, IL-12 and NO assays were also performed at the same time-points (24 and 72 hours) of the peritoneal macrophage-infection index (PMI) the susceptibility of peritoneal macrophage of these primates to *L. (L.) i. chagasi*-infection was based in these two immune-biological parameters of infection, which have been regarded of pivotal importance to analyze the macrophage resistance against *Leishmania*-infection[3,20].

Taking into account these criteria, it was initially considered that two primate species had some potential susceptibility to *ex vivo L. (L.) i. chagasi*-infection: *A. azarae infulatus* and *C. goeldii*, with FPMI of 82.5 and 66, respectively. However, it should not be omitted that these two rates were not different ($p > 0.05$) from those of *C. penicillata* (60.5) and *S. sciureus* (57.5) monkeys and were only different ($p < 0.05$) from *C. jacchus* (33.5), which presented the lowest FPMI. Thus, if the PM-susceptibility to infection is based only in the FPMI, the first impression would be that these four primate species with the highest FPMI rates, *A. azarae infulatus* (82.5), *C. goeldii* (66), *C. penicillata* (60.5) and *S. sciureus* (57.5), would have equivalent degrees of PM-susceptibility to *L. (L.) i. chagasi*-infection and would all be indicated for *in vivo* studying of AVL. Thus, it seems reasonable to assume that neither PMI nor FPMI showed significant differences that should indicate any primate species as candidate for *in vivo* studying AVL. In contrast, another conclusion regarding *C. jacchus* that should be indicated as an experimental model for studying the cellular immune mechanisms related to resistance against infection.

Another point of consideration was the originality of this approach, which used the peritoneal macrophage from five different Neotropical primates to study its susceptibility to *L. (L.) i. chagasi*-infection; indeed, the findings obtained in this work were difficult to be compared with others, once the majority of studies in this area has been made with BALB/c mice peritoneal macrophage, as the target cell to be infected[8,14]. Besides some studies using dermotropic *Leishmania* sp., PINELLI *et al.*[30] had used a cellular lineage of canine macrophage (030-D) to evaluate the role of NO in the control of *L. (L.) infantum*-infection, the etiological agent of visceral leishmaniasis in some European countries of Mediterranean Basin and a leishmanial parasite closely related to *L. (L.) i. chagasi*; the findings of these authors have confirmed that NO production in the cultured macrophage cells exerts a crucial decrease on the intracellular growth of the parasite. In another study using canine peritoneal macrophage, MADEIRA *et al.*[18] have demonstrated the infection of the same cellular lineage by *L. (L.) i. chagasi*, *L. (L.) amazonensis* and *L. (V.) braziliensis* during consecutive six days in RPMI culture, encouraging this approach to be used in screening assays for testing the sensitivity of these parasites against new drugs for treatment of canine leishmaniasis. Thus, the present work represents an original approach providing pioneer results on the interaction of *L. (L.) i. chagasi* with peritoneal macrophage from New World primates, which may also be used as an approach assay for studying the immune mechanisms involved with *Leishmania*-infection.

With regard to TNF-α, IL-12 and NO macrophage responses, which play in a synergy manner a resistant innate immune response against *Leishmania*-infection[41], it was observed that each one of these immune mechanisms have been recorded in the *L. (L.) i. chagasi*-infected peritoneal macrophage from all Neotropical primates examined. Furthermore, it should also be emphasized the pivotal role of TNF-α in developing the immune macrophage resistance against infection, in view of its regular response in four of five monkeys between 24 to 72 hours of experiment: *C. jacchus* 145/122 pg/mL, *C. penicillata* 154/130 pg/mL, *S. sciureus* 164/104 pg/mL and *A. azarae infulatus* 154/104 pg/mL, with exception *C. goeldii* (38/83 pg/mL). Considering that TNF-α has been regarded as a pro-inflammatory cytokine with a crucial role in mediating host protection against *Leishmania*-infection[17,37,38], these findings allowed speculation that TNF-α response was responsible for the significant ($p < 0.05$) PMI decrease found in these four primates (*C. jacchus* 55/12, *C. penicillata* 83/38, *S. sciureus* 77/38 and *A. azarae infulatus* 128/37 pg/mL), with exception *C. goeldii* 78/54 pg/mL ($p > 0.05$).

On the other hand, IL-12 response was also a prominent cytokine in this study, mainly regarding those two primates with the highest FPMI, *A. azarae infulatus* and *C. goeldii*, which showed at 72 hours of infection, the higher levels of IL-12 than those of *C. jacchus*, *C. penicillata* and *S. sciureus*, suggesting a delayed IL-12 response in attempting to increase the macrophage resistance against the parasite replication. This appears in according with the major role for IL-12 cytokine that bridges the innate and acquired immune response, leading T-cell response toward to Th1 cytokine profile[1,23].

The NO-mediated leishmanicidal activity, derived from the inducible nitric oxide synthesis (iNOS), has widely been recognized either in experimental infection or in human disease as a major cellular defensive mechanism against the amastigote-intracellular growth of *Leishmania* sp.[12,28,36]. However, for this activity the TNF-α and IL-12 production is necessary, which will then provide the macrophage activation and the subsequent NO response[6,40]. In this way, two major findings related to NO response should be taken into consideration: first, it was demonstrated, at 24 hours of infection, a very important NO response in the PM-cultures of *C. jacchus* that monkey species with the lowest FPMI and with an expressive TNF-α response; second, it was observed, at 72 hours of infection, an expressive recovery of NO response in those primates which have showed low NO levels at 24 hours of infection, mainly in *C. goeldii* which has also showed significant IL-12 response, thus, suggesting in both situations the role of either TNF-α or IL-12 cytokines in the developing of macrophage resistance.

Taking all these findings together, as well as prior results on the *in vivo* susceptibility of *C. apella* monkey to experimental infection by *L. (L.) i. chagasi*[10], there appears to be enough evidence to suggest that these New World primates have developed resistant innate immune response mechanisms capable of controlling the parasite intracellular growth within macrophages, contradicting their use as experimental model of susceptibility to the infection by *L. (L.) i. chagasi*.

RESUMO

Susceptibilidade do macrófago peritoneal de diferentes espécies de primatas neotropicais para a infecção *ex vivo* por *Leishmania (L.) infantum chagasi*

Este estudo examinou a susceptibilidade do macrófago peritoneal (PM) dos primatas neotropicais: *Callithrix jacchus*, *Callithrix penicillata*, *Saimiri sciureus*, *Aotus azarae infulatus* e *Callimico goeldii* para a infecção *ex vivo* por *Leishmania (L.) infantum chagasi*, o agente etiológico da leishmaniose visceral americana (LVA), como método de triagem para avaliar o potencial desses primatas como modelo de estudo da LVA. A susceptibilidade do PM para a infecção foi investigada através do índice de infecção do PM (PMI) a intervalos de 24, 72 horas e, ainda, pela média dessas taxas (FPMI), assim como, pelas respostas do TNF-α, IL-2 (ELISA de captura) e óxido nítrico (NO) (método de Griess). Às 24hs da infecção experimental, o PMI do primata *A. azarae infulatus* (128) foi maior que aqueles de *C. penicillata* (83), *C. goeldii* (78), *S. sciureus* (77) e *C. jacchus* (55). Às 72hs, houve uma redução significativa do PMI de quatro primatas: *A. azarae infulatus* (128/37), *C. penicillata* (83/38), *S. sciureus* (77/38) e *C. jacchus* (55/12), com exceção de *C. goeldii* (78/54). O FPMI dos primatas *A. azarae infulatus* (82.5) e *C. goeldii* (66) foi maior que do primata *C. jacchus* (33.5), porém, não foi maior que dos primatas *C. penicillata* (60.5) e *S. sciureus* (57.5). A resposta do TNF-α foi mais regular nos quatro primatas que reduziram o PMI no intervalo de 24-72hs: *C. jacchus* (145/122 pg/μL), *C. penicillata* (154/130 pg/μL), *S. sciureus* (164/104 pg/μL) e *A. azarae infulatus* (154/104 pg/μL), com exceção de *C. goeldii* (38/83 pg/μL). A resposta de IL-12 foi, principalmente, marcante nos primatas *A. azarae infulatus* e *C. goeldii*, os quais apresentaram as maiores taxas do FPMI, e a resposta do NO foi maior no primata *C. goeldii*, em especial no intervalo de 72hs. Estes achados sugerem, fortemente, que estes primatas neotropicais parecem ter desenvolvido mecanismos resistentes de resposta imune inata capaz de controlar o crescimento intracelular da infecção por *L. (L.) i. chagasi* no macrófago, o que não encoraja o uso destes primatas como modelo de estudo da LVA.

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REFERENCES

1. Abdi K. IL-12: the role of p40 versus p75. Scand J Immunol. 2012;56:1-11.

2. Anuradha PR, Pal R, Zehra K, Katyiar JC, Sethi N, Bathia G, *et al.* The Indian langur: preliminary report of a new nonhuman primate host for visceral leishmaniasis. Bull World Health Organ. 1992;70:63-72.

3. Awasthi A, Mathur RK, Saha B. Immune response to *Leishmania* infection. Indian J Med Res. 2004;119:238-58.

4. Ayres M, Ayres Jr M, Ayres DL, Santos AS. Bioestat 4.0: aplicações estatísticas nas áreas das Ciências Biológicas e Médicas. Belém: Sociedade Civil Mamirauá, Brasília: CNPq; 2004.

5. Bogdan C. Nitric oxide and the immune response. Nat Immunol. 2001;2:907-16.

6. Bogdan CM, Röllinghoff M, Diefenbach A. The role of nitric oxide in innate immunity. Immunol Rev. 2000;173:17-26.

7. Broderson JR, Chapman WL Jr, Hanson WL. Experimental visceral leishmaniasis in the owl monkey. Vet Pathol. 1986;23:293-302.

8. Campos MB, de Castro Gomes CM, de Souza AA, Lainson R, Corbett CEP, Silveira FT. *In vitro* infectivity of species of *Leishmania (Viannia)* responsible for American cutaneous leishmaniasis. Parasitol Res. 2008;103:771-6.

9. Carneiro LA, Campos MC, Gomes CMC, Pires RN, Brandão J, Jennings YL, *et al.* Evaluation of the infectivity of New World *Leishmania* sp. to cultured macrophage of the monkey *Cebus apella* (Primates: Cebidae). In: Third World Congress on Leishmaniosis; 2005 April 10-15; Palermo, Sicily; 2005. Abstract Book. p. 126.

10. Carneiro LA, Silveira FT, Campos MB, Brígido MCO, Gomes CMC, Corbett, CEP, *et al.* Susceptibility of *Cebus apella* monkey (Primates: Cebidae) to experimental *Leishmania (L.) infantum chagasi*-infection. Rev Inst Med Trop Sao Paulo. 2011;53:45-50.

11. Chapman WL, Hanson WL. Visceral leishmaniasis in the squirrel monkey (*Saimiri sciureus*). J Parasitol.1981;67:740-1.

12. Gantt KR, Goldman TL, McCormick ML, Miller MA, Jeronimo SMB, Nascimento ET, *et al.* Oxidative responses of human and murine macrophages during phagocytosis of *L. chagasi*. J Immunol. 2001;167: 893-901.

13. Gicheru MM, Olobo JO, Kariuki TM, Adhiambo C. Visceral leishmaniasis in vervet monkeys: immunological responses during asymptomatic infections. Scand J Immunol. 1995;41:202-8.

14. Gomes CMC, Silveira FT, Laurenti MD, Costa JML, Tomokane TY, Souza MGT, *et al.* Biological behavior of *Leishmania (Leishmania) amazonensis* isolated from different clinical forms of American tegumentar leishmaniasis in Balb/c mice. Rev Inst Med Trop São Paulo 2003;45(Suppl 13):64.

15. Green SJ, Meltzer MS, Hibbs JB Jr, Nancy CA. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an l-arginine dependent killing mechanism. J Immunol. 1990;144:278-83.

16. Lainson R, Shaw JJ. Evolution, classification and geographical distribution. In: Peters W, Killick-Kendrick R, editors. The Leishmaniases in Biology and Medicine. London: Academic Press; 1987. v. 1, p. 1-120.

17. Liew FY, Parkinson C, Millott S, Severn A, Carrier M. Tumor necrosis factor (TNF-α) in leishmaniasis. I. TNF-α mediates host protection against cutaneous leishmaniasis. Immunology. 1990;69:570-3.

18. Madeira M, Barbosa-Santos E, Marzochi M. Experimental infection of canine peritoneal macrophages with visceral and dermotropic *Leishmania* strains. Mem Inst Oswaldo Cruz. 1999;94:645-8.

19. Maia C, Rolão N, Nunes M, Gonçalves L, Campino L. Infectivity of five different types of macrophages by *Leishmania infantum*. Acta Tropica. 2007;103:150-5.

20. Malla N, Mhajan RC. Pathophysiology of visceral leishmaniasis – some recent concepts. Indian J Med Res. 2006;123:267-74.

21. Marsden PD, Cuba CC, Vexenat A, Costa e Silva M, Costa e Silva A, Barreto AC. Experimental *Leishmania chagasi* infections in the marmoset *Callithrix jacchus jacchus*. Trans R Soc Trop Med Hyg. 1981;75:3124-5.

22. Mattner F, Di Padova K, Alber G. Interleukin-12 is indispensable for protective immunity against *Leishmania major*. Infect Immun. 1997;65:4378-83.

23. Michel MY, Fathy FM, Hegazy EH, Hussein ED, Eissa MM, Said DE. The adjuvant effects of IL-12 and BCG on autoclaved *Leishmania major* vaccine in experimental cutaneous leishmaniasis. J Egypt Soc Parasitol. 2006;36:159-76.

24. Misra A, Dube A, Naik S. Immune responses in normal Indian langur monkey (*Presbytes entellus*); a primate model for visceral leishmaniasis. J Med Primatol. 2004;33:65-9.

25. Misra A, Dube A, Srivastava B, Sharma P, Srivastava JK, Katiyar JC, *et al.* Successful vaccination against *Leishmania donovani* infection in Indian langur using alum-precipitated autoclaved *Leishmania major* with BCG. Vaccine. 2001;19:3485-92.

26. Misra A, Dube A, Srivastava JK, Sharma P, Katiyar JC, Naik S. Establishment of asymptomatic *Leishmania donovani* infection in Indian langurs (*Presbytis entellus*) through intradermal route. Indian J Exp Biol. 2002;40:605-8.

27. Moll H, Flohé S, Rollinghoff M. Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antigen-specific T-cell immune response. Eur J Immunol. 1995;25:693-9.

28. Murray HW, Nathan CF. Macrophage microbicidal mechanisms *in vivo:* reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. J Exp Med. 1999;189:741-6.

29. Olobo JO, Gicheru MM, Anjili CO. The African Green Monkey model for cutaneous and visceral leishmaniasis. Trends Parasitol. 2001;17:588-92.

30. Pinelli E, Gebhard D, Mommaas AM, Van Hoeiji M, Langermans JA, Ruitenberg EJ, *et al.* Infection of canine macrophage cell line with *Leishmania infantum*: determination of nitric oxide production and anti-leishmanial activity. Vet Parasitol. 2000;92:181-9.

31. Rocha FJ, Schleicher U, Mattner J, Alber G, Bogdan C. Cytokines, signaling pathways, and effector molecules required for the control of *Leishmania (Viannia) braziliensis* in mice. Infect Immun. 2007;75:3823-32.

32. Silveira FT, Corbett CEP. *Leishmania chagasi* Cunha & Chagas, 1937: indigenous or introduced? A brief review. Rev Pan-Amaz Saúde. 2010;1:143-7. (DOI: 10.5123/S2176-62232010000200018).

33. Silveira FT, Lainson R, Corbett CE. Leishmaniose tegumentar americana. In: Belda W Jr, Di Chiacchio N, Criado PR, editors. Tratado de dermatologia. São Paulo: Atheneu; 2010. v. 1, p. 1423-55.

34. Silveira FT, Lainson R, Shaw JJ, Garcez LM, Souza AAA, Braga R, *et al.* Leishmaniose cutânea experimental. I. Sobre a susceptibilidade do primata *Cebus apella* (Cebidae) à infecção pela *Leishmania (Viannia) lainsoni* Silveira, Shaw e Ishikawa, 1987. Rev Soc Bras Med Trop. 1989;22:125-30.

35. Silveira FT, Lainson R, Shaw JJ, Garcez LM, Souza AAA, Braga R, *et al.* Leishmaniose cutânea experimental. II. Aspectos evolutivos da infecção no primata *Cebus apella* (Cebidae) pela *Leishmania (V.) braziliensis* e *L. (L.) amazonensis*. Rev Soc Bras Med Trop. 1990;23:5-12.

36. Stenger S, Donhauser N, Thüring H, Röllinghoff M, Bogdan C. Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. J Exp Med. 1996;183:1501-14.

37. Theodos C, Povinelli L, Molina R, Sherry B, Titus RG. Role of tumor necrosis factor in macrophage leishmanicidal activity *in vitro* and resistance to cutaneous leishmaniasis *in vivo*. Infect Immun. 1991;59:2839-42.

38. Titus RG, Sherry B, Cerami A. Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis. J Exp Med. 1989;170: 2097-104.

39. Walton BC, Shaw JJ, Lainson R. Observations on the *in vitro* cultivation of *Leishmania braziliensis*. J Parasitol. 1997;63:1118-9.

40. Weinberg JB. Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review. Mol Med. 1998;4:557-91.

41. Wilhelm P, Ritter U, Labbow S, Donhauser N, Röllinghoff M, Bogdan C, *et al.* Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF. J Immunol. 2001;166:4012-9.

42. World Health Organization. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the control of leishmaniases. Geneva: WHO; 2010.

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