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Analysis using canine peripheral blood for establishing *in vitro* conditions for monocyte differentiation into macrophages for *Leishmania chagasi* infection and T-cell subset purification

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ABSTRACT

Canine visceral leishmaniasis (CVL) is a parasitic disease endemic in many countries, and dogs present as the major natural reservoir of the parasite, *Leishmania chagasi* (syn. *L. infantum*). Biomarkers in the canine immune system is an important technique in the course of developing vaccines and treatment strategies against CVL. New methodologies for studying the immune response of dogs during *Leishmania* infection and after receiving vaccines and treatments against CVL would be useful. In this context, we used peripheral blood mononuclear cells (PBMCs) from healthy dogs to evaluate procedures related to (i) establishment of *in vitro* conditions of monocytes differentiated into macrophages infected with *L. chagasi* and (ii) purification procedures of T-cell subsets ($CD4^+$ and $CD8^+$) using microbeads. Our data demonstrated that after 5 days of differentiation, macrophages were able to induce significant phagocytic and microbicidal activity after *L. chagasi* infection and also showed increased frequency of parasitism and a higher parasite load. Although

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N-acetyl-β-D-glucosaminidase (NAG) levels presented similar levels of macrophage culture and *L. chagasi* infection, a progressive decrease in myeloperoxidase (MPO) levels was a hallmark over 5 days of culture. High purity levels (>90%) of CD4 and CD8 T cells were obtained on a magnetic separation column. We concluded that monocytes differentiated into macrophages at 5 days and displayed an intermediate frequency of parasitism and parasite load 72 h after *L. chagasi* infection. Furthermore, the purification system using canine T-lymphocyte subsets obtained after 5 days of monocyte differentiation proved efficient for CD4 or CD8 T-cell purification (≥90%). The *in vitro* analysis using *L. chagasi*-infected macrophages and purified T cells presented a prospective methodology that could be incorporated in CVL vaccine and treatment studies that aim to analyze the microbicidal potential induced by specific $CD4^+$ and/or $CD8^+$ T cells.

1. Introduction

Leishmaniasis is endemic in 88 countries in tropical and subtropical regions of the Old and New Worlds, with more than 350 million cases being visceral leishmaniasis (VL) (Desjeux, 2004). Infected dogs have a high density of cutaneous parasites, and they are the main domestic reservoir of *Leishmania chagasi* (syn. *Leishmania infantum*) contributing to the propagation of the parasite (Deane and Deane, 1962). Thus, the current strategy for managing VL in humans centers on the detection and elimination of seropositive dogs alongside vector control and therapy for individual cases (Tesh, 1995).

A key goal in the control of canine visceral leishmaniasis (CVL) has been the development of vaccines with high protective capability to interrupt the cycle of parasite transmission (Reis et al., 2010). Assessments of vaccine safety and anti-CVL efficacy generally require a long follow-up, stretching into years of study (Giunchetti et al., 2007, 2008; Roatt et al., 2012). In this context, the development of methodological strategies that enable optimal evaluation of the dog's immune system would be highly relevant. Such tests could be included in clinical trials vaccine against CVL, so that the time needed for the experiments could be reduced. This would likely reduce the costs of experimentation using the dog model as well as provide a more rational way of selecting candidate vaccines against CVL.

Macrophages play an important role in the control of *Leishmania* infection in distinct experimental models. It is well established that macrophages participate in killing parasites through mechanisms that depend on reactive oxygen and nitrogen intermediates. However, the mechanisms by which macrophages kill *Leishmania* in dogs have not been investigated as thoroughly (Rodrigues et al., 2007). The immune response against *Leishmania* sp. is highly dependent on the microbicidal action of macrophages, which are actually the host cell target of this protozoan; however, they have full capacity for antigen presentation and establishment of an effective response against the parasite (Pinelli et al., 1999).

Thus, to develop new approaches for analyzing the immune response of naturally *L. chagasi*-infected dogs or dogs immunized against CVL, *in vitro* co-culture systems with macrophages and purified T-lymphocytes would be useful. However, there is so far no standardized methodology for this purpose, and these tests usually only involve a system with peripheral blood mononuclear cells (PBMCs) without purified T-lymphocyte subsets (Holzmuller et al., 2005; Rodrigues et al., 2007, 2009). The development of additional methodologies for evaluating the immune system in veterinary medicine, especially in experimental dog models, is required. Such an advance would contribute to the identification of biomarkers related to interactions between innate and adaptive immune responses of dogs. In this context, we aimed to further analyze the immune response by using standardized methodologies for a co-culture system of canine *L. chagasi*-infected macrophages and for obtaining purified $CD4^+$ and $CD8^+$ T cells. This approach could contribute to identifying specific immune response biomarkers for developing a resistance or susceptibility profile in CVL, which could be used in both vaccine and treatment strategies against the parasite.

2. Materials and methods

2.1. Animals

Healthy mongrel dogs, both sexes with a mean age of 7 months, born and raised in a kennel at the Center of Animal Science, Federal University of Ouro Preto, were used in the experiments of (i) establishment of *in vitro* conditions of monocytes differentiated into macrophages infected with *L. chagasi* ($n = 5$) and (ii) purification procedures of T-cell subsets ($CD4^+$ and $CD8^+$) using microbeads ($n = 12$). The animals received all the appropriate health management before entering the experiment, having received anti-helmintic treatment (plus Chemital®, Chemitec Agro-Veterinary LTDA., BRA) and vaccination against rabies (Tecpar, BRA), distemper, adenovirus type 2, coronavirus, parainfluenza, parvovirus, and *Leptospira* (HTLP 5/CV-L Vanguard®, Pfizer, BRA). The study protocol was approved by the Ethical Committee for the Use of Experimental Animals of the Universidade Federal de Ouro Preto, Ouro Preto – MG, Brazil.

2.2. Parasites

This study used a wild-type strain of *L. chagasi* (C46) isolated from an infected dog of Governador Valadares, MG, and previously characterized in hamsters (Moreira et al., 2012). This strain was grown in culture medium NNN/LIT (Sigma Chemical Co., USA) supplemented with 20% fetal bovine serum (FBS) inactivated (Cultilab, BRA), plus penicillin (200 U/mL) and streptomycin (100 μg/mL), at pH 7.4 and incubation temperature of 23 °C. Parasites used for

in vitro tests were removed from culture at the stationary phase, at the seventh passage.

2.3. Obtaining PBMCs

Blood samples from the five healthy dogs, 20 mL each, were collected in heparinized tubes intended for obtaining PBMCs. The whole blood volume collected was placed in a mixture of Ficoll–Hypaque (Sigma Chemical Co., USA, density: 1.119 g/mL) and Ficoll–Hypaque (Sigma Chemical Co., USA, density: 1.077 g/mL) at a 1:3 ratio (Ficoll/blood) in sterile polystyrene conical bottom tubes (Falcon™, Corning®, USA). All samples were centrifuged at 700 × *g* for 80 min at 22 °C. The ring of mononuclear cells collected at the Ficoll–Hypaque interface was transferred to another tube with 40 mL of Falcon sterile 1× PBS containing 10% FBS. This tube was centrifuged two times at 400 × *g* for 10 min at 4 °C. After the supernatant was discarded, the cells were resuspended in 1 mL of cell culture medium RPMI 1640. Cells were counted in a Neubauer hemocytometer chamber to determine the numbers of monocytes or lymphocytes per milliliter.

2.4. Establishment of culture conditions of circulating monocytes differentiated into macrophages (Mϕ) with enhanced microbicidal activity

After counting cells in a Neubauer chamber, we calculated the percentage of monocytes that were plated at 5×10^5 monocytes/well using 24-well plates (Thermo Fisher Scientific Inc., NUNC, USA), on circular coverslips (15 mm; Glasscyto, BRA). Cultures were established using RPMI supplemented with 20% fetal calf serum (FCS) and 20% macrophage colony-stimulating factor (M-CSF) medium and incubated at 37 °C/5% CO_2. The M-CSF was obtained from supernatant of cultures of L929 immortalized cells. After 24 h, the wells were gently washed to removed nonadherent cells, which were then transferred to new 48-well plates (Thermo Fisher Scientific Inc., NUNC, USA) and grown for 4 days in RPMI/20% FCS, at which time purification of $CD4^+$ and $CD8^+$ T lymphocytes was undertaken. To determine the timing of monocyte differentiation into macrophages with high phagocytic and microbicidal activity, distinct conditions were analyzed in duplicate. Monocytes differentiating into macrophages were evaluated from 2 to 5 days of culture. In all conditions, the cells were infected with 5×10^6 of *L. chagasi* promastigotes in the stationary phase, using a 10:1 ratio (10 parasites per macrophage). Each well was washed gently 3 h after infection and cultures were maintained to assess microbicidal activity 24, 48, 72, and 96 h postinfection. For the rate of parasitic infection, we counted the numbers of amastigotes in 200 macrophages. Thus, the total number of amastigotes was divided by the total number of infected macrophages in order to obtain the average number of amastigotes per macrophage.

2.5. Determination of N-acetyl-β-D-glucosaminidase (NAG)

NAG analysis served as an indicator of cellular activation levels after *in vitro* infection for various differentiation times of monocytes and macrophages. Supernatant from macrophages cultured for 2–5 days was submitted to *in vitro* infection with *L. chagasi* promastigotes, and after 3 h secretion of NAG was evaluated at various time points after infection. The NAG levels were evaluated in a 96-well plate (Thermo Fisher Scientific Inc., NUNC, USA) using 100-μL supernatant samples, in duplicate, diluted in 400 μL of citrate (0.1 M citric acid, pH 4.5). The assay was initiated with the addition of 100 μL of the substrate p-nitrophenyl N-acetyl-D-glucosaminide (Sigma Chemical Co., USA) diluted in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4, pH 4.5) at a final concentration of 2.24 mM and submitted to incubation at 37 °C for 30 min. The reaction was terminated by the addition of 100 μL of 0.2 M glycine buffer (0.8 M glycine, 0.8 M NaCl and NaOH, pH 10.6). The plate was read in a spectrophotometer at 405 nm. The content of the supernatants were calculated from a standard curve based on the expression activity of NAG.

2.6. Determination of myeloperoxidase (MPO)

The MPO evaluation was intended to determine the presence of polymorphonuclear cells in cultures at 2–5 days of differentiation, as an additional control for culture purity. Thus, 50 μL of the supernatant was placed, in duplicate, in 96-well plates (Thermo Fisher Scientific Inc., NUNC, USA), to which 100 μL of HCl tetramethylbenzidine (TMB; Promega Corporation, USA)/H_2O_2 was subsequently added. The plate was then incubated at 37 °C for 6 min, and the reaction was terminated by addition of 100 μL of 4 M H_2SO_4 to each well. The enzyme activity was determined colorimetrically using a plate reader (Bio-Tek EL 808 Ultra Microplate reader, USA) at a wavelength of 450 nm and is expressed as optical density.

2.7. Purification of $CD4^+$ and $CD8^+$ T lymphocytes by MACS® separation

Purification of $CD4^+$ and $CD8^+$ T lymphocytes was performed using a total of 12 healthy control dogs. A 20-mL peripheral blood sample was collected from each animal to obtain PBMCs for use in Ficoll–Hypaque (Sigma Chemical Co., USA) density gradient centrifugation (Section 2.3). After the first separation on the Ficoll–Hypaque gradient, the PBMCs were maintained for 24 h for adhesion of monocytes. After this period, nonadherent cells were separated into additional cultures for 4 days, for a total of 5 days in culture. At this time, lymphocytes were submitted for further purification by the same Ficoll–Hypaque method. $CD4^+$ and $CD8^+$ T lymphocytes were isolated using magnetic beads (Miltenyi Biotec Inc., USA) by positive selection using anti-CD4 or anti-CD8-FITC (fluorescein isothiocyanate) antibodies (AbD Serotec, UK) and microbeads coated with anti-FITC. Briefly, a cell suspension was prepared at a concentration of 6×10^7 cells in a 1-mL tube in isolation buffer containing PBS 1×, pH 7.2, 0.5% BSA, 2 mM EDTA). Monoclonal antibodies (CD4 or CD8-FITC) were added to 2 μL/mL of total lymphocytes, and incubated at room temperature (RT) for 15 min. Then, magnetic microbeads were added to 10 μL/mL lymphocytes and incubated for 15 min at RT. The

cell suspension was loaded onto a MACS® column (Miltenyi Biotec Inc., USA), which was placed in the magnetic field of a MACS® separator. The magnetically labeled $CD4^+$ or $CD8^+$ cells were retained on the column. The unlabeled cells ran through, thus this cell fraction was depleted of $CD4^+$ or $CD8^+$ cells. After removal of the column from the magnetic field, the magnetically retained $CD4^+$ or $CD8^+$ cells were eluted as the positively selected cell fraction by washing the magnetic column with 15 mL of isolation buffer.

The purity of $CD4^+$ and $CD8^+$ T cells was evaluated by flow cytometry on a FACSCalibur instrument (Becton Dickinson, USA) interfaced to an Apple G3 workstation. Cell-Quest software (Becton Dickinson, USA) was used for both data acquisition and analysis. A total of 20,000 events were acquired for each preparation. Flow cytometric analysis was performed using canine whole blood leukocytes that were selected on the basis of their characteristic forward (FSC) and side (SSC) light-scatter distributions. Following FSC and SSC gain adjustments, the lymphocytes were selected by gating on the FSC *versus* SSC graph. Fluorescence was evaluated from FITC spectra (anti-CD4 and anti-CD8 antibodies) on FL1 in dot plot representations. A marker was set as an internal control for nonspecific binding in order to encompass >98% of unlabeled cells, and this marker was then used to analyze data for individual animals. The results are expressed as the percentage of positive cells within the selected gate for cell surface markers presenting CD4 or CD8.

2.8. Statistical analysis of data

Statistical analysis was performed using instrumental support of the software GraphPad Prism 5.0 (Prism Software, USA). Data normality was demonstrated by the Kolmogorov–Smirnoff test. The analyses of the macrophage cultures (% of infection and number of amastigotes), NAG, and MPO were performed by ANOVA employing repeated measures (paired). Data were considered statistically significant when the p value was <0.05.

3. Results

3.1. Morphological changes

During the cultivation period, changes were observed in cultures of monocytes adhered to cover slips that differentiated into macrophages, 2, 3, 4, and 5 days after culture began (Fig. 1). At 2 and 3 days of differentiation, even after wells were washed, large numbers of granulocytes as well as mononuclear cells remained attached (Fig. 1A and B). In contrast, monocytes differentiated into macrophages by the fourth day of culture already demonstrated morphological changes such as increased size, cytoplasm vacuolation, and irregular shape (Fig. 1C). On the fifth day of maturation, these morphological changes remained, and there was an increase in cell size, number of nuclei (giant cells), and cytoplasm vacuolation (Fig. 1D).

3.2. Monocytes differentiated into macrophages after 5 days of culture had increased frequency of parasitism and higher parasite load

The phagocytic ability of monocytes that had differentiated into macrophages was assessed 3 h after *L. chagasi* promastigotes were used to infect monocytes at 2–5 days of differentiation (Fig. 2). These monocytes were then analyzed 24, 48, 72, and 96 h after *L. chagasi* infection. As shown in Fig. 2A, the percentage of macrophages infected by *L. chagasi* was statistically higher ($p < 0.05$) based on the length of time monocytes had differentiated into macrophages. Thus, monocytes that had differentiated for 5 days of culture showed a significantly higher percentage of *L. chagasi* infection (78.4 ± 0.6) in relation to the other times evaluated: 2 days (54.9 ± 0.7), 3 days (56.2 ± 2.9), and 4 days (67.6 ± 2.6).

Similarly, higher parasite loads were observed based on the time period of monocyte differentiation into macrophages (Fig. 2B). With 5 days of differentiation, there was a significantly enhanced number of amastigotes/macrophage (5.3 ± 0.6), when compared with other times: 2 days (2.5 ± 0.1), 3 days (2.6 ± 0.4), and 4 days (3.8 ± 0.5).

3.3. Macrophages cultured for 5 days and 72 h postinfection showed an intermediate profile for the frequency of the parasitism and parasite burden

Monocytes differentiated into *Mϕ* for 2 days showed statistically ($p < 0.05$) lower frequency of *L. chagasi*-infected macrophages at 96 h (51.2 ± 0.9) in relation to 24 h (56.1 ± 1.3) and 48 h (55.5 ± 2.0) (Fig. 3A). Fig. 3B showed increased frequency of parasitism at 24 h (54.1 ± 4.1) compared with 48 h (44.6 ± 3.8), 72 h (43.6 ± 3.7), and 96 h (42.3 ± 2.6) ($p < 0.05$).

Fig. 3C showed lower frequency of parasitism occurred at 96 h (46.8 ± 4.9) compared with 72 h (48.5 ± 4.4). Additionally, lower frequency of parasitism was described at 48 h (53.0 ± 7.3), 72 h (48.5 ± 4.4), and 96 h (46.8 ± 4.9) compared with 24 h (63.9 ± 2.4). We observed a reduced frequency of *L. chagasi*-infected macrophages at 96 h (48.0 ± 6.1) in comparison with both 72 h (53.5 ± 8.4) and 48 h (56.0 ± 1.4; Fig. 3D). Moreover, lower frequency of *L. chagasi*-infected macrophages was observed at 48 h (56.0 ± 1.4), 72 h (53.5 ± 8.4), and 96 h (48.0 ± 6.1) in relation to 24 h (74.0 ± 1.3). The Fig. 3E–H showed a similar profile as described for the frequency of *L. chagasi*-infected macrophages based on the different differentiation times.

3.4. NAG indicated that macrophages at 2–5 days of maturation displayed similar profiles of activation 24–96 h after internalization of L. chagasi

The analysis by NAG evaluation of lysosomal hydrolase levels from macrophages showed significant differences ($p < 0.05$) only after 4 days of differentiation (Fig. 4). A decreased NAG level at 72 h (47.2 ± 1.7) was observed in relation to 24 h (56.5 ± 2.0). For the other differentiation durations and time points postinfection, the pattern of release of enzyme in culture supernatants was similar.

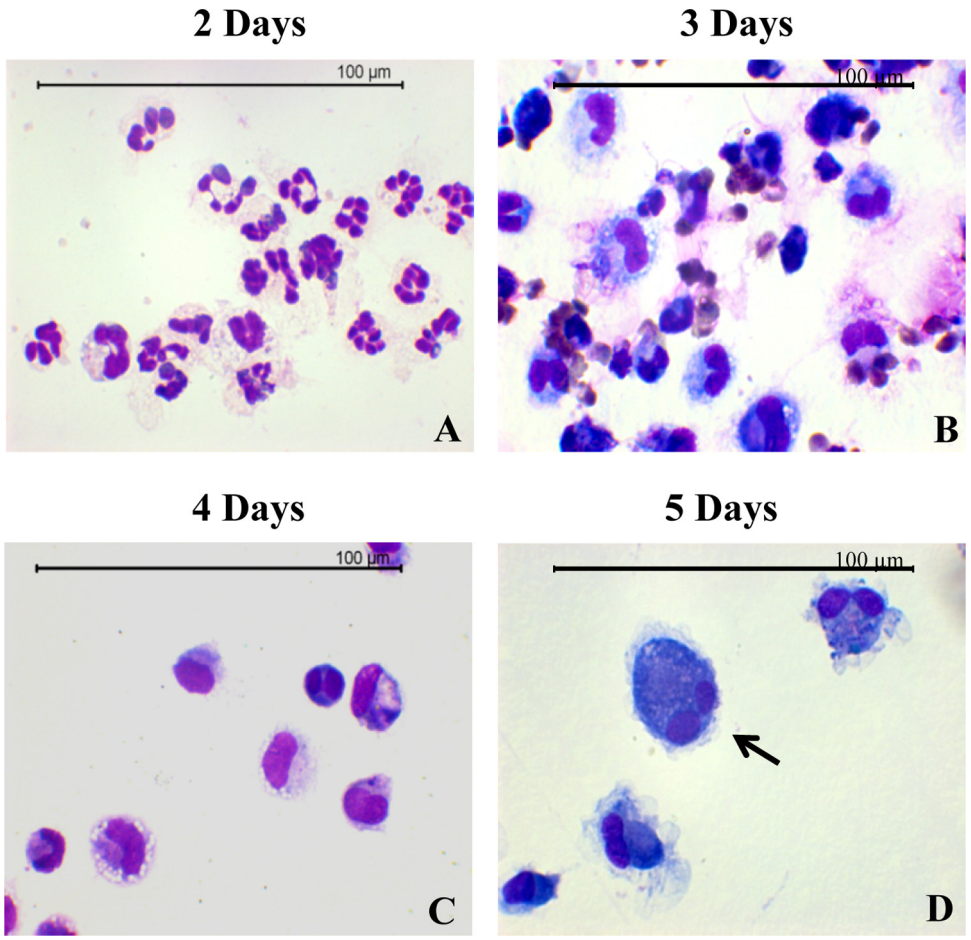

Fig. 1. Photomicrographs of cultured monocytes differentiated into macrophages (*Mϕ)* and stained with hematoxylin and eosin, at 2–5 days of culture, using peripheral blood mononuclear cells (PBMCs, up to ×100 oil immersion). Cultures of 2 (A; predominantly polymorphonuclear cells) and 3 (B) days for differentiation of monocytes into macrophages. (C) Cultures of 4 days for differentiation of monocytes into macrophages present a marked reduction in the number of granulocytes as well as morphological changes such as vacuolated macrophages. (D) Five-day cultures for differentiation of monocytes into macrophages display morphological changes in macrophages such as increased cell size, cytoplasm vacuolization, and the presence of multinucleated giant cells (arrow).

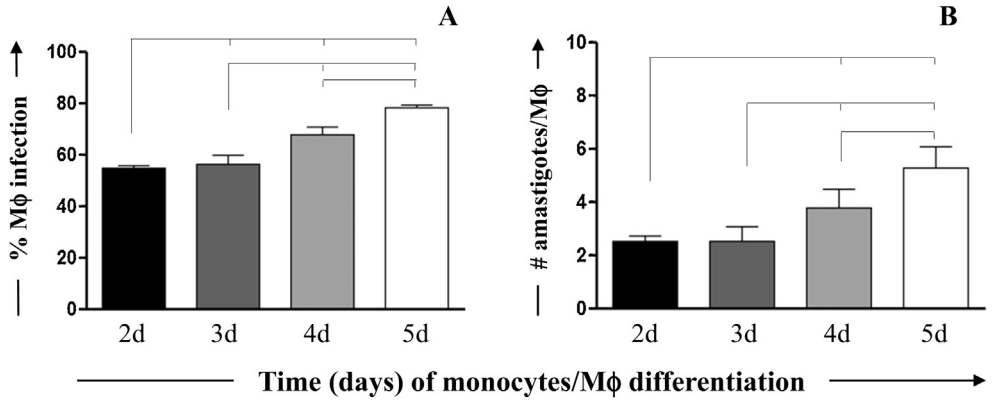

Fig. 2. Time differentiation of monocytes into macrophages for analysis of the ability to internalize *L. chagasi* and microbicidal activity 3 h after infection *in vitro*, where the ratio of infection *L. chagasi*/macrophage was 10:1. The results are shown for 2, 3, 4 and 5 days of monocyte incubation in culture. The *x*-axis represents time of differentiation for monocytes/macrophages in days (d): (2d ■), (3d ■), (4d ■), and (5d □). The *y*-axis on the left in the figure represents the average percentage and standard deviation of infection by *L. chagasi* in a total of 200 macrophages; on the right, the *y* axis represents the average number and standard deviation of amastigotes by macrophages infected with *L. chagasi*. The connecting lines between the bars indicate statistical differences ($p < 0.05$).

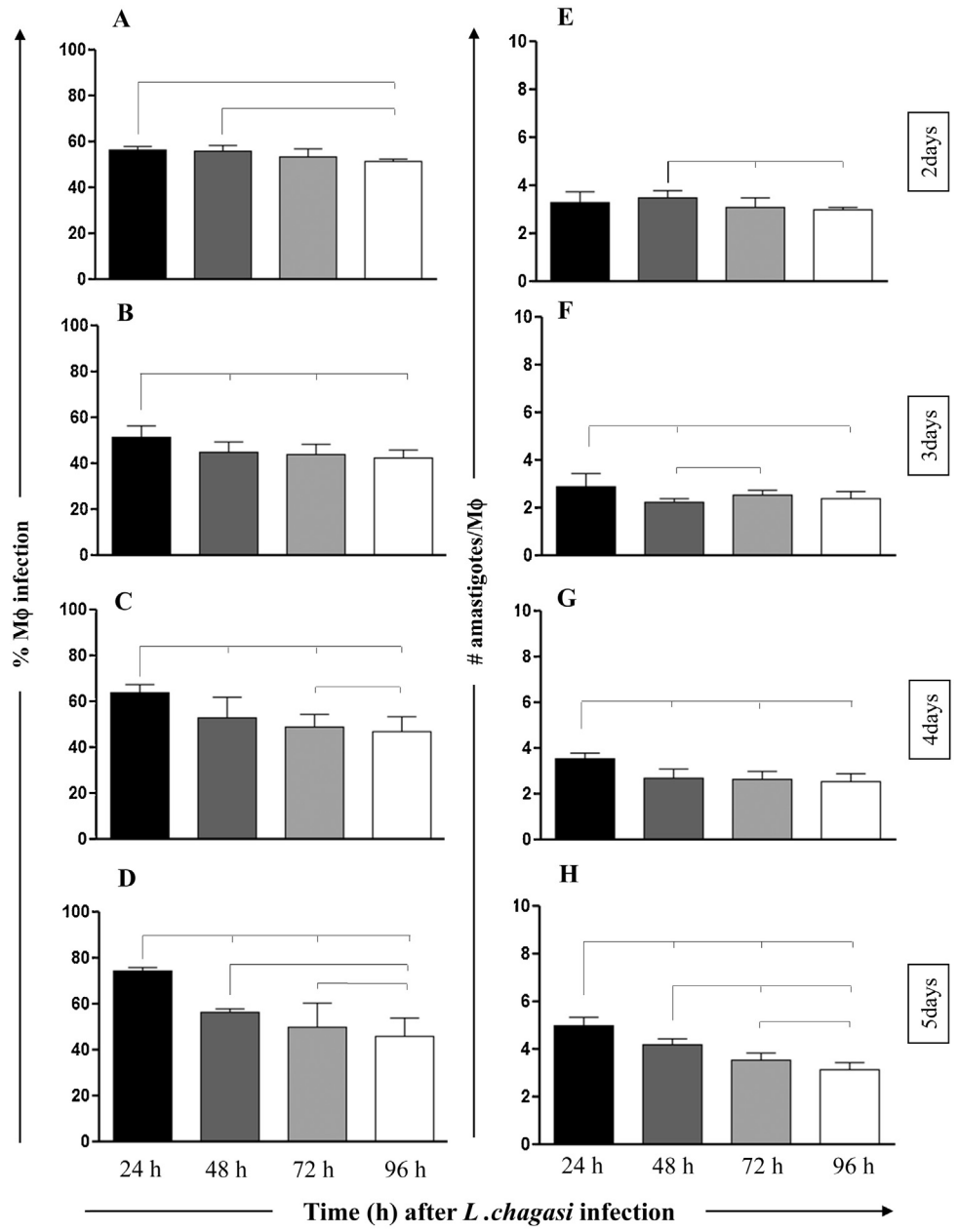

Fig. 3. Analysis of different times (24, 48, 72, and 96 h) after internalization of *L. chagasi* by monocytes that differentiated into macrophages for different times (2, 3, 4, and 5 days) to study their ability to internalize *L. chagasi* and their microbicidal activity, considering the rate of infection *L. chagasi*/macrophage as 10:1. The *x*-axis of figure represents the periods of assessments after internalization of *L. chagasi* promastigotes by macrophages in hours (h): (24 h ■) (48 h ■), (72 h ■) and (96 h □). The *y*-axis on the left of the figure represents the average percentage and standard deviation of macrophages infected with *L. chagasi* in a total of 200 macrophages evaluated (A–D), while on the right the *y*-axis represents the mean number of amastigotes and standard deviation for macrophage infection by *L. chagasi* (E–H). The connecting lines between the bars indicate statistical differences ($p < 0.05$).

3.5. Progressively decreased levels of MPO from monocyte/macrophage culture supernatants over 5 days of culture was the hallmark of macrophage differentiation after 3 h of Leishmania infection

Three hours after infection, MPO levels were significantly reduced for monocytes that had differentiated for 4 days (0.3 ± 0.1) and 5 days (0.2 ± 0.01) in relation to those cultured for 2 days (0.02 ± 0.3), and for 5 days (0.2 ± 0.01) in relation to 4 days (0.3 ± 0.1) ($p < 0.05$). These data suggest the development of a culture with a high degree of purity, given that this enzyme is secreted primarily by granulocytes containing azurophilic granules. Furthermore, it should be noted that given the short life of these PMNCs,

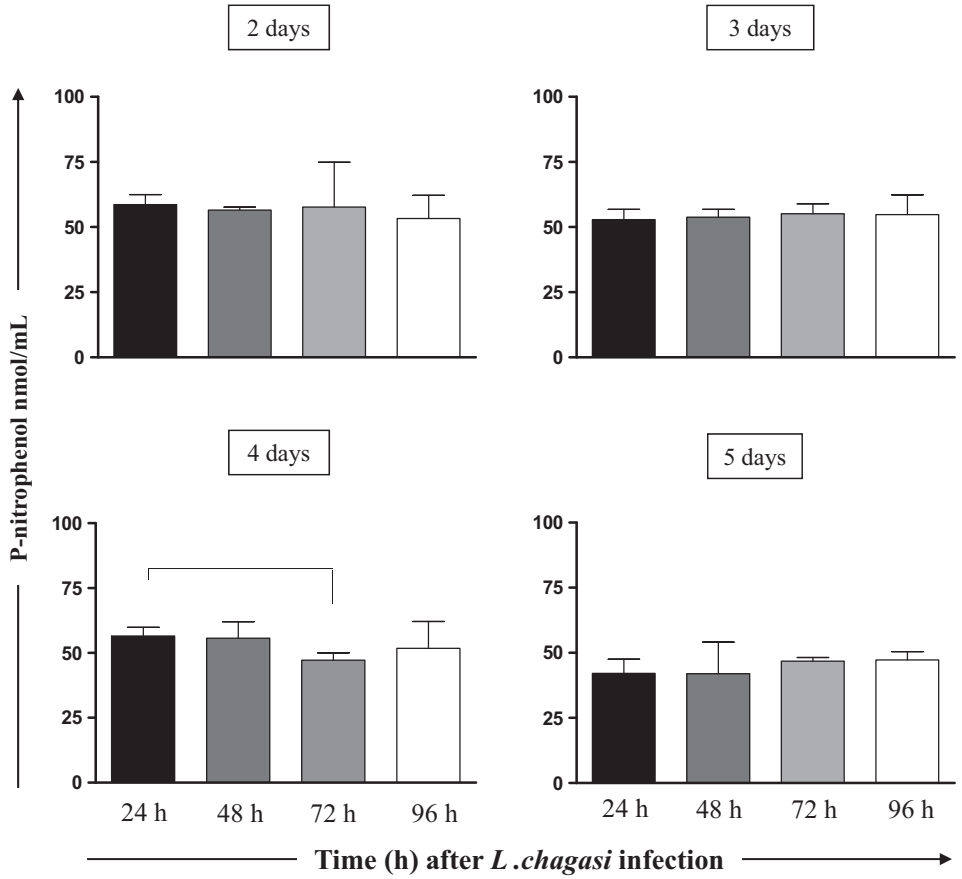

Fig. 4. Analysis of levels of the enzyme N-acetyl-D-glucosaminidase (NAG) in the culture supernatant, at different times (24, 48, 72, and 96 h) after internalization of *L. chagasi* by monocytes differentiated into macrophages for different times (2, 3, 4, and 5 days), the ratio of infection parasite/macrophage 10:1. The *x*-axis of the figure represents the periods of assessments after internalization of promastigotes of *L. chagasi* by macrophages in hours (h): (24 h ■), (48 h ■), (72 h ■), and (96 h □). The *y*-axis of figure represents the average levels and standard deviation of P-nitrophenol in nmol/mL. The connecting lines between the bars indicate statistical differences ($p < 0.05$).

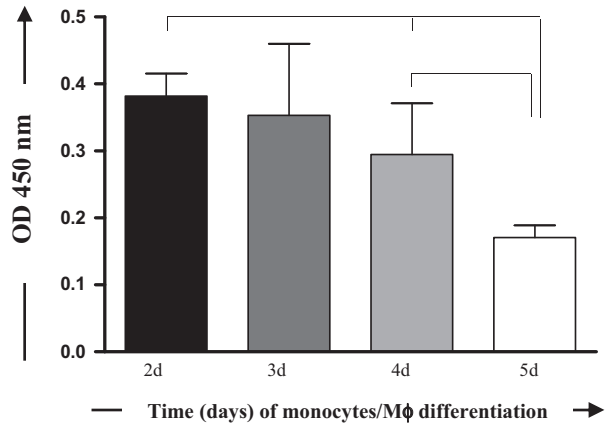

Fig. 5. Analysis of levels of the enzyme myeloperoxidase (MPO) in the culture supernatant, after internalization of *L. chagasi* (3 h) by monocytes differentiated into macrophages for different times (2, 3, 4 and 5 days); the infection rate of parasite/macrophage was 10:1. The *x*-axis represents time in figure differentiation of monocytes/macrophages in days (d): (2 d), (3 d), (4 d) and (5 d). The *y*-axis of figure represents the optical density (OD) mean and standard deviation for the MPO analysis. The connecting lines between the bars indicate statistical differences ($p < 0.05$).

they are almost certainly at an apoptotic stage on the fifth day of culture (Fig. 5).

3.6. Purification of CD4 and CD8 T cells

High purity levels of subpopulations of $CD4^+$ and $CD8^+$ T (≥90%) were obtained through the protocol described in this study's methodology, which took into account the large amount of circulating granulocytes in the peripheral blood of dogs. Graphs with different profiles before and after PBMC purification are shown in Fig. 6A–F. As shown in Fig. 6A, Ficoll gradient 1.077 was used to demonstrate a lower percentage of monocytes (10.2%) compared with the results from Ficoll gradients 1119 and 1077 (17.4%, Fig. 6B). Fig. 6C shows the PBMC profile from the culture plates on the fifth day of culture, which had substantial cell debris and 79% lymphocytes. In contrast, in Fig. 6D, where the lymphocytes were passed again in double Ficoll (1.119 and 1.077), the levels of lymphocytes were higher (91.7%). The percentage of lymphocyte purity using anti-CD4 or anti-CD8 antibodies and the sorting using magnetic column demonstrated high levels for CD4

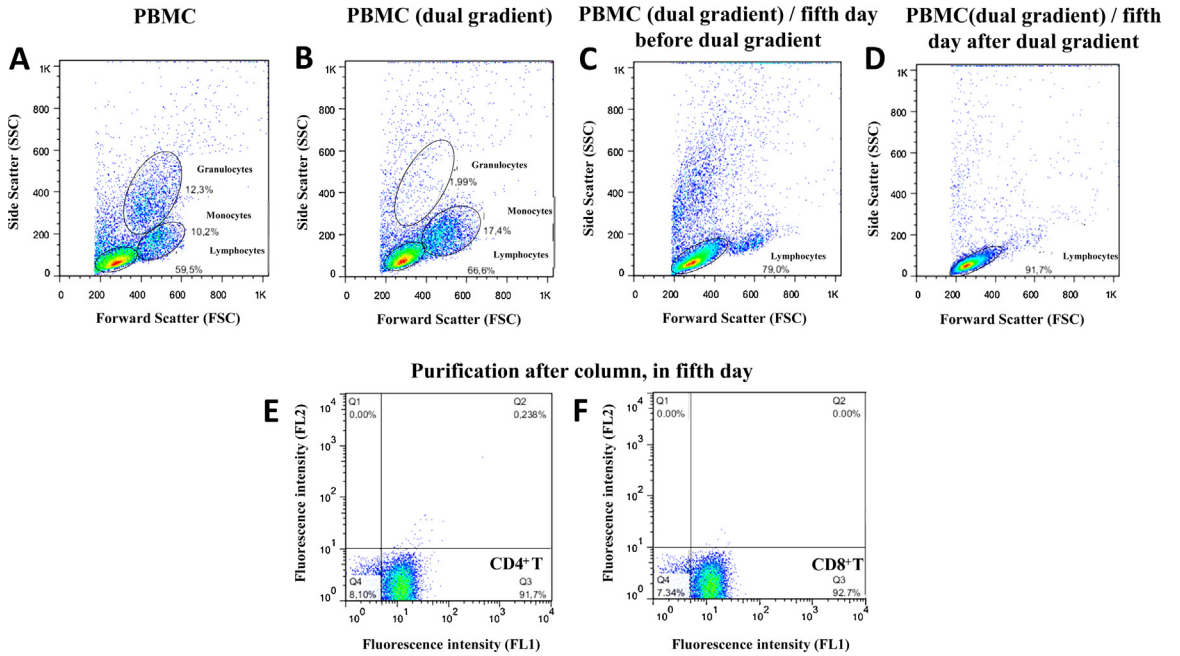

Fig. 6. Procedures illustrating the PBMCs obtained from distinct strategies based on Ficoll density gradients (A–D) used to establish the purity percentage of T-cells $CD4^+$ (E) and $CD8^+$ (F). A–D display PBMCs that were selected on the basis of their characteristic forward (FSC) and side (SSC) light-scatter distributions. Different FSC *versus* SSC graphs demonstrate PBMC pattern obtained after application of the whole blood leukocytes in the Ficoll gradient 1.077 density (A); or after application of the whole blood leukocytes in the Ficoll gradient 1.077 and 1.119 density (B); or after application of the whole blood leukocytes in the Ficoll gradient 1.077 and 1.119 density and maintained in culture during 5 days (C); or after application of the whole blood leukocytes in the Ficoll gradient 1.077 and 1.119 density and maintained in culture during 5 days and resubmitted to another purification using Ficoll gradient 1.077 and 1.119 density (D). The percentage of lymphocyte purity are demonstrated after selecting by gating on the FSC *versus* SSC graph (D), displaying the purity frequency of cell markers anti-CD4 (E) and anti-CD8 (F) FITC (FL-1) labeled.

(91.7%, Fig. 6E) and CD8 (92.7%, Fig. 6F) T-cell purification.

4. Discussion

The immune response against *Leishmania* sp. is highly dependent on the microbicidal action of macrophages, which, although the host target cells of this protozoan, have full capacity for antigen presentation and establishment of an effective response against the parasite (Pinelli et al., 1999). This methodology could be employed in immunogenic studies during testing of candidate vaccines against CVL. Thus, the microbicidal ability of antigen-specific CD4 or CD8 T cells co-cultured with *Leishmania*-infected macrophages could be investigated in dogs during testing of vaccines or treatment strategies against CVL.

Our results indicated that differentiated macrophages after 5 days of culture induced increases in both phagocytic and microbicidal activity (Figs. 1–3). Moreover, only at this time point was it possible to observe multinucleated giant cells and vacuolation of the cytoplasm. These results were encouraging for macrophages at this stage of maturation being satisfactory for application in *in vitro* experiments using *L. chagasi* infection. Furthermore, the differentiation of peripheral blood monocytes into macrophages permits obtaining cells less invasively than puncture through the peritoneal compartment (Zhang et al., 2008; Sampaio et al., 2007).

From the morphological point of view, the presence of multinucleated giant cells from cell fusion in cultures of monocytes differentiated into macrophages is reported in humans. However, it is known that a variety of inflammatory conditions can generate these cells (Gerberding and Yoder, 1993). These cells were previously reported in canine macrophages in the 1970s, in the studies of Ho and Babiuk (1979), however, the cell fusion occurred only in cultures after 4 weeks. In addition, they proposed a virtually pure culture, after 10 days of culture, from which macrophages can be maintained for up to 2 months under *in vitro* conditions. However, it is noteworthy that the longer the duration of culture, the greater the chances of contamination by different microorganisms. Therefore, it would be helpful to standardize these cultures so that experiments could be performed more quickly. In this context, Goto-Koshino et al. (2011) described a standardized method using canine macrophages with 7 days of differentiation, for studying of phagocytic ability with latex microspheres.

There is no consensus about the period of cell maintenance in culture, which may range up to 14 days (Wardley et al., 1980), for incubation of monocytes to be used as macrophages. Bueno et al. (2005) attempted to optimize obtaining larger numbers of macrophages derived from canine peripheral blood monocytes, comparing results of cell cultures kept in teflon flasks separated by a Ficoll gradient. In this study after 10 days of culture, it was possible

to obtain 84.17% canine macrophages. Furthermore, the rates of *L. chagasi*-infected macrophages after 24 and 72 h of infection were 75.93% and 76.70%, respectively. These results were similar to those of Panaro et al. (1998). However, except for the results 24 h after internalization of *L. chagasi* observed in the current study (Fig. 3, infection rate of 74.1%), the results obtained 72 h after infection by Bueno et al. (2005) are not in accordance with the present study. We found a gradual reduction in the frequency of parasitism and parasite load (number of amastigotes/*Mϕ*) based on the time after *L. chagasi* infection, especially for monocytes with a longer period of cell differentiation (5 days). Thus, it became evident that monocytes differentiated after 5 days into macrophages showed greater microbicidal ability, probably due to their stage of maturation.

Indirect dosages of lysosomal enzyme NAG showed that the cells were activated at least 24 h after *L. chagasi* infection, since we showed constant levels of this enzyme throughout the period of analysis (96 h) (Fig. 4). However, with the exception of macrophages of 4 days of differentiation, in which enzyme levels were significantly reduced at 72 h post-infection ($p < 0.05$), the enzyme levels were similar at other times. These results are consistent with those obtained by Kausalya et al. (1996) who found the release pattern of NAG from peritoneal macrophages from BALB/c was similar, with a peak release only after 21 days post *L. donovani* infection. Moreover, in the study by Chakraborty and Das (1989), infection with *L. donovani* peritoneal macrophages from hamsters resulted in a drastic reduction in the levels of NAG during the 96 h following infection. Furthermore, the analysis of NAG levels has not been reproduced with increased dead parasites in the cell culture medium, indicating that inhibition of enzyme levels is related to the characteristics of living parasites (Chakraborty and Das, 1989). The higher microbicidal activity is closely related to the number of activated macrophages, hence with high levels of secretion of lysosomal hydrolases (Akporiaye et al., 1983). Moreover, Meagher et al. (1992) described a apoptotic neutrophils study used in addition to macrophages were not sufficient to induce high levels of NAG compared to zymosan. These data indicate that apoptotic neutrophils in the cultures at 5 days may not interfere with the secretion of the lysosomal enzyme due to the phagocytic activity of macrophages.

In fact, in the present study the morphological characteristics described for different macrophage differentiation times indicated the presence of granulocytes was very low (<5%). Additionally, the results related to morphologic analysis, phagocytosis, microbicidal activity, enzymatic NAG and MPO activity, and the previous reports in the literature confirm that the ideal culture condition of canine monocyte differentiation into macrophages is obtained after 5 days of *in vitro* monocyte incubation.

The canine immune system has several peculiarities, especially in relation to the number of circulating granulocytes in the blood stream. Neutrophils present high expression of the $CD4^+$ molecule (Williams, 1997), and this feature interferes with the purification of $CD4^+$ T cells with high purity using typical methods of separation. Thus, using peripheral blood samples and performing $CD4^+$ T-cell separation, increased contamination by canine neutrophils cannot be avoided. The best alternative for establishing a purification system was to carry it out on the fifth day of monocyte differentiation, when lower levels of granulocytes are present. This strategy allowed an increased performance of $CD4^+$ or $CD8^+$ purity level (≥90%) using magnetic column methodology (Fig. 6).

5. Conclusions

The data presented here describe the ideal conditions for *in vitro* differentiation of monocytes, derived from canine peripheral blood, into macrophages. Based on our data presented here, we concluded that monocytes differentiate into macrophages over the course of 5 days and displayed an intermediate frequency of parasitism and parasite load 72 h after *L. chagasi* infection. At this time, the inclusion of purified CD4 and/or CD8 T cells in infected macrophages culture would be useful for analyzing the impact of modulation in *in vitro* parasitism. Furthermore, the purification system using canine T-lymphocyte subsets after 5 days of monocyte differentiation proved to be efficient for obtaining cultures permitting high CD4 or CD8 T-cell purity (≥90%). Thus, the use of co-culture systems employing canine monocytes differentiated into macrophages and purified $CD4^+$ and/or $CD8^+$ T cells may contribute to the analysis of the adaptive immune response in dogs. This methodology could be incorporated in vaccine and treatment studies against CVL that aim to analyze the microbicidal potential induced by specific $CD4^+$ and/or $CD8^+$ T cells.

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