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Despite Leishvaccine and Leishmune[®] trigger distinct immune profiles, their ability to activate phagocytes and CD8⁺ T-cells support their high-quality immunogenic potential against canine visceral leishmaniasis

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Summary Phenotypic features of peripheral blood leukocytes have been investigated as a pre-requisite to characterize the protective immunity attributed to both Leishvaccine and Leishmune[®]. Our results showed that either those vaccine were accompanied by distinct profiles on innate immune compartment. While Leishvaccine promoted early changes in phenotypic features of neutrophils and eosinophils with late involvement of monocytes, Leishmune[®] induced early and persistent activation of neutrophils and monocytes, without changes on eosinophil activation status. Regarding the adaptive immunity, Leishvaccine sponsored a mixed profile, associated with phenotypic changes of T and B-lymphocytes. Major phenotypic changes in CD4⁺ T-cells with transient activation of CD8⁺ T-cell, besides decreased frequency of B-cell expressing

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CD32 were the hallmark of Leishvaccine. In contrast, Leishmune[®] was associated with phenotypic changes in T-lymphocytes, particularly in CD8⁺ T-cells, and selective up-regulation of CD3⁺CD5^{Low}CD8⁺ cells. We hypothesized that this dissimilar alteration in immunological events would represent phenomenon directly related with the molecular nature of these vaccines besides the distinct adjuvants employed. However, it is important to emphasize that both immunobiologicals are able to activate phagocytes and CD8⁺ T-cells and therefore could be considered priority vaccines with a high-quality immunogenic potential against CVL.

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Introduction

Leishmania (Leishmania) infantum chagasi is the etiological agent of zoonotic visceral Leishmaniasis, an important re-emergent canine zoonose worldwide [1].

The elimination of seropositive dogs, vector control surround peridomestic environment and systematic treatment of human cases is recommended in Brazil as the current strategy for managing the disease control [1]. In the past 5 years more than 160,000 seropositive dogs have been eliminated in Brazil [2] but it is not universally acceptable, mainly due to ethical reasons [3], its low impact in situation of endemic transmission [4] and partially because of the resistance by dog owners to acquiesce in culling their infected pets [5,6].

The development of a protective vaccine against zoonotic visceral leishmaniasis has been recommended by WHO as important tool in the control of canine visceral Leishmaniasis (CVL) and also for an effective eradication of diseases [7,8]. Several candidates for canine vaccines against *L. (L.) infantum chagasi* infection have been proposed and include from live/killed *Leishmania* parasites (first-generation) to purified *Leishmania* antigens or live recombinant bacteria expressing *Leishmania* antigens (second generation) as well as antigen-encoding DNA plasmids (third generations) [9].

The Leishmune[®], a second-generation vaccine, have been recently licensed in Brazil and become commercially available. It is the first registered vaccine against CVL composed of purified fraction named fucose mannose ligand (FML), isolated from *L. donovani* promastigotes and uses saponin as adjuvant [10,11]. This formulation proved to be safe protective and highly immunogenic for dogs [12,13]. These Phase III trials reported the efficacy of Leishmune[®] with protection ranging from 92 to 95%, highlighting the long-lasting and strong immunoprophylactic effect against CVL. Leishmune[®] immunogenicity have been reported by its ability to trigger a specific humoral and a potent cellular immune response, as vaccines presented, even 3.5 years after vaccination high anti-FML seropositivity besides positive intradermal reactions [13]. Additionally, it has been stated that Leishmune[®] vaccines showed significant increase percentage of circulating CD8⁺ T-cells as expected for QuilA saponin vaccines. More recently, it has been reported the immunotherapeutic potential of Leishmune[®] on CVL, using a new formulation with increased adjuvant concentration have demonstrate that treated animals have the ability to up-regulate the frequency of CD4⁺ T-cells following *Leishmania*-specific lymphoproliferation assay *in vitro*, highlighting the potential of Leishmune[®] in eliciting a potent cellular immune response [14].

Although some encouraging results have been reported by the use of purified fractions from purified *Leishmania* extracts [11,12,15], the use of vaccines prepared from whole parasites antigens extracts still remain a reliable perspective considering their broad spectrum of antigenicity, cost and safety, and a number of such vaccines have been tested [16,17].

Phase I and II clinical trials have demonstrated enhanced *in vitro* lymphocyte proliferation and significant protection against infection with *Leishmania* in Brazilian dogs that had received merthiolated ultrasound-disrupted *Leishmania* promastigotes of together with Bacillus Calmette-guérin (BCG) as adjuvant. Moreover, strong cellular proliferation to soluble *Leishmania* antigens has also been reported in dogs vaccinated with autoclaved *Leishmania* promastigotes also using BCG as adjuvant [18]. More recently, it has been reported that a killed *Leishmania* vaccine with saponin adjuvant elicited a strong antigenicity related to the increase humoral immune response together with outstanding upregulation of CD8⁺ T-lymphocytes and antigen-specific CD8⁺ T-cell response [19].

Despite immunoprophylaxis of CVL has become an important control strategy and protective immunity have been reported following the use of whole parasites antigens extracts as well as purified *Leishmania* antigens, the precise immunological events triggered by these immunobiological tools still remains to be elucidated. It is important to mention that most previous studies did not report details on the immunological status of canine vaccines, probably due to the lack of specific reagents and standardized protocol to investigate the canine immune response. Aiming to further focus on this issue, herein, we have performed a detailed flow cytometry study in order to characterize the kinetic of changes in phenotypic features of peripheral blood leukocytes triggered by Leishvaccine and Leishmune[®] as the prototypes of first and second-generation vaccines against CVL, focusing on both innate and adaptive immune response, using a paired and unified experimental vaccination regimen.

Our results demonstrated that Leishvaccine and Leishmune[®] vaccines lead to distinct impact in peripheral blood leukocytes associated with the innate and the adaptive immune response. In particular, Leishvaccine was associated with a mixed cellular and humoral immune response, whereas Leishmune[®] showed to trigger a more selective cellular immune response. We hypothesized that both the antigenic nature and the adjuvant employed on each vaccination regimen represent the major key for the differences observed between Leishvaccine and Leishmune[®] vaccines.

Materials and methods

Animals

Twenty-four healthy German Shepherd dogs, 16 males and 8 females, age ranging from 18 to 60 months, were maintained at the kennel of Polícia Militar de Minas Gerais, Brazil during the entire experimental procedures. Prior to the inclusion in this study, all animals were treated for intestinal helminthic infections and immunized against parvovirus, leptospirosis, distemper, parainfluenza and hepatitis. All animals received drinking water and a balanced feed given *ad libitum* and were maintained in quarantine before the inclusion in the study. The dogs included in this study were selected based on their negative serological results in the enzyme-linked immunosorbent assay (ELISA, Biomanguinhos, FIOCRUZ, RJ, Brazil) used as a reference standard test for the diagnosis of CVL.

All procedures in this study were according to the guidelines set by the Brazilian Animal Experimental College (COBEA). This study was approved by the Ethical Committee for the use of Experimental Animals (CETEA) of the Universidade Federal de Minas Gerais, Brazil.

Vaccination

Dogs were divided into two groups named Leishvaccine and Leishmune® with twelve animals each. Leishvaccine consisted of *Leishmania (L.) amazonensis* (strain IFLA/BR/1967/PH8) antigenic preparation obtained as described by Mayrink et al. [18] using BCG (Fundação Ataulfo de Paiva, RJ, Brazil) as adjuvant. The Leishmune® consisted of *Leishmania donovani* purified fucose manose ligand (FML), uses saponin as adjuvant and was commercially obtained from FortDodge® manufacturer.

Dogs in the Leishvaccine group were immunized throughout a complete vaccination regimen that included three subcutaneous doses of the vaccine with an interval of 21 days between each. The first dose corresponded to 0.6 ml of Leishvaccine (360 µg of protein) plus 0.4 ml of BCG (400 µg of protein) as adjuvant. The second dose corresponded to 0.6 ml of Leishvaccine (360 µg of protein) plus 0.3 ml of BCG (300 µg of protein) as adjuvant. The third dose corresponded to 0.6 ml of Leishvaccine (360 µg of protein) plus 0.2 ml of BCG (200 µg of protein) as adjuvant.

Dogs in the Leishmune® were submitted to a complete vaccination regimen as recommended by the manufacturer (FortDodge®, Campinas, SP, Brazil), which included three subcutaneous doses of 1.0 ml of vaccine with an interval of 21 days between each dose.

Blood samples

Whole EDTA blood samples were collected at four consecutive periods including: before vaccination and 1 week after the 1st, 2nd and 3rd dose of each vaccine, corresponding to T0, T1, T2 and T3, respectively.

Samples constituted of 5 ml of canine whole peripheral blood using EDTA as the anticoagulant (final concentration of 1 mg/ml) collected by trained professional at the ken-

nel of the 4° Cia da Polícia Militar de Minas Gerais, PMMG, Minas Gerais, Brazil. All samples were maintained at room temperature up to 12 h prior to processing.

Complete hemogram was performed by conventional techniques using an automated blood cell analyzer (ADVIA® 60, Bayer HealthCare, Tarrytown, NY, USA).

Monoclonal antibodies

A range of cell surface markers that define major and minor canine leukocytes subpopulations were used, including anti-canine CD3-RPE 1:10 (mouse IgG1, clone CA17.2A12), anti-canine CD4-FITC or RPE 1:320 (rat IgG2a, clone YKIX302.9), anti-canine CD5-FITC 1:160 (rat IgG2a, clone YKIX322.3), anti-canine CD8-FITC or RPE 1:40 (rat IgG1, clone YCATE55.9), anti-canine B-cell-RPE 1:160 (mouse IgG1, clone CA2.1D6), anti-human CD14-PE-Cy5 1:40 (mouse IgG2a, clone TuK4), all purchased from Serotec (Oxford, UK).

Aiming to further characterize the activation status of canine peripheral blood leukocytes, a set of cell surface markers were analyzed using anti-mouse MHC I-FITC 1:20 (mouse IgG2b, clone 2G5), anti-canine MHC II-FITC 1:80 (rat IgG2a, clone YKIX334.2), anti-human CD18-RPE 1:6 (rat IgG2b, clone YFC118.3) and anti-human CD32-FITC 1:6 (mouse IgG1, clone AT10), all purchased from Serotec (Oxford, UK). Although virtually almost all nucleated cells express MHC I [20], canine granulocytes were weakly labeled with anti-MHC I 2G5 monoclonal antibody. In contrast with other species, MHC II molecule is expressed by all canine peripheral blood mononuclear cells [20], with no reference to expression by canine granulocytes. A small percentage of neutrophils express MHC I and MHC II and may reflect an antigenic priming-immunological event as previously reported for canine lymphocytes [21]. CD32 is the low affinity receptor for aggregated IgG (FcγRII), constitutively expressed on granulocytes, monocyte and B-cells. CD32 mediates endocytosis, cytotoxicity and immunomodulation [22]. Two major subclasses of FcγRII have been described in humans FcγRIIa and FcγRIIb [23]. In dog, a single report has addressed the analysis of CD32 expression by peripheral blood granulocytes [24]. Although Lilliehöök et al. [24] have reported that anti-CD32 antibodies label percoll-purified neutrophils but not percoll-purified eosinophils, a small percentage of canine unpurified neutrophils and eosinophils express CD32. The glycoprotein CD18 is an adhesion molecule of the complex LFA-1, ICAM-1 ligand crucial for leukocytes' ability to bind on the endothelial cells and further transmigration into the extravascular tissue. CD18 is virtually expressed by all circulating canine granulocytes [24].

Immunophenotyping of canine whole blood leukocytes

Immunophenotyping analyses of canine peripheral blood through flow cytometry were carried out as follow: in 12 mm × 75 mm polystyrene tubes, 30 µl of fresh whole blood were incubated at room temperature (RT) for 30 min in the dark in the presence of 30 µl of fluorochrome-labeled anti-canine cell surface marker monoclonal antibodies (mAbs) previously diluted in PBS–0.5%BSA (phosphate buffered saline 0.15 M, pH 7.2 supplemented with 0.5% of

bovine seric albumin and 0.1% of sodium azide). After incubation, the erythrocytes were lysed by adding 3 ml of lysis solution (FACS brand lysing solution; Becton Dickinson San Diego, CA, USA) followed by incubation for 10 min at RT. Canine whole blood leukocytes were then washed twice with 2 ml of PBS (phosphate buffered saline 0.15 M, pH 7.2) and centrifuged at 400 g for 10 min at RT. After the washing procedures, labeled cells were then fixed for 30 min at RT, with 200 μ l of FACS FIX solution (10.0 g/l paraformaldehyde; 10.2 g/l sodium cacodylate and 6.65 g/l sodium chloride, pH 7.2) before analysis in the cytometer. The stained cells were stored at 4–8 °C up to 24 h before cytofluorometric analysis. Each assay included an internal control for autofluorescence in which the cells were incubated in the presence of PBS–0.5%BSA.

Flow cytometry data storage and analysis

Flow cytometric measurements were performed on a FAC-Scan instrument (Becton Dickinson, Mountain View, CA) interfaced to an apple G3 FACStation. The Cell-Quest software package was used in both data acquisition and analysis. A total of 10,000 events were acquired for each preparation. Canine whole blood leukocytes were first identified on the basis of their specific forward (FSC) and side (SSC) light-scatter properties. Following FSC and SSC gain adjustments; the lymphocytes were detected based on their characteristic FSC versus SSC gain distribution.

The neutrophils and lymphocytes were selected based on their characteristic FSC versus SSC gain distribution and their phenotypic features analyzed on dual color graphs to evaluate their fluorescence spectra for FITC and R-PE on FL1 \times FL2 dot plots.

Eosinophil gating strategy was essentially based on their autofluorescence using non-related FL-3 channel versus FSC. A specific scatter gate using anti-CD14 TC versus SSC dot plot combination was performed for selective analysis of monocytes identified as SSC^{Low}CD14^{High} cells.

The results were expressed as percentage of positive cells within the selected gate, for cell surface markers presenting bimodal distribution and the results expressed as percentage of gated cells. For this purpose, a marker was set on the

internal control for unspecific binding, in order to confine over 98% of the unlabeled cells. This marker was used in all data analysis for a given sample to determine the percentage of positive cells. The variables expressed as percentage of gated cells included MHC1⁺, MHCII⁺ and CD32⁺ cells within gated neutrophils, eosinophils or monocytes besides CD3⁺, CD4⁺, CD3⁺CD5^{Low}, CD8⁺, CD4⁺CD18⁺, CD8⁺CD18⁺, B-cells and CD32⁺ B-cells within gated lymphocytes.

Semi-quantitative analyses were also performed to evaluate differential expression of cell surface markers presenting unimodal distribution and the results were expressed as mean fluorescence intensity (MFI) on a log scale. The variables expressed as MFI included CD18 by gated neutrophils eosinophils and monocytes besides MHCII by gated CD4⁺ and CD8⁺ lymphocytes.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 4.03 software package (San Diego, CA, USA). Considering the nonparametric nature of all data sets, the Wilcoxon matched pairs test was used to access significant differences on the phenotypic features during vaccination protocol by comparing for each immunobiological used the post-vaccination timing points (T1, T2 and T3) with the starting point (T0). Spearman's rank correlation was also used to evaluate associations between specific phenotypic features. In all cases, the differences were considered significant when the probabilities of equality, *p*-values, were *p* < 0.05.

Results

Vaccinated dogs did not display any significant changes in their hematological profile following Leishvaccine and Leishmune[®] vaccination regimens

Aiming to characterize the impact of the Leishvaccine and Leishmune[®] on major hematological features, whole EDTA blood samples were collected before and after the 1st, 2nd and

Table 1 Hematological profile of German shepherd dogs following Leishvaccine and Leishmune[®] vaccination regimens

Hematological parameters	Immunobiological tool							
	Leishvaccine				Leishmune [®]			
	T0	T1	T2	T3	T0	T1	T2	T3
White blood cells ^a	12.5 \pm 1.9	13.5 \pm 2.3	13.9 \pm 2.6	14.4 \pm 3.7	13.7 \pm 2.6	15.0 \pm 3.1	15.1 \pm 3.8	15.6 \pm 3.2
Granulocytes ^a	4.6 \pm 1.9	5.6 \pm 2.1	5.2 \pm 1.8	6.0 \pm 1.8	5.0 \pm 1.8	6.6 \pm 2.5	6.6 \pm 2.7	6.2 \pm 1.8
Monocytes ^a	3.4 \pm 0.8	3.6 \pm 0.7	3.4 \pm 0.8	3.7 \pm 1.4	3.8 \pm 0.7	3.9 \pm 0.9	3.6 \pm 1.0	4.2 \pm 1.2
Lymphocytes ^a	4.5 \pm 1.4	4.4 \pm 1.1	5.4 \pm 1.9	4.8 \pm 1.4	4.8 \pm 1.0	4.4 \pm 0.9	5.0 \pm 1.3	5.2 \pm 1.7
Red blood cells ^a	6.4 \pm 0.7	6.6 \pm 0.4	6.7 \pm 0.6	6.5 \pm 0.8	6.3 \pm 0.8	6.7 \pm 0.4	7.1 \pm 0.4	6.8 \pm 0.5
Hemoglobin ^b	15.7 \pm 1.7	15.8 \pm 1.8	15.6 \pm 1.7	15.3 \pm 1.7	15.3 \pm 2.1	16.6 \pm 1.7	16.4 \pm 0.9	15.6 \pm 1.1
Hematocrit ^c	48.2 \pm 5.4	48.9 \pm 5.5	48.4 \pm 5.5	47.0 \pm 4.7	46.9 \pm 6.5	51.1 \pm 5.4	50.9 \pm 2.9	47.3 \pm 3.6

^a Results are expressed as mean number of cells/mm³ \pm S.E.

^b Hemoglobin is expressed as g% \pm S.E.

^c Hematocrit as % of whole blood \pm S.E.

3rd dose of each vaccine and complete hemogram was performed by conventional techniques (Table 1). Data analysis throughout the vaccination regimen did not demonstrate any significant differences in the hematological features, including white blood cells and major leukocytes subpopulations (granulocytes, monocytes and lymphocytes) as well as red blood cells, hemoglobin and hematocrit.

Dogs immunized with Leishvaccine or Leishmune® vaccines exhibited overall similar phenotypic changes on circulating neutrophils throughout the vaccination regimen

Kinetic analysis of phenotypic changes in circulating neutrophils was performed throughout the vaccination regimen with Leishvaccine or Leishmune®. Our data demonstrated that dogs immunized with Leishvaccine displayed a transient decreased percentage of MHCII⁺ neutrophils early at T1 during the vaccination regimen (Fig. 1, upper left panel), whereas the Leishmune® vaccines showed a persistent decrease in the percentage of MHCII⁺ neutrophils throughout the vaccination regimen as observed as T1, T2 and T3 (Fig. 1, upper right panel).

Down regulation of CD32 parallel with lower CD18 expression was also the hallmark of the phenotypic changes on

neutrophils during the entire immunization procedure (T1, T2 and T3) with either Leishvaccine or Leishmune® (Fig. 1, lower left and right panels). On the other hand, persistent up regulation of MHCII was observed throughout the vaccination regimen at T1, T2 and T3, despite the immunogenic tool used (Fig. 1, upper left and right panels).

Together, our data demonstrated that both immunobiological tools triggered an overall similar phenotypic change on circulating neutrophils following the vaccination regimen.

Leishvaccine triggered selective phenotypic changes on circulating eosinophils, with later input on monocytes, whereas Leishmune® vaccines display early and persistent changes monocytes with no phenotypic changes on circulating eosinophil

Kinetic analysis of phenotypic changes in circulating eosinophils and monocytes was performed following vaccination regimen with Leishvaccine or Leishmune®. Our data demonstrated that only dogs immunized with Leishvaccine display changes in eosinophil phenotypic features, with down-regulation of CD32⁺ cells at T2 and T3 and up-regulation of CD18 expression at T1 and T2 (Fig. 1, left

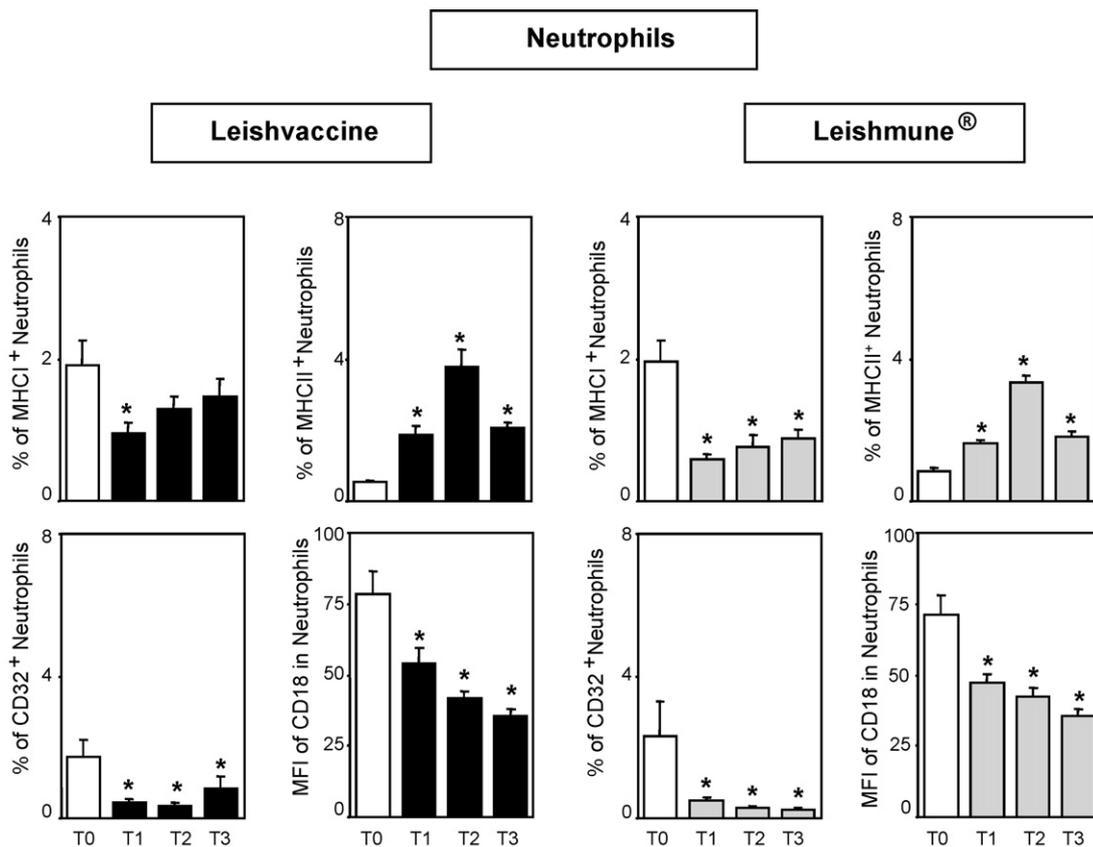


Figure 1 Immunophenotypic profile of peripheral blood NEUTROPHILS in German Shepherd dogs, following Leishvaccine (■) and Leishmune® (■) vaccination regimens. Neutrophils were selected based on their characteristic FSC versus SSC gain distribution and their phenotypic features analyzed on dual color FL1/FITC versus FL2/R-PE dot plots. The results were expressed as percentage of positive cells \pm S.E. (MHCII⁺, MCHII⁺ and CD32⁺ cells) within gated neutrophils and as mean fluorescence intensity (MFI) of CD18 expression \pm S.E. by gated neutrophils. Significant differences at $p < 0.05$ are indicated by asterisk for the comparison between T1, T2 and T3 with those unvaccinated paired control dogs (T0 = □).

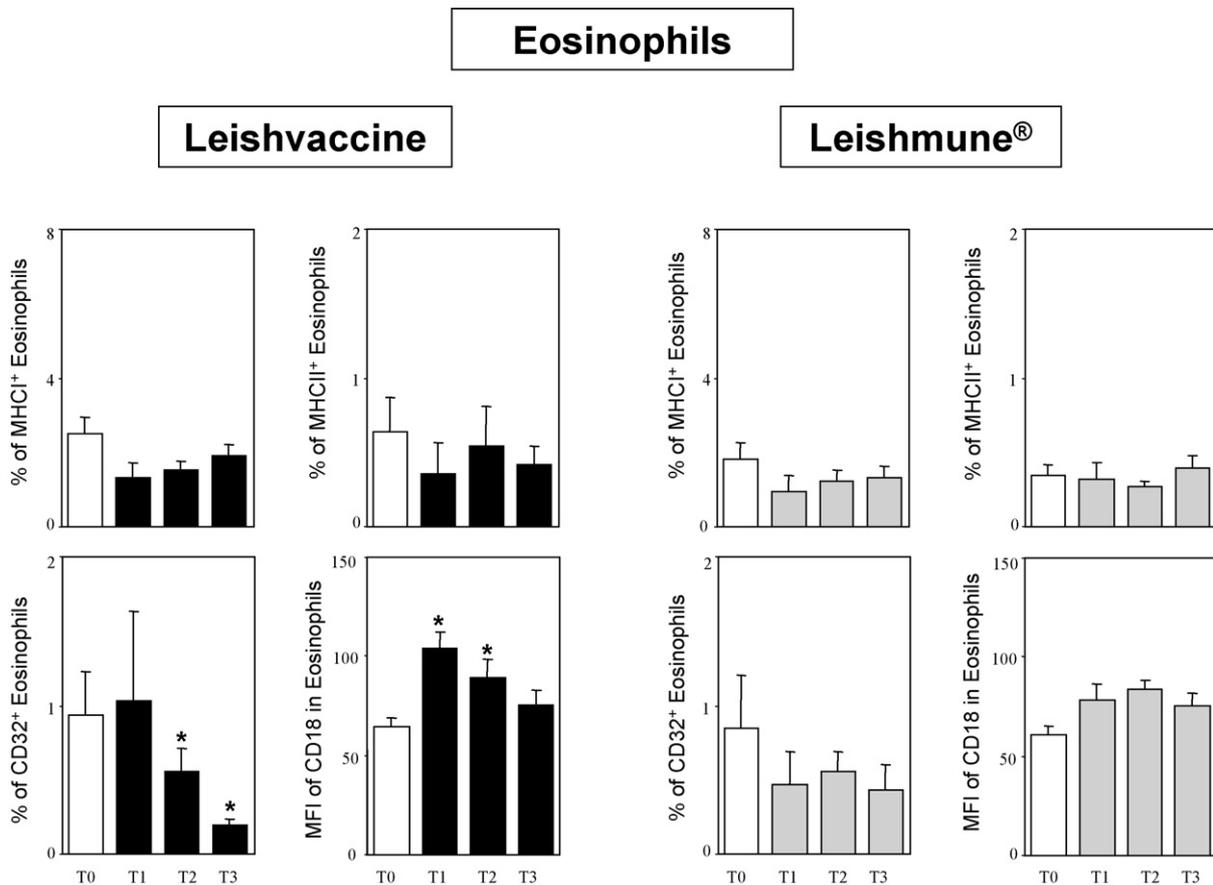


Figure 2 Phenotypic aspects of circulating EOSINOPHILS in German Shepherd dogs, following Leishvaccine (■) and Leishmune® (□) vaccination regimens. Eosinophil gating strategy was essentially based on their autofluorescence using non-related FL-3 channel versus forward scatter (FSC) and their phenotypic features analyzed on dual color FL1/FITC versus FL2/R-PE dot plots. The results are expressed as percentage of positive cells \pm S.E. (MHCII⁺, MHCII⁺ and CD32⁺ cells) within gated eosinophils and as mean fluorescence intensity (MFI) of CD18 expression \pm S.E. by gated eosinophils. Significant differences at $p < 0.05$ are indicated by asterisk for the comparison between T1, T2 and T3 with those unvaccinated paired control dogs (T0 = □).

panels). No significant differences were observed in the percentage of MHCII⁺ and MHCII⁺ eosinophils throughout the vaccination regimen, regardless the immunobiological tool (Fig. 2, upper panels).

Analysis of phenotypic features of circulating monocytes revealed down-regulation of MHCII following both vaccination regimen at T2 and T3 with selective earlier impact in dogs vaccinated with Leishmune® as observed at T1 (Fig. 3, top panels). No significant differences were observed in the percentage of MHCII⁺ and CD32⁺ monocytes throughout the vaccination regimen, regardless the immunobiological tool (Fig. 2). Punctual down-regulation of CD18 expression by monocytes was observed at T3 following immunization with Leishvaccine (Fig. 3, left lower panel).

Leishvaccine sponsored a mixed profile associated with major phenotypic changes in CD4⁺ T and B-lymphocytes, whereas Leishmune® promoted a selective T-cell dependent profile particularly associated with up-regulation of CD8⁺ T-cells

Analysis of the adaptive immunity compartment revealed that while Leishvaccine sponsored a mixed profile, asso-

ciated with phenotypic changes of T and B-lymphocytes, Leishmune® vaccination was typically associated a T-cell dependent immunity.

Downregulation of circulating B-cells with consecutive up-regulation of T/B cell ratio was observed at T2 following Leishvaccine immunization (Fig. 4, left upper panels). On the other hand, up-regulation of T-cells with parallel increase on circulating CD8⁺ T-cells were the outstanding phenotypic feature at T2 following Leishmune® vaccination (Fig. 4, right panels).

Additional analysis of the activation status of T-lymphocyte subsets as well as the B-cell modulator molecule CD32 was also performed following Leishvaccine and Leishmune® intervention (Fig. 5). Major changes in CD4⁺ T-cells (CD18 and MHCII) with transient activation of CD8⁺ T-cells (CD18 and MHCII) besides decreased frequency of B-cell with lower frequency of CD32⁺ B-cells was the hallmark of Leishvaccine immunization (Fig. 5, left panels). On the other hand, Leishmune® vaccination was associated with phenotypic changes in T-lymphocytes, with recurrent up-regulation of CD18⁺ CD8⁺ T-cells and decreased levels of MHCII⁺ CD8⁺ T-cells at T2 and T3 and transient down-regulation of MHCII⁺ CD4⁺ T-cells at T3.

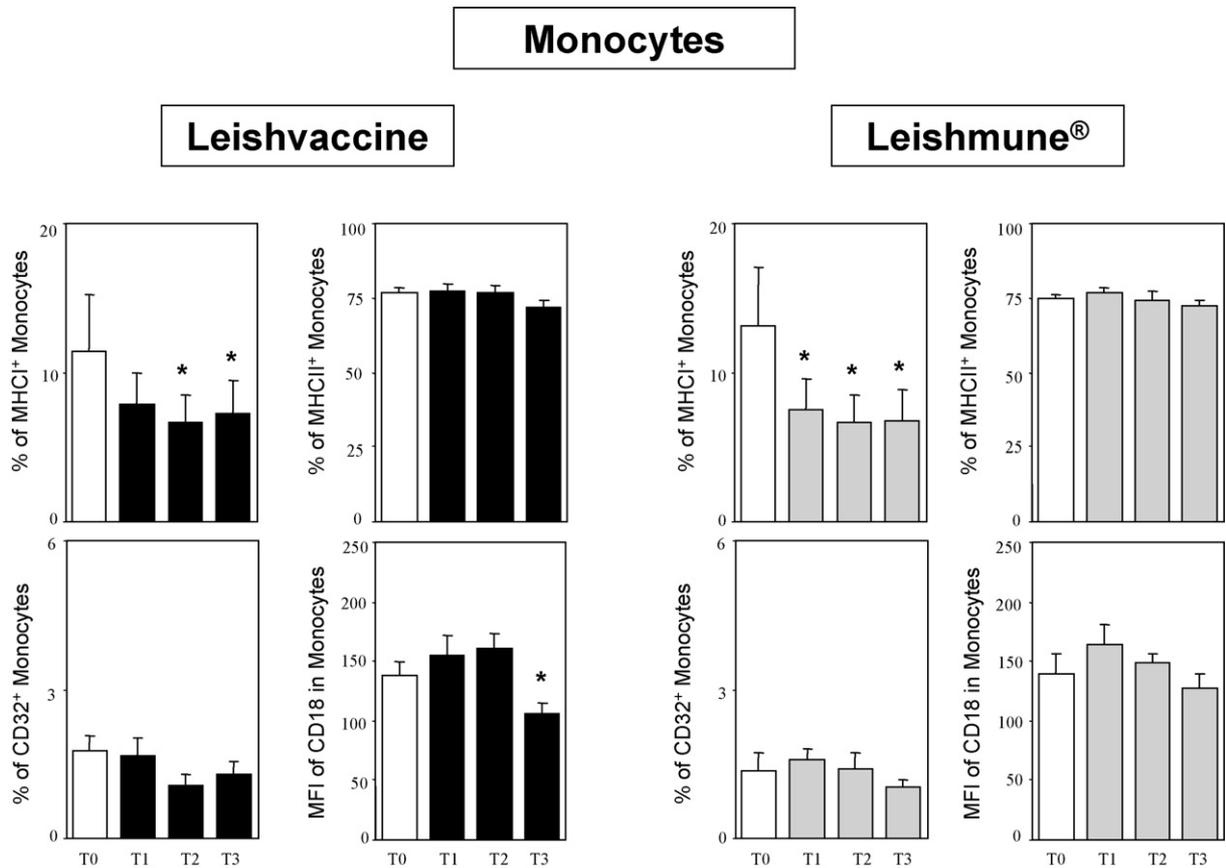


Figure 3 Immunophenotypic features of peripheral blood monocytes in German Shepherd dogs, following Leishvaccine (■) and Leishmune® (▒) vaccination regimens. A specific scatter gate using anti-CD14 TC versus SSC dot plot combination was performed for selective analysis of monocytes identified as SSC^{Low}CD14^{High} cells and their phenotypic features analyzed on dual color FL1/FITC versus FL2/R-PE dot plots. The results are expressed as percentage of positive cells \pm S.E. (MHCII⁺, MCHII⁺ and CD32⁺ cells) within gated monocytes and as mean fluorescence intensity (MFI) of CD18 expression \pm S.E. by gated monocytes. Significant differences at $p < 0.05$ are indicated by asterisk for the comparison between T1, T2 and T3 with those unvaccinated paired control dogs (T0 = □).

Up-regulation of CD3⁺CD5^{Low}CD8⁺ cells is selectively observed in Leishmune® vaccines

Recent advances in canine immunological tools including the accessibility of monoclonal antibodies labeled with distinct fluorochromes have allowed the multiparametric analysis of a range of cell subsets that might impact the canine immune response. Herein, we have observed that a singular T-cell subset expressing low density of CD5⁺ cell surface marker, but an overall unaltered expression of CD3 could be identified following double labeled staining protocol. Additionally, we have characterized this cell population and characterized that all CD5^{Low} cells are indeed CD8⁺ T-cells but not CD4⁺ T-cells (Fig. 6, dot plot charts).

Aiming to characterize the role of these cells we have followed their frequency in the canine peripheral blood after Leishvaccine and Leishmune® vaccination. Our data demonstrated a selective up-regulation of CD3⁺CD5^{Low} T-cells particularly at T2 following Leishmune® vaccination. Additional analysis have further addressed the positive correlation between CD3⁺CD5^{Low} T-cells and the percentage of circulating CD8⁺ T-cells following Leishmune® vaccination.

Discussion

The canine visceral leishmaniasis is currently expanding markedly worldwide, mainly in Brazil, where the typical rural outline has shifted recently toward a progressive urbanization. In endemic areas in Brazil, the prevalence of CVL reported ranges from 5 to 35% [25]. This large prevalence besides the fact that both asymptomatic and symptomatic dogs are equally infectious to the vectors, mainly due to the intense cutaneous parasitism observed in these animals, one of the current strategy for managing the disease includes the detection and elimination of seropositive dogs [1,26]. However, the selective elimination of seropositive dogs has been pointed an expensive and drastic tool difficult to be implemented with a major impact in the general society [27,28]. Thus, it has been considered that the implementation of chemotherapeutic procedures as well the use of a protective vaccine against *L. (L.) infantum chagasi* would represent the most effective alternative to control CVL spreading. Most studies focusing of therapeutic strategies have failed to achieve a consistent parasitological cure in CVL [29,30].

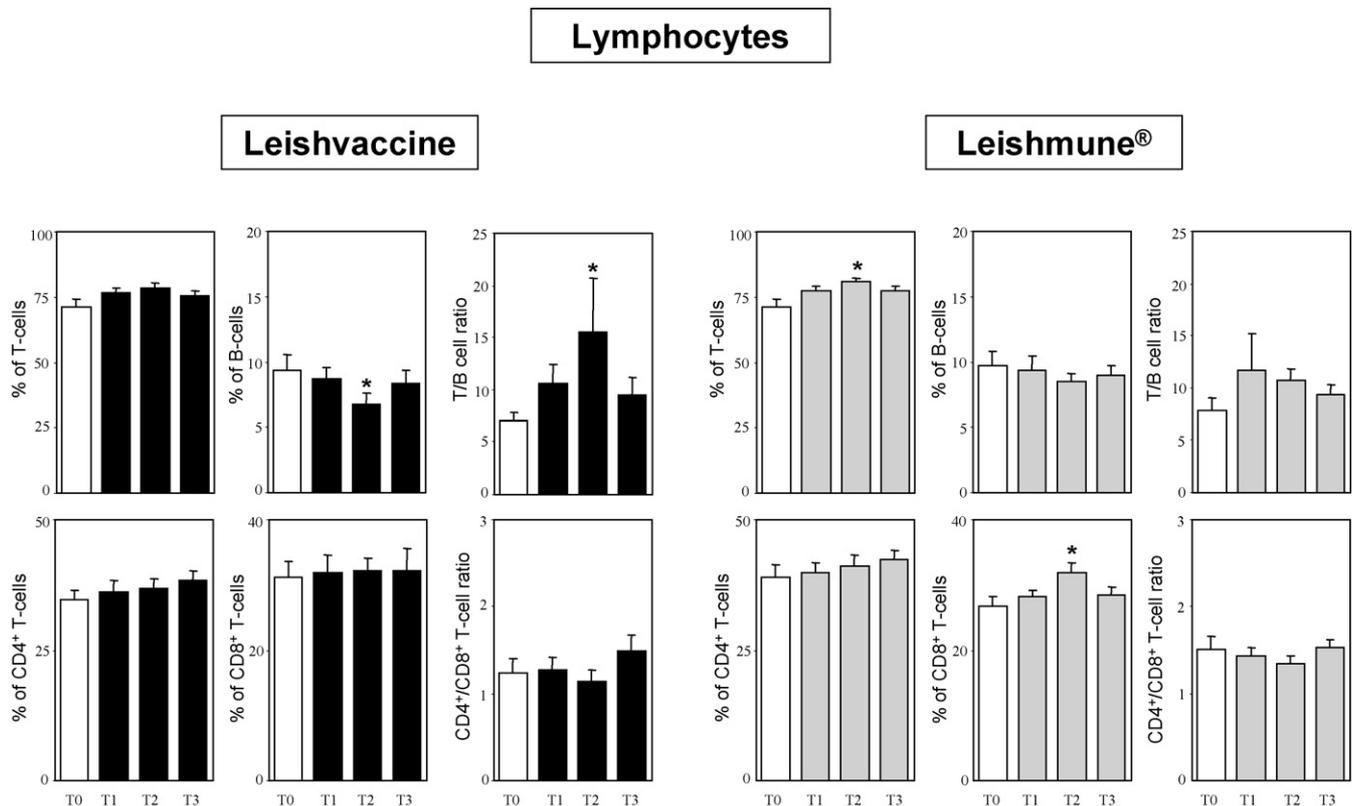


Figure 4 Major peripheral blood LYMPHOCYTES subpopulations in German Shepherd dogs, following Leishvaccine (■) and Leishmune® (■) vaccination regimens. Lymphocytes were selected based on their characteristic FSC versus SSC gain distribution and their phenotypic features analyzed on dual color FL1/FITC versus FL2/R-PE dot plots. The results are expressed as percentage of positive cells ($CD3^+$, $CD4^+$, $CD8^+$ and B-cell maker $^+$ lymphocytes) and cell ratio ($CD3^+/B$ -cell and $CD4^+/CD8^+$) \pm S.E. within gated lymphocytes. Significant differences at $p < 0.05$ are indicated by asterisk for the comparison between T2 with those unvaccinated paired control dogs (T0=□).

All these features pointed to immunoprophylaxis as a promising alternative for CVL prevention [15]. Several studies have reported the potential of different *Leishmania* antigens to trigger immunoprotective mechanisms against CVL. The use of whole *L. amazonensis*, formerly named Leishvaccine [31] and *L. braziliensis* antigens [19] as well purified *L. donovani* antigens [9], currently available as Leishmune® have been proposed as a first and second-generation vaccine candidate for CVL, respectively. Most studies, including clinical trials with vaccine candidates, specially Leishvaccine or Leishmune®, suggested their potent capacity to prevent *L. (L.) infantum chagasi* infection in dogs [12,13,19,31]. Considering the importance of a vaccine for the control of CVL and the lack of studies regarding the cellular and molecular basis underlying the effectiveness of vaccination, the present work attempted to perform a detailed longitudinal phenotypic analysis of the innate and adaptive compartment of canine peripheral blood as a pre-requisite to understand the immunological mechanisms related to immunogenicity elicited by Leishvaccine or Leishmune®.

Our results showed that interventions with either Leishvaccine or Leishmune® vaccine were accompanied by distinct profiles regarding the innate immune compartment. While Leishvaccine induced early phenotypic changes in neutrophils and eosinophils with late involvement of

monocytes, Leishmune® induced early and persistent phenotypic changes on neutrophils and monocytes, without alteration in eosinophil activation status. In this study, we have assumed that the increased percentage of MHC1 $^+$ and MHCII $^+$ cells usually refer to an activation event, with the up-regulation of MHC1 and MHCII reflecting higher ability of interaction with $CD8^+$ T-cells and $CD4^+$ T-cells, respectively. Therefore, our findings highlighting the down-regulation of MHC1 in neutrophils and monocytes besides the up-regulation of MHCII in neutrophils may reflect that following Leishvaccine and Leishmune® vaccination, the neutrophils and monocytes showed higher ability to interact with $CD4^+$ T-cells. As both immunobiological displayed ability to induce phenotypic changes on both $CD4^+$ and $CD8^+$ T-cells, the activation of $CD8^+$ T-cells may be an event that counts with the involvement of other antigen presenting cells.

The higher frequency of MHCII $^+$ neutrophils is in consonance with the lower percentage of $CD32^+$ neutrophils, since $CD32$ is usually associated with neutrophil activation via immunocomplex (IC) and upon stimulation the $CD32/IC$ pair is internalized, which reflected the lower frequency of $CD32^+$ neutrophils observed following vaccination. Moreover, the lower expression index of $CD18$ observed in circulating neutrophils may indirectly reflect that the activated neutrophils expressing high $CD18$ density are

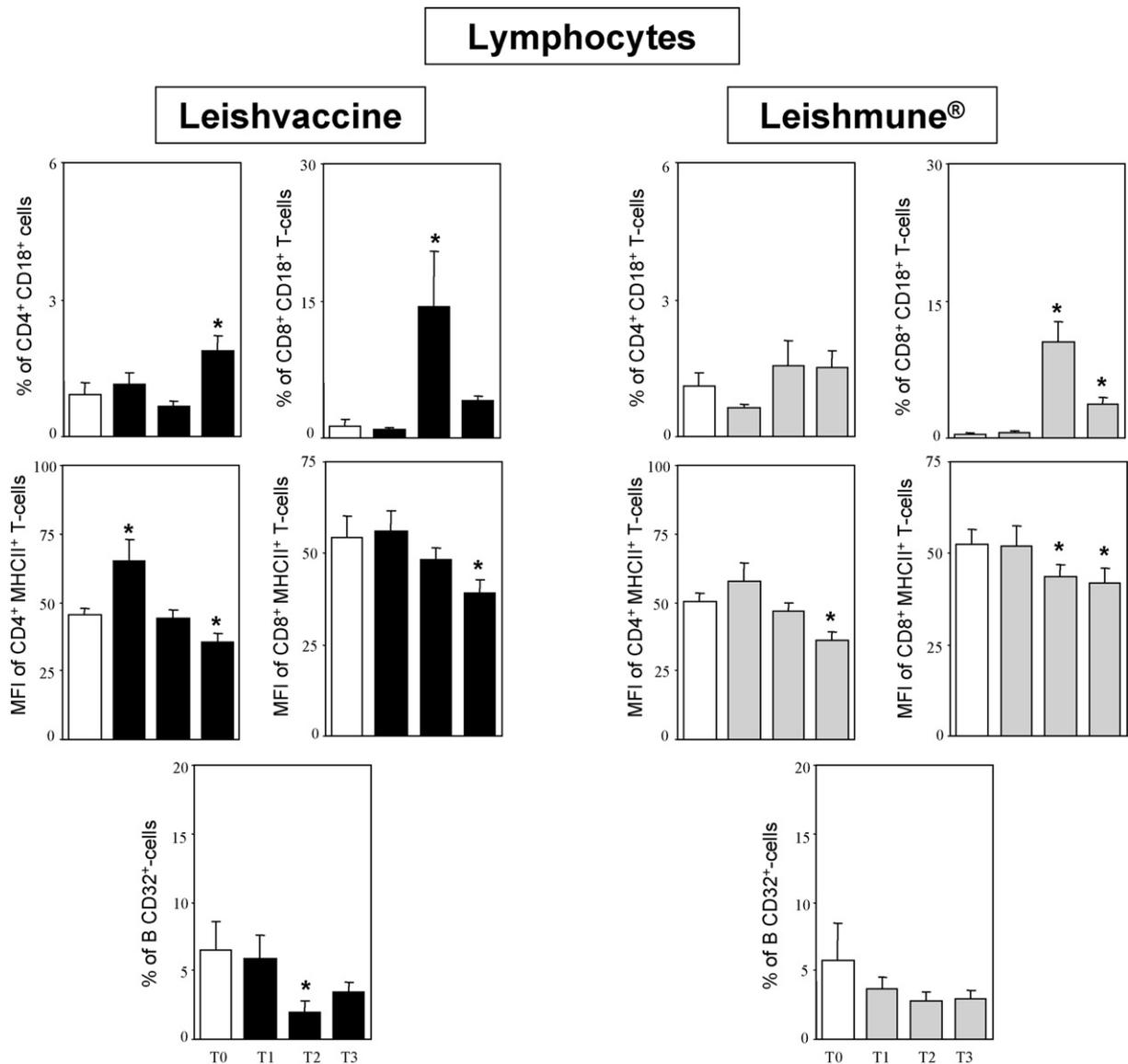


Figure 5 Cell surface phenotypic status of peripheral blood LYMPHOCYTES in German Shepherd dogs, following Leishvaccine (■) and Leishmune® (▒) vaccination regimens. Lymphocytes were selected based on their characteristic FSC versus SSC gain, following additional phenotypic analysis on dual color FL1/FITC versus FL2/R-PE dot plots. The results are expressed as percentage of CD4⁺CD18⁺, CD8⁺CD18⁺ and CD32⁺ B-cells ± S.E. as well as mean fluorescence intensity (MFI) of MHCII expression in CD4⁺ and CD8⁺ cells ± S.E. within gated lymphocytes. Significant differences at $p < 0.05$ are indicated by asterisk for the comparison between T1, T2 and T3 with those unvaccinated paired control dogs (T0 = □).

transmigrated into adjacent tissues following Leishvaccine and Leishmune® vaccination.

The multiplicity of interactions induced by the whole crude *L. amazonensis* antigen would be the basis for the broader phenotypic change observed following Leishvaccine immunization. On the other hand, the purified nature of FML antigen in Leishmune® corroborated with a more selective activation of innate immunity cells. Therefore, we hypothesized that the distinct molecular nature of Leishvaccine and Leishmune® plays a pivotal role triggering innate immunity cells.

It has been proposed that the antigen recognition by the innate immunity cells may involve multiple mechanisms [32]. The reliable detection of antigenic determinants would

be more or less complex depending of the antigen molecular heterogeneity [32]. Several recognition strategies have already been proposed. The pattern-recognition strategy is based on the detection of a limited set of conserved molecular patterns (PAMPs) that are unique to the microbial world and invariant among entire classes of pathogens. The targets of the PAMPs are named toll-like receptors (TLRs). There are at least 10 mammalian TLRs expressed by different cell populations. To date, the pattern of TLR expression by human leukocytes demonstrated that neutrophils express TLR1 through TLR10, except TLR3, monocytes express TLR1 through TLR8, except TLR3 whereas eosinophils express TLR1, TLR4, TLR7, TLR9, TLR10 [32]. Recently, Bazzocchi et al. [33] have described the partial sequence of the

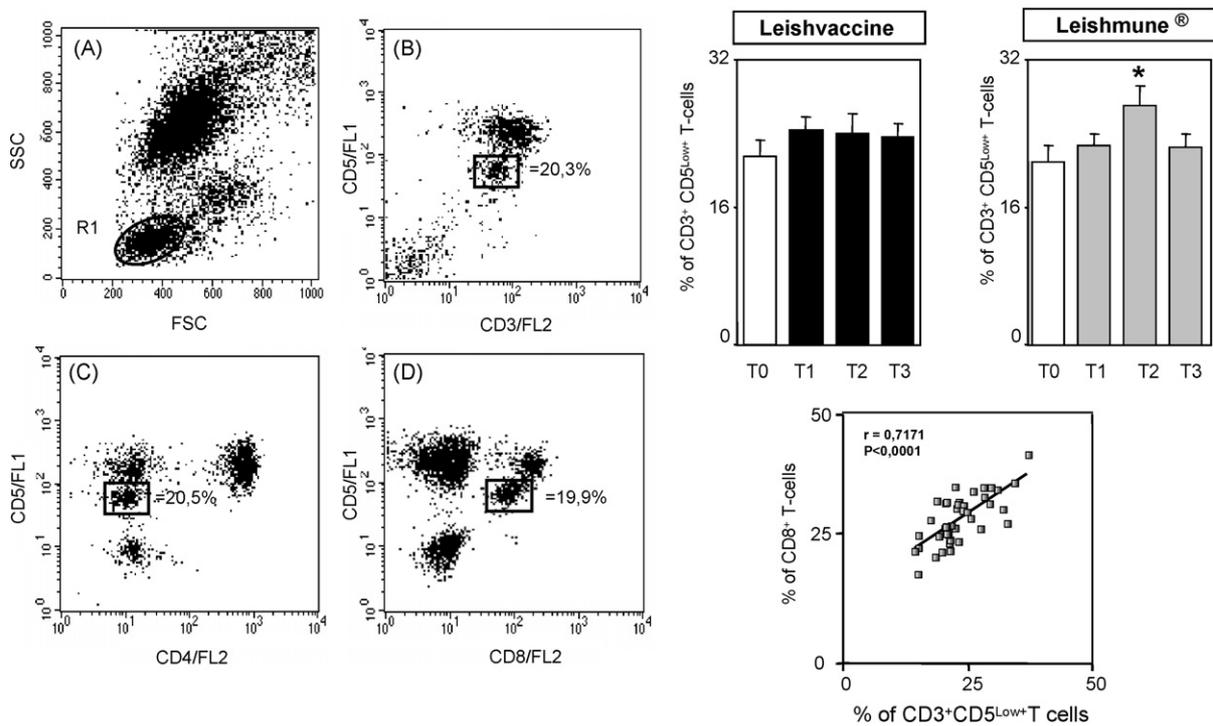


Figure 6 Immunophenotypic features of a novel peripheral blood LYMPHOCYTE subpopulation, referred as $CD3^+CD5^{Low}CD8^+$ in German Shepherd dogs. Representative density plots distributions allowed the selection of total lymphocytes selected based on their characteristic FSC versus SSC distribution (ellipse in A) and further selection of $CD3^+CD5^{Low}$ cells on dual color FL1/FITC versus FL2/R-PE dot plots (rectangle in B). Parallel immunophenotypic staining confirmed that all $CD5^{Low}$ co-express the CD8 do not express the CD4 cell marker as observed on dual color FL1/FITC versus FL2/R-PE dot plots (rectangle in C and D, respectively). Analysis of $CD3^+CD5^{Low}CD8^+$ following Leishvaccine (■) and Leishmune® (□) vaccination regimens highlighted the selective increase of this novel lymphocytes in the peripheral blood of subpopulation Leishmune® vaccines. The results are expressed as percentage of $CD3^+CD5^{Low}$ cells \pm S.E. within gated lymphocytes. Significant differences at $p < 0.05$ are indicated by asterisk for the comparison between T2 with those unvaccinated paired control dogs (T0 = □). Further statistical analysis revealed the tide correlation between the frequency of $CD3^+CD5^{Low}$ cells and the circulating $CD8^+$ cells in Leishmune® vaccines throughout the vaccination regimen. The results are expressed as scattering of individual values and the Spearman correlation indices (r and p -values) are shown on graphs. Connecting lines illustrates the positive correlation index.

gene coding for canine TLR-2 and show that TLR2 mRNA is constitutively expressed by canine peripheral blood polymorphonuclear cells. Furthermore, using a cross-reactive anti-human TLR2 antibody, these authors have demonstrated that TLR2 protein is expressed by granulocytes, monocytes, but slightly on lymphocytes, with no specific reference to neutrophils and eosinophils. Interestingly, Becker et al. [34] have demonstrated that *Leishmania* LPG is able to bind to TLR2 and activate the innate immune system and thereby increasing the effective destruction of the parasite, suggesting that TLR2 pathway may represent a relevant loop to trigger anti-*Leishmania* protective immunity.

In general, TLR ligands have been shown to be excellent vaccine adjuvants in animal studies, promoting development of robust antigen-specific humoral and cellular responses, including cytotoxic T-cell responses [35]. Thus, their clinical potential as vaccine adjuvants has been considered by a number of laboratories. The use of TLR4 and TLR9 agonists, ligands for TLR3, TLR5 and TLR7/8 are currently being investigated in phase 1 and 2 for human vaccine trials against several pathogens [35]. In this context, it has been demonstrated that BCG is able to trigger TLR2 and TLR4 stimulatory action [36]. It is possible that besides the complex antigen

formulation the ability of BCG to trigger TLR2 and TLR4 may count to the broader spectrum of innate immunity activation observed following Leishvaccine immunization. However, it is important to mention that although the engagement of TLR signaling pathways is a promising mechanism for boosting vaccine responses, questions of efficacy, feasibility and safety remain the subject of active investigation.

Regardless of the scarcity of information concerning the TLRs expression by canine peripheral blood cells as well as the interaction between *Leishmania* antigens with canine TLRs, herein, we speculate that the FML, similarly to LPG is most likely to interact with innate immunity cells throughout TLR2 and therefore selectively stimulate neutrophils and monocytes, but not eosinophils. On the other hand, the complex antigenic nature in Leishvaccine would lead to an interaction with a wide range of TLRs and thus trigger the activation of several leukocyte subsets within the innate immune compartment, with involvement of neutrophils, monocytes and also eosinophils. It is important to mention that despite the distinct profile triggered by Leishvaccine and Leishmune® vaccines, both immunobiologicals are capable to stimulate relevant innate immunity cells involved in leishmanicidal activities, such as neutrophils and monocytes,

that enable them to elicit a protective immune response against *Leishmania* infection.

Evidences are being accumulated, suggesting that the innate immune response plays a pivotal role during host resistance against intracellular parasitic infections. This response would act both in controlling pathogen growth during the early stages of infection as well as in driving the cytokine microenvironment in which parasite-specific T-cells are primed [37,38].

The activation of phagocytes, the main targets of *Leishmania*, represents one of the first events linked to the innate immune response to intracellular infection [39,40]. Upon their activation, neutrophils and monocytes are recruited to inflammatory foci where secrete cytokines, mainly IL-12. It has a very important role in stimulating cytotoxicity and IFN- γ production by NK cells as well as on the development of specific type 1 T-cell-mediated immunity [39,40]. This microenvironment up regulates the anti-*Leishmania* activity, increasing the phagocytosis and parasite killing through mechanisms such as those related to oxidative burst [41,42]. Rousseau et al. [43] showed that during the early phase of the infection, neutrophils play an important role in controlling *L. infantum* burden in the spleen. Furthermore, in *L. donovani*-infected C57BL/6 mice, the depletion of the neutrophils induced an important enhancement of parasite growth in both liver and spleen [44]. Indeed, these data associated with our results reinforce the hypothesis that the involvement of the innate immune, elicited by both vaccines, could be important in the development of the protective immune response during *Leishmania* challenge.

Although there is a consensus that the phagocytic cells are crucial to *Leishmania* elimination and infection resolution, data from experimental models have suggested that the effective and consistent activation of phagocytes require an effective adaptive cellular immune response [45]. Furthermore, it is relevant point out that the adaptive cell memory following vaccination is important to successful vaccination. Aiming to evaluate the involvement of the adaptive immunity compartment, we have further focused on the changes in the phenotypic profile of circulation T and B-cells following Leishvaccine and Leishmune® vaccination. Our major findings showed that while the Leishvaccine triggered a mixed immunological profile, inducing phenotypic changes in both T and B-lymphocytes, the Leishmune® prompted a selective pattern on cellular adaptive immune response, selectively linked with T-cell activation.

In fact, our data demonstrated that Leishvaccine elicited a consecutive activation of T-cells and B-lymphocytes, with early activation of CD4⁺ T-cells (CD4⁺MHCII⁺) and a later activation of B-cells (CD32 in B-cells) and CD8⁺ T-cells (CD8⁺CD18⁺). It has been proposed by Reis et al. [46] that sustained T-cell compartment in the peripheral blood associated with higher activation status of circulating lymphocytes, as demonstrated by higher expression of MHCII is an important event underlying the protective immune response during ongoing asymptomatic CVL. In fact, Cobbold & Metcalfe [21] developed a pioneer investigation regarding the MHCII molecule expression by canine cells. These authors observed that, in contrast with other species, all canine circulating lymphocytes constitutively express the MHCII molecule. It has been proposed that increased expression of MHCII may reflect an antigenic priming-related

immunological event [21]. The increased MHCII expression on CD4⁺ T-cells following Leishvaccine immunization might reflect their increased ability of antigen presentation leading to effective activity of the immune system supporting the hypothesis that these cells are prone to be primed by *Leishmania* antigens and may contribute to mount a vigorous protective immune response. These findings are in agreement with those reports that the activation of CD4⁺ T-lymphocytes is essential for the establishment of the immunological network needed to trigger an effective cellular and humoral immune response to protein antigen, referred as T-dependent antigens [47]. The outstanding increase in the CD18 expression by CD8⁺ T-cells at T2 following vaccination is the major phenotypic features that indicate the ability of both immunobiological to trigger CD8⁺ T-cells, and therefore the major finding that support their use as high-quality immunogenic device against CVL. We believe that the down-regulation of MHCII observed in CD4⁺ and CD8⁺ T-cells at T3 after Leishvaccine and Leishmune® vaccination may represent a compensatory immunoregulation mechanism that is triggered to overcome the immune response following antigenic booster.

Despite no references concerning the role of canine CD32 molecule, data provide for experimental model as well as investigations in humans have already demonstrated that the downregulation of CD32 expression by B-cells may be associated with an enhanced immunoglobulin synthesis [48]. These data are in agreement with our results that showed decrease in the percentage of CD19⁺CD32⁺ cells, beside higher levels of IgG (data not shown). Further, it has been demonstrated that the level of expression of CD32 on human B cells is not uniform, but depended on activation status [49]. Moreover, TLRs signals can be delivered to B cells directly [50] and as previously discussed would represent a potential mechanism elicited by the whole *Leishmania* antigenic nature in Leishvaccine. On the other hand, the Leishmune® led to a preferential involvement of CD8⁺ T-cells, with no phenotypic changes on circulating B-lymphocytes, suggested a possible activation of a selective pathway of cooperation between antigen-presenting cells (APCs) and cytotoxic T-cells.

In a innovative flow cytometric platform analysis for canine cell surface markers, we have performed a dual label protocol and described for the first time, that the dogs presented in their peripheral blood two distinct T-cell subsets based on the differential density of CD5 expression by CD3⁺ cells, referred as CD3⁺CD5^{High} and CD3⁺CD5^{Low}. In a pioneer investigation in the field of canine immunological studies, we have further characterized that all CD3⁺CD5^{Low} T-cells co-express the CD8 cell surface marker but not the CD4 molecule. Indeed, we have characterized that the CD8⁺ T-cells in the canine peripheral blood can be segregated into two major cell subsets, CD8⁺CD5^{High} and CD8⁺CD5^{Low}. Interestingly, our data demonstrated that Leishmune® led to a parallel increase in the percentage of circulating CD8⁺ T-cells and CD3⁺CD5^{Low} T-cells. Additional correlation analysis further confirms these findings. It is possible that FML is prone to be presented throughout cross-priming of APCs' MHC I molecules that in consonance with co-stimulatory signals on APCs would efficiently activate CD8⁺ T-cells. Antigen presentation by professional APCs to cytotoxic CD8⁺ T cells can occur via two processing routes

named “direct” and “cross-priming” pathways [51]. Cross-presentation of exogenous antigens in the context of MHC I molecules has recently attracted a lot of research interest because it may prove crucial for vaccine development. This alternative pathway has been implicated in priming CD8⁺ T-cell responses to pathogens *in vivo*. In cross-presentation, the internalized antigens can be processed through diverse intracellular routes [51]. Further studies are currently under investigation in our laboratory to further characterize the functional aspects of these CD3⁺CD5^{Low}CD8⁺ T-cells, major focusing on their cytokine profile. Recently, studies have focused on the role of CD8⁺ T-cells and their relevance in the protective mechanisms during ongoing CVL as well as in the vaccine design against CVL [52,53]. It is important to highlight that in CVL CD8⁺ T-cells have been associated with protection and asymptomatic disease [46,52]. Indeed, it has been shown that CD8⁺ T-cells are crucial in controlling the primary *Leishmania* infection by shifting the early Type 2 toward a Type 1 immune response [54,55,56]. Moreover, Pinelli et al. [52] postulated that besides cytokine production, it is also possible that CD8⁺ T-cells participate in the control of *Leishmania* through cytotoxic mechanisms that eliminate infected macrophages. Therefore, both mechanisms, including the production of IFN- γ and the destruction of the parasitized host cells by *Leishmania*-specific T-cells play a pivotal role in resistance to visceral leishmaniasis. The ability of CD8⁺ T-cells to participate in protective mechanism have been also reported in experimental models for cutaneous *L. major* leishmaniasis, with the demonstration that CD8⁺ T-cells participate in the control of *Leishmania* throughout IFN- γ production as well as by up-regulating of IFN- γ production by CD4⁺ T-cells [57,58]. These authors suggested a cross-talk between these two T-cell subsets [57,58].

Besides the molecular nature of vaccinal antigen, another interesting approach usually required during the development of first and second-generation vaccines is the search for more potent vaccine adjuvants [59,60]. Vaccines require optimal adjuvants including immunopotentiator and delivery systems to offer long-term protection from infectious diseases in animals and man. Initially, it was believed that adjuvants are responsible for promoting strong and sustainable antibody responses. Now it has been shown that adjuvants influence the isotype and avidity of antibody and also affect the properties of cell-mediated immunity. Saponin and BCG based adjuvants have the ability to stimulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity [59,61,62]. These can be used to improve the immune response to vaccine antigens for several different purposes, including: (1) increase of the immunogenicity of purified antigens; (2) enhancement of the speed and duration of the immune response; (3) modulation of antibody avidity and specificity; (4) stimulation of cell mediated immunity and others [60].

Herein, we have found that Leishvaccine led to significant changes in the activation status of innate and adaptive immunity cells, characterizing a broader spectrum of immunogenicity. We hypothesized that the BCG-based adjuvant associated with the complex whole crude *L. amazonensis* antigen formulation used in Leishvaccine could potentiate the establishment of a mixed type of immune response. On the other hand, the saponin-based adju-

vant used in Leishmune[®] would prompt a selective cellular immune response as previously reported [63]. These data are in agreement with our results that showed a preferentially involvement of phagocytes and CD8⁺ T-cells in Leishmune[®] vaccines. It is likely to speculate that saponin would provide optimal condition for FML uptake by APCs’ making it prone to be presented throughout MHC I molecules as proposed in the cross-priming theory [51].

Altogether, our data demonstrated that Leishvaccine and Leishmune[®] induced distinct phenotypic changes in the peripheral blood innate and adaptive immunity compartments, with the latter inducing a more selective involvement of the cellular immune response. We believe that this dissimilar activation of immunological events would represent phenomenon directly related with the molecular nature of these vaccines besides the distinct adjuvants employed. However, it is important to emphasize that both immunobiologicals are able to activate phagocytes and CD8⁺ T-cells and therefore could be considered priority vaccines with a high-quality immunogenic potential against CVL.

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