Alteration in mononuclear cell subpopulations in dogs immunized with gentamicin-attenuated *Leishmania infantum*

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SUMMARY

The impact of immunization with gentamicin-attenuated *Leishmania infantum* (H-line) on the immunophenotypic profile of popliteal lymph node (PLN) and peripheral blood mononuclear cells (PBMCs) of dogs was assessed by flow cytometry and immunohistochemistry. Compared with the dogs infected with *L. infantum* wild-type (Group WT), there was a significantly higher percentage of $CD4^+$, $CD44^+$ T cells and $CD14^+$, MHC-II^+ cells and a lower percentage of $CD4^+$ $CD25^+$ regulatory T cells in PLN of the immunized dogs with *L. infantum* H-line (Group H). The percentage of $CD4^+$ and $CD8^+$ T cells in PBMCs of immunized dogs was higher than that in dogs of Group WT. The CD4:CD8 ratio in PLN of dogs of Group H was significantly higher than that in dogs of Group WT. A significantly higher percentage of $CD21^+$ B cells and a lower percentage of $CD79b^+$ cells were found in PLN of the immunized dogs compared with dogs of Group WT. Immunohistochemical investigation showed no parasites in the PLN of immunized dogs whereas there were parasites in the PLN of 60% of dogs infected with *L. infantum* WT. In this study, the immunophenotypic profile of mononuclear cells of the immunized dogs correlates with cellular immunity.

INTRODUCTION

Leishmania infantum (*L. infantum*) is an obligatory intracellular protozoan and causative of visceral leishmaniasis in humans and dogs. Control of parasites in dogs leads to a reduced prevalence of disease in humans (Palatnik-de-Sousa *et al.* 2001). No effective and safe vaccine against canine visceral leishmaniasis (CVL) is currently available and control of the disease by chemotherapy is compromised because existing drugs are toxic and drug resistance is prevalent. We previously described the generation of an attenuated line of *L. infantum* (*L. infantum* H-line) which was selected by culturing promastigotes *in vitro* under pressure of gentamicin (Daneshvar *et al.* 2003). Gentamicin, an aminoglycoside antibiotic, is produced by micromonospora (Weinstein *et al.* 1963*a*, *b*). It is widely used for the treatment of bacterial infections and shows a broad antibacterial spectrum of action (Edson and Terrell, 1999). The precise mechanism of bactericidal activity of aminoglycosides is not fully understood, but hypotheses include disruption of ribosomal activity by breaking up polysomes, misreading of mRNA during protein synthesis and production of abnormal or non-functional proteins (Chambers, 2001). A quantitative proteomic analysis revealed the expression of a group of proteins which mediate the response to reactive oxygen intermediates in *L. infantum* H-line which had changed compared with wild-type parasite (Daneshvar *et al.* 2012). We showed that the attenuated *L. infantum* is more susceptible to oxidative challenge, a change which may explain the loss of virulence (Daneshvar *et al.* 2012). Thus *L. infantum* H-line invaded but was unable to survive within bone marrow-derived macrophages of BALB/c mice *in vitro*, whereas *L. infantum* wild-type (WT) survived and multiplied within infected macrophages (Daneshvar *et al.* 2003). Moreover, the attenuated line of *L. infantum* failed to spread to, and within, the visceral organs of BALB/c mice (Daneshvar *et al.* 2009). Previously we reported that *L. infantum* H-line induced a cellular immunity which protected dogs against wild type parasites (Daneshvar *et al.* 2010). In addition, no clinical signs and histopathological abnormalities were found in the dogs immunized with the *L. infantum* H-line (Daneshvar *et al.* 2009). Several studies have shown that the

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absence of an adequate T cell response leads to insufficient control of the parasite and consequent appearance of clinical signs of disease with high parasite burdens in visceral organs of symptomatic dogs. Conversely, asymptomatic dogs are characterized by a self-limiting disease in the presence of protective cellular immunity (Guarga *et al*. 2000; Reis *et al*. 2009, 2010). Moreover, low levels of $CD4^+$ and $CD8^+$ T cells, CR2 ($CD21^+$) B cells and $CD14^+$ monocytes in the peripheral blood mononuclear cells (PBMCs), is associated with higher parasite density in the bone marrow as seen in symptomatic dogs (Reis *et al*. 2006*a*). CD4 + T cells from symptomatic dogs respond to antigen stimulation during the earlier stages of infection but lose this ability as the dogs progress to clinically disseminated disease (Boggiatto *et al*. 2010). In contrast, a high level of $CD8^+$ T cells reported in the PBMCs of asymptomatic dogs was associated with control of parasitism (Reis *et al*. 2006*a*).

In the present study, the impact of *L. infantum* H-line on the immunophenotypic profile of popliteal lymph node (PLN) and PBMCs of dogs was assessed. The proportions of $CD4^+$, $CD8^+$, $CD4^+$ $CD25^+$, $CD44^+$ T cells and $CD21^+$, $CD79b^+$ B cells and MHC-II^+ (HLA-DR), $CD14^+$ cells of dogs immunized with the attenuated line were analysed. Moreover, the influence of tissue burden parasitism in the PLN of dogs was investigated.

MATERIALS AND METHODS

Parasite

Promastigotes of *L. infantum* JPCM5 (MCAN/ES/98/LIM-877), were cultivated in complete haemoflagellate minimal essential medium (HOMEM) (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (HI-FCS) (Labtech International), as described elsewhere (Mallinson and Coombs, 1989). *L. infantum* H-line was generated in the same medium supplemented with 10% (v/v) HI-FCS and gentamicin (Sigma-Aldrich) at 20 μg/ml (Daneshvar *et al*. 2003). Briefly, amastigotes of *L. infantum* derived from a spleen of an infected hamster were transferred into complete HOMEM medium and incubated at 25 °C, where they differentiated into promastigotes over 72 h. These were transferred into complete HOMEM with or without gentamicin and were subcultured routinely at 25 °C. Cultures grown in the absence of gentamicin were maintained in parallel to those with the antibiotic, to confirm that attenuation was not simply the result of long-term cultivation. Stationary-phase promastigotes of the attenuated line or *L. infantum* WT were harvested after a total of 48 sub-passages by centrifugation and washed twice in phosphate-buffered saline (PBS, pH 7·4); the pellet was suspended at 10^9 cells/ml in PBS.

Dogs

Thirty healthy semi-German shepherd dogs, between 6 months and 1 year old were used in this study. All dogs were checked by a veterinary surgeon for clinical sign of leishmaniasis and were shown to be negative for the presence of anti-leishmanial antibody by immunoflourescence assay (IFA) test, and also negative for *Leishmania* DNA by PCR of PBMCs. The protocols used for animal handling were approved by the animal care committee of Kerman University of Medical Sciences. The welfare of the dogs was closely monitored by a veterinary surgeon. The dogs had previously been vaccinated against canine parvovirus and rabies and were also treated with the anthelmintic drugs praziquantel and pyrantel. The animals were accommodated in indoor kennels with windows covered with deltamethrin-sprayed, double anti-mosquito nets. The dogs were allocated into 3 Groups in a double-blind randomized fashion. Two Groups (10 dogs per Group) were injected intradermally (i.d.) with 100 μl of the suspension of stationary-phase promastigotes of *L. infantum* H-line (Group H) or *L. infantum* WT (Group WT). Control Group (Group C) (10 dogs) was injected with PBS. The experiment was terminated after 12 months infection to ensure that the control dogs did not suffer unnecessarily. The animals were anaesthetized by intravenous injection of thiopental sodium 33% (5 ml/kg) (Tafuri *et al*. 2001) at the end of the study. The PLN was collected in aseptic conditions for flow cytometry and immunohistochemical examinations.

Leishmania *soluble antigen (LSA)*

LSA was prepared from stationary-phase promastigotes of *L. infantum* WT. Cells were harvested by centrifugation and washed 3 times with ice-cold (PBS). The pellet was re-suspended in PBS (10^7 cells/ml) and sonicated at 4 °C for 1 min at 12-mm peak amplitude. The suspension was finally centrifuged at 10 000 ***g*** and 4 °C for 15 min. The protein concentration in the supernatant was determined by the method described by Bradford (1976) and frozen as 1 mg/ml aliquots at 20 °C until use.

Cell preparation

Heparinized blood samples taken from the peripheral veins of dogs were diluted with an equal volume of PBS and layered on Ficoll-Hypaque (Sigma). After centrifugation at 350 ***g*** for 40 min at room temperature, the layer containing PBMC fraction was collected. Fragments of PLNs were excised and then teased to obtain single-cell suspensions. PBMCs or PLN cells were washed twice with PBS and adjusted to 5×10^6 cells/ml in complete RPMI-1640 supplemented with 10% FCS, penicillin G (100 U/ml),

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Table 1. Monoclonal and polyclonal antibodies used in flow cytometry

Antigen	Antibody specific	Dilution	Isotype	Source
A: Monoclonal antibodies				
CaCD4	Canine CD4	1:100	Rat IgG 2a	Abcam
CaCD8	Canine CD8	1:100	Rat IgG 1	Abcam
CaCD79b	Canine CD79b FITC-conjugated	1:10	Rat IgG 1	Abcam
CaCD44	Canine CD44 PE-conjugated	1:10	Mouse IgG 1	Abcam
CaCD25	Canine CD25 PE-conjugated	1:10	Mouse IgG 1	eBioscience
CaCD21	Canine CD21 PE-conjugated	1:60	Mouse IgG 1	VMRD
CaMHC-II	Canine MHC-II PE-conjugated	1:60	Mouse IgG 1	VMRD
B: Polyclonal antibodies				
Ig	Anti Rat IgG (H + L) FITC-conjugated	1:200	Rabbit	Abcam
Ig	Anti Rat IgG F(ab)2 PE-conjugated	1:50	Goat	Abcam
Ig	Anti Mouse IgG F(ab)2 PE-conjugated	1:50	Goat	Abcam

streptomycin sulphate (100 μg/ml) (Life Technologies, USA) with LSA (50 μg/ml). The cells were incubated at 37 °C in 5% CO_2 95% air for 24 h and then harvested.

Flow cytometric analysis

Cell surface and cytoplasmic antigens were identified by flow cytometric analysis based on the method described by Murphy *et al.* (1996). The cells were washed twicw with PBSW (PBS with 0·5% bovine serum albumin (BSA) and 0·1% sodium azide), counted, and then re-suspended at 1×10^6 per microcentrifuge tube. The cells were stained with the monoclonal antibodies (mAbs) listed in Table 1 for 40 min at 4 °C. After washing with 2 ml of PBSW, the cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythin (PE) conjugated to secondary antibodies (Table 1). After 2 washes with PBSW, the cells were fixed in 2·8% formaldehyde-PBS. The cells were analysed by flow cytometery with FluMax software. Negative controls included isotype mAbs substituted for mAbs listed in Table 1 and secondary antibodies used alone without primary antibodies.

Immunohistochemical finding

An immunohistochemical investigation was performed to evaluate the number of $CD14^+$ cells and parasite burden in the PLN of dogs. Briefly, 5 μm sections were cut, deparaffinized slides were hydrated and incubated in 4% hydrogen peroxide (H_2O_2) (30 v/v) in 0·01 M PBS, pH 7·2. The tissue sections were incubated with goat serum (diluted 1:100) for 30 min and then incubated overnight at 4 °C with the primary monoclonal antibody anti-canine CD14 (VMRD, USA) (15 μg/ml in PBS). After washing, the secondary antibody (diluted with PBS at 1:100) was applied, and incubated for 60 min at room temperature. The slides were visualized by incubation with 3,3′-diaminobenzidine-tetrahydrochloride (DAB) (Sigma, St Louis, MO, USA) and H_2O_2 in 0·1 M imidazole, pH 7·1 for 5 min. Finally, the slides were dehydrated, cleared, counterstained with haematoxylin and eosin (HE), and mounted under coverslips. $CD14^+$ cells with a positive brown immunocytochemical reaction were counted in 15 high-power fields (ocular grit = 0·0625 mm^2) on cross-sections by light microscopy (Ensinger *et al.* 2010). The parasite burden in the PLN of dogs was evaluated as described by Tafuri *et al.* (2004) as follows. The slides were incubated with goat serum (diluted 1:100). Serum from a dog infected with *L. infantum* WT (IFAT titre 1:320), diluted 1 in 100 in PBS, was applied as the primary antibody and incubated for 22 h at 4 °C. The slides were then incubated with biotinylated anti-mouse and anti-rabbit antibody (Dako, LSAB2 kit, USA) and subsequently with the streptavidin peroxidase complex (Dako, LSAB2 Kit, USA) for 20 min at room temperature. The slides were visualized as described above and the number of macrophages containing amastigotes with a visible nucleus and a brown immunocytochemical reaction were counted as positive. A semi-quantitative study was carried out by optical microscopy (110×). Parasite load was measured as: (+) discrete, (++) moderate and (+++) intense (Xavier *et al.* 2006).

Statistics analysis

Statistical analysis was performed using SPSS software. Parametric ANOVA and Student's *t*-test test were used to assess significant differences between flow cytometry and IHC data, respectively. A *P*-value of 0·05 or less was considered as a significant difference.

RESULTS

Characterization of immune cells

Flow cytometric analysis was carried out to investigate the proportions of $CD4^+$, $CD8^+$, $CD4^+$ $CD25^+$, $CD44^+$ T cells and $CD21^+$, $CD79b^+$ B cells and MHC-II expression in the samples from the dogs.

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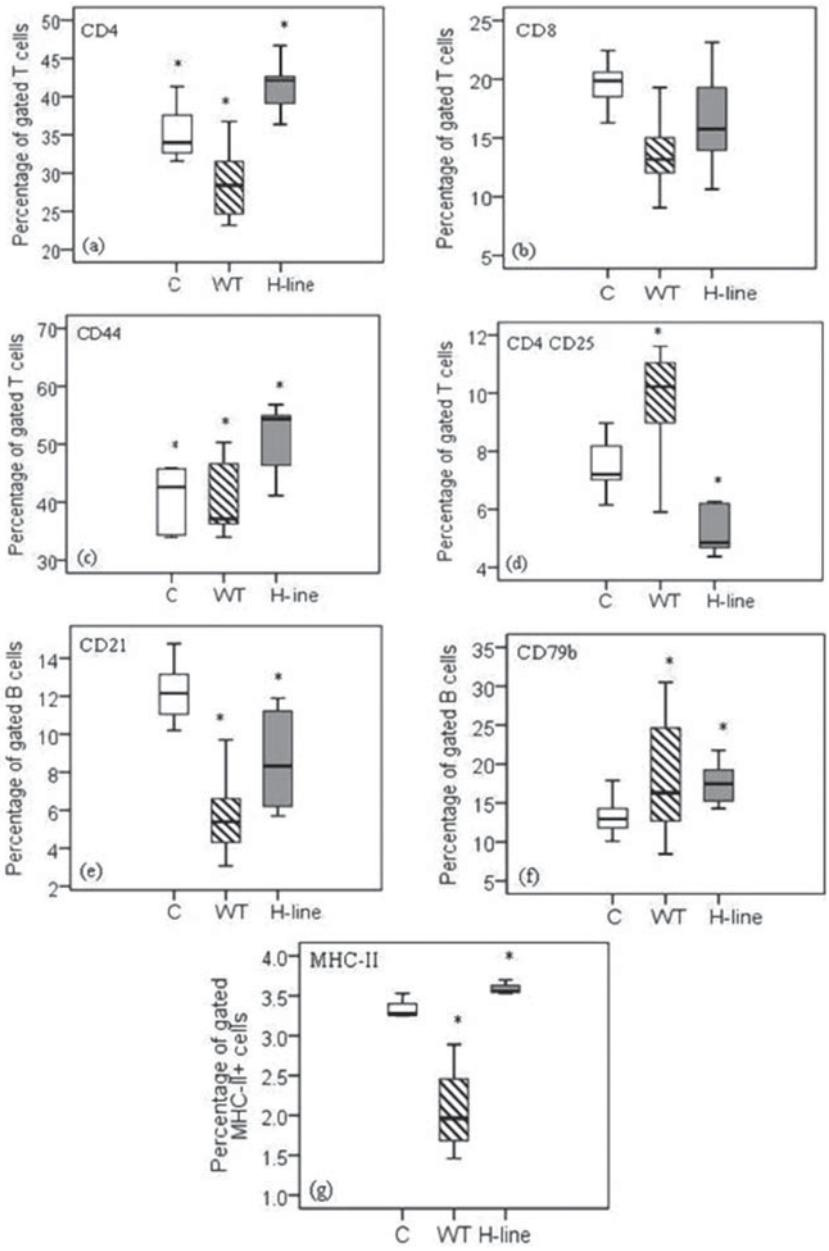

Fig. 1. Percentage of immunophenotype cells in the PLN of dogs infected with *Leishmania infantum* WT or dogs immunized with *L. infantum* H-line or healthy control dogs. The cells were re-stimulated with LSA *in vitro*. Asterisk represents significant difference.

The percentages of $CD4^+$ T cells in the SLA-stimulated PLN cells and PBMCs in dogs of Group H were significantly higher than those in dogs from other groups (Figs 1a and 2a respectively; $P=0$). There was no significant difference between the percentages of $CD8^+$ T cells in PLN of dogs of Group H with that of dogs of Group WT or Group C dogs (Fig. 1b). But a significant difference was found between the higher percentage of $CD8^+$ T cells in PBMCs of immunized dogs compared with that in dogs of Group WT and healthy dogs (Fig. 2b; $P=0$ and $P<0{\cdot}003$ respectively). The CD4/CD8 ratio in the PLN of Group H dogs was significantly higher than that in the dogs of Group WT ($P<0{\cdot}005$). The percentage of $CD44^+$ cells in PLN of dogs immunized with the attenuated parasite was significantly higher than that in dogs of Group WT and Group C (Fig. 1c; $P<0{\cdot}01$). The percentage of $CD4^+$ $CD25^+$ T cells in PLN of Group H dogs decreased notably compared with that in dogs of Group WT (Fig. 1d; $P=0$). The percentages of $CD21^+$ B cells in PLN and PBMCs were significantly higher in the immunized dogs after 12 months post-immunization and re-stimulated with LSA *in vitro* compared to the dogs infected with WT parasites (Figs 1e and 2c respectively; $P=0$). In contrast, the percentage of $CD79b^+$ B cells in PBMCs of dogs of Group H was significantly lower than that in dogs of Group WT and healthy dogs (result not shown; $P=0$ and $P<0{\cdot}005$ respectively). There was no significant

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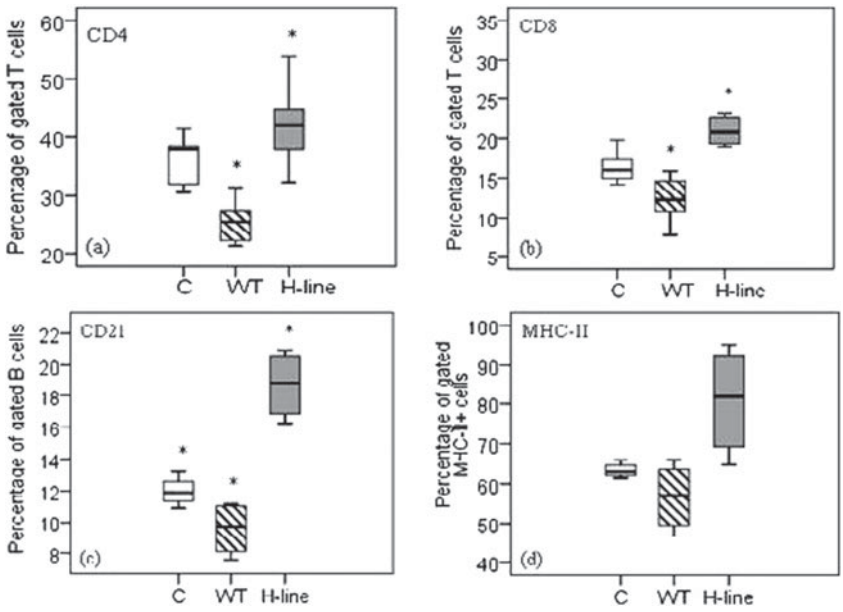

Fig. 2. Percentage of immunophenotype of PBMCs of dogs infected with *Leishmania infantum* WT or dogs immunized with *L. infantum* H-line or healthy control group. The cells were re-stimulated with LSA *in vitro*. Asterisk represents significant difference.

difference between the percentage of $CD79b^+$ B cells in the PLN of immunized dogs compared with that of control and dogs infected with WT parasites (Fig. 1f). *L. infantum* WT caused down-regulation of expression of MHC-II in antigen presenting cells (APCs) in PLN and PBMCs of dogs (Figs 1g and 2d; $P<0{\cdot}001$ and $P<0{\cdot}01$ respectively) compared with healthy control dogs. There was no significant change in MHC-II expression in PLN cells and PBMCs between immunized and control healthy dogs. MHC expression on the APCs in PLN of dogs immunized with the attenuated parasite was significantly higher than that in dogs infected with WT parasites (Fig. 1g; $P=0$).

Immunohistochemical finding

In the cell culturing process, the $CD14^+$ macrophages within the PLN spread and stuck on the surface of the tissue-culture plate. The number of cells isolated from the plates was low for flow cytometric analysis. Thus, the immunohistochemical method was performed to measure the subpopulation of $CD14^+$ cells and parasite burden in the dogs immunized with *L. infantum* H-line compared with dogs of Group WT. The mean number of $CD14^+$ cells per field was $4{\cdot}3\pm3{\cdot}2$ in dogs of Group WT and $11{\cdot}2\pm4{\cdot}2$ in dogs of Group H, indicating that there was significantly lower in dogs of Group WT (Fig. 3; $P<0{\cdot}01$). Moreover, no amastigotes were found in the PLN of immunized dogs 12 months post-immunization (Fig. 4a). In contrast, amastigotes were found in the PLN tissue section of six dogs (60%) of Group WT (Fig. 4b). Parasites were found in 3 dogs as (+), 2 dogs as (++) and 1 dog as (+++).

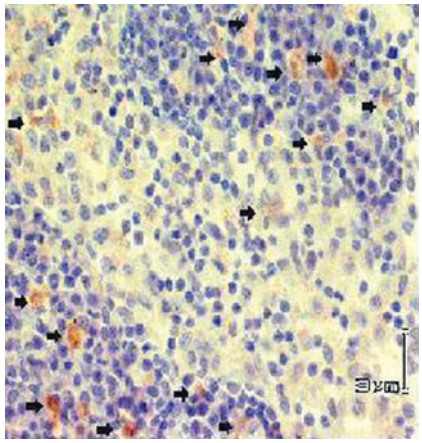

Fig. 3. Immunohistochemical labelling of CD14, a marker for macrophages in the PLN of a dog immunized with *Leishmania infantum* H-line. Avidin–biotin–peroxidase complex method.

DISCUSSION

In the present study we characterized the immunophenotypic profile of mononuclear cells, including lymphocyte proportions, in PBMCs and PLN of dogs immunized with the attenuated parasites compared with dogs infected with wild-type parasites or uninfected dogs. The parasite burden was also investigated in PLN tissue sections. We previously described the generation of *L. infantum* H-line, which was attenuated by culturing of promastigotes in the pressure of gentamicin. In prokaryotic cells, aminoglycosides bind directly to the ribosomal A site and cause misreading of mRNA during protein synthesis that leads to production of abnormal or non-functional proteins (Woodcock *et al.* 1991;

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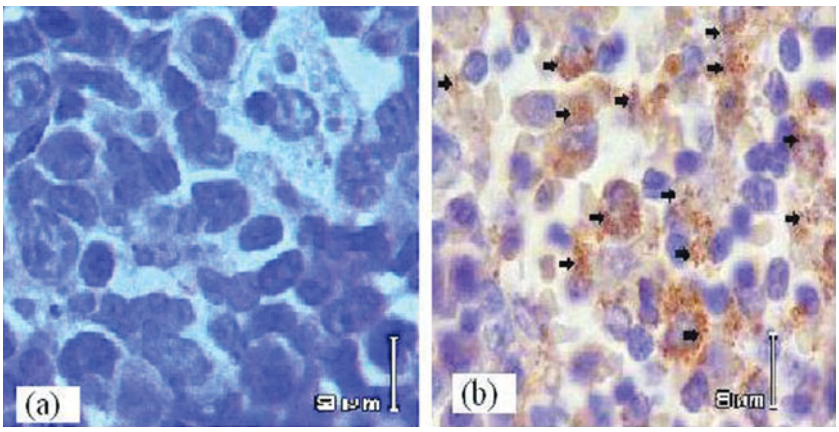

Fig. 4. Immunohistochemical labelling of numerous amastigotes within macrophages in the PLN of a dog immunized with *Leishmania infantum* H-line (a) or a dog infected with *L. infantum* WT (b). Avidin–biotin–peroxidase complex method.

Yoshizawa *et al*. 1998; Walter *et al*. 1999). Aminoglycosides exhibit anti-leishmanial activity, although the mechanism of action is not understood (Tekos *et al*. 2000). We reported that thiol-redoxin is significantly lower in *L. infantum* H-line as compared to *L. infantum* WT. Thiol-redox is critical for *Leishmania* parasites which are exposed to an oxidative burst when they encounter their mammalian macrophage host cell (Wilson *et al*. 1994; Goyal *et al*. 1996; Dumas *et al*. 1997). We also reported that *L. infantum* H-line invaded but was unable to survive within bone marrow-derived macrophages of BALB/c mice *in vitro*, whereas *L. infantum* WT survived and multiplied within these macrophages (Daneshvar *et al*. 2003). In addition, no parasites were found in the visceral organs of dogs immunized with *L. infantum* H-line (Daneshvar *et al*. 2009). In order to assess the immunophenotypic profile of PLN cells and PBMCs of the difference dogs *Leishnania* specific, the cells were exposed to LSA to induce a secondary immune response (Belkaid, 2003; Araújo *et al*. 2009). In the present study, we found that numbers of $CD4^+$ T cells in dogs immunized with the *L. infantum* H-line were significantly higher than those in dogs infected with wild-type parasites. It has been reported that $CD4^+$ T cells play a key role in controlling parasite growth and susceptibility to infection is associated with loss of *L. infantum*-specific $CD4^+$ T cell function (Pinelli *et al*. 1994; Strauss-Ayali *et al*. 2005; Boggiatto *et al*. 2010). Several studies have demonstrated that $CD8^+$ T cells play an important role in the development of a protective immunity and are required for the effective clearance of *Leishmania* (Stern *et al*. 1988; Pinelli, 1997). In agreement with these studies, our results showed that the population of $CD8^+$ T cells in the dogs immunized with the attenuated parasites was higher than that in the dogs of Group WT. We previously reported that *L. infantum* H-line inhibited IL-10 production and induced a cellular immunity that protected dogs against wild-type parasites (Daneshvar *et al*. 2010). In the present study we found that the percentage of $CD4^+$ $CD25^+$ T cells significantly decreased in the PLN of dogs immunized with *L. infantum* H-line compared with that in dogs infected with wild-type parasites. These results are consistent with other studies that showed that $CD4^+$ $CD25^+$ T cells are capable of producing IL-10 and play an important role during the early phase of visceral leishmaniasis in dogs (Belkaid, 2003; Rodrigues *et al*. 2009). It has been also reported that the reduction in $CD4^+$ $CD25^+$ T cells in lymph nodes induced a protection against leishmaniasis (Felix De Lima *et al*. 2010). Non-Treg $CD4^+$ T cells are often divided into 2 major subpopulations that can be designated naïve phenotype ($CD4^+$ $CD25^-$ $CD44^-$) and memory-phenotye ($CD4^+$ $CD25^-$ $CD44^+$) T cells (Surh and Sprent, 2008; Day, 2008). Memory $CD44^+$ T cells are important in the development of a protective immune response to *Leishmania* sp (Sanchez *et al*. 2004; Kedzierski *et al*. 2009). This observation is in agreement with our result that the percentages of $CD44^+$ T cells in the PLN and PBMCs of the dogs immunized with *L. infantum* H-line were significantly higher than those in dogs infected with wild-type parasites.

The present study shows the percentage of $CD21^+$ B lymphocytes in vaccinated dogs was significantly higher than that in dogs infected with *L. infantum* WT. In canine VL, previous studies revealed a negative correlation between the LN CD21+ B cell frequency and parasite burden in symptomatic dogs (Reis *et al*. 2006*a*,*b*). Cabral *et al*. (2008) reported that *L. infantum* tryparedoxin is a potent modulator of B cell activity and induces IL-10 in VL. A quantitative proteomic analysis revealed that the most prominent changes were in expression of thiol-redox active enzyme tryparedoxin peroxidase in *L. infantum* H-line compared with wild-type parasite (Daneshvar *et al*. 2012). Thus, the high percentage of $CD21^+$ B cells observed in the immunized dogs could be related to a reduction in trypanothione reductase activity in

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the attenuated line of *L. infantum*. In contrast, the percentage of $CD79b^+$ cells in the dogs immunized with the attenuated parasites was significantly lower than that in dogs of Group WT. Our results are in agreement with this observation that mature B cells with CD21, IgM and cytoplasmic CD79b receptors, differentiate toward plasma cells and lead to the loss CD21 and IgM receptors (Chu and Arber, 2001; Wilkerson *et al*. 2005).

One of the immune evasion strategies associated with the *Leishmania* parasite could be a down-regulation of MHC class II on the APCs (Reiner *et al*. 1988; Papadogiannakis *et al*. 2005). In the present study, we found expression of $MHC\text{-}II^+$ in APCs of dogs infected with wild-type parasites significantly down-regulated compared with healthy control dogs ($P<0{\cdot}001$). Whereas, $MHC\text{-}II^+$ expression in the dogs immunized with H-line was higher that in dogs of Group WT ($P=0$). Consistent with our results, Reis *et al*. (2006*a*) have demonstrated that asymptomatic dogs display enhanced expression of MHC-II on circulating lymphocytes besides lower overall tissue parasitism (Reis *et al*. 2006*b*).

In the cell culturing process, macrophages spread and stick on the solid membranes, and on these cells an immunohistochemical investigation was performed to measure the population of $CD14^+$ macrophages in the PLN of the dogs. Our results showed that $CD14^+$ cells in dogs infected with wild-type parasites were significantly less numerous than that in dogs immunized with *L. infantum* H-line. No parasites were found in the PLN of dogs immunized with *L. infantum* H-line whereas parasites were found in 60% dogs of Group WT and in one of them this was intense. Several studies showed that the immunohistochemical method is highly efficient at revealing the presence on intact parasites in tissues of dogs (Ferrer *et al*. 1988; Lima *et al*. 2004; Giunchetti *et al*. 2008*a*). Moreira *et al*. (2007) used IHC in the PLN of symptomatic, oligosymptomatic and asymptomatic dogs with a specificity of 100% and a sensitivity of 92·68%, 60% and 73·91%, respectively. Down-regulation of CD14 and MHC-II in the dogs infected with *L. infantum* WT was associated with parasitism whereas dogs with a higher expression of MHC-II and CD14 were the immunized dogs without a parasite tissue load. Our results are in agreement with reports of this association between a low parasite burden with an enhanced expression of MHC-II. In contrast, in a high parasite burden, there is a reduced expression of MHC-II (Papadogiannakis *et al*. 2005; Giunchetti *et al*. 2008*b*; Saridomichelakis *et al*. 2009). Our major findings re-emphasize the role of cellular immunity in the immunized dogs.

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