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Relationship between Canine Visceral Leishmaniosis and the *Leishmania (Leishmania) chagasi* Burden in Dermal Inflammatory Foci

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Summary

The skin is the first point of contact with organisms of the genus *Leishmania* from sand fly vectors, and apparently normal skin of sick dogs harbours amastigote forms of *Leishmania chagasi*. In relation to canine visceral leishmaniosis (CVL), the ear skin was examined in 10 uninfected dogs (UDs) and in 31 dogs dogs naturally infected with *L. chagasi*. The infected animals consisted of 10 symptomless dogs (SLDs), 12 mildly affected dogs (MADs) and nine affected dogs (ADs). A higher parasite burden was demonstrated in ADs than in SLDs by anti-*Leishmania* immunohistochemistry ($P<0.01$), and by Leishman Donivan Unit (LDU) indices ($P = 0.0024$) obtained from Giemsa-stained impression smears. Sections stained with haematoxylin and eosin demonstrated a higher intensity of inflammatory changes in ADs than in SLDs ($P<0.05$), and in the latter group flow cytometry demonstrated a correlation ($P = 0.05/r = 0.7454$) between the percentage of $CD14^+$ monocytes in peripheral blood and chronic dermal inflammation. Extracellular matrix assessment for reticular fibres by staining of sections with Masson trichrome and Gomori ammoniacal silver demonstrated a decrease in collagen type I and an increase in collagen type III as the clinical signs increased. The data on correlation between cellular phenotypes and histological changes seemed to reflect cellular activation and migration from peripheral blood to the skin, mediated by antigenic stimulation. The results suggested that chronic dermal inflammation and cutaneous parasitism were directly related to the severity of clinical disease.

Keywords: dog; kala azar; *Leishmania chagasi*; parasitic infection

Introduction

Visceral leishmaniosis (VL; kala azar) is endemic in 87 countries, and approximately 90% of VL cases recorded worldwide occur in Bangladesh, Brazil, India and Sudan. Brazil is responsible for 90% of the VL records from the American continent (Monteiro *et al.*, 1994).

One of the first epidemiological surveys in Brazil was conducted by Chagas *et al.* (1938) in the region of Abaeté, state of Pará, where infection rates of 1.48% in man and 4.49% in dogs were found. Later, Deane and Deane (1962) reported the role of the dog and the fox

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(*Disicyon vetulus*) as domestic and sylvatic reservoirs, respectively.

Earlier reports of canine visceral leishmaniosis (CVL) described various macroscopical skin lesions (e.g., desquamation, alopecia, pustular dermatitis, ulcerative dermatoses and nodular disease, the type of which depended on the immune response (Adler and Theodor, 1932; Cunha, 1938; Torres, 1941; Ferrer *et al.*, 1988). The skin was considered by Abranches *et al.* (1991) to be an important reservoir compartment for parasites in healthy and sick *Leishmania*-infected dogs and the important role of dogs in VL transmission is supported by the high parasite loads found in the skin of infected animals (Deane and Deane, 1962).

Histopathological changes in the skin of *Leishmania*-infected dogs consist of variable degrees of focal or diffuse inflammatory infiltrate in the dermis, and variable numbers of plasma cells, macrophages (parasitized or not by amastigotes of *Leishmania chagasi*), lymphocytes and isolated neutrophils (Torres, 1941; Santos *et al.*, 2004; Solano-Gallego *et al.*, 2004). Changes in the extracellular matrix (ECM) in *Leishmania amazonensis*-infected mice are characterized by decreased collagen type I, increased collagen type III, and reduced fibronectin and laminin (Abreu-Silva *et al.*, 2004).

Histological tissue changes in CVL are probably triggered by the type of host immune response. Symptomless VL dogs are believed to produce a T-helper (Th) 1-mediated response. Clinically affected VL dogs, on the other hand, show a Th 2-mediated response, their peripheral blood mononuclear cells being unable to produce interferon (IFN)-γ in the presence of parasite antigens (Pinelli *et al.*, 1994). Bourdoiseau *et al.* (1997) reported the occurrence of immunosuppression associated with diminished numbers of $CD4^+$ T lymphocytes and $CD21^+$ B lymphocytes.

Investigations on cutaneous immunopathology in CVL might contribute to a better understanding of events related to kala azar morbidity and to the human disease (Nieto *et al.*, 1999; Moreno and Alvar, 2002). The present study was therefore designed to investigate the relationship between CVL and parasite burden as seen in the ear skin of dogs with different clinical forms of *L. chagasi* infection.

Materials and Methods

Animals

Thirty-one dogs naturally infected with *L. chagasi* and 10 uninfected dogs (UDs; controls) were obtained from the Zoonosis Control Centre, Belo Horizonte City Council. The dogs, of either sex, were aged 2–6 years. The UDs were confirmed as negative by parasitological examination and by an indirect fluorescent antibody test (IFAT) for *anti-Leishmania* IgG (Biomanguinhos Kit; FIOCRUZ-RJ, Brazil), titres of <40 indicating freedom from VL. The VL dogs, selected on the basis of IFAT titres of >40, were classified clinically according to signs of infection (Mancianti *et al.*,1988) as: symptomless dogs (SLDs; $n = 10$); mildly affected dogs (MADs; $n = 12$), with a maximum of three clinical signs; or affected dogs (ADs; $n = 9$), with more than three clinical signs. The study was approved by the Ethical Committee for the use of Experimental Animals, Universidade Federal de Minas Gerais.

Collection and Examination of Ear Skin Samples

The dogs were euthanatized by an intravenous overdose of barbiturate. Samples of ear skin were fixed in 10% neutral buffered formalin for (1) routine histopathological examination of sections stained with haematoxylin and eosin (HE), Masson trichrome and Gomori ammoniacal silver, and (2) anti-*Leishmania* immunohistochemistry.

Evaluation of parasite density in terms of the Leishman Donovan Unit (LDU) index was carried out by light microscopy on Giemsa-stained impression smears prepared from fragments of ear skin, the LDU index being the number of *Leishmania* amastigotes per 1000 nucleated cells (Stauber, 1956).

Parasite density was also evaluated immunohistochemically, as described by Tafuri *et al.* (2004). Briefly, serum from a dog naturally infected with *L. chagasi* (IFAT titre $>1{:}40$), diluted 1 in 100 in 0.01 M phosphate-buffered saline (PBS), was applied as the primary antibody. The slides were then incubated with biotinylated anti-mouse and anti-rabbit antibody (LSAB2 Kit; Dako, Carpinteria, CA, USA), which cross-reacts with canine serum immunoglobulins (Tafuri *et al.*, 2004), and subsequently with the streptavidin–peroxidase complex (LSAB2 Kit; Dako). The reaction was "visualized" with diaminobenzidine (DAB; Sigma, St Louis, MO, USA) and hydrogen peroxide. Finally, the slides were dehydrated, cleared, counterstained with Harris's haematoxylin, and mounted under coverslips.

The dermal inflammatory pattern and the cell population were evaluated histologically on HE-stained sections. The inflammatory infiltrate was graded according to Solano-Gallego *et al.* (2004), as follows: −, no inflammatory infiltrate; +, isolated foci of inflammatory cells; ++, isolated to coalescing areas of inflammatory infiltrate; +++, diffuse areas of inflammatory infiltrate.

Parasite density, assessed semi-quantitatively in sections labelled immunohistochemically for amastigotes, was based on the average number in five fields (×400) in areas with inflammatory infiltrate, in accordance

with a modification of the methods described by Ridley and Ridley (1983). The results were recorded as: −, none; +, light density, (1–100); ++, moderate density (101–300); +++, high density (>300).

Extracellular matrix (ECM), assessed in sections stained with Masson trichrome (for collagen I) and Gomori ammoniacal silver (for collagen III), was classified as:–, normal distribution of collagen I and III; +, either slight reduction of collagen I or slight increase of collagen III; ++, either moderate reduction of collagen I or moderate increase of collagen III; and +++, striking reduction of collagen I and striking increase of collagen III.

Flow Cytometry Analysis

Immunophenotyping analyses of peripheral blood by flow cytometry was performed as described by Reis *et al.* (2005). Briefly, 1 ml of whole blood with EDTA was subjected to pre-fixation and erythrocyte lysis by the slow addition of 13 ml of lysis solution (FACS Lysing solution; Becton Dickinson) followed by incubation for 10 min at room temperature (RT). After centrifugation (450 ***g***, at RT for 10 min), the pellet was resuspended in 500 µl PBS containing fetal bovine serum 10%.

In 96 well, "U" bottom plates (LIMBRO Biomedicals, Aurora, OH, USA), 30 µl of pre-fix leucocyte suspension were incubated at RT for 30 min in the dark with 30 µl of anti-canine cell surface marker antibodies. These monoclonal antibodies (mAbs) which define canine cell phenotypes, included purified rat anti-dog Thy-1 (Rat-IgG2b – Clone YKIX337.217), anti-dog CD5 (Rat-IgG2a – Clone YKIX322.3), anti-dog CD4 (Rat-IgG2a – Clone YKIX302.9), anti-dog CD8 (Rat-IgG1 – Clone YCATE55.9), fluorescein isothiocyanate (FITC)- labelled mouse anti-human-CD21 (Mouse-IgG1—Clone IOBla) and PE/Cy-5-conjugated mouse anti-human-CD14 (Mouse-IgG2a – Clone TÜK4), used in indirect and direct immunofluorescence procedures. The unlabelled mAbs and anti-CD14 mAbs were purchased from SEROTEC (Oxford, UK), and anti-CD21 from Immunotech (Marseille, France).

When purified mAbs were used, the cells were also incubated, under the same conditions, with 60 µl of previously diluted FITC-conjugated sheep anti-rat IgG antibody. Before flow cytometric data collection and analysis, labelled cells were fixed for 30 min with 200 µl of FACS FIX Solution (paraformaldehyde 10.0 g/l, sodium cacodylate 10.2 g/l and sodium chloride 6.65 g/l, pH 7.2). The results were expressed as the percentage of positive cells within the selected lymphocyte gate (Thy-1$^+$, CD5$^+$, CD4$^+$, CD8$^+$ and CD21$^+$) or of ungated leucocytes (CD14$^+$). The latter were also expressed as absolute counts taking into account the white blood cell values for each animal.

Statistical Analysis

This was performed with the Prism 3.0 software package (Prism Software, Irvine, CA, USA). The Kruskal-Wallis test was used to compare insensitivity between groups in both the immunohistochemical (anti-*Leishmania*) and histopathological approaches. χ^2 analysis was used for the LDU index. The Spearman test was performed for strategy correlation between peripheral blood phenotype and histological/parasitological status. $P<0.05$ was considered significant.

Results

Clinical Evaluation

Table 1 shows the frequency of occurrence of the various clinical signs in the groups MADs and ADs. The most frequently observed signs were weight loss, onychogryphosis, localized or diffuse ulcers, dry exfoliative dermatitis and hepatosplenomegaly.

Histological, Immunohistochemical and LDU Results

These are shown in Table 2. In terms of frequency of occurrence of chronic inflammation of the dermis, SLDs, MADs and ADs all differed significantly ($P<0.05$) from UDs (controls). Fig. 1 (A, D, G and J) shows the normal appearance of ear skin, as seen in the UDs. In all infected animals, sparse cellular infiltrates in the superficial and deep dermis consisted mainly of plasmacytes, with smaller numbers of macrophages and lymphocytes. In this context, MADs and ADs (both groups clinically affected) showed evident chronic

Table 1
Clinical signs recorded in mildly affected dogs (MADs) and affected dogs (ADs)

Clinical signs	*Number (and %) of dogs showing each stated sign*	
	MADS (n = 12)	*ADs (n = 9)*
Localized alopecia	5 (45.4)	4 (44.4)
Diffuse alopecia	0 (0)	2 (22.2)
Furfuraceous dermatitis	1 (9.1)	3 (33.3)
Opaque cornea	2 (18.2)	1 (11.1)
Localized ulcers	7 (63.6)	3 (33.3)
Diffuse ulcers	1 (9.1)	5 (55.5)
Paresis of limbs	0 (0)	1 (11.1)
Keratoconjunctivitis	2 (18.2)	2 (22.2)
Loss of weight	2 (18.2)	8 (88.9)
Onychogryphosis	6 (54.5)	8 (88.9)
Hepatosplenomegaly	4 (36.4)	6 (66.7)

The study also included a group of 10 symptomless dogs (SLDs).

Table 2
Assessment of chronic dermal inflammation and heavy parasite burden in the ear skin of uninfected dogs (UDs), symptomless dogs (SLDs), mildly affected dogs (MADs) and affected dogs (ADs)

Sign of infection in ear skin	*Number (and %) of dogs showing each stated sign*			
	UDs (n = 10)	*SLDs (n = 10)*	*MADs (n = 12)*	*ADs (n = 9)*
Chronic dermal inflammation	1 (10)	7 (70)*	10 (83.3)*	9 (100)*
Parasite burden				
ALIH	0	3 (30)	6 (50)	9 (100)†
LDU	0	4 (40)	8 (66.6)‡	9 (100)†

ALIH, as judged by anti-*Leishmania* immunohistochemical assay. LDU, as judged by Leishman Donovan index
*Significantly different from UDs ($P < 0.05$).
†Significantly different from SLDs ($P < 0.05$).
‡Based on examination of 11 of the 12 dogs.

inflammation. In ADs (Fig. 1C, F, I and L), the inflammation intensity was greater than in SLDs (Fig. 1B, E, H and K). As the clinical picture progressed, there was a reduction of collagen type I (Fig. 1G, H and I) and an increase in collagen type III (Fig. 1J, K and L). Semi-quantitative histological analysis of sections stained by Masson trichrome or Gomori ammoniacal silver (Fig. 2) revealed that the percentage of ADs showing severe changes was higher than that of UDs and SLDs ($P < 0.05$). Moreover, in terms of chronic dermal inflammation, the percentage of animals showing severe changes was higher in the MAD and AD groups than in the SLD and UD groups ($P < 0.05$) (Fig. 2).

The anti-*Leishmania* immunohistochemical assay and LDU index showed that the percentage of animals with heavy parasite burdens in the ear skin was higher in the AD group than in the SLD group ($P < 0.05$) (Table 2). Assessment of the parasite burden by the LDU index revealed the following mean (and standard deviation; SD) values: SLDs, 25.3 (57.8); MADs 196.9 (338.8); and ADs 1346.1 (2431.0). The LDU index revealed a higher parasitic burden in ADs than in SLDs ($P < 0.05$). When the parasite burden was evaluated by anti-*Leishmania* immunohistochemical assay, higher parasite burdens were demonstrated in ADs than in SLDs ($P < 0.01$) (Fig. 2).

Relationship between Chronic Dermal Inflammation and Immunophenotyping of Peripheral Blood

In ADs, the absolute counts of $CD14^+$ cells were significantly lower than in UDs, and there was a positive correlation between the low percentages of monocytes and chronic dermal inflammation ($P = 0.025$/ $r = 0.7454$) (Fig. 3A, B, C). There was no significant correlation between chronic dermal inflammation and Thy-1^+, $CD5^+$, $CD8^+$ and $CD21^+$ cells, despite a suggestion of increased numbers of $CD8^+$ lymphocytes in dogs with only a slight inflammatory infiltrate (Fig. 4).

Discussion

The clinical outcome of CVL range from symptomless infection to classical kala azar with a full array of symptoms (Mancianti *et al.*, 1988). In the present study, the main clinical signs observed were localized or diffuse ulcers, loss of weight, onychogryphosis and hepatosplenomegaly, signs similar to those reported by Lima *et al.* (2004). In VL endemic regions these signs are relevant to the differential diagnosis of canine skin diseases.

Some authors (Adler and Theodor, 1932; Cunha, 1938, Tafuri *et al.*, 2001) reported that the dermal inflammatory infiltrate in CVL consisted of mononuclear cells surrounding sebaceous follicles. In the present study, dogs naturally infected with *L. chagasi* had plasmohistiocytic- and lymphocyte-like cellular infiltrates (data not shown), but no histiocytic granuloma such as those reported by Solano-Gallego *et al.* (2004) and Santos *et al.* (2004).

The observed reorganization of the ECM, with a reduction of collagen type I and increase of collagen type III (reticular fibres), was related to the degree of tissue destruction produced by inflammatory processes and to parasite burden, as prevously reported by Abreu-Silva *et al.* (2004). In the present study, the degree of inflammatory infiltration was related to the parasite burden in the skin.

The study showed differences between the groups of infected dogs (SLDs, MADs and ADs) and the control group (UDs) in terms of inflammatory infiltrate. The infiltrate was mainly distributed diffusely in superficial and deep dermis but was sometimes concentrated in perifollicular sites. Solano-Gallego *et al.* (2004) reported a focal pattern of inflammatory infiltrate

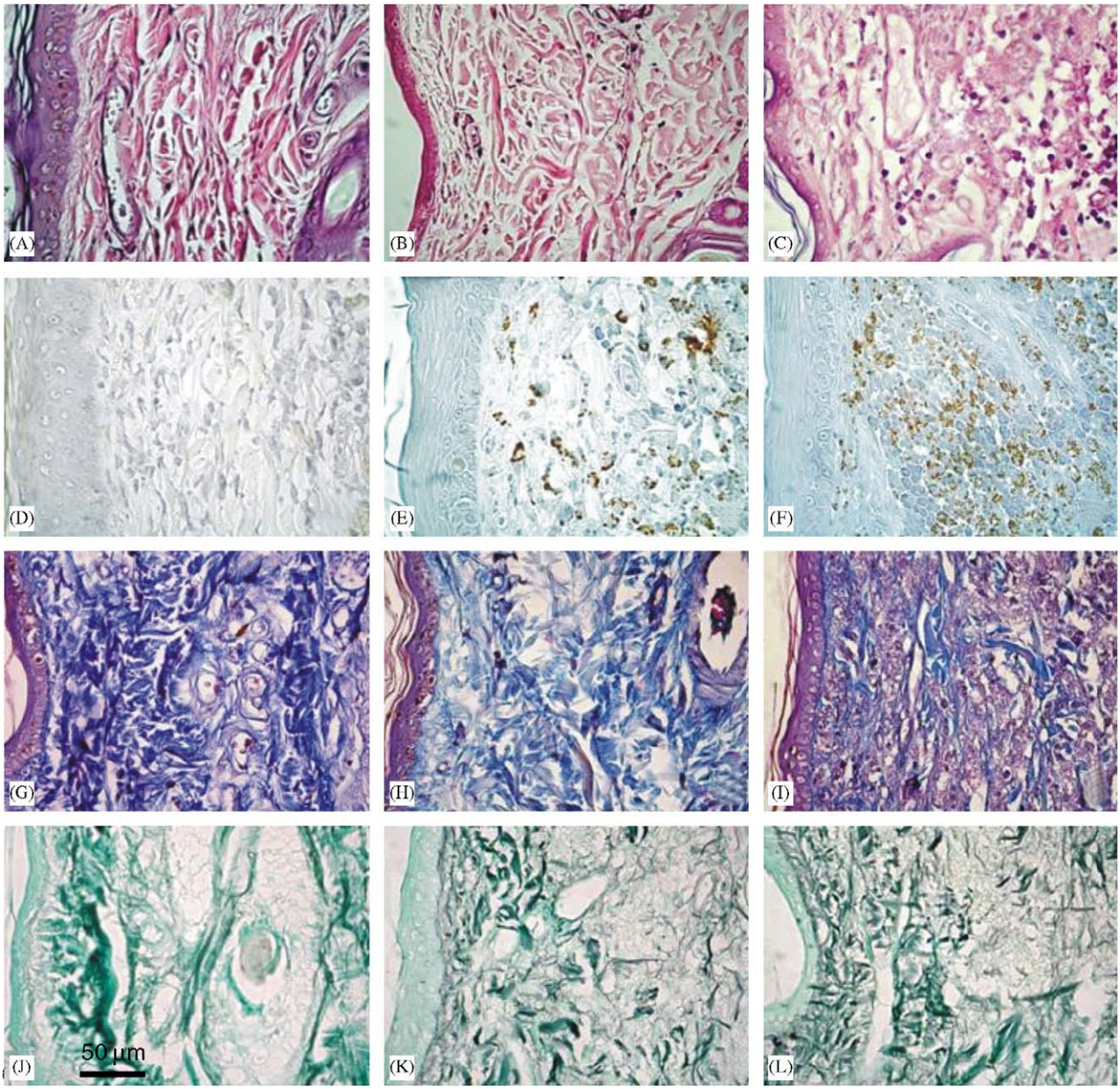

Fig. 1. A–L Photomicroscopy of ear skin of uninfected dogs (A, D, G and J), symptomless infected dogs (B, E, H and K) and clinically affected dogs (C, F, I and L). HE staining (A, B and C). Anti-*Leishmania* immunohistochemistry (D, E and F). Masson trichrome staining (G, H and I). Gomori ammoniacal silver staining (J, K and L). Bar, 50 µm (applies to all photographs). As canine visceral leishmaniosis progresses, there is an increase in inflammation, parasite burden and collagen type III, and a reduction in collagen type I.

suggestive of perifollicular dermatitis. It should be emphasized that although the frequency of occurrence of inflammatory infiltrates was similar in SLDs and SDs, the latter group showed a higher inflammatory density. Solano-Gallego *et al.* (2004) observed a greater degree of cutaneous inflammation in the nose of clinically infected dogs than in symptomless dogs; in the latter, the skin showed no significant changes.

Santos *et al.* (2004) reported that in most dogs with localized inflammatory infiltrate it was not possible to demonstrate cutaneous parasitism. The same study showed a correlation between parasitism, which ranged from moderate to dense, and diffuse and granulomatous inflammatory patterns. In the AD group, the present study revealed an intense, diffuse dermal inflammatory infiltrate, with a high parasite burden. (Figs 1 and 2). In addition, the inflammatory infiltrates were more striking in ADs than in SLDs. Similar observations were made by Martínez-Moreno *et al.* (1995), Tarantino *et al.* (2001) and Solano-Gallego *et al.*,

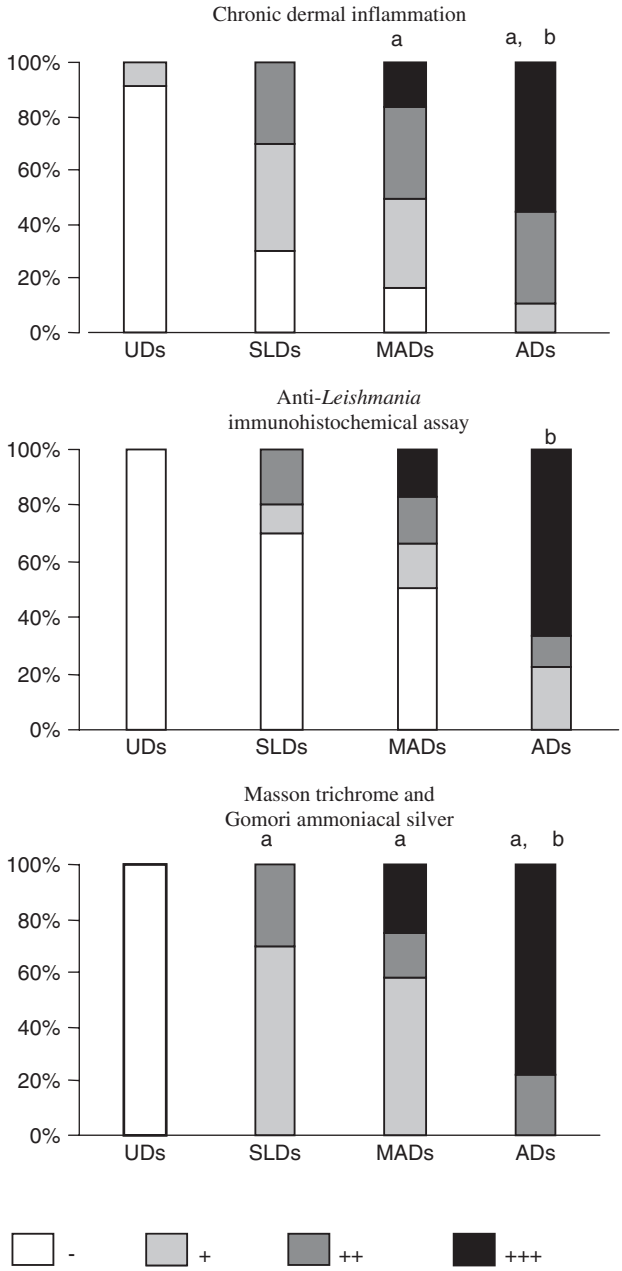

Fig. 2. Chronic dermal inflammation and parasite burden (immunohistochemical assay) in ear skin (white bar [−], absent; grey bar [+], mild; dark bar [++], moderate; black bar [+++], intense. Masson and Gomori staining (white bar [-], normal distribution of collagen I and III; grey bar [+], slight reduction of collagen I and slight increase of collagen III; dark bar [++], moderate reduction of collagen I and moderate increase of collagen III; black bar [+++], striking reduction of collagen I and striking increase of collagen III. "a", significantly different ($P<0.05$) from UDs; "b", significantly different ($P<0.05$) from SLDs.

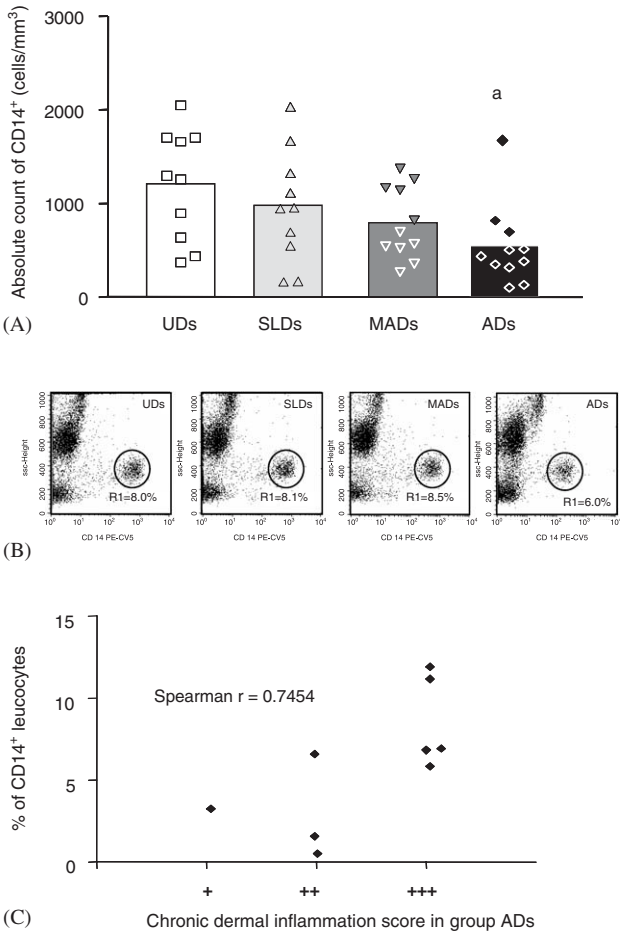

Fig. 3. (A–C) Analysis of CD14$^+$ cells within peripheral blood leucocytes from dogs naturally infected with *L. chagasi*. (A) Data analysis of CD14$^+$ cells was performed on symptomless (SLDs, △), mildly affected dogs (MADs, ▼), affected dogs (ADs, ◆) and uninfected dogs (UDs, □). Single flow cytometry platform was used to determine the frequency and the absolute counts of CD14$^+$ cells expressed as scattering of individual values of CD14$^+$ cells/mm^3. (B) Representative side scatter (SSC) versus fluorescence type 3 (CD14Cy-5PE) dot plots illustrating the lower frequency of CD14$^+$ cells in ADs than in UDs ($P<0.05$). Region statistics were used for data analysis, and the results are expressed as percentage of positive cells within ungated leucocytes. (C) Correlation (Spearman $r=0.7454$) between the percentage of CD14$^+$ leucocytes and the chronic dermal inflammation score in the group of ADs.

(2004). The LDU index and anti-*Leishmania* immunohistochemical assay for cutaneous parasitism gave similar results, suggesting that the former method represents a reliable and inexpensive method for evaluating the cutaneous parasite burden.

As observed by Pinelli *et al.* (1994, 1995) and Bourdoiseau *et al.* (1997), symptomless dogs would seem to be more resistant than clinically affected dogs, probably as the result of more efficient cell activation, resulting in limited inflammatory infiltration.

In ADs, the correlation ($r=0.7454$) observed between CD14$^+$ monocytes and chronic dermal

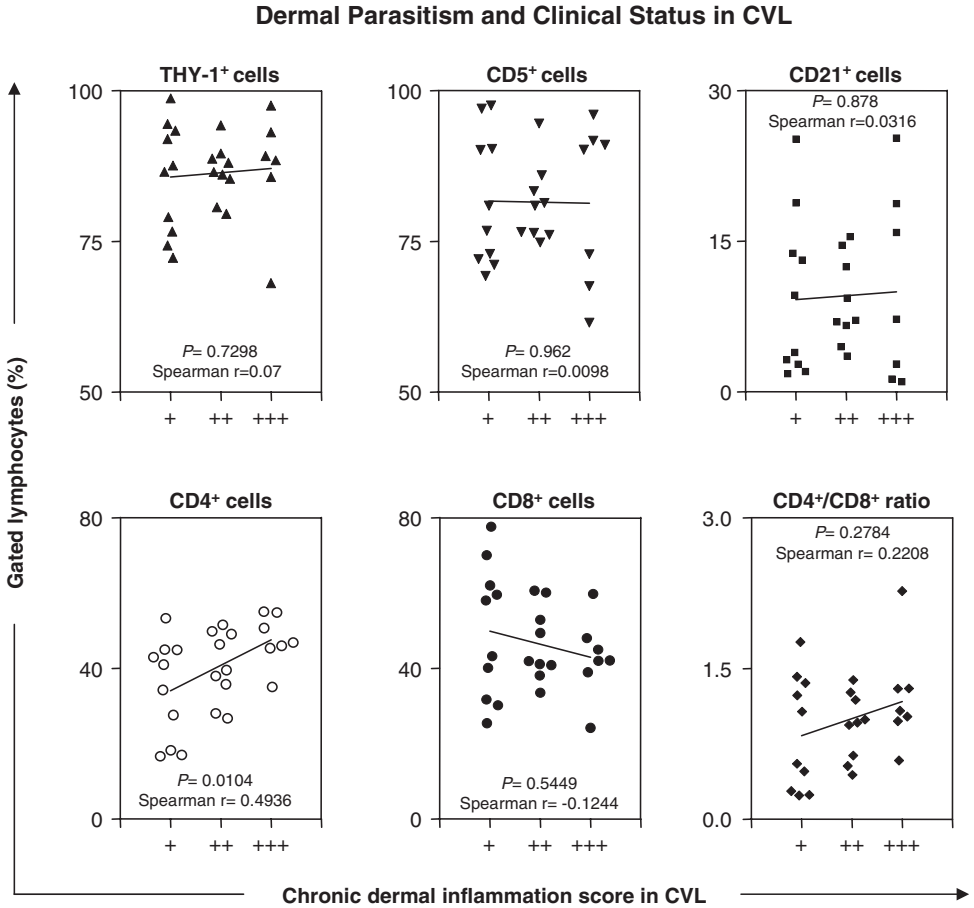

Fig. 4. Correlation between chronic dermal inflammation score (+; ++; +++) with the percentage of Thy-1$^+$ (▲), CD5$^+$ (▼), CD21$^+$ (■), CD4$^+$ (○), CD8$^+$ cells (●) and CD4/CD8 cell ratio (◆), within gated peripheral blood lymphocytes from *L. chagasi* infected dogs. The results are expressed as scattering of individual values. Spearman correlation indices (r) at $P<0.05$ are shown on graphs. Connecting lines illustrate positive and negative correlation indices.

inflammation probably reflected activation of such peripheral blood cells and their migration to the dermis, where they participated in the striking inflammatory reaction (Fig. 3). However, this cell population probably made little contribution to the resistance in view of the high morbidity and parasite burden shown by ADs.

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