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THE EFFECT OF AN IMMUNE MODULATING DIET ON IMMUNE SYSTEM OF DOGS NATURALLY INFECTED BY *LEISHMANIA INFANTUM*

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INTRODUCTION

Canine leishmaniasis (CL) is a systemic parasitic disease, endemic in Mediterranean countries. It has been demonstrated that the immune system plays a key role in the development and outcome of *Leishmania* infection in the dog and in the response to the treatment, although this response is not well understood. Several mechanisms account for the control of the immune response. Regulatory systems include mechanisms intrinsic to the antigen activation and to T cell differentiation, but they are also mediated by regulatory suppressor populations, as represented by the CD4+CD25+FoxP3+ T subset (Tregs). The energy/metabolic status has been described to significantly modify the immune response as well as the immune tolerance control in human and animal models. Moreover malnutrition is a risk factor for the development of visceral leishmaniasis. The aim of this study has been to assess the impact of immune modulating diet intake on the immunological state of dogs naturally infected by *L. infantum.*

MATERIAL AND METHODS

Forty dogs naturally infected by *L. infantum* (20 males and 20 females, 5-9 years old) from the Campania region (South Italy) were enrolled with the owner's consent. Twenty dogs were submitted to a treatment with meglumine antimoniate (50 mg/kg, subcutaneous, twice daily, for 1 month) in addition to an immune modulating diet for 6 months **(Group I)** and 20 dogs were submitted to a therapy with meglumine antimoniate (50 mg/kg, subcutaneous, twice daily, for 1 month) in combination with allopurinol (10 mg/kg, oral, twice daily, for 6 months) in addition to a standard feed, for 6 months **(Group II)**. Clinical diagnosis of CL was confirmed by amastigotes detection in lymph nodes or bone marrow aspirate smears, by positive indirect fluorescent antibody test (≥1:160) and by PCR. Occurrence of other infectious agents (*Ehrlichia canis, Anaplasma phagocytophilum, Babesia canis, Dirofilaria immitis, etc.)* was excluded in all dogs. Twenty clinically healthy dogs (negative for serological, parasitological and molecular examinations) were also used as controls. Anti-CD3,-CD4,-CD8,-CD45, and isotype-matched mAbs were purchesed from Serotec Ltd, London, UK. Intracellular detection of Foxp3 was performed using a cross-reactive, directly conjugated murine Foxp3 antibody (Clone FJK-16s, eBioscience, San Diego, CA). CD8+ and CD4+ T cell subsets were identified by a combination of canine anti-CD3 together with anti-CD4 or anti-CD8 mAbs. Besides we assessed the presence of pro-inflammatory T cells: intracellular staining with mAbs recognizing dog IFN-γ and IL-4 or isotype-matched controls (Serotec) was performed by a fixing/permeabilization kit (Caltag, Burlingame, CA). Flow cytometry and data analysis were performed by *FACScalibur* apparatus and *CellQuest analysis software* (Becton Dickinson, Mountain View, CA). Statistical analysis was performed by Mann-Withney test and Spearman's rank correlation (*GraphPad Prism,* San Diego, CA, USA). Results were considered significant at $p < 0.05$.

Figures 1 - A and B. Clinical status before and after the treatment **(Group I)**

Figures 2 - A and B. Clinical status before and after the treatment **(Group II)**

RESULTS

We focused our investigation on T lymphocytes. As shown in figure 3A and B, at the time of enrolment the percentage of CD8+CD3+ T lymphocytes was slightly increased if compared to healthy dogs. In addition, after 3 and 6 months from the treatments (pharmacological therapy + Immune modulating diet = group I, and pharmacological therapy alone = group **II**) the CD8+CD3+ T lymphocytes percentage of both groups was maintained constantly high, as compared to healthy controls. In this regard, it is worth noting that the percentage of CD8+CD3+ T lymphocytes was significantly increased after 6 months in group I. This observation is confirmed by the decreased CD4+/CD8+ ratio (data not shown). The trend line of absolute numbers confirmed observations on percentages (data not shown).

We also analyzed the level of Tregs in a cohort of CL infected dogs. Figure 4A and B refers to the comparative analysis of pathological cohort (I and II groups) and the healthy controls. A significant reduction of Tregs in the peripheral blood of both groups of infected dogs was observed at the time of enrolment (figure 4A and B), while a relevant increase of percentage of Tregs was observed in the group I after 3 and 6 months. In this regard, the Tregs became similar to the level observed in healthy dogs.

To assess whether the immunological profile by us described, might be related to the induction of a specific cytokine profile in T lymphocytes, we analysed the IFN-γ and IL-4 production in T lymphocytes of both groups. As shown in figure 5A and B, at the time of enrolment the percentage of Th1 T cells (specifically producing IFN-γ and negative for IL-4) is slightly increased in infected animals as compared to controls, while after 6 months the percentage of Th1 T cells appears to be significantly increased only in the group I (figure 5A). Intriguingly, the production of IL-4 by CD4+ T lymphocytes decreased only in the group I after 6 month (figure 6A).

CONCLUSIONS

Our preliminary observations suggest as an immune modulating diet could intriguingly interfere with the asset of CD8+ T lymphocyte effectors and of T regulatory cells, as well as with pro-inflammatory cytokine secretion. In this regard, our diet appeared to potentiate the pharmacological treatment. Such observations confirmed the relevance for CD8+ T lymphocytes in the control of intracellular-parasites. In this regard, the increased CD8+ T (combined to a slightly polarisation versus Th1 secretion) could be correlated to the immune response against parasite in untreated infected dogs. It is of note that the percentage of CD8+ T lymphocytes significantly increased after 6 months from the treatment and in presence of immune-modulating diet (group I). After 3 and 6 months from treatment (group I), we also observed a recover of Tregs percentage, if compared to healthy dogs. This observation, combined to the decrease of Th1 cells, could be mainly correlated to the reduction of pro-inflammatory effects in the peripheral tissues. Our diet appears to potentiate the pharmacological effects. It is of note that the CD8+ T percentage remains constantly high in both groups and it could evidence the control of chronic CL disease spreading.

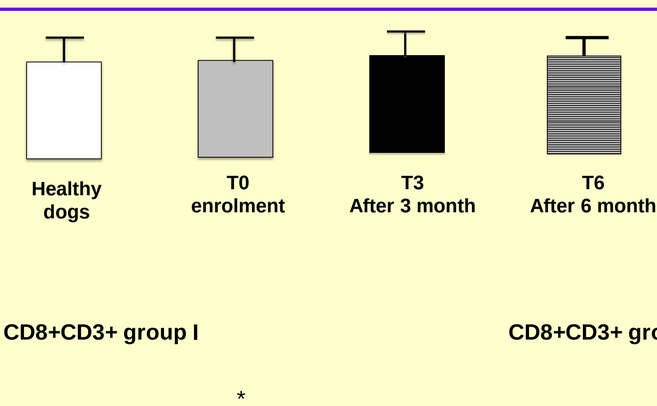

Figure 3 – A and B. Evaluation of CD8 T lymphocyte percentage by immunofluorescence and Flow Cytometry

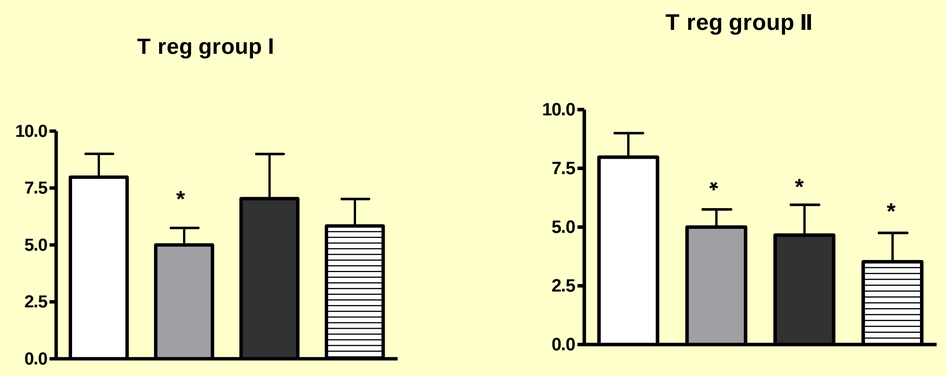

Figure 4 – A and B. Evaluation of Tregs percentage by immunofluorescence and Flow Cytometry

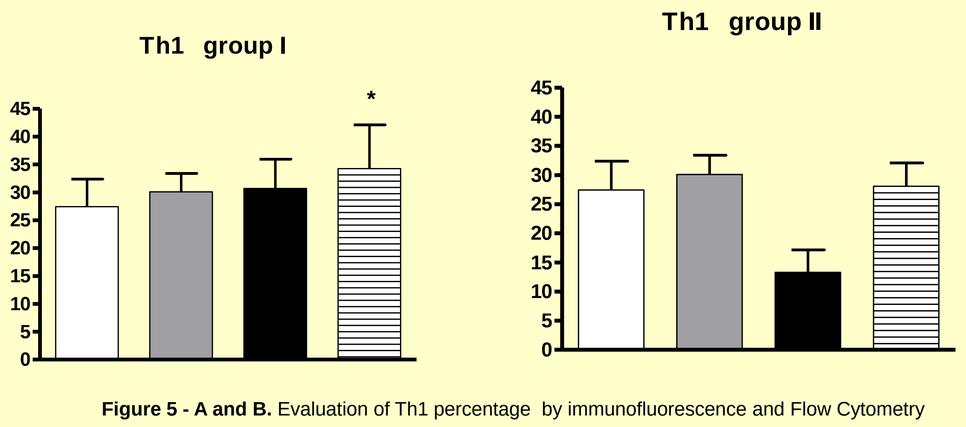

Figure 5 - A and B. Evaluation of Th1 percentage by immunofluorescence and Flow Cytometry

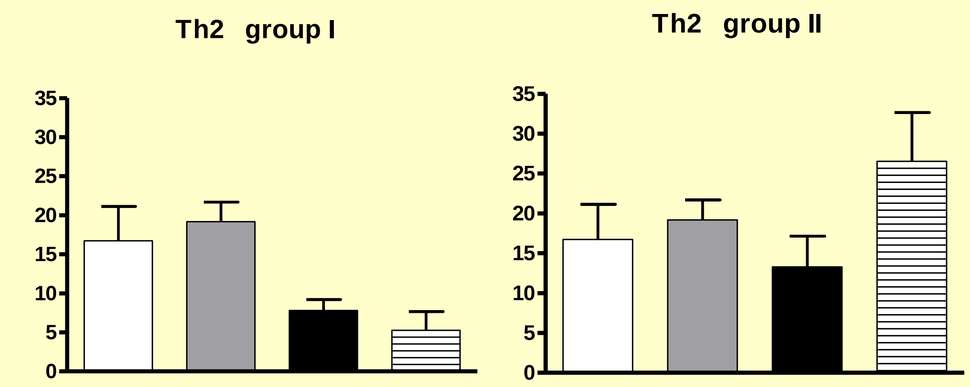

Figure 6 – A and B. Evaluation of Th2 percentage by immunofluorescence and Flow Cytometry

Bibliography available from the author, by e-mail

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