CHARACTERIZATION OF THE IMMUNE RESPONSE TO *LEISHMANIA* IN BLOOD DONORS FROM MADRID (SPAIN)

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INTRODUCTION

Leishmaniasis comprises a wide spectrum of human diseases ranging from self-limited cutaneous leishmaniasis to potentially fatal visceral infection.
Human visceral leishmaniasis (VL) caused by *Leishmania infantum* is endemic in the Mediterranean basin, Asia and Latin America. Clinical cases represent only a fraction of those infected and the percentage of asymptomatic individuals is underestimated. To our knowledge, there is no previous report identifying and characterizing healthy individuals who had contact with *L.infantum* from the hypo-endemic area of Madrid (Spain).

OBJECTIVE

The aim of the present study was to identify asymptomatic leishmaniasis subjects in the Madrid area and for the first time, verify the nature of *Leishmania*-specific immune responses that are elicited in comparison to naive individuals.

METHODS

Subjects: Blood from 261 subjects having no history of VL were obtained at the Centro de Transfusion de la Comunidad de Madrid from blood donors that accepted to participate in the study.
Asymptomatic individuals for leishmaniasis were defined based on the T-cell proliferation assay against *Leishmania* antigens (stimulation index>2).
Proliferation assays: Lymphocyte proliferation was evaluated in 96 well plates using 2×10^5 PBMCs stimulated for 5 days with soluble leishmanial antigen (SLA) at 10 mg/mL or PHA (10 mg/ml). Supernatants were harvested 120 hours after *in vitro* stimulation and maintained at -20°C until use. Results were expressed as stimulation index (SI) (OD of SLA stimulated cells/unstimulated).
Cytokine measurement in supernatant: Cytokines were measured using the BD Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit.
Phenotype of blood: 50 µl of blood were incubated with MoAbs to stain T, B and NK population by using BD TriTEST™ for 30 min at 4ºC in the dark. Erythrocytes were lysed and cells were washed twice and resuspended in fixation buffer. Data acquisition and analysis were performed by 4-color flow cytometry FACS Calibur and using Cell Quest software (Becton Dickinson).
ELISA: 96-well polystyrene plates were sensitized with SLA from *L. infantum* (10µg/ml). Coated plates were then blocked and treated successively with serum samples and peroxidase-labelled antibodies to total human IgG or subclasses (IgG1, IgG2, IgG3, IgG4) and IgM. Reactions were developed with H_2O_2, *o*-phenylene diamine substrate, stopped with 1N HCl and the optical densities read at 490 nm. Cut-off values were determined as the mean of naïve subjects±3SD.
Real time PCR: 100µl of blood were subjected to classical phenolchloroform DNA extraction and ethanol precipitation. The DNA obtained was diluted in 100 µl distilled sterile water and stored at 4°C until use. Real time hot Start PCR was performed in LC FastStart DNA Master (Roche Diagnostic) using an external standard curve with a detection limit of 0.1 parasites/reaction. Specificity of the products was checked with the melting peak of the PCR products obtained.
Statistical analysis: Results were analyzed by Student's *t*-test. Statistical significance was assigned to P < 0·05.

RESULTS

Of the 261 donors studied, we have found 11 (4,2%) with a lymphoproliferative IS > 2 in response to SLA (Figure 1). Asymptomatic subjects significantly presented higher levels of IFN-γ and TNF-α in culture supernatant than naives (Figures 2 and 3, Tables 1 and 2). No significant differences in the peripheral blood T, B, and NK-lymphocyte subsets were found between groups (Figure 4). All individuals were negative by parasitological and serological techniques (Figure 5, Tables 3 and 4).

Lymphoproliferation

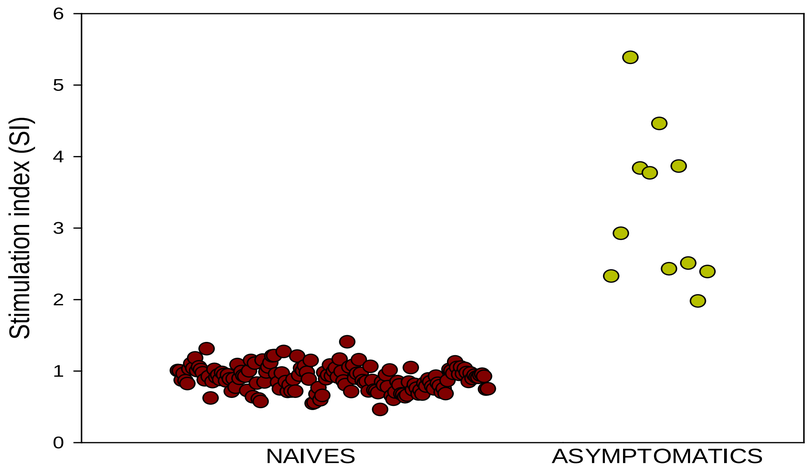


Figure 1. Proliferative response of PBMCs to *L. infantum* SLA from 261 individuals of the *Leishmania* endemic area of Madrid.

Of 261 blood tested, 11 asymptomatic subjects showed antigen specific proliferation to SLA. Stimulation with PHA (data not shown) produced high lymphoproliferative responses in both groups.

Cytokine production

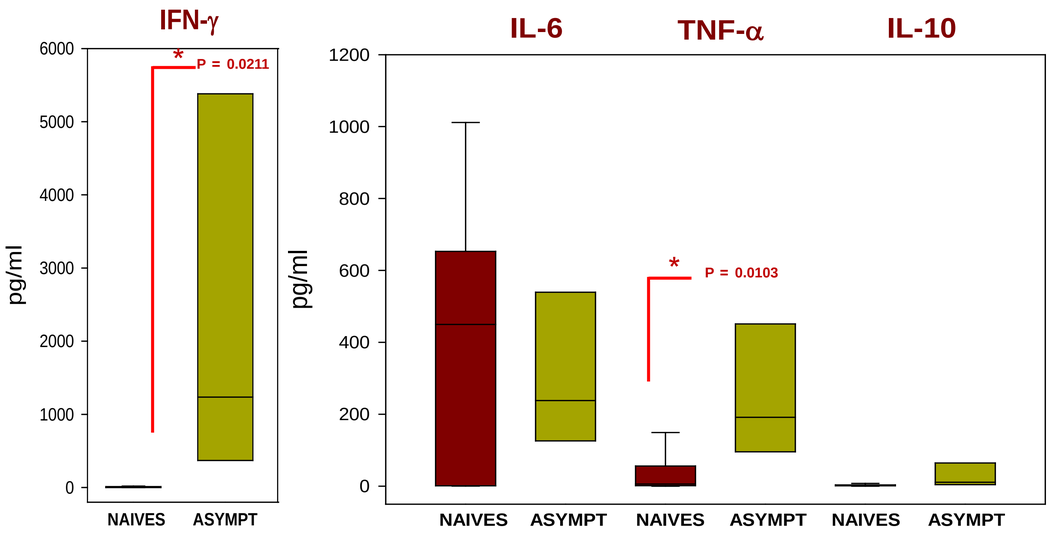


Figure 2 and 3. Median of IFN-γ, IL-6, TNF-α and IL-10 production in PBMC culture supernatants from 11 asymptomatic (ASYMPT) and 11 naive subjects to SLA from *L. infantum*.

Asymptomatics

	IFN-γ	IL-6	TNF-α	IL-10
Mean	2.551,12	322,99	256,86	87,83
SD	4.347,34	221,63	297,34	116,85

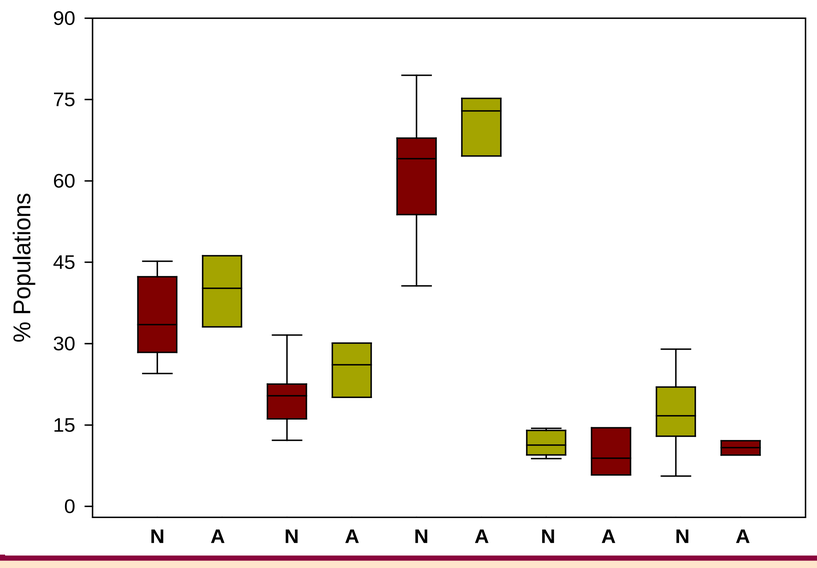
Naives

	IFN-γ	IL-6	TNF-α	IL-10
Mean	6,85	446,07	7,64	14,87
SD	7,66	307,92	15,17	41,61

Tables 1 and 2. Mean of cytokine production in PBMC culture supernatants from Asymptomatic and naive individuals to *L. infantum* SLA.

The stimulation with SLA leishmanial antigen produced high levels of IFN-γ and TNF-α cytokines and low IL-10 production in PBMC from asymptomatic individuals, indicating a preferential Th1-like response to *Leishmania.*

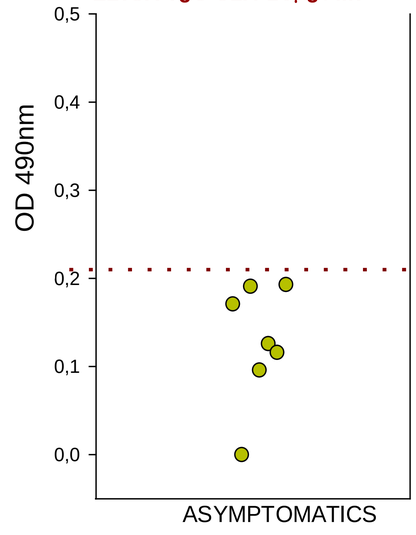
Peripheral blood lymphocytes phenotyping



The differences found in the percentage of T, B and NK lymphocytes were not significantly different in asymptomatic individuals compared to naives.

Figure 4. Phenotype of blood cells from naive (N) and asymptomatic (A) individuals

ELISA SLA



Asymptomatics	Total IgG	IgG1	IgG2	IgG3	IgG4	IgM
Mean	0,128	0,003	0,016	0,028	0,053	0,012
SD	0,068	0,007	0,015	0,018	0,027	0,017

Naives	Total IgG	IgG1	IgG2	IgG3	IgG4	IgM
Cut-off	0,210	0,080	0,100	0,120	0,120	0,070

Tables 3 and 4. Levels of SLA antibodies in the sera of the screened blood bank subjects

SLA- specific IgG, IgG1, IgG2, IgG3, IgG4 and IgM antibodies were not found in the individuals screened. The seroprevalence for *Leishmania* infection found in the blood bank from Madrid was 0%.

Figure 5. SLA-specific IgG antibodies

CONCLUSIONS

In the hypoendemic area of Madrid (Spain), asymptomatic individuals for leishmaniasis were characterized by undetectable levels of *Leishmania*-specific serum antibodies, absence of parasitemia and for a high production of IFN-γ and TNF-α from SLA-stimulated PBMCs. Our study has shown that as much as 4% of blood donors in the Madrid area elicited specific cellular response to *L. infantum*, which is associated with a Th1-like response to *Leishmania*.
The usefulness of these immunological markers will be further evaluated in order to identify cryptic/asymptomatic infection in endemic areas.

ACKNOWLEDGMENTS

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