IMMUNOLOGICAL PROFILE OF ASYMPTOMATIC LEISHMANIASIS INDIVIDUALS AFTER VISITING ENDEMIC AREAS IN PERU

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INTRODUCTION

❖ Leishmaniasis comprises a group of diseases that mainly affect poor people around the world, occurs in four continents and is considered to be endemic in 88 countries. In Peru, the main clinical manifestation is the Cutaneous Leishmaniasis (CL) and Mucosal Leishmaniasis (ML), also known as American Tegumentary Leishmaniasis (ATL). The disease affects between 6000 and 9000 persons annually in Peru (Direccion General de Epidemiologia, Ministerio de Salud del Peru).

❖ In Peru, ATL is principally caused by *Viannia* subgenus where *L. (V.) peruviana, L. (V.) braziliensis* and *L. (V.) guyanensis* cause the majority of the CL and ML reported cases. *L. (V.) lainsoni* was also described (Lucas, C. *et al.* 1998).

❖ Clinical outcome after infection with *Leishmania* parasite relies on several factors involving (i) the host (genetics background, immunological response); (ii) the parasite (infecting specie, strain pathogenicity); (iii) the vector; and (iv) the enviromental. Among those, it is well known that host immune response plays a central role.

❖ Previous reports indicate that protective immune response, necessary to control Leishmaniasis, is characterized by the predominance of a Th1 immune response: IFN-γ and TNF-α production, mediators of parasite killing through macrophage activation. On the other hand, a Th2 type response could lead to the disease progression and non-healing lesions, when cytokines such as IL-4 and IL-10 (maintains latent infection by down-modulating the IFN-γ production) are evoked.

❖Natural resistance to leishmanisis, supported by self-cured or asymptomatic cases in endemic areas, constitute evidence that host is capable to develop protective immunity against the parasite.

❖ Under this context, the aim of this work was to study the cellular and humoral immune response in asymptomatic leishmaniasis subjetcs and to compare their response with the developed by active CL and ML patients, our findings will contribute to assess immune factors contribution to disease outcome.

METHODS

Blood Samples: A total of 65 individuals from the highlands of Cuzco, Peru, were enrolled in the study. Serum and peripheral blood mononuclear cells were isolated from blood samples from individuals with CL (n=7), ML (n=12) and those who visited endemic area but without any sign of the disease (n=24). Nine naive subjects that never visited endemic areas were included as control. ***Cell culture:*** Isolated PBMC were resuspended at a concentration of 2×10^6 cells/ml in supplemented RPMI, plated in 96 well tissue culture plates at a final concentration of 2×10^6 cells/well and incubated for 5 days at 37˚ C, 5% CO2. Stimulation was done by addition of local *L. braziliensis* SLA (soluble leishmania antigen) at 10 µg/ml. PBMC were stimulated with PHA (10 µg/ml) for 3 days as positive controls. Supernatants were harvested 120 hours after in vitro stimulation and maintained at -20˚ C until use. ***Proliferation assays:*** Lymphoproliferation was carried out by evaluation of [3H] thymidine incorporation in beta counter after a 5 hour pulse of cell cultures with [3H]thymidine (1 µCi/well). Incorporation of radioactive label was measured by scintillation. Results are expressed as stimulation index (SI) defined as counts per minute (cpm) (mean in wells containing antigen) divided by background (cpm mean in non-stimulated wells). Cut-off values were determined as the mean of naïve subjects±3SD. ***Leishmania Antigens:*** Local SLA was prepared from stationary phase promastigotes of *L. braziliensis.* Parasites were lysed by 10 Freeze (-90˚ C)-Thaw (37˚ C) cycles and then sonicated and centrifuged at 14,000×*g* for 10 min. The supernatant was used at a final concentration of 10 µg/ml. ***Cytokine measurement in supernatant:*** Cytokines were measured using the BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit. ***ELISA:*** ELISA was carried out as follows, 96-well polystyrene plates were sensitized with SLA from *L. braziliensis* [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 and IgG3). 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. ***Statistical analysis:*** Results were analysed by one-way analysis of variance (anova), Kruskal–Wallis test, using GraphPad Prism software. Statistical significance was assigned to P < 0·05.

Table 1. Characteristics of the enrolled groups

Characteristics	Subjects: Naive	Enrolled as ASY[a]	CL	ML
n	9	24	7	12
Age (years)*	36.7 ± 18.6 (22 - 46)	34.0 ± 12.7 (22 - 50)	22.4 ± 6.8 (18 - 35)	38.1 ± 9.4 (19 - 50)
Sex (Male:Female)	2:7	9:15	7:0	10:2
N° lesions*	0	0	3.5 ± 4.8 (1 - 17)	2.6 ± 2.0 (1 - 7)
N° days in endemic area*	0	2188.2 ± 4133.5 (3 - 14600)	135.7 ± 107.5 (45 - 365)	450.8 ± 819.6 (45 - 2920)
Positive lymphoproliferative response SI>2.47	0/9 0.00%	13/24 54.17%	7/7 100%	12/12 100%

* Mean ± SD (range)
a Individuals who visited *Leishmania* endemic area without any sign ofdisease

RESULTS

Asymptomatic individuas were defined as individuals without disease who showed T-cell proliferation against *Leishmania* antigens. They represented 54% of those who visited endemic areas (SI median=4.85). ASY presented low levels of IFNγ, TNFα and IL10 (medians= 18.60, 6.83 and 31.50, respectively), but a remarkably low IFNγ/IL10 ratio (median=0.32). ELISA for IgG isotypes showed that IgG3 and IgG1 were detected in 100% and 15.38% of ASY, respectively; while IgG2 was absent in these individuals. These levels of cytokines and IgG isotypes were considerably lower than corresponding values from CL/ML patients (p<0.01). Exacerbated pro-inflammatory response were found in CL and ML (Medians: SI=77.60 and 22.20; IFNγ=3259.48 and 4,673.70; TNFα=66.30 and 155.90; IFNγ/IL10 ratio=61.27 and 64.31 respectively). IgG1 and IgG3 were detected in most of the CL/ML samples, whereas IgG 2 titres were found with lower reactivity.

Cellular response

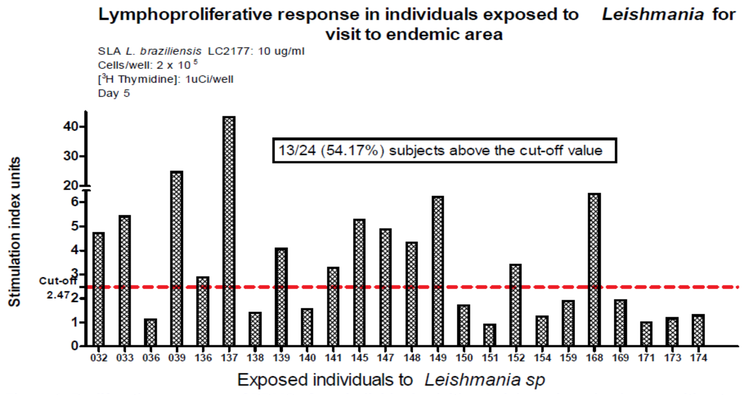
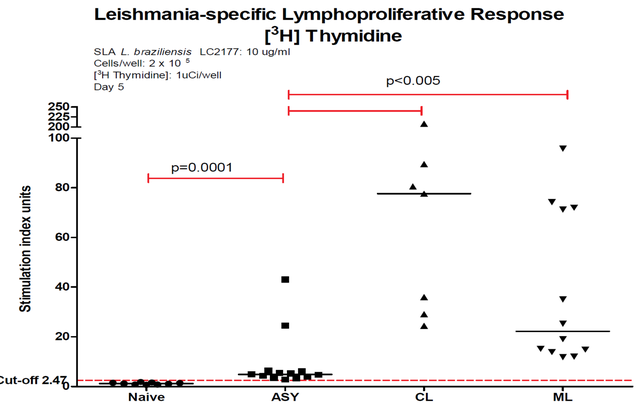
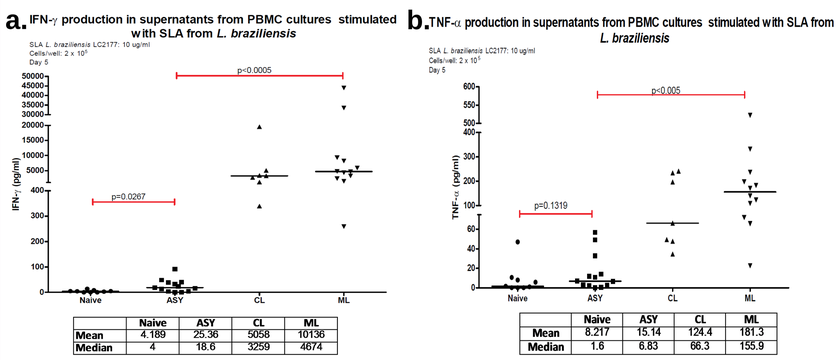


Figure 1. Proliferative response of PBMCs from individuals visiting *Leishmania* endemic area without any sign of disease to SLA from *L. braziliensis*. Cut-off is indicated by red dashed line.



	Naive	ASY	CL	ML
Mean	1.188	9.085	77.85	38.4
Median	1.14	4.85	77.6	22.2

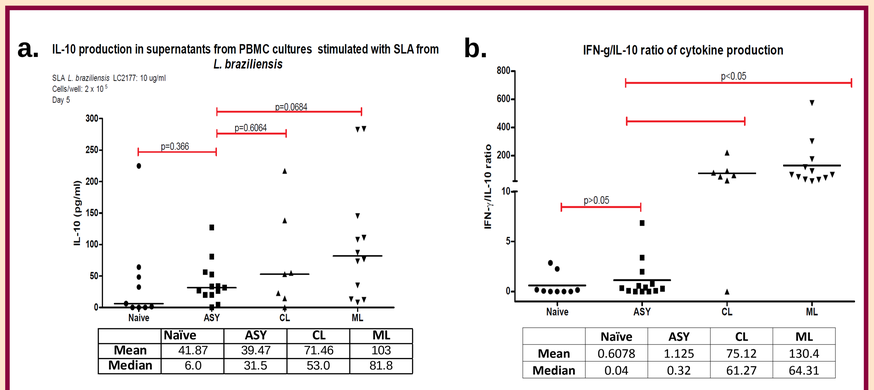
Figure 2: Lymphoproliferative response meassure by [3H] Thymidine incorporation in response to SLA from *L. braziliensis*. Each point represents a subject. Medians are indicated by horizontal lines.



	Naive	ASY	CL	ML
Mean	4.189	25.36	5058	10136
Median	4	18.6	3259	4674

	Naive	ASY	CL	ML
Mean	8.217	15.14	124.4	181.3
Median	1.6	6.83	66.3	155.9

Figure 3: IFN-γ (a) and TNF-α (b) production in PBMC culture supernatants from active CL and ML, asympyomatic (ASY) and naïve individuals to SLA from *L. braziliensis* . Each point represents a subject. Medians are indicated by horizontal lines

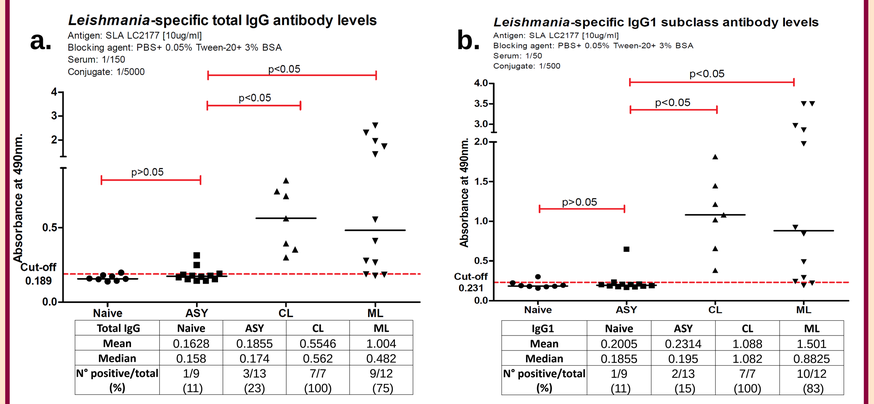


	Naïve	ASY	CL	ML
Mean	41.87	39.47	71.46	103
Median	6.0	31.5	53.0	81.8

	Naïve	ASY	CL	ML
Mean	0.6078	1.125	75.12	130.4
Median	0.04	0.32	61.27	64.31

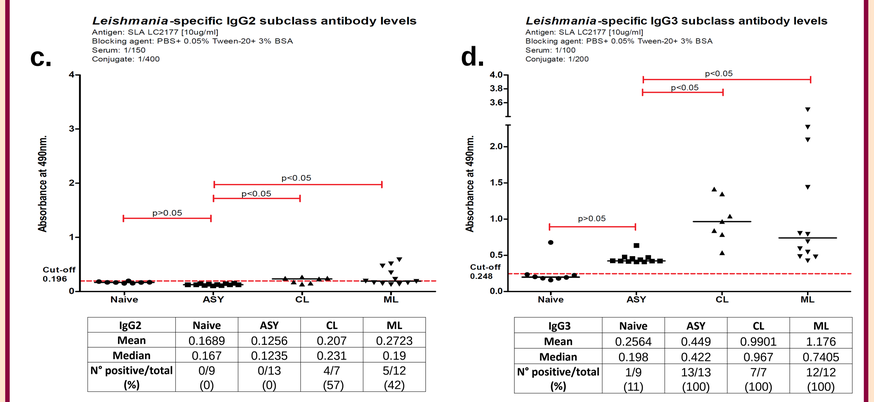
Figure 4: IL-10 production in PBMC culture supernatants (a) and IFN- γ /IL-10 ratio (b) from active CL and ML, ASY and naïve individuals to SLA from *L. braziliensis* . Each point represents a subject. Medians are indicated by horizontal lines

Humoral response



Total IgG	Naive	ASY	CL	ML
Mean	0.1628	0.1855	0.5546	1.004
Median	0.158	0.174	0.562	0.482
N° positive/total (%)	1/9 (11)	3/13 (23)	7/7 (100)	9/12 (75)

IgG1	Naive	ASY	CL	ML
Mean	0.2005	0.2314	1.088	1.501
Median	0.1855	0.195	1.082	0.8825
N° positive/total (%)	1/9 (11)	2/13 (15)	7/7 (100)	10/12 (83)



IgG2	Naive	ASY	CL	ML
Mean	0.1689	0.1256	0.207	0.2723
Median	0.167	0.1235	0.231	0.19
N° positive/total (%)	0/9 (0)	0/13 (0)	4/7 (57)	5/12 (42)

IgG3	Naive	ASY	CL	ML
Mean	0.2564	0.449	0.9901	1.176
Median	0.198	0.422	0.967	0.7405
N° positive/total (%)	1/9 (11)	13/13 (100)	7/7 (100)	12/12 (100)

Figure 5 : Comparison of *Leishmania*-specific total IgG (a), IgG1 (b), IgG2 (c) and IgG3 (d) antibodies in different Leishmaniasis clinical manifestations. Results are expressed as the optical densities at 490 nm. Each point represents a subject. Medians are indicated by horizontal lines. Dashed lines, cutoff optical densities.

CONCLUSIONS

Immune response in ASY was characterized by both, moderate cell proliferative response and production of IFN-γ and TNF-α when compared with CL/ML, despite similar IL-10 production in these groups. Furthermore, it is interesting to note the presence of IgG3 and absence of IgG1 in ASY, whereas both are present in CL/ML patients. This fact might suggest that other factors different than IL-10 could be involved in the modulation of Th1 response in ASY.

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