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In Vitro Evaluation of a Soluble *Leishmania* Promastigote Surface Antigen as a Potential Vaccine Candidate against Human Leishmaniasis

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Abstract

PSA (Promastigote Surface Antigen) belongs to a family of membrane-bound and secreted proteins present in several *Leishmania* (*L.*) species. PSA is recognized by human Th1 cells and provides a high degree of protection in vaccinated mice. We evaluated humoral and cellular immune responses induced by a *L. amazonensis* PSA protein (LaPSA-38S) produced in a *L. tarentolae* expression system. This was done in individuals cured of cutaneous leishmaniasis due to *L. major* (CCLm) or *L. braziliensis* (CCLb) or visceral leishmaniasis due to *L. donovani* (CVLd) and in healthy individuals. Healthy individuals were subdivided into immune (HHR-*Lm* and HHR-*Li*: Healthy High Responders living in an endemic area for *L. major* or *L. infantum* infection) or non immune/naïve individuals (HLR: Healthy Low Responders), depending on whether they produce high or low levels of IFN- γ in response to *Leishmania* soluble antigen. Low levels of total IgG antibodies to LaPSA-38S were detected in sera from the studied groups. Interestingly, LaPSA-38S induced specific and significant levels of IFN- γ , granzyme B and IL-10 in CCLm, HHR-*Lm* and HHR-*Li* groups, with HHR-*Li* group producing TNF- α in more. No significant cytokine response was observed in individuals immune to *L. braziliensis* or *L. donovani* infection. Phenotypic analysis showed a significant increase in CD4⁺ T cells producing IFN- γ after LaPSA-38S stimulation, in CCLm. A high positive correlation was observed between the percentage of IFN- γ -producing CD4⁺ T cells and the released IFN- γ . We showed that the LaPSA-38S protein was able to induce a mixed Th1 and Th2/Treg cytokine response in individuals with immunity to *L. major* or *L. infantum* infection indicating that it may be exploited as a vaccine candidate. We also showed, to our knowledge for the first time, the capacity of *Leishmania* PSA protein to induce granzyme B production in humans with immunity to *L. major* and *L. infantum* infection.

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Introduction

Leishmania (*L.*) are intracellular protozoa that cause a wide spectrum of human diseases, ranging from self-healing cutaneous to lethal visceral leishmaniasis. Cutaneous leishmaniasis (CL) is widely distributed in the Americas, North Africa, the Middle East and Central Asia, causing considerable morbidity [1]. In Tunisia, CL is zoonotic and is mainly due to *L. major* [2]. In Peru, *L. braziliensis* is responsible for nearly all the zoonotic CL cases [3].

Anthroponotic Visceral Leishmaniasis (VL) due to *L. donovani* is mainly distributed in the Indian subcontinent with over 300 000 annual cases [4], whereas zoonotic VL caused by *L. infantum* is present mainly in Mediterranean basin, Middle east, central Asia and Brazil with up to 50 000 annual cases [5–7]. Treatment of leishmaniasis mainly relies on chemotherapy and the control of the disease is challenged by serious side effects of existing drugs and the widespread emergence of drug resistant strains. Several lines of evidence are in favor of the feasibility of a vaccine in humans.

Table 1. Study population.

Country	Human groups*	Number of individuals	Parasite species
India	CVLd	16	<i>L. donovani</i>
	aVLd	25	<i>L. donovani</i>
	HLR-I	19	
Peru	CCLb	8	<i>L. braziliensis</i>
	aCLb	21	<i>L. braziliensis</i>
	HLR-P	10	
Tunisia	CCLm	25	<i>L. major</i>
	HHR-Lm	20	<i>L. major</i>
	HHR-LiT	18	<i>L. infantum</i>
	aVLiT	12	<i>L. infantum</i>
	HLR-T	16	
France	HHR-LiF	8	<i>L. infantum</i>
	HLR-F	28	
Spain	HHR-LiS	9	<i>L. infantum</i>
	aVLiS	4	<i>L. infantum</i>
	HLR-S	9	

The recruitment and sampling collection (186 donors) of different groups (patients, cured, immunes without clinical symptoms and naives) were performed in endemic and non-endemic areas in each country.

*CVLd: Cured Visceral Leishmaniasis due to *L. donovani* (India), aVLd: active Visceral Leishmaniasis due to *L. donovani* (India), HLR-I: Healthy Low Responders from India, CCLb: Cured Cutaneous Leishmaniasis due to *L. braziliensis* (Peru), aCLb: active Cutaneous Leishmaniasis due to *L. braziliensis* (Peru), HLR-P: Healthy Low Responders from Peru, CCLm: Cured Cutaneous Leishmaniasis due to *L. major* (Tunisia), HHR-Lm: Healthy High Responders living in an endemic area for *L. major* (Tunisia), HHR-LiT: Healthy High Responders living in an endemic area for *L. infantum* (Tunisia), aVLiT: active Visceral Leishmaniasis due to *L. infantum* (Tunisia), H-T: Healthy Low Responders from Tunisia, HHR-LiF: Healthy High Responders living in an endemic area for *L. infantum* (France), HLR-F: Healthy Low Responders from France, HHR-LiS: Healthy High Responders living in an endemic area for *L. infantum* (Spain), aVLiS: active Visceral Leishmaniasis due to *L. infantum* (Spain), HLR-S: Healthy Low Responders from Spain.

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antibiotic Nourseothricin (Jena Bioscience, Germany) to create the recombinant pF4X1.4-*La*PSA-38S plasmid. The resulting construct, encoding a full sequence of *La*PSA-38S secreted protein fused to a C-terminus His₆ tag. *L. tarentolae* promastigotes were successfully maintained in continuous culture by successive passages of 5×10^5 flagellates/ml every week into 10 ml of completely chemically defined medium (CDM/LP) free of serum, macromolecules, proteins and cell contaminants as previously described [39,40]. For stable integration of the expression cassette into the 18S ribosomal RNA (ssu) locus, 10 µg of pF4X1.4sat1 plasmid containing *La*PSA-38S gene was digested by *Sma*I restriction enzyme. Transfections of *L. tarentolae* promastigotes were performed by electroporation in 2 mm cuvettes using a Gene Pulser II (Biorad) and a single pulse (5–6 msec) with the settings 450 V and 450 µF. After pulse, cells were transferred into a fresh CDM/LP medium. 100 µg/ml of Nourseothricin was added 24 h after electroporation to select stable transformants. After a week of culture only nourseothricin-resistant cells survived. Transgenic cells were selected as single colonies on the supplemented CDM/LP-agar medium containing 100 µg/ml Nourseothricin. To confirm the integration of the *La*PSA-38S containing cassette into the *Leishmania* genome, PCR was performed on the genomic DNA of wild type and transgenic cells with *ssu* forward primer F3001 and *aprt* reverse primer A1715 (Jena bioscience, Germany). Mass culture and protein purification were manufactured by Virbac in GMP conditions. Culture amplifications were performed on CDM/LP medium. The culture supernatant containing *L. amazonensis* excreted secreted *La*PSA-38S, released by the parasite during its growth, was recovered at the late stationary phase of growth. The only proteins contained in the medium were of

parasitic origin and were found with their native conformation because they were naturally excreted-secreted by *Leishmania* parasites. Excreted secreted PSA was purified from concentrated culture supernatant by Ni-NTA affinity chromatography.

4. Preparation of total soluble Leishmania promastigote antigens (TSLA)

All antigen extracts were prepared from promastigote stationary phase parasite cultures of *L. donovani*, *L. braziliensis*, *L. major* and *L. infantum*. Each consortium partner used local TSLA (*L. major* TSLA in Tunisia, *L. infantum* TSLA in France and Spain, *L. braziliensis* TSLA in Peru and *L. donovani* TSLA in India) and TSLA from *L. donovani* (TSLA Ldd8) (MHOM/IN/80/Ldd8Cl₂ *L. donovani* strain (Ldd8)), as a standard preparation. TSLA were obtained from washed parasites in 1x phosphate-buffered saline (PBS), centrifuged at 1000×g/10 min at 4°C and supernatants were removed. The pellets were resuspended in lysis buffer (50 mM Tris/5 mM EDTA/HCl, pH7. 1 mL/1×10⁹ parasites), subjected to three rapid freeze/thaw cycles followed and to three pulses of 20 seconds/40W with sonicator. Samples were centrifuged at 5000×g for 20 min at 4°C, and supernatants were collected, aliquoted and stored at –80°C until use. Protein quantification was performed using Bradford method.

5. Cell culture and stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from blood by density centrifugation through Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

The cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, 100 IU/mL penicillin, 100 µg/mL strepto-

mycin, 2 mM L-glutamin, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate and 1X amino acid non essential. Briefly, cells were plated in 96 well (TPP, Switzerland) and were kept with media alone (unstimulated) or stimulated with 10 μ g/mL of Phytohemagglutinin (PHA) (Sigma-Aldrich) as a positive control or with TSLA (10 μ g/mL) or with LaPSA-38S (10 μ g/mL), in a 5%CO₂ humidified atmosphere at 37°C for 5 days.

6. Cytometric Bead Array assay (CBA)

IFN- γ , granzyme B, tumor necrosis factor-alpha (TNF- α) and Interleukin-10 (IL-10), were detected and quantified from culture supernatants (50 μ l) of PBMC exposed for 120 h to the following stimuli: PHA-M (10 μ g/ml), TSLA (10 μ g/ml) or LaPSA-38S (10 μ g/ml), using the BD CBA Human Soluble Protein Flex Set system and according to the instructions of the manufacturer (BD Biosciences). It should be noted that granzyme B was not analyzed in individuals immune to *L. braziliensis* infection. In order to quantitate samples, the BD CBA Human Soluble Protein Flex Standard was performed for each cytokine and in each experiment. Data were acquired by flow cytometry (FACSCalibur or FACSCanto) using 2-color detection. Flow Cytometric Analysis Program Array (FCAP Array; BD Biosciences) software was required for analyzing samples [41].

7. Cell phenotyping

Freshly isolated PBMC were stimulated with PMA (50 ng/ml)/ionomycin (10⁻⁶ M) or PHA at 10 μ g/ml for 6 h (positive control) or with TSLA at 10 μ g/ml or with LaPSA-38S at 10 μ g/ml for 120 h and, or kept with medium alone. Cells were treated with GolgiStop (BD Biosciences) for the last 6 hours of culture, then washed and incubated with antibodies: FITC CD3, PerCPcy5.5 CD4, APC-H7 CD8 or PerCPcy5.5 CD8, and PE-Cy7 CD69 or PE CD69 (BD Biosciences), for 20 minutes at 4°C. For intracellular IFN- γ detection, cells were fixed and permeabilized using BD Cytoperm/cytofix plus kit (BD Biosciences) according to manufacturer's instructions and labeled with PE-anti-IFN- γ mAb (intracellular formulation) (BD Biosciences). Analysis was performed with FACSCalibur using CellQuest Pro software or FACS canto II flow cytometer using DIVA software. FITC mouse IgG1, PerCPcy5.5 mouse IgG1, mouse IgG1PE, PE mouse IgG2a (intracellular formulation) (Biosciences) were used as isotype controls for acquisition by FACSCalibur. For acquisition by FACSCanto, BD CompBeads Set Anti-Mouse Ig, κ (Anti-Mouse Ig, κ /Negative Control (FBS) Compensation Particles Set) (BD Biosciences) were used.

8. Detection of antibodies specific for LaPSA-38S

An enzyme-linked immunosorbent assay (ELISA) test was performed to detect specific antibodies to TSLA Ldd8 and LaPSA-38S in individual serum samples from the different studied groups (Table 2). In order to improve antibodies detection, ELISA protocol was optimized by combining antigens concentrations and anti-serum dilutions. Briefly, 96 wells plates (NuncMaxisorp Immuno Plates, PolyLabo, Strasbourg, France) were coated with 100 μ l/well of 2 or 5 μ g/ml LaPSA-38S and TSLA overnight at 4°C. Plates were washed three times with PBS, 0.1% Tween 20 (PBS-T), pH 7.4 and blocked with 200 μ l/well of PBS containing 0.1% Tween 20 and 3% BSA for 1 h at 37°C. After washing with PBS-T, diluted sera (1/100, 1/150 and 1/200 in PBS-T) were added (100 μ l/well) and incubated for 1h30 at 37°C. Plates were then washed with PBS-T and 100 μ l/well of diluted HRP-conjugated anti-human IgG in washing buffer were added for 30 min at 37°C (1/5000). Plates were developed with the 100 μ l/well of Sigma Fast o-phenylene diamine dihydrochloride (OPD) for

Table 2. Frequency of CD4+ and CD8+ T cells after SLA and LaPSA-38S stimulation.

	PMA/iono or PHA			SLA			LaPSA-38S		
	Groups	CCLm	HHR-Lm	HHR-LIS	HHR-S	CCLm	HHR-Lm	HHR-LIS	HHR-S
CD4+T cells	CD4+T	64,4 \pm 5,97	56,75 \pm 6,01	51,2 \pm 5,01	44,8 \pm 10,22	22,9 \pm 5,91	20,6 \pm 6,59	27 \pm 6,00	25,15 \pm 6,88
	CD8+T	25,15 \pm 6,88	27 \pm 6,00	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59
CD8+T cells	CD8+T	25,15 \pm 6,88	27 \pm 6,00	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59
	CD8+T	25,15 \pm 6,88	27 \pm 6,00	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59	20,6 \pm 6,59

Phenotypic analysis of LaPSA-38S and SLA-specific CD4+ and CD8+ was performed for PBMC among CD3+ population, in groups in which we observed a significant and specific LaPSA-38S-induced IFN- γ response (CCLm: n = 7, HHR-Lm: n = 6, HHR-LIS: n = 7). Naive individuals (HHR-S: n = 14) were also analyzed. Data are means percentage \pm SD.
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15 min. The reaction was stopped with 50 µl/well of 2 N HCl. The absorbance was measured at 492 nm. All sera were tested in duplicate/triplicate, and the mean value was recorded.

9. Statistical analysis

Data from all partners were collected into a unique database using the open source software Epidata 3.1 (Epidata Association, Denmark <http://www.epidata.dk/>) and checked for consistency. Individuals with missing data or exclusion criteria were deleted. Data analysis was then performed with Stata statistical software (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP.). We used non-parametric statistical tests because of the low number of individuals in some groups and of the heterogeneity of the standard deviation between groups. Results are expressed as mean ± standard deviation (SD) and a p-value of <0.05 was considered significant in all cases. In the statistical analysis of cytokine level and phenotyping, we used Wilcoxon signed-rank test to compare median levels of cytokines or percentage of cells producing IFN-γ after different stimulations in paired data. Kruskal-Wallis rank test was used for inter-groups analysis on normalized data after deducting the non-stimulated value. Correlations were performed using pairwise correlations of variance test. For humoral response analysis, we used Kruskal-Wallis rank test to compare the median production of total IgG between different stimulations. We estimated a threshold of positivity for each country by calculating a cut-off using the following formula:

$$\tau_{\text{pos}} = \text{mean}(\text{level in naif group}) + 3\text{sd}(\text{level in naif group}).$$

Individuals with a level of IgG after stimulation higher than τ_{pos} were considered as positive responders. For the statistical analysis of sub-classes, results were compared only for positive individuals

Results

1. Production of LaPSA-38S in *L. tarentolae*

In this study, we generated a recombinant *L. tarentolae* strain expressing exogenously the *L. amazonensis* LaPSA-38S gene. The resulting construct encoded a full sequence of LaPSA-38S secreted protein fused to a C-terminus His₆ tag. LaPSA-38S protein purity was assessed by SDS-PAGE using 12% polyacrylamide Ready Gels (Bio-Rad) under reducing and non reducing conditions and after silver staining. Immobilized Ni²⁺ affinity chromatography of culture supernatant containing excreted/secreted extracts from recombinant *L. tarentolae*, yielded a preparation featuring a purified recombinant LaPSA-38S protein migrating as a 45 kDa species in SDS-PAGE (Figure 1). The extra band observed in figure 1 was identified as a dimer form of LaPSA-38S as suggested by a proteomic study of purified LaPSA-38S using a combination of 1D gel electrophoresis and LC-MS/MS (coupling between liquid chromatography and tandem mass spectrometry) (Bras-Gonçalves et al., manuscript in preparation).

2. IFN-γ, granzyme B, TNF-α, and IL-10 levels in response to LaPSA-38S protein in immune individuals

The supernatants of TSA or LaPSA-38S-stimulated PBMC were assayed for IFN-γ, granzyme B, TNF-α and IL-10 production (Figure 2, Figure 3). PBMC were isolated from (i) individuals cured from *L. major* (CCLm), *L. braziliensis* (CCLb) or *L. donovani* (CVLd) infection, (ii) healthy individuals living in endemic areas and showing high IFN-γ response to TSA, considered as

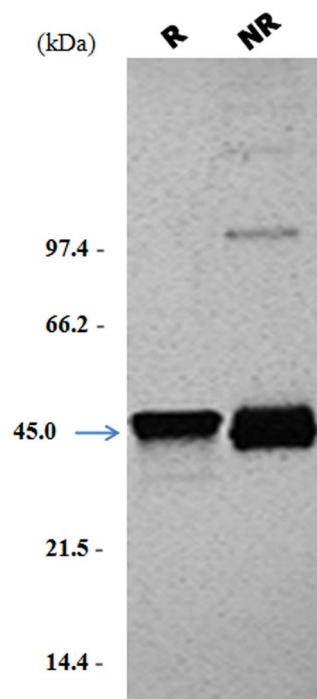


Figure 1. SDS-PAGE and silver staining of purified recombinant La-PSA38S protein expressed in *L. tarentolae*. Purified La-PSA38S was separated by electrophoresis in a 12% SDS-PAGE and silver stained under reducing (R) and non reducing (NR) conditions. The arrow indicates the position of La-PSA-38S (45 kDa) and Molecular Weight markers were marked in kDa.

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immune (HHR-*Lm*: Healthy High Responder living in endemic area to *L. major* infection and HHR-*Li*, HHR living in endemic area to *L. infantum* infection) and (iii) healthy individuals living in low or non-endemic areas and showing low or no IFN-γ response to TSA, considered as non immune/naive (HLR-T: Healthy Low Responders from Tunis; HLR-F from France; HLR-S from Spain; HLR-I from India and HLR-P from Peru). HHR-*Li* individuals were recruited from three different areas (France: HHR-*LiF*; Spain: HHR-*LiS*; Tunisia: HHR-*LiT*). LaPSA-38S induced significant (when compared to non stimulated cultures, $p \leq 0.003$) and specific (when compared to corresponding naive groups, $p \leq 0.01$) levels of IFN-γ in PBMC cultures of CCLm, HHR-*Lm*, HHR-*LiT* and HHR-*LiS* (Mean ± SD: 410.9 ± 913.1 pg/ml; 297.2 ± 306.9 pg/ml; 297.2 ± 423.2 pg/ml; and 127.3 ± 79.1 pg/ml, respectively) (Figure 3a). No significant IFN-γ levels were detected in HHR-*LiF* or in naive groups HLR-T, HLR-F or HLR-S. Low but significant IFN-γ levels were observed in CCLb and CVLd but also in their related naive groups (HLR-P and HLR-I, respectively) (Figure 3a). LaPSA-38S also induced significant and specific ($p \leq 0.04$) granzyme B levels in CCLm (235.7 ± 448.5), HHR-*Lm* (311.7 ± 345.9) and HHR-*LiS* (492.8 ± 451) (Figure 3b). Granzyme B was not observed in naive individuals. The levels of IFN-γ and granzyme B were significantly higher in the supernatants of all immune groups (CCLm: 3027.1 ± 1356.4 and 2852.2 ± 2035.9; CCLb: 740.2 ± 1145.6, granzyme B was not tested; CVLd: 740.1 ± 824.4 and 1511.1 ± 1549.5; HHR-*Lm*: 3182.3 ± 1198.4 and 2608.5 ± 1818.1; HHR-*LiT*: 3311.8 ± 818.3 and 3873.6 ± 3401.8; HHR-*LiF*: 778.8 ± 299.3 and 364.5 ± 377.9; HHR-*LiS*: 2946.3 ± 3773.7 and 7478 ± 5902.6; respectively) compared to naives ($p \leq 0.007$) after

TSLA Ldd8 stimulation (Figure 2a and b). It should be noted that no significant differences were observed in IFN- γ and granzyme B responses between local TSLA and TSLA Ldd8 (Figure 2a and b). Significant TNF- α levels ($p \leq 0.02$) were observed in CCLb (158.5 ± 216.2), CVLd (183.9 ± 271.4) and HHR-LiS (54.7 ± 46), but also in some naive groups (HLR-P: 122.2 ± 72 and HLR-I: 253.7 ± 227.3) after LaPSA-38S stimulation (Figure 3c). TSLA Ldd8 induced significant and specific TNF- α production ($p \leq 0.03$) in the same groups but also in HHR-LiT (CCLb: 149.3 ± 333.5 ; CVLd: 47.2 ± 50.8 ; HHR-LiS: 275.2 ± 152.8 and HHR-LiT: 529.2 ± 359.9) (Figure 3c). PBMC from all immune and naive groups showed significant levels of IL-10 ($p \leq 0.02$) in response to LaPSA-38S (immune groups: CCLm: 218.5 ± 184.3 ; CCLb: 797.8 ± 1110 ; CVLd: 74.1 ± 54.9 ; HHR-Lm: 233.4 ± 241.7 ; HHR-LiT: 104.5 ± 56.2 ; HHR-LiF: 87.9 ± 37.9 ; HHR-LiS: 175.7 ± 244.8 ; naive groups: HLR-T: 73.5 ± 54.2 ; HLR-P: 449.4 ± 432.6 ; HLR-I: 78.8 ± 83.6 ; HLR-F: 71.8 ± 38.3 ; HLR-S: 24.5 ± 17.6) (Figure 3d). IL-10 levels were significantly higher in CCLm, HHR-Lm and HHR-LiT compared to corresponding naive groups (HLR-T) ($p \leq 0.009$). No IL-10 was observed in response to TSLA. No difference was observed in the cytokine levels between immune groups, except for LaPSA-38S-induced granzyme B levels which were significantly higher in HHR-LiS compared to CCLm and HHR-Lm ($p = 0.02$ and 0.04 , respectively).

Interestingly, strong positive correlations were found between IFN- γ and granzyme B production in CCLm group ($r = 0.82$; $p = 0.0000$) and between granzyme B and IL-10 production in HHR-LiS group ($r = 0.7$; $p = 0.03$), in response to LaPSA-38S. IFN- γ and granzyme B productions were also highly correlated in response to TSLA Ldd8 in CCLm and HHR-Li groups ($r = 0.67$; $p = 0.0002$ and $r = 0.76$; $p = 0.01$; respectively).

3. Phenotyping results

Phenotypic analysis of LaPSA-38S and TSLA Ldd8-specific CD4+ and CD8+ cells producing IFN- γ was performed in groups in which we observed a significant LaPSA-38S-induced IFN- γ response (CCLm: $n = 7$, HHR-Lm: $n = 6$, HHR-LiS: $n = 7$) and in naive individuals (HLR-S: $n = 14$) (Figure 4).

We showed a significantly higher percentage of CD4+ T cells producing IFN- γ (mean \pm SD: $0.14 \pm 0.21\%$) among activated CD4+ T cells ($57.4 \pm 13.9\%$), after LaPSA-38S stimulation, only in CCLm individuals, in comparison to non stimulated cultures ($p = 0.049$), (Figure 4, table 2). A strong positive correlation was found between the percentage of IFN- γ -producing CD4+ T cells and IFN- γ production in cell culture supernatants assessed by CBA analysis, in response to LaPSA-38S antigen ($r = 0.97$, $p = 0.0003$). No significant increase was observed in the percentage of IFN- γ -producing CD8+ T cells in tested immune groups, in response to LaPSA-38S, compared to non stimulated cultures (Figure 4). A significant increase was showed in the percentage of IFN- γ -producing CD4+ T cells in CCLm ($0.37 \pm 0.47\%$) and HHR-LiS (0.87 ± 0.74) and of IFN- γ -producing CD8+ T cells in HHR-LiS (0.53 ± 0.4), compared to non stimulated cultures ($p = 0.02$), in response to TSLA Ldd8 stimulation (Figure 4).

4. Humoral response to LaPSA-38S

Anti-LaPSA-38S total IgG responses were analyzed by using ELISA tests in sera from (i) patients with active disease (aVLd, aVLiT, aVLiS, aCLb), (ii) cured individuals (CVLd, CCLm, CCLb), (iii) healthy individuals with high IFN- γ response to TSLA Ldd8 as immune with no clinical symptoms (HHR-Lm, HHR-LiT, HHR-LiF, HHR-LiS) and healthy individuals with low IFN- γ response to TSLA Ldd8 as naive controls (Table 3). The cut-off

value of reactivity for LaPSA-38S antigen was calculated as the mean plus 3 SD of the OD values observed in naive controls from each country. These cut-off values allowed us to distinguish between positive and negative sera and consequently to estimate performance parameters of each ELISA test (Table 3). Globally, the results indicated that LaPSA-38S antigen does not constitute a target for the humoral response in the different studied groups. The percentage of positivity was very low ranging from 0% to 12.5%. However, an exception was observed with the aVLiS group where LaPSA-38S was recognized by 50% of tested sera. In contrast and as expected, highest positivity was observed using TSLA antigen. Best results were obtained with sera from individuals with active VL diseases which 100% react with TSLA Ldd8.

Discussion

Few vaccine candidates have progressed beyond the experimental stage in animal models of leishmaniasis (mice, hamsters). This is consistent with the difficulty in developing a suitable animal model that reproduces the features of the human disease. Direct extrapolation of data from animal models to human disease is often controversial and the protective immune responses controlling experimental infection may not reflect those required to prevent leishmaniasis in endemic areas. The purpose of this study was to evaluate the ability of a PSA antigen to elicit an *ex vivo* cellular immune response in PBMC of non exposed and previously exposed human subjects with and without evidence of protective immunity. The rationale is that antigens that elicit immune responses correlating with protection or cure (e.g. significant IFN- γ production in previously exposed and cured individuals) may be good candidates for prophylactic and/or therapeutic vaccines.

We have previously demonstrated that naturally ESA purified from culture supernatants of *L. infantum* promastigotes formulated with an adjuvant were able to induce a long lasting Th1-mediated protection against experimental and natural canine VL [19,32,37,38]. Interestingly, a native soluble *L. infantum* PSA has been identified as the active constituent of ESA that reproduce the observed protective immunity when used as a vaccine in dogs (unpublished data). PSA is present in both promastigote and amastigote stages and is over-expressed in stationary phase parasites [19,32,33]. It is highly conserved in its N-terminal and C-terminal parts, among *Leishmania* species, which is a requisite for ensuring cross-species protection (unpublished data). PSA isolated from *L. amazonensis* promastigotes as well as recombinant vaccinia viruses expressing this protein have been demonstrated to elicit a protective immune response against infection with *L. amazonensis* in BALB/c mice [28,42]. Moreover, PSA-2 polypeptides purified from *L. major* and plasmid DNA encoding PSA-2, but not recombinant PSA-2 purified from *E. coli*, provided significant protection against *L. major* infection in BALB/c mice [29,43]. Our aim was to identify a human vaccine candidate targeting *Leishmania* species that cause CL and VL. We used a recombinant native *L. amazonensis* PSA (LaPSA-38S), as a potential candidate vaccine. Despite the fact that the amino sequence alignment of LaPSA-38S with PSA proteins from several *Leishmania* species, shows that these proteins are not 100% identical, the N-terminal and C-terminal regions present highly conserved segments. However, the number of Leucine Rich Repeat motifs located in the central region was variable [19]. Nevertheless, these proteins belong to the same unique PSA family according to various well known criteria which constitute the PSA family unique signature [19]. Thus, all PSA might be considered as highly similar regarding their protein primary structure since they correspond to the same architecture

a

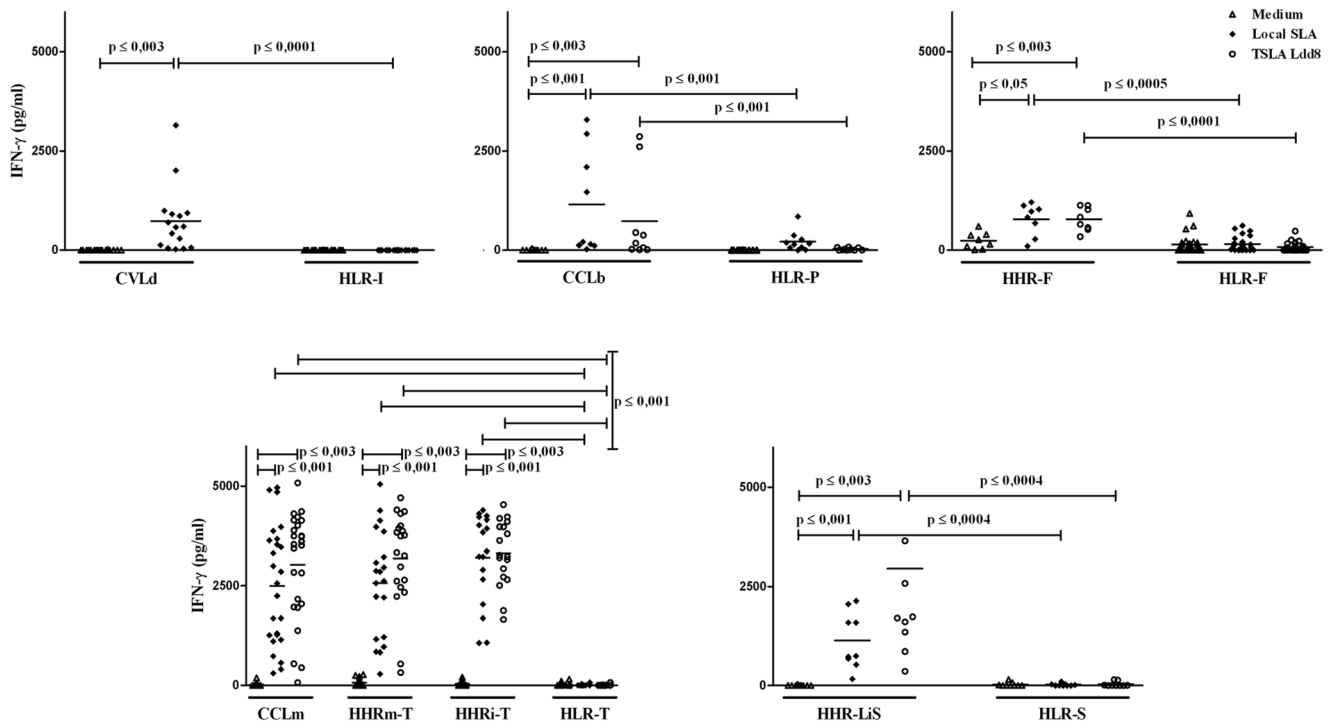
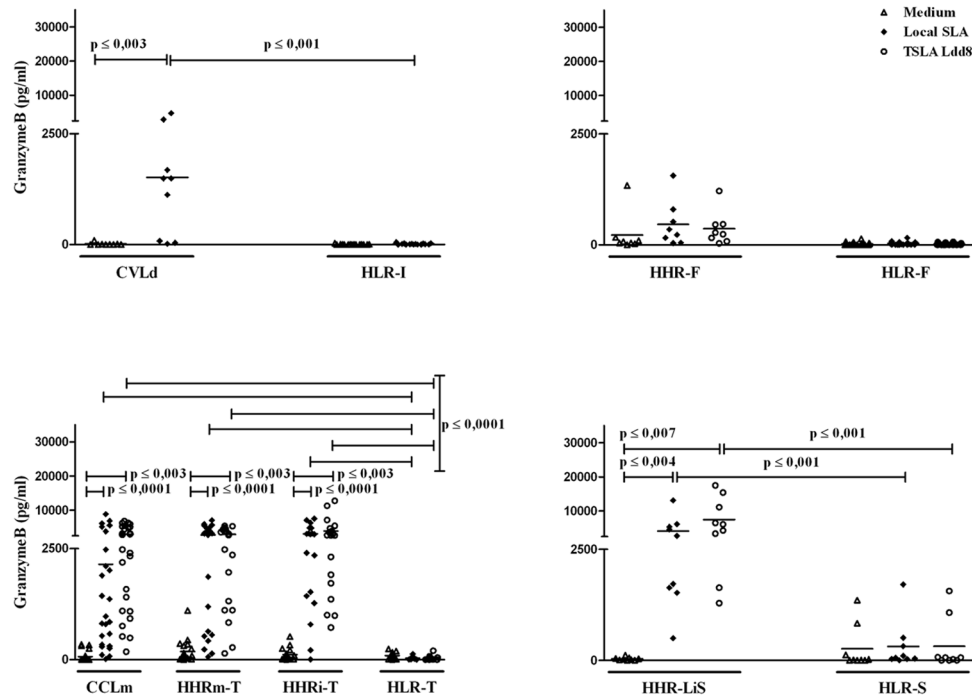
**b**

Figure 2. Total Soluble *Leishmania* antigen (TSLA) specific IFN- γ , granzyme B, TNF- α responses. IFN- γ (Fig. 2a), granzyme B (Fig. 2b), were detected and quantified from culture supernatants of PBMC exposed for 120h to local TSLA (10 μ g/ml) and TSLA Ldd8 (10 μ g/ml), by Cytokine Beads Array test (CBA) using Flow cytometry. Statistically significant differences between stimulated and non stimulated cultures and between groups ($p \leq 0.03$) are showed.
doi:10.1371/journal.pone.0092708.g002

depicted by a precise domain organization which is well conserved among PSA from different *Leishmania* species [19].

LaPSA-38S was produced in a non-pathogenic *L. tarentolae* recombinant expression system. This expression system allows the conservation of *Leishmania*-type post-translational modifications including glycosilation, phosphorylation and disulfide bond formation. Moreover, the *L. tarentolae* expression system, in addition to being non-pathogenic to mammals, is an ideal expression system to produce native proteins of *Leishmania* [44]. A multi-disciplinary consortium has been set-up including groups from Europe (France and Spain for *L. infantum* infection) and from different endemic countries where both VL and CL are highly prevalent (India for *L. donovani*, Peru for *L. braziliensis* and Tunisia for *L. major* and *L. infantum* infections). We demonstrated that LaPSA-38S induced similar significant and specific levels of IFN- γ in individuals immune to *L. major* and *L. infantum* infection. Healthy

high IFN- γ responders had equivalent responses to the cured group. A non specific IFN- γ response to LaPSA-38S stimulation was observed in *L. braziliensis* or *L. donovani* immune individuals. Analysis of the phenotypes of LaPSA-38S-stimulated T cells showed a significant increase in the percentage of IFN- γ producing CD4+ T cells detected only in cured individuals. Furthermore, a strong positive correlation was found between the percentage of IFN- γ -producing CD4+ T cells and the IFN- γ production determined by CBA. Data on the PSA immunogenicity in humans are scarce. One study has shown that PSA from *L. amazonensis* was able to stimulate T cell proliferation in leishmaniasis patients [45]. To our knowledge, only one study reported that PSA from *L. major* was able to induce strong proliferative responses and high IFN- γ and TNF- α levels in individuals with a past history of self-healing CL [30]. Healing from human leishmaniasis is generally associated with an appropriate Th1 response characterized by IFN- γ

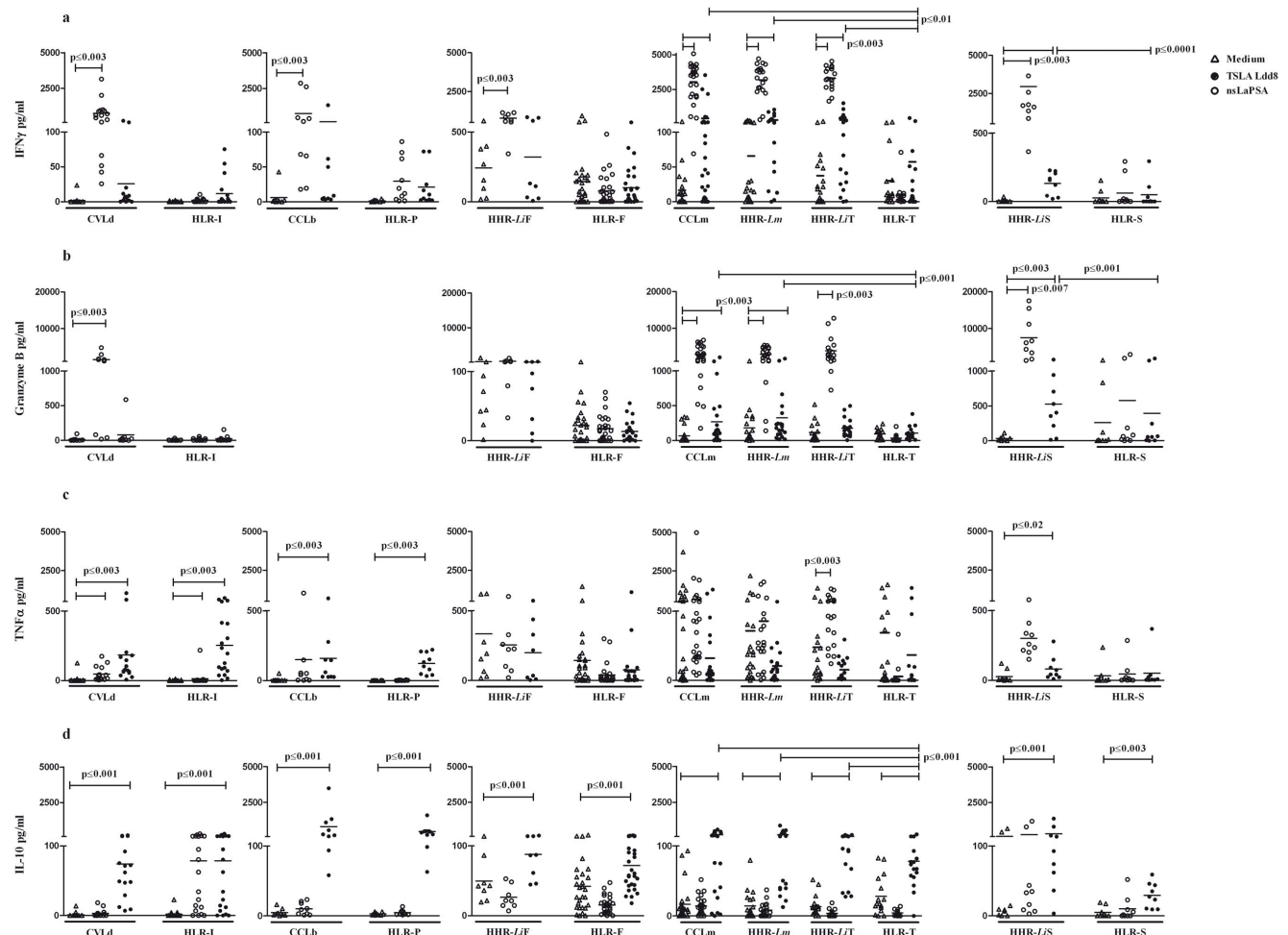


Figure 3. LaPSA-38S specific IFN- γ , granzyme B, TNF- α and IL-10 responses. IFN- γ (Fig. 3a), granzyme B (Fig. 3b), TNF- α (Fig. 3c) and IL-10 (Fig. 3d) were detected and quantified from culture supernatants of PBMC exposed for 120 h to SLA (10 μ g/ml) or LaPSA-38S (10 μ g/ml) using Cytokine Beads Array test (CBA). Data were analyzed by Flow cytometry. PHA (10 μ g/ml) was used for all cultures as positive control (data not shown). Statistically significant differences between stimulated and non stimulated cultures ($p \leq 0.003$) and between groups ($p \leq 0.01$) are showed.
doi:10.1371/journal.pone.0092708.g003

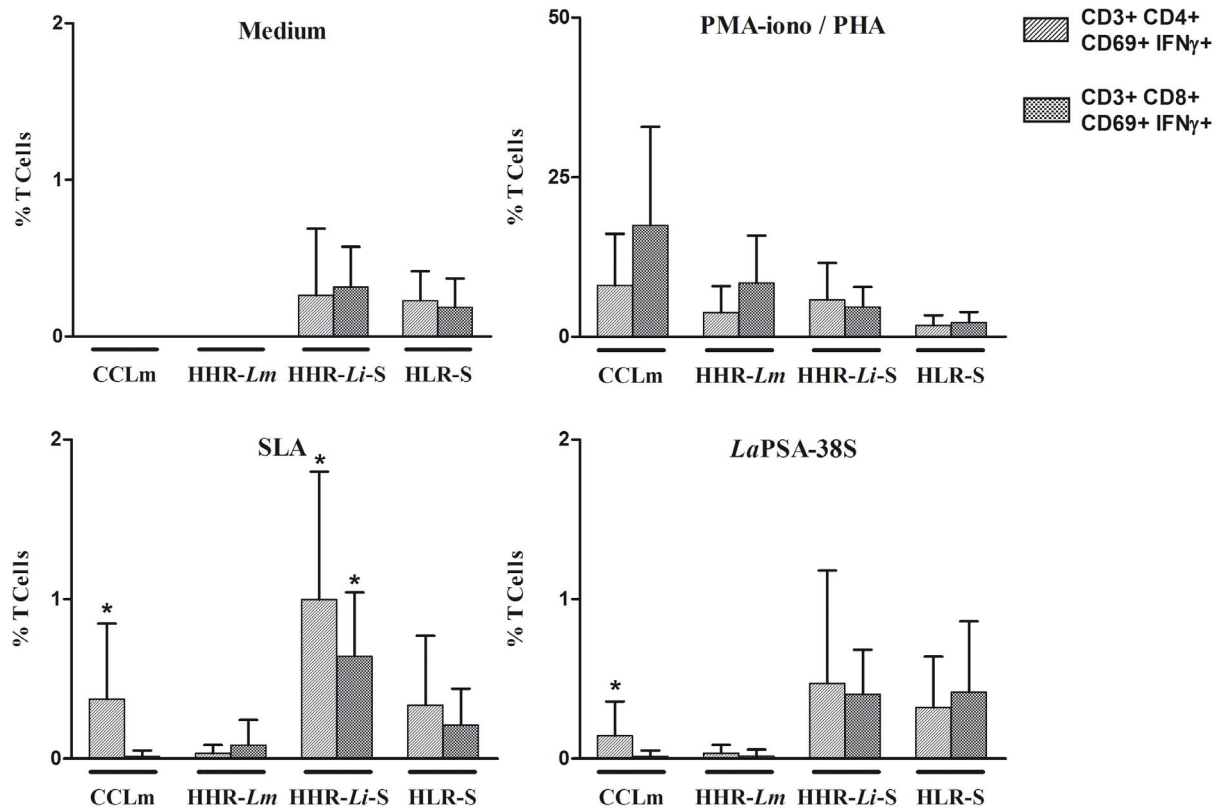


Figure 4. Phenotype of IFN- γ producing cells. PBMC were stimulated with PMA (50 ng/ml)/ionomycin (10^{-6} M) or PHA (10 μ g/ml) for 6 h (positive controls), TSA Ldd8 (10 μ g/ml) or LaPSA-38S (10 μ g/ml) for 120 h. For intracellular IFN- γ detection, cells were treated with Golgistop for the last 6 hours of culture, fixed and permeabilized using BD Cytoperm/cytofix kit, and analyzed by flow cytometry. Results represent the frequency of IFN- γ producing cells within the CD3⁺CD4⁺CD69⁺ and CD3⁺CD8⁺CD69⁺ cell populations. * Statistically significant differences from stimulated and non stimulated cultures ($p < 0,05$).
doi:10.1371/journal.pone.0092708.g004

Table 3. Total IgG prevalence against LaPSA-38S in different endemic areas of leishmaniasis.

Endemic Area	Ig	Group of individuals	Tested sera	Percentage of positive sera (%)	
				SLA	LaPSA
India	Total IgG	CVLd	13	92,3	-
		aVLd	25	100	8
		HLR-I	9	-	-
Peru	Total IgG	CCLb	9	-	-
		aCLb	21	4,8	-
		HLR-P	10	-	-
Tunisia	Total IgG	CCLm	19	68,4	5,3
		HHR-Lm	20	15	5
		HHR-Li	15	40	6,7
		aVLiT	12	100	-
		HLR-T	17	5,9	5,9
France	IgG	HHR-Li F	8	12,5	12,5
		HLR-F	28	-	3,5
Spain	IgG	HHR-Li S	9	-	-
		HLR-S	9	-	11,11
		aVLiS	4	100	25

The cut-off value of reactivity for LaPSA-38S antigen was calculated as the mean plus 3 SD of the OD values observed in naive controls from each country. These cut-off values allowed us to distinguish between positive and negative sera and consequently to estimate performance parameters of each ELISA test.
doi:10.1371/journal.pone.0092708.t003

production. Thus, our finding showing that *LaPSA*-38S stimulates high levels of IFN- γ in individuals with immunity to *L. major* or *L. infantum* infection, suggests that this protein may be associated with protective immunity. With regard to IL-10 production, we showed that, in addition to its Th1 response-inducing activity, *LaPSA*-38S induces significant IL-10 levels in individuals with immunity to *L. major* or *L. infantum* infection, and to a lesser extent in naïve groups. IL-10 is an anti-inflammatory cytokine with broad immune-regulatory functions, produced by different cell populations including dendritic cells, macrophages, Th2 and different regulatory CD4⁺ T subsets [46]. The regulatory effects of IL-10 on immune responses and pathology have been frequently reported [47]. Heterogeneity of CD4⁺ T cell cytokine response has complicated the definition of the immune correlates of vaccine-mediated protection [48]. Recently, the balance between pro-inflammatory IFN- γ /TNF- α and regulatory IL-10 cytokines has been shown to be involved in the outcome of human leishmaniasis and in the prediction of vaccine success [17]. Therefore, the capacity of *LaPSA*-38S to induce IL-10 does not exclude this protein as a potential candidate vaccine. We also detected significant and specific levels of granzyme B in *L. major* and *L. infantum* immune individuals. Very few reports have investigated the involvement of granzyme B in human leishmaniasis. While an increase of granzyme B activity have been associated with a good prognosis in human CL caused by *L. major* or *L. mexicana*, [49,50], CD8⁺ granzyme B⁺ T cells have been reported to mediate tissue injury in CL caused by *L. braziliensis* [51]. Granzyme B is a serine protease expressed in human T CD8⁺ and T CD4⁺ cells as well as in Treg cells [52]. It has been first implicated in apoptosis of target cells [53]. Recently, several studies have highlighted a new inflammatory role of granzyme B through the regulation of cytokine expression and processing and regulatory T cells activity [53–56]. To our knowledge, this is the first description of a *Leishmania* antigen-induced granzyme B response in humans immune to *Leishmania* infection. The increase of granzyme B, in response to a heterologous prime boost vaccination of mice using the LACK p36 antigen suggests its contribution to the disease control [57]. Our data also show strong positive correlations between granzyme B and IFN- γ levels in response to *LaPSA*-38S, in individuals with cured *L. major* CL, which could be associated with protective immunity.

We also showed in our study that *LaPSA*-38S induced a mixed Th1 and Th2/Treg cytokine profile in individuals with immunity to *L. major* and *L. infantum* infection but not in individuals with immunity to *L. braziliensis* or *L. donovani*. Data on conserved *Leishmania* antigens enabling cross-species protection were reported in murine models [58–60]. However, absence of cross-species protection was more frequently described [61–63]. The absence of cross-protection between *Leishmania* species could be explained by the biological differences between *Leishmania* species and by the level of sequence homology of the *Leishmania* proteins used. Indeed, while the *L. major* and *L. infantum* proteins-coding sequences are conserved, the new world *L. braziliensis* proteins-coding sequences are more divergent [64]. Finally, humoral responses analysis results clearly show that *LaPSA*-38S antigen did not induce a significant antibody response in individuals that display different clinical manifestations and forms of leishmaniasis. These results

are in accordance with other studies describing reactivities of human VL sera to PSA antigen [32]. However, in our case, we cannot exclude that this low recognition may be due to the amino-acids sequence divergence between *LaPSA*-38S (used in our experiment) and PSA from *L. major*, *L. infantum* and *L. donovani*. In conclusion, we have demonstrated here that, in spite of differences in disease forms induced by *L. major* and *L. infantum*, the *LaPSA*-38S protein was able to induce a similar Th1 and Th2/Treg profile in individuals immune to infection by these two species. To our knowledge, this is the first description of PSA-induced IFN- γ , granzyme B, TNF- α and IL-10 response in humans with immunity against *L. major* and *L. infantum* infection. The capacity of *LaPSA*-38S to induce mixed Th1 and Th2/T regulatory responses is an argument for the use of this protein as a potential candidate vaccine. Our approach to investigate the ability for a given antigen, to elicit cellular protective responses in human populations from different endemic areas may help to select vaccine candidates and to prioritize antigens for clinical development of a subunit vaccine against leishmaniasis.

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References

- Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, et al. (2012) Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7: e35671.
- Salah AB, Kamarianakis Y, Chlif S, Alaya NB, Prastacos P (2007) Zoonotic cutaneous leishmaniasis in central Tunisia: spatio temporal dynamics. *Int J Epidemiol* 36: 991–1000.
- Lucas CM, Franke ED, Cachay MI, Tejada A, Cruz ME, et al. (1998) Geographic distribution and clinical description of leishmaniasis cases in Peru. *Am J Trop Med Hyg* 59: 312–317.
- Stauch A, Sarkar RR, Picado A, Ostyn B, Sundar S, et al. (2011) Visceral leishmaniasis in the Indian subcontinent: modelling epidemiology and control. *PLoS Negl Trop Dis* 5: e1405.

5. Aoun K, Jeddi F, Amri F, Ghrab J, Bouratbine A (2009) [Current epidemiological data on visceral leishmaniasis in Tunisia]. *Med Mal Infect* 39: 775–779.
6. den Boer M, Argaw D, Jannin J, Alvar J (2011) Leishmaniasis impact and treatment access. *Clin Microbiol Infect* 17: 1471–1477.
7. Marty P, Pomares C, Michel G, Delaunay P, Ferrua B, et al. (2011) [Mediterranean visceral leishmaniasis]. *Bull Acad Natl Med* 195: 181–188.
8. Sacks D, Noben-Trauth N (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol* 2: 845–858.
9. Mansueto P, Vitale G, Di Lorenzo G, Rini GB, Mansueto S, et al. (2007) Immunopathology of leishmaniasis: an update. *Int J Immunopathol Pharmacol* 20: 435–445.
10. Nylen S, Gautam S (2010) Immunological perspectives of leishmaniasis. *J Glob Infect Dis* 2: 135–146.
11. Carvalho EM, Correia Filho D, Bacellar O, Almeida RP, Lessa H, et al. (1995) Characterization of the immune response in subjects with self-healing cutaneous leishmaniasis. *Am J Trop Med Hyg* 53: 273–277.
12. Kharazmi A, Kemp K, Ismail A, Gasim S, Gaafar A, et al. (1999) T-cell response in human leishmaniasis. *Immunol Lett* 65: 105–108.
13. Sassi A, Louzir H, Ben Salah A, Mokni M, Ben Osman A, et al. (1999) Leishmanin skin test lymphoproliferative responses and cytokine production after symptomatic or asymptomatic *Leishmania major* infection in Tunisia. *Clin Exp Immunol* 116: 127–132.
14. Ajdary S, Alimohammadian MH, Eslami MB, Kemp K, Kharazmi A (2000) Comparison of the immune profile of nonhealing cutaneous Leishmaniasis patients with those with active lesions and those who have recovered from infection. *Infect Immun* 68: 1760–1764.
15. Coler RN, Goto Y, Bogatzki L, Raman V, Reed SG (2007) Leish-111f, a recombinant polypeptide vaccine that protects against visceral Leishmaniasis by elicitation of CD4⁺ T cells. *Infect Immun* 75: 4648–4654.
16. Kushawaha PK, Gupta R, Sundar S, Sahasrabudhe AA, Dube A (2011) Elongation factor-2, a Th1 stimulatory protein of *Leishmania donovani*, generates strong IFN- γ and IL-12 response in cured *Leishmania*-infected patients/hamsters and protects hamsters against *Leishmania* challenge. *J Immunol* 187: 6417–6427.
17. Singh OP, Stober CB, Singh AK, Blackwell JM, Sundar S (2012) Cytokine responses to novel antigens in an Indian population living in an area endemic for visceral leishmaniasis. *PLoS Negl Trop Dis* 6: e1874.
18. Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. *J Immunol* 172: 2731–2738.
19. Devault A, Banuls AL (2008) The promastigote surface antigen gene family of the *Leishmania* parasite: differential evolution by positive selection and recombination. *BMC Evol Biol* 8: 292.
20. Murray PJ, Spithill TW, Handman E (1989) The PSA-2 glycoprotein complex of *Leishmania major* is a glycosylphosphatidylinositol-linked promastigote surface antigen. *J Immunol* 143: 4221–4226.
21. Lohman KL, Langer PJ, McMahon-Pratt D (1990) Molecular cloning and characterization of the immunologically protective surface glycoprotein GP46/M-2 of *Leishmania amazonensis*. *Proc Natl Acad Sci U S A* 87: 8393–8397.
22. Hanekamp T, Langer PJ (1991) Molecular karyotype and chromosomal localization of genes encoding two major surface glycoproteins, gp63 and gp46/M2, hsp70, and beta-tubulin in cloned strains of several *Leishmania* species. *Mol Biochem Parasitol* 48: 27–37.
23. Murray PJ, Spithill TW (1991) Variants of a *Leishmania* surface antigen derived from a multigenic family. *J Biol Chem* 266: 24477–24484.
24. Symons FM, Murray PJ, Ji H, Simpson RJ, Osborn AH, et al. (1994) Characterization of a polymorphic family of integral membrane proteins in promastigotes of different *Leishmania* species. *Mol Biochem Parasitol* 67: 103–113.
25. Beetham JK, Myung KS, McCoy JJ, Wilson ME, Donelson JE (1997) Glycoprotein 46 mRNA abundance is post-transcriptionally regulated during development of *Leishmania chagasi* promastigotes to an infectious form. *J Biol Chem* 272: 17360–17366.
26. Jimenez-Ruiz A, Boceta C, Bonay P, Requena JM, Alonso C (1998) Cloning, sequencing, and expression of the PSA genes from *Leishmania infantum*. *Eur J Biochem* 251: 389–397.
27. Kedzierski L, Montgomery J, Bullen D, Curtis J, Gardiner E, et al. (2004) A leucine-rich repeat motif of *Leishmania* parasite surface antigen 2 binds to macrophages through the complement receptor 3. *J Immunol* 172: 4902–4906.
28. McMahon-Pratt D, Rodriguez D, Rodriguez JR, Zhang Y, Manson K, et al. (1993) Recombinant vaccinia viruses expressing GP46/M-2 protect against *Leishmania* infection. *Infect Immun* 61: 3351–3359.
29. Handman E, Symons FM, Baldwin TM, Curtis JM, Scheerlinck JP (1995) Protective vaccination with promastigote surface antigen 2 from *Leishmania major* is mediated by a TH1 type of immune response. *Infect Immun* 63: 4261–4267.
30. Kemp M, Handman E, Kemp K, Ismail A, Mustafa MD, et al. (1998) The *Leishmania* promastigote surface antigen-2 (PSA-2) is specifically recognised by Th1 cells in humans with naturally acquired immunity to *L. major*. *FEMS Immunol Med Microbiol* 20: 209–218.
31. Boceta C, Alonso C, Jimenez-Ruiz A (2000) Leucine rich repeats are the main epitopes in *Leishmania infantum* PSA during canine and human visceral leishmaniasis. *Parasite Immunol* 22: 55–62.
32. Beetham JK, Donelson JE, Dahlin RR (2003) Surface glycoprotein PSA (GP46) expression during short- and long-term culture of *Leishmania chagasi*. *Mol Biochem Parasitol* 131: 109–117.
33. Handman E, Osborn AH, Symons F, van Driel R, Cappai R (1995) The *Leishmania* promastigote surface antigen 2 complex is differentially expressed during the parasite life cycle. *Mol Biochem Parasitol* 74: 189–200.
34. McMahon-Pratt D, Traub-Cseko Y, Lohman KL, Rogers DD, Beverley SM (1992) Loss of the GP46/M-2 surface membrane glycoprotein gene family in the *Leishmania braziliensis* complex. *Mol Biochem Parasitol* 50: 151–160.
35. Myung KS, Beetham JK, Wilson ME, Donelson JE (2002) Comparison of the post-transcriptional regulation of the mRNAs for the surface proteins PSA (GP46) and MSP (GP63) of *Leishmania chagasi*. *J Biol Chem* 277: 16489–16497.
36. Lincoln LM, Ozaki M, Donelson JE, Beetham JK (2004) Genetic complementation of *Leishmania* deficient in PSA (GP46) restores their resistance to lysis by complement. *Mol Biochem Parasitol* 137: 185–189.
37. Lemesre JL, Holzmüller P, Cavaleyra M, Gonçalves RB, Hottin G, et al. (2005) Protection against experimental visceral leishmaniasis infection in dogs immunized with purified excreted secreted antigens of *Leishmania infantum* promastigotes. *Vaccine* 23: 2825–2840.
38. Lemesre JL, Holzmüller P, Gonçalves RB, Bourdoiseau G, Hugnet C, et al. (2007) Long-lasting protection against canine visceral leishmaniasis using the LiESAP-MDP vaccine in endemic areas of France: double-blind randomised efficacy field trial. *Vaccine* 25: 4223–4234.
39. Lemesre JL (1994) Methods for the culture in vitro of different stages of tissue parasites. International publication n° WO94/226899. World Intellectual Property Organization.
40. Merlen T, Sereno D, Brajon N, Rostand F, Lemesre JL (1999) *Leishmania* spp: completely defined medium without serum and macromolecules (CDM/LP) for the continuous in vitro cultivation of infective promastigote forms. *Am J Trop Med Hyg* 60: 41–50.
41. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, et al. (2004) Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clin Immunol* 110: 252–266.
42. Champis J, McMahon-Pratt D (1988) Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. *Infect Immun* 56: 3272–3279.
43. Sjolander A, Baldwin TM, Curtis JM, Handman E (1998) Induction of a Th1 immune response and simultaneous lack of activation of a Th2 response are required for generation of immunity to leishmaniasis. *J Immunol* 160: 3949–3957.
44. Breidling R, Klingner S, Callewaert N, Pietrucha R, Geyer A, et al. (2002) Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. *Protein Expr Purif* 25: 209–218.
45. Burns JM Jr, Scott JM, Carvalho EM, Russo DM, March CJ, et al. (1991) Characterization of a membrane antigen of *Leishmania amazonensis* that stimulates human immune responses. *J Immunol* 146: 742–748.
46. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR (1993) Interleukin-10. *Annu Rev Immunol* 11: 165–190.
47. Jankovic D, Kugler DG, Sher A (2010) IL-10 production by CD4⁺ effector T cells: a mechanism for self-regulation. *Mucosal Immunol* 3: 239–246.
48. Alexander J, Brombacher F (2012) T helper1/t helper2 cells and resistance/susceptibility to leishmania infection: is this paradigm still relevant? *Front Immunol* 3: 80.
49. Boussofara T, Louzir H, Ben Salah A, Dellagi K (2004) Analysis of granzyme B activity as a surrogate marker of *Leishmania*-specific cell-mediated cytotoxicity in zoonotic cutaneous leishmaniasis. *J Infect Dis* 189: 1265–1273.
50. Hernandez-Ruiz J, Salaiza-Suazo N, Carrada G, Escoto S, Ruiz-Remigio A, et al. (2010) CD8 cells of patients with diffuse cutaneous leishmaniasis display functional exhaustion: the latter is reversed, in vitro, by TLR2 agonists. *PLoS Negl Trop Dis* 4: e871.
51. Santos CS, Boaventura V, Ribeiro Cardoso C, Tavares N, Lordelo MJ, et al. (2013) CD8(+) Granzyme B(+) Mediated Tissue Injury vs. CD4(+)IFN γ (+) Mediated Parasite Killing in Human Cutaneous Leishmaniasis. *J Invest Dermatol* 133: 1533–1540.
52. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, et al. (2004) Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* 104: 2840–2848.
53. Vignali DA, Collison LW, Workman CJ (2008) How regulatory T cells work. *Nat Rev Immunol* 8: 523–532.
54. Afonina IS, Tynan GA, Logue SE, Cullen SP, Bots M, et al. (2011) Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of IL- α . *Mol Cell* 44: 265–278.
55. Hiebert PR, Granville DJ (2012) Granzyme B in injury, inflammation, and repair. *Trends Mol Med* 18: 732–741.
56. Magnani CF, Alberigo G, Bacchetta R, Serafini G, Andreani M, et al. (2011) Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *Eur J Immunol* 41: 1652–1662.
57. Dondji B, Deak E, Goldsmith-Pestana K, Perez-Jimenez E, Esteban M, et al. (2008) Intradermal NKT cell activation during DNA priming in heterologous prime-boost vaccination enhances T cell responses and protection against *Leishmania*. *Eur J Immunol* 38: 706–719.
58. Aguilar-Be I, da Silva Zardo R, Paraguai de Souza E, Borja-Cabrera GP, Rosado-Vallado M, et al. (2005) Cross-protective efficacy of a prophylactic

- Leishmania donovani* DNA vaccine against visceral and cutaneous murine leishmaniasis. *Infect Immun* 73: 812–819.
59. Goto Y, Bhatia A, Raman VS, Vidal SE, Bertholet S, et al. (2009) *Leishmania infantum* sterol 24-c-methyltransferase formulated with MPL-SE induces cross-protection against *L. major* infection. *Vaccine* 27: 2884–2890.
 60. Ramirez L, Santos DM, Souza AP, Coelho EA, Barral A, et al. (2013) Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis. *Vaccine* 31: 1312–1319.
 61. Melby PC, Yang J, Zhao W, Perez LE, Cheng J (2001) *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. *Infect Immun* 69: 4719–4725.
 62. Salay G, Dorta ML, Santos NM, Mortara RA, Brodskyn C, et al. (2007) Testing of four *Leishmania* vaccine candidates in a mouse model of infection with *Leishmania* (*Viannia*) *braziliensis*, the main causative agent of cutaneous leishmaniasis in the New World. *Clin Vaccine Immunol* 14: 1173–1181.
 63. Tonui WK, Titus RG (2007) Cross-protection against *Leishmania donovani* but not *L. Braziliensis* caused by vaccination with *L. Major* soluble promastigote exogenous antigens in BALB/c mice. *Am J Trop Med Hyg* 76: 579–584.
 64. Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, et al. (2007) Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nat Genet* 39: 839–847.