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RESEARCH ARTICLE

Leishmania donovani promastigote phosphoproteins stimulate helper T-cells and macrophage functions for parasite killing

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Abstract

Visceral Leishmaniasis involves invasion of macrophages by infective *Leishmania* promastigotes. *Leishmania* causes severe suppression of cell signaling components, expression of MHC Class II molecules, TNF-alpha (which is critical in inducing anti-leishmanial response), interleukin-12 (the major Th1 polarizing factor), and IFN-γ. Moreover, a dysfunctional signaling mechanism makes the infected macrophages unresponsive to the T-cell derived activation factors. Nitric oxide (NO), a crucial leishmanicidal component of macrophages is also down-regulated. The present study was undertaken with an aim to identify components of *L. donovani* that could induce a potentially protective immune response. Four phosphoprotein components of *L. donovani*, namely pp63, pp41, pp39 and pp29 were used to restimulate peripheral blood mononuclear cells (PBMNCs) from VL patients. Their respective impacts on HLA-DR expression, T-cell population, cytokine response and NO production were studied using flow cytometry and ELISA techniques. The study identified a few components of *L. donovani* promastigote which have a potential to induce and activate a Th1 response. The pp63 had a remarkable impact on HLA-DR and IFN-γ expression. It also stimulated NO production to a considerable extent. The pp41 fraction enhanced Th1 cytokine production and TNF-alpha expression. The most promising candidate to elicit a wholesome anti-*Leishmania* response was pp29 that up-regulated IFN-γ production, decreased IL-4 expression and potentially induced macrophages for TNF-α and NO production.

Introduction

Leishmaniases include a wide spectrum of diseases caused by the intracellular parasite of genus *Leishmania*.[1-4] Visceral leishmaniasis (VL) or kala-azar (KA) is a disease of the reticuloendothelial system caused by *L. donovani, L. chagasi or L. infantum*. It is characterized by fever, hepatosplenomegaly, peripheral blood cytopenias and progressive wasting. VL may lead to death if untreated [5, 6]. In endemic areas subclinical infection with *L. donovani* has also been reported [5, 6, 7].The infective flagellated promastigotes of *Leishmania* invade the macrophages, convert into aflagellar amastigotes and proliferate within the macrophages causing progressive infection [8]. To ensure survival *Leishmania* is known to down-regulate macrophage activation. This involves severe downregulation of cell signaling components such as Ca^{2+} -dependent PKC, JAK/STAT, MAPK and p38 among others [9]. Expression of MHC Class II molecules is severely inhibited [9-16], causing defective antigen presentation [17]. Tumor necrosis factor alpha (TNF-α), which is critical in inducing anti-leishmanial response, is suppressed. The production of interleukin-12 (IL-12) is reported to be inhibited both *in vitro* [11, 14, 18] and *in vivo* [13, 19] upon infection with metacyclic promastigotes of *Leishmania*. Neutralization of IL-12 leads to sustained suppression of interferon gamma (IFN-γ) production from helper T cells (Th-cells) causing serious exacerbation of infection [20, 21]. Moreover, a dysfunctional signaling mechanism makes the infected macrophages unresponsive to the T-cell derived activation factors [22]. Nitric oxide (NO), a crucial

leishmanicidal component of macrophages, is also reported to be down-regulated in leishmaniasis [14, 23].

Immunity in patients with active VL is characterized by strong humoral response and there is little evidence of a specific cellular immunity [5, 7, 24, 25, 26]. Antibodies, however, appear to play no role in protection and in fact are often associated with non-healing forms of the disease. There is strong clinical and experimental evidence that resolution of leishmanial infection depends primarily upon cell-mediated immunity [27-37]. The proinflammatory cytokines, IL-12 and IFN-γ, secreted by Th-1 CD4+ cells are associated with resolution of disease [36, 38-45]. On the other hand, a Th2-mediated immune response, characterized by the production of IL-4, IL-5, IL-10 and others is usually associated with progression of the disease [40, 46]. Resolution of disease involves activation of macrophages by IFN-γ and generation of reactive oxygen products and nitric oxide (NO). A central role for NO in disease resolution has been shown in several studies [47, 48, 49, 50]. The ability of IFN-γ primed macrophages to kill parasites is dependent upon TNF-α and that the parasites have been reported to induce TNF-α production [51].

However, little is known about the parasite antigens involved in eliciting such a response. Development of protective strategies against leishmaniasis is dependent upon identification of such components of the parasite that may mediate activation and induction of a protective response.

The present study identifies few such component of *Leishmania donovani* promastigote that show potential to induce a protective immune response.

Material and Methods

Source of leishmanial antigen:

L. donovani parasites were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO) supplemented with 20% heat inactivated fetal calf serum (GIBCO Life Technology, India), 20mM L-Glutamine, 100 units/ml of penicillin and 50 µg/ ml of gentamycin at pH 7.4. Soluble Leishmania antigen (SLA) was prepared from late log phase promastigotes after few passages in liquid culture. Briefly, 200 x 10^6 promastigotes per ml were washed thrice in 5 ml of cold sterile PBS. After 5 cycles of freeze and thaw, the suspension was centrifuged at 10,000xg for 20 min. at 4°C and the supernatant containing soluble *Leishmania* antigens were collected and stored at -70°C till further use. Protein concentration was measured by Lowry's method. 63kDa, 41kDa, 39kDa and 29kDa phosphoprotein antigens (pp63, pp41, pp39 and pp29 respectively) used in the study were derived from *L. donovani* promastigotes (MHOM/IN/80/Dd8). Briefly, cultured promastigotes were harvested in the late log phase (6-7 days), washed thrice in cold phosphate buffered saline, pH 7.2-7.4 (PBS) and lysed by boiling for 5 min. in sample buffer. Antigen extract thus prepared were subjected to SDS-PAGE using 12.5% resolution under reducing conditions.[52] Gel slices containing the respective antigenic proteins were then placed into separate micro centrifuge tubes, grinded and incubated in extraction buffer. Gel slurry were transferred to Nanosep MF device that contained Bio-inert modified nylon with polypropylene filtrate receiver and spun. Lower chambers containing the respective proteins in buffer were collected, while upper chambers of gel were rinsed and spun again to get any unseparated specific protein left in the process. 500µl samples of 100µg/ml protein solutions containing pp63, pp41, pp39 and pp29 respectively were centrifuged at 5000xg in Nanosep device to a final volume of 50 µl. resulting retentates were checked for size and concentrated samples were recovered in two washes of 20 µl de-ionized water for use into downstream application at later stages in the study.

Subjects:

A total of 25 subjects (aged between 17 to 40 years) of both genders were studied after obtaining their informed consent. Measurement of body temperature, body weight, liver and spleen size, total and differential WBC count, haemoglobin, blood sugar, serum creatinine and prothrombin, ECG and chest X-rays were performed in all cases. All subjects presented characteristic signs and symptoms of VL infection and diagnosis was confirmed by presence of *L. donovani* in the Giemsa stained spleen aspirate and positive serology (DAT). The serological result was further confirmed by PCR. All the healthy controls were negative for all disease symptoms and matched the diseased cases by gender and age (± 2 years). Any subject who had been treated for VL in the past were excluded from the study.

Immunological evaluation of the T-lymphocyte reactivity to the leishmanial antigen was carried out before commencement of treatment. These evaluations included T-cell subpopulation analysis (CD4 versus CD8), which was later corroborated with cytokine level (IFN-γ and IL-4) in culture supernatants after *Leishmania* specific stimulation with whole *L. donovani* SLA or with SDS PAGE fractionated *Leishmania* antigens (pp63, pp41, pp39 and pp29). Blank controls were included in all experiments that were not given any specific stimulation.

Lymphocyte isolation and *in vitro* cultures:

Peripheral Blood Mononuclear Cells (PBMNCs) were isolated from each of the blood samples by centrifugation through Ficoll Paque™ (Emerson Biosciences, Sweden) gradients. The crude isolates of PBMNCs were purified by washing in Phosphate Buffer Saline (PBS, Bangalore Genei, India) and then centrifuged (800xg, 15 min.) over Histopaque. The PBMNC collected from the layer immediately above

Histopaque were washed thrice in PBS before being used further. All cultures were carried out using RPMI 1640 supplemented with 2mM L-glutamine, 10% heat inactivated LPS/endotoxin free Fetal Calf Serum (GIBCO, Life Technology, India), 5 x 10^5 mM 2-mercaptoethanol and antibiotics (100U/ml Penicillin, 50 µg/ml Gentamycin and 100 µg/ml Streptomycin) (Himedia Laboratories Pvt. Ltd., India).

For isolation of macrophages the PBMNCs were cultured for 48-72 hours at 5% CO_2 to allow transformation of monocytes into macrophages and their subsequent adherence to the culture vessel walls. Following plate adherence, lymphocytes (non-adherent cells in supernatant) and macrophages (adherent cells) were separated. RPMI-1640 (pre-warmed to 37°C) was added to the adherent cells and these were scraped using a cell scraper. Ice cold PBS (pH 7.4) was added to the cell suspension followed by centrifugation, at 400xg, 10 mins. The macrophage count was adjusted to 10 x 10^6 cells/ml using complete RPMI-1640 medium supplemented with 10-15% FCS.

Immunofluorescent staining to determine expression of M H C Class II molecules and TNF-α after antigen stimulation:

Macrophages from VL patients were incubated with fluorescein isothiocyanate (FITC) labeled HLA-DR antibody for 20 minutes at 4°C for *ex vivo* staining of macrophages for surface MHC Class II molecules. The antibodies used were anti-CD69-PE and anti-HLA-DR-FITC. After staining the preparations were washed with 0.09% sodium azide PBS, fixed with 200µl of 2% formaldehyde in PBS and kept at 4°C until data were acquired using a fluorescence-activated cell sorter (FACS). For the evaluation of the impact of antigen specific stimulation on HLA-DR expression cells were pulsed with *L. donovani* parasite (50:1 ratio) and cultured in the presence of 20µg of the phosphoproteins antigens. Unstimulated control cultures were set up in parallel to obtain the background data in the absence of any particular stimulation. All cultures were maintained at 37°C in a water saturated air atmosphere containing 5% CO_2 in air. Cultured cells were later stained with the labeled antibodies as indicated before. Macrophages were analysed for the expression of intracellular TNF-α as described below. Briefly, about 2 x 10^6 cells were cultured in 24-well plates in 1 ml cultures for 24 hours with either medium alone (negative control) or the specific phosphoproteins (20 µg/ml). Brefeldin A (1 µg/ml) was added during the last 4 hours of culture to impair protein secretion by the golgi complex. The cells were later harvested using ice-cold PBS plus azide and fixed with 2% formaldehyde. The fixed cells were permeabilized with a solution of saponin (0.1%) and stained using anti-TNF- α-FITC antibodies by incubating with the antibody solution for 30 min at 4°C. Isotype control panels were run in parallel to all experiments. Unbound antibodies were removed by washing the cells twice with wash buffer before each sample was resuspended in 450µl stain buffer containing 1% paraformaldehyde for examination of fluorescence in a FACS Calibur flow cytometer.

Immunofluorescent staining to determine intracellular Th1/ Th2 cytokines expression after antigen stimulation:

PBMNCs were analyzed for the expression of intracellular cytokines as described below. Briefly, about 2.0x10^6 cells were cultured in 24- well plates in 1 ml cultures for 24 h with either medium alone, or with the different *L. donovani* phosphoprotein antigens (at 20 µg/ml final concentration). After stimulation period of two hours all cultures, except the control, were pulsed with *Leishmania donovani*. Brefeldin-A (1µg/ml) was added to culture during the last 4-hrs of culture to impair protein secretion by the golgi complex. The cells were later harvested using ice cold PBS plus azide, stained for CD4 surface markers using anti-CD4-PE antibodies and fixed with 2% formaldehyde. The fixed cells were permeabilized with a solution of saponin and stained for IFN-γ using FITC-labeled antibodies. Isotype control panels (FITC and PE-labeled immunoglobulin control antibodies were run in parallel to all experiments. Unbound antibodies were removed by washing the cells twice with wash buffer before each sample was re-suspended in 450 µl stain buffer containing 1% paraformaldehyde for examination of fluorescence in a FACS Calibur flow cytometer.

7. Flow cytometric analysis of samples

Macrophages/monocytes and lymphocytes were analyzed for the expression of surface markers and intracellular cytokines using the Cell Quest software on a FACS Calibur flow cytometer. Limits for the quadrant markers were always set based on negative populations and isotype controls. All analyses on lymphocytes were performed using the lymphocyte gate and blastlymphocyte gate [37] on either FSC versus SSC plot or FL-3 versus SSC plot (in case ofCD3-PerCP used as pan-lymphocyte marker). For analysis of CD4 positive lymphocytes, quadrants were always set for CD4 high populations so as not to include CD4 low-positive monocytes and macrophages.

8. **Estimation of nitric oxide in culture supernatants**

Macrophage cultures were set up as previously described for determining the effect of phosphoprotein antigens on nitric oxide (NO) release. NO in the culture supernatants was measured at different time intervals (6 hr, 24 hr and 30 hr post stimulation with the antigen) by a colorimetric detection of nitrite (oxidation product of nitric oxide) as an azo dye product of the Griess reaction. Griess assay method was performed according to Ding et al. (Ding et al, 1990)[53]. Briefly, the culture supernatant and the mixture of Greiss reagent [1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5%

H_3PO_4] were mixed at 1:1 ratio and further incubated for 20 min at room temperature, and the O.D was determined at 550nm by ELISA reader (BioRad). The results were expressed in mM nitrite using $NaNO_2$ diluted in culture medium as standard.

The lower limit of sensitivity of the nitrite assay was 0.08µM/ml. The degree of stimulation or suppression of the macrophage activity for nitric oxide production was calculated as the stimulation index (SI), where

SI = Amount of NO in the presence of test antigen / amount of NO in the control

9**. Statistics**

Experiments were performed in triplicates for each set of antigens and time points with at least two different measurements for each sample. Data are provided as means±SEM or means±SD, the number of independent experiments. Data were tested for significance by using the paired Student t-test. Differences were considered statistically significant when P values were <0.05.

Results

1. **Expression of TNF-α by macrophages.**

Figure I-A indicates the comparative expression of TNF-α in macrophages obtained from healthy subjects and VL patients and also the impact of different phosphoproteins fractions on its expression. As is evident from the figure, the percentage of TNF-α expressing cells was significantly low in active VL cases as compared to their healthy counterparts. Stimulation with *L. donovani* phosphoproteins induced some changes in the expression of this monokine. While pp63 and pp41 failed to induce a significant impact, pp39 appeared to inhibit its expression in infected macrophages. However, the pp29 fraction had the most promising impact on frequency of TNF-α positive cells. It increased from 6.9% (±1.01) in the control to 15.18% (±1.67) upon pp29 specific stimulation ($p<0.02$).

2. **Expression of M H C class II molecules:**

In active VL patients, there was reduced expression of HLA-DR on macrophages. As compared to a healthy background expression of 22.86(±1.21)% HLA-DR+ macrophages, patients infected with *Leishmania donovani* exhibited 10.96 (±1.04) % HLA-DR+ macrophages ($p<0.001$). Stimulation of patient macrophages with *Leishmania* phosphoproteins was capable of overcoming this suppression in HLA-DR expression to a large extent (figure I-B). There was almost 3-fold increase on stimulation with pp63 and pp29 fractions (37.50 ±0.87% and 32.81 ±1.74%, respectively) ($p<0.001$). pp41 stimulation enhanced the expression to 27.03 (±0.68) % cells while pp39 stimulation led to an enhanced expression of HLA-DR by 23.81(±1.94) % macrophages.

3. **Expression of Th1/Th2 cytokines by C D 4+ T-cells:**

Further the cytokine secretion pattern of the lymphocytes was studied with regard to interferon (IFN)-γ and interleukin (IL)-4, the characteristic cytokines of the Th1 and Th2 subtypes of helper T cells. The total number of CD4+ T-cells was almost similar in control and antigen-stimulated cultures (figure II-B). There was high production of IL-4 from helper T cells of active VL patients as reflected by the higher percentage of CD4+ T cells positive for IL-4 (3.11± 0.69 %). On the other hand, only 1.87(±0.52) % CD4+ cells expressed IFN-γ in such patients. An analysis of the blast lymphocytes showed the presence of mainly CD4+ cells (>90%). Out of these, 35 (± 0.68) % cells responded with the expression of IFN-γ on SLA stimulation, whereas, 57.75% showed an IL-4 response.

It was observed that the phosphoprotein antigens could potentially alter the cytokine production pattern of the patient PBMNCs. The pp41 fraction led to a remarkable increase in IFN-γ positive cells, with upto 4.78(± 0.11) % CD4+ cells producing the cytokine ($p<0.002$). Although small, there was also an increase in IFN-γ producing cells other than CD4+ T cells (data not shown). Analysis of the blast lymphocytes revealed that stimulation with pp41 antigen had a positive impact on pro-inflammatory response. 82.24 (± 1.35) % of the proliferating lymphocytes were IFN-γ positive ($p<0.001$) whereas, the population of IL-4 producing cells decreased to 18.92 (±0.21) %. A remarkable down-regulatory impact on IL-4 production was also evident from the decreased CD4+ IL-4 producing population (0.58 ±0.13%) ($p<0.001$).

Similarly, the pp29 fraction stimulated 2.53 (± 0.15) % CD4+ cells for IFN-γ production and 55.93 (±3.44) % blast lymphocytes were IFN-γ positive ($p<0.01$). There was a slight decrease in IL-4 producing cells ($p<0.002$) as evident from (figure II C & D).

pp39 fraction had a marginal down-regulatory effect on both IFN-γ and IL-4 production by lymphocytes (figure II-C&D).

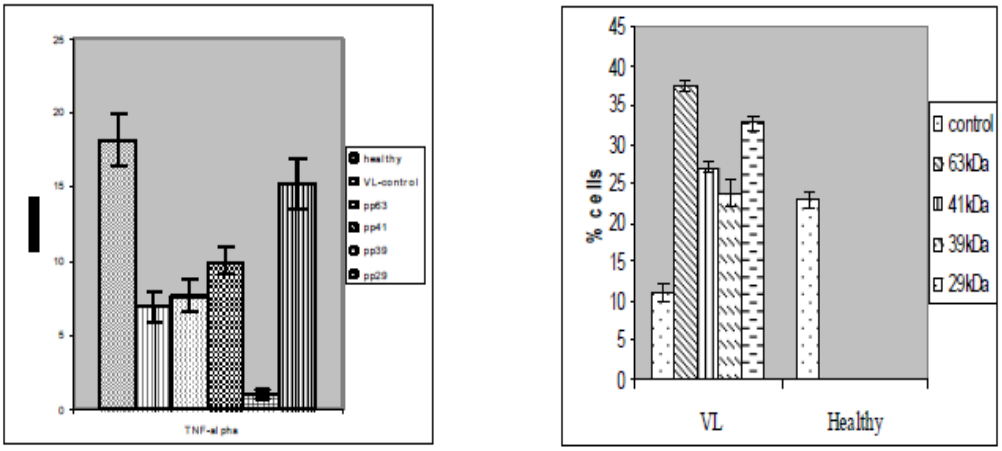
Stimulation with pp63 antigen led to an increase in IFN-γ production and slight decrease in IL-4 production from CD4+ lymphocytes. 6.03 (±0.95) % CD4+ cells were found to be positive for IFN-γ and blast lymphocyte population positive for IFN-γ also increased up to 82.56 (± 3.42) %($p<0.001$).

4. **NO production by macrophages:**

The amount of nitric oxide produced was measured in terms of nitrite (oxidation product of nitric oxide) using the Griess reaction. The level of nitrite in the sera of kala-azar patients was slightly higher than that in healthy subjects. However, when the lymphocyte populations from both the groups were cultured there was reduced

production of nitric oxide from the *Leishmania* infected macrophages as compared to the healthy subjects.

Further, there was little nitrite production up to the first 6 hours and increased greatly from 6 to 24 hours and then further up to 30 hours of culture (figure III). Such a trend in nitrite production kinetics has been indicated before [54]. Stimulation with the different phosphoprotein antigens led to differential effects on the peneration of this leishmanicidal factor. A significant increase in the levels of nitric oxide production was observed following stimulation with pp29 of *Leishmania.* The concentration increased from 25.5(±1.07) micromoles/ml in control to 57(±1.87) µM/ml ($p<0.01$) (stimulation index=2.23). The pp63 antigen also had a stimulatory effect upon nitric oxide production with a stimulation index of 1.72. The amount of nitric oxide produced increased to 44 µM/ml ($p<0.01$). The other antigens (pp41 and pp39) failed to exert an effect on nitric oxide production.



A B

Figure I: Comparative profiles of expression of intracellular TNF-alpha (A) and surface HLA-DR (B) molecules on macrophages derived from healthy subjects and VL patients following specific stimulation.

The suppression in expression of these molecules on VL infection is evident. Some of the *L.donovani* phosphoproteins were capable of eliciting an enhanced response when VL patient macrophages were stimulated with them at a final concentration of 20µg/ml. The impact made by pp29 fraction is notable.

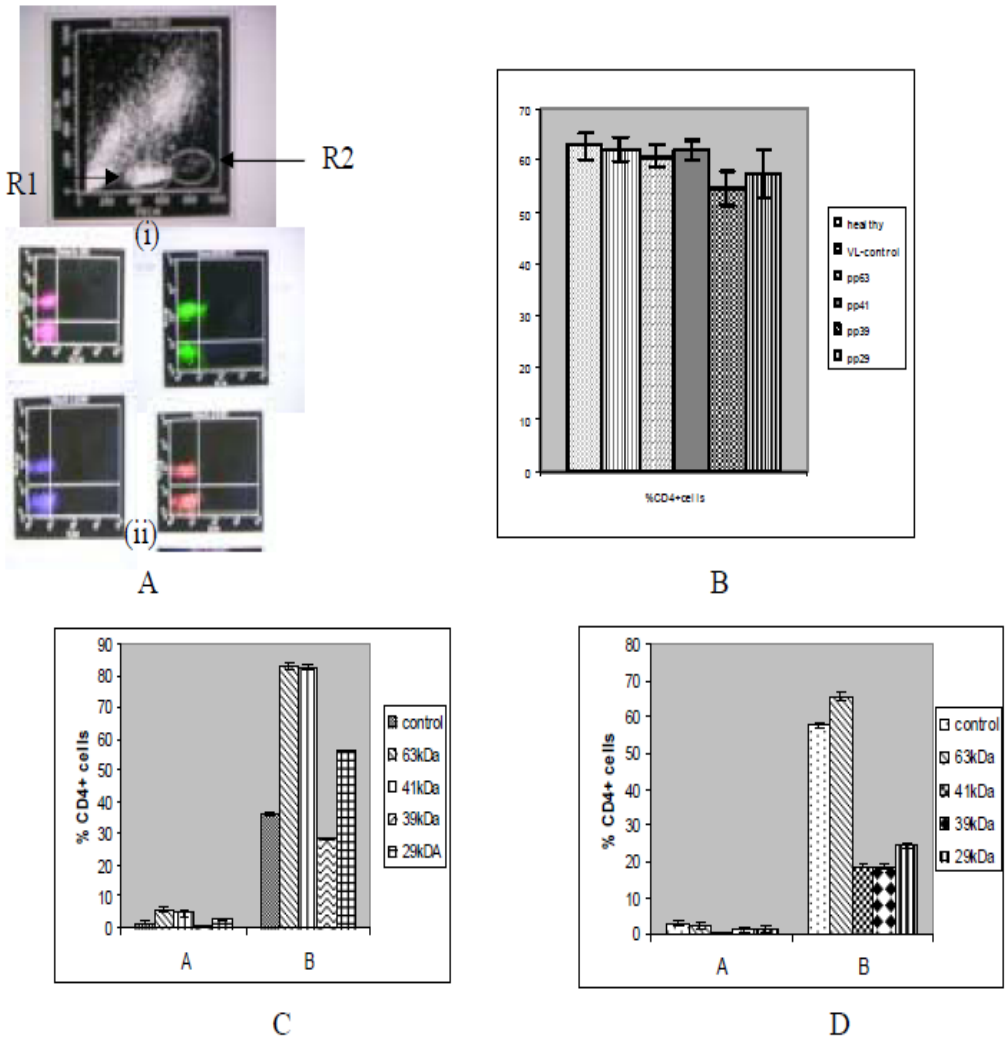


Figure II: Impact of *L.donovani* phosphoproteins on Th1/Th2 cytokine response on CD4+ T-cells from VL patients.

A(i) An FSC versus SSC plot showing lymphocyte (R1)and blast lymphocyte (R2) gates.

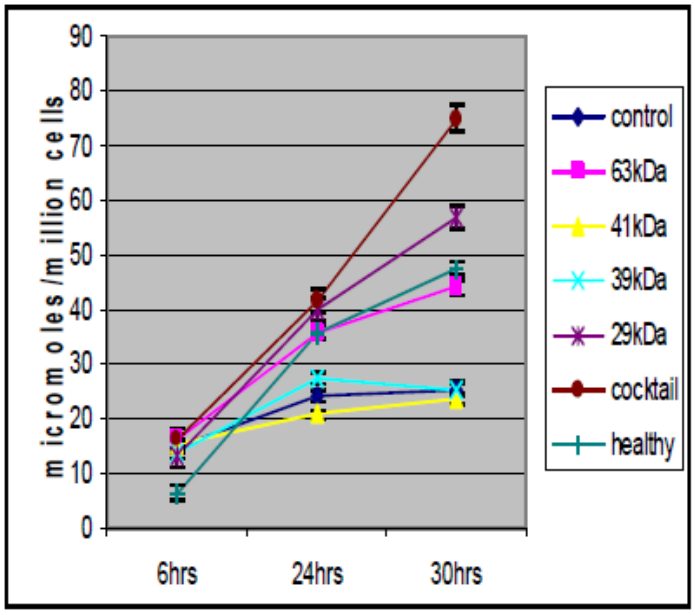
(ii) FACS plots indicating different subpopulations in variously stimulated cells (clockwise from upper left: control, pp63, pp41, pp29 stimulated)

(quadrants: UL: CD4single positive cells; UR:CD4 IFN-gamma double positive cells; LL: negative cells; LR: IFN-gamma single positive cells.

B: profile of CD4+ population in VL (control) and upon different antigen stimulation.

C: effect of antigen stimulation upon IFN-gamma expression by CD4 +T-cells.

D : effect of antigen stimulation upon IL-4 expression by CD4+ T-cells.



stimulant↓	time interval		
	6hrs	24hrs	30hrs
medium	15±1.12	24±0.98	25.5±1.07
63kDa	16.5±1.08	36±1.15	44±1.23
41kDa	15.5±0.78	21±0.09	23.5±0.86
39kDa	13.5±1.62	27.5±1.45	25.5±1.33
29kDa	13±2.02	40±1.96	57.2±1.87
cocktail	16.5±1.67	41.5±1.32	75±0.99

Figure III: The impact of *L.donovani* phosphoprotein stimulation on nitric oxide production by macrophages obtained from peripheral blood of VL patients. There was little production of NO until the first six hours, it increased rapidly from 6 hours to 24 hours upto 30 hours.

Stimulant	VL patient	Healthy
control	6.9±1.01	18.18±1.69
pp63	7.69±1.06	-
pp41	10±0.96	-
pp39	0.95±0.37	-
pp29	15.18±1.67	-

A

stimulant	VL	Healthy
control	10.96 ±1.04	22.86 ±1.21
63kDa	37.50 ±0.87	
41kDa	27.03 ±0.68	
39kDa	23.81± 1.74	
29kDa	32.81 ±0.93	

B

Stimulant	%CD4+ cells
control	61.94 ±2.19
63kDa	60.69 ±2.10
41kDa	61.99 ±1.92
39kDa	54.52 ±6.09
29kDa	57.31 ±4.66

C

stimulant	A	B
control	1.87 ±0.52	35.94 ±0.68
63kDa	6.03 ±0.95	82.56 ±1.06
41kDa	4.78 ±0.11	82.24 ± 1.35
39kDa	0.48 ±0.13	28.50 ±0.11
29kDA	2.53 ±0.15	55.93 ±0.27

D

stimulant	A	B
control	1.87 ±0.52	35.94 ±0.68
63kDa	6.03 ±0.95	82.56 ±1.06
41kDa	4.78 ±0.11	82.24 ± 1.35
39kDa	0.48 ±0.13	28.50 ±0.11
29kDA	2.53 ±0.15	55.93 ±0.27

E

Table I: Data obtained with the control and antigen-stimulated cultures of peripheral blood mononuclear cells of VL patients compared to healthy subjects. Values denoted as mean ± standard deviation.

A: Comparative effects on intracellular TNF-alpha expression by macrophages.

B: Comparative effects on surface HLA-DR (MHC ClassII molecules) expression on macrophages.

C: Comparative impact on total CD4+ T-cell population

D: Impact upon CD4+ T-cells for the production of interferon gamma (Th1)
A: lymphocyte gate
B: blast lymphocyte gate

E: Impact upon CD4+ T-cells for the production of ilterleukin-4 (Th2)
A: lymphocyte gate
B: blast lymphocyte gate

Discussion

The immunological profile of active VL cases found in our study corresponded well with the other reports. There was a general reduction in IFN-γ expression by Th-cells, decreased antigen presenting capabilities (low HLA-DR expression on macrophages) [9- 16] and low NO production. *Leishmania* infection is reported to proceed with a down regulation of NO production by disrupting the membrane potential (Vm) during the invasion process by altering host cell K+ channel activity [23]. Other studies have suggested involvement of various mechanisms like post-translational events [55], activation of phosphotyrosine phosphatases [56] which lead to suppressed iNOS expression and/or activity resulting in reduced NO production. Our data showing reduced production of nitric oxide in macrophages from kala-azar patients in comparison to their healthy counterparts reinstates this observation.

Immunity to leishmanial infection is mediated through cellular mechanisms involving sensitized T-lymphocytes and their lymphokines acting on infected macrophages [27-29, 57]. Clinical and experimental evidences suggest that resolution of leishmanial infection is dependent upon cell-mediated immunity [27-29] leading to activation of macrophage to eliminate the parasites. Recovery from infection confers immunity to reinfection [58-59] and moreover, subclinical or asymptomatic infection with *L. donovani* can result in detectable *Leishmania*-specific cellular immune response [5-7]. The characteristic increase in antibody response is associated with leishmanial infections. The presence of a remarkably heterogenous T-cell response in individuals, in the absence of any obvious immuno-dominant antigen, raises issues concerning the identification of protective T-cell antigens. Since these individual T-cell responses arise from sensitization during the course of active infection, it is possible that each of the parasite antigens might contribute in part to the control and resolution of infection and to the immunity to reinfection that cured patients acquire.

Identification of antigens that stimulate IFN-γ production is further predictive of those antigens that might be involved in immunity. In experimental models lymphokine production, specifically IFN-γ production is associated with activation of macrophages and killing of intracellular parasites. A clear Th1/Th2 pattern of disease development demonstrated for *L. major* and *L. mexicana* has not been observed in VL caused by *L. donovani* in mice and humans [60-62] and the role of IL-4 in disease susceptibility is yet to be determined. However, IL-4 is reported to abrogate the effect of IFN-γ and act antagonistically to this protective cytokine [63]. In addition, cure of active visceral disease is associated with restoration of T-cell responsiveness and IFN-γ production [44], and activation of macrophage leishmanicidal components [17, 64].

Designing protective strategies against leishmaniases requires identification of parasite components that can stimulate such protective effector functions in T-cells and macrophages. As evident from our studies, pp63 had a remarkable impact on induction of IFN-γ production by CD4 T-cells which was also reflected in upregulated HLA-DR expression. In fact, all the tested antigenic fractions seemed to make a positive impact on HLA-DR expression. pp63 failed to influence TNF-alpha expression but nonetheless, it stimulated NO production to a considerable extent. This could be indicative of a TNF-independent; IFN-γ mediated NO production process being stimulated either in macrophages [10, 65] or from other cells like neutrophils [66].

The pp41 fraction had an enhancing effect on Th1 cytokine production and also on TNF-α expression. Although these factors appeared physiologically insufficient to induce increased NO generation, the potential of this phosphoprotein fraction of *L. donovani* to affect immunological responses, skewing them towards a protective phenotype should not be overlooked.

Out of the four phosphoproteins examined, pp39 appeared to be negatively influencing the protective responses and hence seemed of little value in protection against VL.

The most promising candidate identified in the study was pp29. This component was capable of eliciting a wholesome anti-leishmania response from the participating cells. It up-regulated IFN-γ production, lowered the antagonistic IL-4 expression and was also effective in inducing macrophages for TNF-α and NO production. pp29 specific stimulation thus appeared competent enough to override the suppression of NO production that could be due to a reversal in PKC impairment known to occur in *Leishmania* infected macrophages [67]. This fraction presented great promise as a protective antigen by virtue of its enhancing effect on almost all essential factors known to generate an anti-leishmania response.

The results presented by us are a definite advancement over the previous reports on *Leishmania* antigens eliciting a protective response in VL. Turhan and colleagues [68] reported a role of 97.4-66kDa molecular weight fractions in inducing IFN-γ response and 66-45kDa fractions in suppressing IL-4 secretion. Laskay and colleagues [69] reported stimulation of TNF-α by molecular weight fractions 43-36, 33-27 & 22 kDa antigens of *Leishmania aethiopica* during active human infection. The two antigens in our study, pp41 and pp29, fall in the same weight range. In fact, our study is further able to denote one fraction that might be responsible for the observations made by them on a range of weights and that this fraction is a phosphoprotein. Some recent reports have suggested a NO stimulating impact of a few *Leishmania* antigens that include the membrane proteins

of *L. donovani* [70], promastigote soluble antigen [73] and a high molecular weight fraction (97.4-68kDa) of *Leishmania* [74-75]. Our study formed a step ahead towards identifying *L. donovani* phosphoprotein antigens with NO enhancing effects.

Our results with these medium to low molecular weight phosphoprotein fractions of *L. donovani* showing impact on CD4 T-cell cytokine response and macrophage functions for parasite elimination, yield some valuable insight into the possible role of these fractions in modulating the immune response against the parasite and in favor of the host.

Several reports in the past have indicated the importance of lower molecular weight fractions of *L. donovani* in preferentially stimulating a protective response [76-77]. These phosphoproteins of *Leishmania donovani* are worth being pursues for further studies regarding leishmaniasis immunoprophylaxis.

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