

Towards Identifying Immunogenic Targets in Visceral Leishmaniasis: Role of 17kDa and 63kDa Phosphoproteins

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Abstract: We observed predominant phosphorylation at Protein Kinase C (PKC- α) residue in seven immunoreactive proteins of virulent *Leishmania donovani* parasites. These phosphoproteins were tested for their ability to stimulate T-cells for secretion of Macrophage Migration Inhibition Factor (MIF) in comparison to crude soluble antigen in Visceral Leishmaniasis (VL) patients. Two of these phosphorylated *L. donovani* antigens led *Leishmania* to present these antigens to Th1 cells, which was obvious from an increased MIF response. These phosphorylated antigens i. e. 63kDa and 17kDa further led to reprogramme CD4 cells for enhanced IFN- γ production. The study suggests that PKC-phosphorylation in *L. donovani* antigens can play a critical role in modulation of signal transduction pathway in T-cells to differentiate into Th1 effectors.

Key words: Protein Kinase C, Macrophage Migration Inhibition Factor, *Leishmania donovani*, Interferon Gamma, Visceral Leishmaniasis.

INTRODUCTION

The eukaryotes are reported to employ a signalling mechanism at tyrosine residue that controls a variety of intracellular events^[1]. Virulent promastigote strain of *Leishmania* also undergo phosphorylation of tyrosine containing proteins and as reported, it is species specific^[2]. Reports on tyrosine related proteins, protein kinase C (PKC), a serine/threonin containing protein are also available which suggest its role in innate macrophage functions and hence thereby, appears to be a key element in the control of infection by *L. donovani*^[3]. The reports presented in this study describe the phosphorylated or dephosphorylated status of differentially expressed *L. donovani* antigen grown initially at 24°C and later subjected to 30°C and 37°C temp. shift in laboratory based experimental set-up. Such heat shock induced changes were further compared with the antigenic pattern shown by natural promastigotes present within *Phlebotomus argentipes*. The investigations were further carried out to assess the antigenic components for their immunological properties.

MATERIALS AND METHODS

Promastigotes pass through a rapid temperature shift from 22-24°C to 37°C while entering in mammalian host through *P. argentipes* the proven vector of Indian Visceral Leishmaniasis. As such, late

log phase *L. donovani* culture (3-4 days) propagated in M-199 medium with 10% FCS was incubated at 24° C and 37° C for 4h. This was done to experimentally mimic those conditions that usually are governed in vivo during conversion of promastigotes into amastigotes. To study antigenic pattern of *Leishmania* during natural infection, *P. argentipes* (10/pool in 50 μ l of distilled water) were collected from three adjacent endemic areas from Bihar province in India. A 12.5% SDS-PAGE^[4] was later applied for proteins of 50 μ g lysates/lane and antigenic pattern of promastigotes at 24, 30 and 37°C and those present within *P. argentipes* was compared. Later, antigens blotted from different experimental stages were probed with anti-PKC- α antibodies (mouse IgG 2b; clone 3, BD Biosciences, USA) in TBS-tween (1:1000; 0.25 μ g/ml final concentration) that followed incubation with IgG-HRP (1:2000 dilution in wash buffer containing 1%BSA) for 1h at room temperature, which were stained with 0.3% diaminobenzidine chemiluminiscent working solution for 1-5 min. (0.3% diaminobenzidine). Colour development was monitored and the reactive bands were calculated for their respective molecular weight size. These antigens with a critical role in signalling, were further tested for their effect on humoral immune response to *L. donovani* for which NCP blotted antigenic fractions were probed with pooled sera in immunoblot^[5]. These sera were obtained from 10 VL subjects in whom infection was confirmed microscopically by demonstration of amastigotes in

spleen biopsy and in whom *Ld* grade ranged from 1+ to 5+. These subjects were also confirmed as VL through PCR and DAT. For the study of T-cell response SDS-PAGE slots of selected antigens were isolated and subjected to second SDS-PAGE gel and subsequently transferred on NCP. The polypeptides on blotted NCP was stained with amido-black (0.5% amido-black, Himedia, India with 25% methanol and 7% acetic acid in distilled water), isolated into PBS and stored at -70°C for further studies. To study the association of T-cells in response to these antigens, $5 \times 10^6/\text{ml}$ peripheral blood mononuclear cells (PBMNC) were pulsed with *L. donovani* promastigotes in responder (PBMNC) to stimulator (*L. donovani* parasite) ratio of 100:1 for two hours at 37°C and later cultured in 96 well round bottomed plates in presence of selected polypeptides ($1\mu\text{g}/100\mu\text{l}$ culture). Control cultures were set up in medium alone or medium containing soluble *Leishmania donovani* $20\mu\text{g}/\text{ml}$ for 48h. All cultures were maintained at 37°C in a water saturated air atmosphere containing 5% CO_2 . Macrophage migration inhibition factor (MIF) was measured as an index of cell mediated immunity^[6]. Two of these protein fragments (17kDa and 63kDa) which showed an augmented T-cell response for MIF were further tested for their abilities to stimulate intracellular $\text{IFN-}\gamma$ production by CD4^+ subpopulation on FACS-Calibur using cell quest software. Briefly, the cells stimulated with 17kDa and 63kDa protein fractions in presence or absence of recombinant IL4 for 18h at water saturated atmosphere in 5% CO_2 incubator. Cultured cells, pre-treated with Brifeldin-A for 6h before harvesting, were fixed, permeabilised and stained with anti CD4 PE and anti- $\text{IFN-}\gamma$ -FITC as previously described^[7].

RESULTS

The results show appearance of 3 proteins upon heat stress from 24°C to 30°C , which remains stable at 37°C . The polypeptides identified ranged in the molecular weight of 6.5 to 87 kDa as determined on the basis of mobility of stander marker. Most of the polypeptides appeared at 24°C were also visible in the SDS-PAGE pattern of *P. argentipes* while few proteins of sand-fly (57kDa, 40kDa, 15kDa etc) did not match Leishmanial pattern under any condition of the heat stress and thus probably represented sand-fly specificity of these proteins (figure-1). Predominant phosphorylation in 15 polypeptides was observed at PKC- α residue (24°C) but 4 of these proteins did not phosphorylate when temperature was shifted to 30°C (Table-1). A new polypeptide of 21kDa molecular size showed significant phosphorylation at 30°C but this antigen suddenly got dephosphorylated at 37°C . Altogether 7 important proteins with significant impact on phosphorylation state was identified which were of molecular size of 63, 39, 32, 26, 24, 19 & 17 kDa. To

assess the immunogenic properties of these critical *Leishmania* antigens, humoral immune response for elicitation of anti-*Leishmania donovani* antibodies in response to these antigens was examined by Western blot. These antigens induced strong antibody response but many antigens which earlier showed poor phosphorylation at PKC α residue also triggered antibody response in sera from VL patients.

In comparison, all phosphoproteins elicited antibody response but this type of response was not seen with majority of antigens where no phosphorylation was observed at PKC α residue.

We later examined cytokine response (MIF & $\text{IFN}\gamma$) to whole or fractionated phosphoprotein *L. donovani* antigens. Increased humoral response in *L. donovani* phospho-proteins was later observed associated with raised T-cell function for MIF response (Table-2). MIF release up to 46.09% was observed after priming of PBMNC with 17kDa ($10\mu\text{g}/\text{ml}$) *L. donovani* polypeptide. This up regulated pattern of MIF expression was also noticed with 63kDa (44.12%), 24kDa (35%), 26kDa (33%) and 19kDa (27.5%). The above response however, was not observed when PBMNC were primed with crude soluble antigen where T-cells secreted insignificant MIF of 16.78%. To further investigate role of these phosphoproteins, $\text{IFN}\gamma$ production by CD4^+ -T-cells induced by them was determined on FACS-Calibur after short-term stimulation.⁷ Eighteen hours culture with these *Leishmania* derived antigens and CSA were analyzed using dual staining and flow cytometry. Analyzing lymphocytes in R1 gate on FSC Vs SSC plot, it was observed that CSA induced a high $\text{IFN-}\gamma$ producing CD4 cells (2.8%) compared to medium (0.8%). When these results obtained with CSA were compared with *Leishmania* derived antigens, there was an increase in frequency of $\text{IFN-}\gamma$ producing cells, which was 5.28% with 63kDa and 5.11% with 17kDa (Figure-2). The response of 17kDa was slightly better as it induced higher frequency of $\text{IFN-}\gamma$ producing cells compared to 63kDa when cultured in presence of recombinant IL4.

DISCUSSION

It is well known that PKC- α , a calcium dependent PKC isoform regulates innate macrophage function, which is involved in the control of infection by *L. donovani*^[3]. Recently it has been shown that VL infection is associated with selective impairment of PKC isoform caused by *Leishmania donovani* parasite which results in dysfunction in Ca^{2+} signalling^[8,9]. Many reports also suggest the involvement of PKC mediated phosphorylation in CD4 cells, which results in proliferation and IL2 production and its receptor

expression^[10,11]. Therefore we speculated that there might be some activation in PKC dependent signalling in certain antigens of *L. donovani*, many of which, we thought can be with pro-host immuno-modulatory action. To characterize the role of *Leishmania* antigens, which may be used to produce a protective immune response, we examined the phosphorylation of different antigens at PKC- α residue and later examined its impact on MIF and IFN- γ response of T-cells. The PKC- α phosphorylation was observed up regulated in 7 *Leishmania donovani* antigens and the increased phosphorylation possibly led *Leishmania* to present these antigens to Th1 cells, which was obvious from increased MIF response. MIF secretion by T-cells was recently shown by our group to act as a prognostic marker of VL infection^[12]. The results highlights the importance of two critical antigens i. e. 63kDa and 17kDa, which were shown better, shaped in reprogramming of CD4 cells to produce IFN- γ in VL patients. The trends obtained further suggest that 17kDa antigen can be a promising immuno-therapeutical target, as there seems to be some mechanism triggered by this antigen in detecting IL4 as a result of which this cytokine was interrupted to prevent the production of IFN- γ up to some extent. It is now well known that outcome of leishmanial infection relies on induction of type-1 response of CD4 cells, which produce IFN- γ ^[13,14]. The study presented here reflects that triggering of PKC in *L. donovani* is important which can enforce re-stimulation in T-cells for Th1 sub-set expansion. The inability of most vaccine formulations to prevent the

infection has been the feature observed for most experimental vaccines against leishmaniasis so far. Hence evidence for a role of 17kDa and 63kDa in immunity to *Leishmania donovani* infection shown in this study is worth pursuing. The information collected on immunogenic properties of these antigens will be useful in studies based on immuno-modulatory therapy and vaccine development.

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Table-1: Characteristics of stable protein fractions under western blot analysis:

	Proteins fractions recognized by sera from VL patients	Proteins fractions not recognized by sera from VL patients
Proteins fractions recognized by PKC- α residue	63, 39, 32, 26, 24, 19 & 17	-
Proteins fractions not recognized by PKC- α residue	87, 71, 49 & 29kDa	77, 65, 43.5, 28, 20.5, 20, 18.5, 18, 15 kDa

*The 7 protein fractions showing strong humoral response were reactive to PKC- α residue, however, 4 immuno-reactive protein fractions did expressed their reactivity to PKC- α residue. 9 protein fractions showed their reactivity neither with patient sera nor with anti PKC- α antibodies.

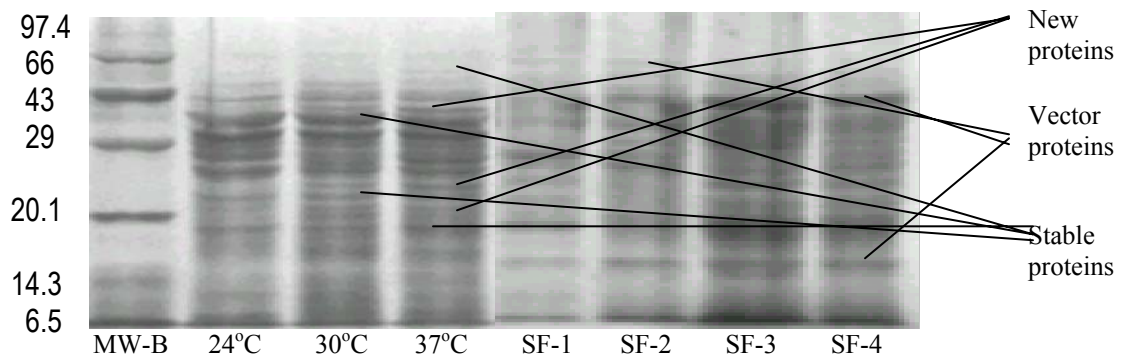


Fig. 1: SDS-PAGE pattern of *L. donovani* isolates cultured at 24°C, 30°C and 37°C in comparison to SDS-PAGE pattern of *P. argentipes* from endemic area

*SDS-PAGE pattern of crude *L. donovani* promastigote cultured at 24°C (L-2) 30°C (L-3) 37°C (L-4), compared to SDS-PAGE pattern of *P. argentipes* lysate (10/pool dissolved in 50 μ l distilled water) collected from VL endemic areas (lane 5-8, labelled as SF1, SF2, SF3, SF4). There are expression of few newly appeared proteins on lane 3 and 4. Majority of proteins are stable on heat shock and also visible in lane 5-8. However some of the proteins of lane 5-8 did not matched with Leishmania pattern of any heat stress condition and thus probably represented sand-fly proteins under this range.

Table-2: MIF response by T-cells from VL and healthy subjects after priming with different immunogenic protein fractions from *L. donovani*

Category	Crude soluble antigen of <i>L. donovani</i>	Fractionated proteins							PHA
		63 kDa	39 kDa	32 kDa	26 kDa	24 kDa	19 kDa	17 kDa	
Visceral Leishmaniasis (n=5)	16.78 ±5.17	44.12 ±7.44	12 ±1.14	14 ±4.24	33 ±2.82	35 ±5.65	27.5 ±4.94	46.09 ±6.05	36.55 ±6.19
Healthy (n=5)	12.12 ±2.61	19.22 ±4.56	9.5 ±3.53	10.5 ±2.12	18 ±2.82	14 ±5.65	9.5 ±3.53	18.9 ±1.27	36.51 ±4.1

Different polypeptide fractions showed differential impact on secretion of macrophage migration inhibition factor by T-cells in vitro.

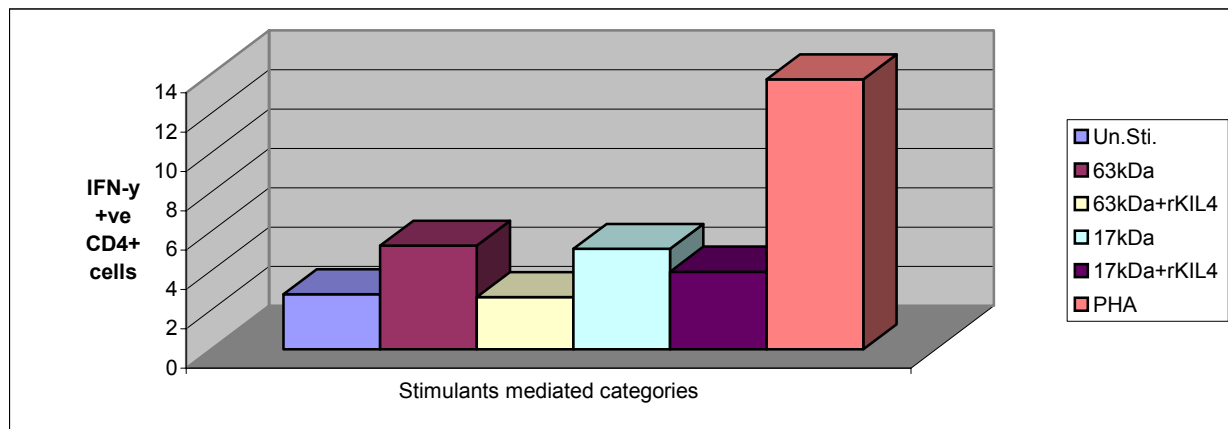


Fig. 2: Frequency of IFN-γ producing CD4+T-cells after stimulation with 63kDa or 17kDa in presence or absence of recombinant IL4.

*Increased frequencies of detectable IFN-γ producing CD4 cells after stimulation with 17kDa and 63kDa *Leishmania* derived antigens in presence or absence of recombinant IL4 and compared to crude soluble *Leishmania* antigen preparation and PHA. The response of 17kDa protein fragment was slightly better in presence of recombinant IL4.

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