Immunomodulatory effect of antigenic fractions of a recent clinical isolate of *L. donovani* on monocytic cell lines

PARUL TRIPATHI, DINESH CHANDRA, SITA NAIK

Department of Immunology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India

Abstract

Nitric oxide (NO) and cytokines are important mediators of the immune response to Leishmania. We have recently reported that following SDS-PAGE separation and transfer of whole L. donovani antigen (strain 2001, a recent clinical isolate from Bihar) into 11 fractions (named F1 to F11; MW range 139-24.2 Kd), only the high molecular weight (MW) fractions (F1 to F4; MW range 134-64.2 Kd) had immunostimulatory activity when tested in leishmania exposed immune individuals. The F1 to F11 fractions were able to induce significant proliferation of peripheral blood mononuclear cells (PBMCs) of leishmania exposed immune individuals and production of variable amounts of IFN-γ, IL-12p40 and IL-10. The present study was undertaken to evaluate the effect of L. donovani promastigotes whole antigen extract (WE) and F1 to F11 fractions on NO induction and production of cytokines IL-12p40, IL-10 and TNF-α by mouse and human macrophage cell lines, respectively. NO production was determined by using Griess reagent and cytokines were measured by ELISA. Antigenic fractions and WE stimulated impressive production of NO and cytokines IL-12, IL-10 and TNF-α; F1 to F4 fractions induced higher levels of NO and monocyte derived cytokines IL-10, TNF-α, IL-12 than fractions F5 to F11, although the difference did not reach statistical significance. The high molecular weight antigenic fractions need to be evaluated in greater depth for their possible role as immunomodulatory agents.

Key words: *nitric oxide, TLRs, Leishmania, macrophage, cytokines, lipopolysaccharide.*

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Introduction

The innate immune system is the first line of defense against microorganisms and initiates a local inflammatory reaction immediately following their entry. Pathogens are recognized through pattern recognition receptors such as toll-like receptors (TLRs), which bind conserved pathogen-associated molecular patterns (PAMPs) on microbes [1]. Ligand engagement through TLRs leads to secretion of inflammatory cytokines and up-regulation of MHC class II molecules and costimulatory molecules, which in turn activates the adaptive arm of the immune response [2, 3]. These receptors are chiefly present on antigen presenting cells (APCs) such as monocytes, macrophages, B cells, dendritic cells [1].

So far eleven TLRs have been identified in humans, which are distinct from each other in their ligand specificity and expression pattern. While the ligand for TLR4 is LPS, TLR2 recognizes a wide variety of PAMPs from bacteria, yeast, fungi, parasites and viruses, TLR9 recognizes unmethylated CpG motifs present in bacterial DNA, TLR3 recognizes double-stranded RNA and TLR7 and 8 recognize single-stranded RNA from viruses. TLRs transduce signals through adaptor proteins such as Myd88 (myeloid differentiation factor 88) and activate various cytokines including that of IFN-α, IL-1, IL-10, IL-12, TNF-α [4]. In order to respond to all pathogens, TLRs may work together to complement or synergize each other's functions as an immune survival network [5, 6].

Correspondence: Dr Sita Naik, Department of Immunology, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Raebareli Road, Lucknow 226014, India. Phone number: 91-522-2668004-8 Extn. 2284 (O), 2285 (R), fax number: 91-522-2668017, 2668973, Email: sitanaik@sgpgi.ac.in

The protozoan parasite *L. donovani*, is the causative agent of human visceral leishmaniasis (VL) and infects cells of the mononuclear phagocytic system, primarily macrophages [7]. Macrophages serve not only as host cells for *Leishmania* but also as APCs modulating the specific cellular immune response and after appropriate activation, as effector cells for killing of the parasites via the production of nitric oxide (NO) [8]. NO, which is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine by NO synthase, is an important mediator of macrophage microbicidal activity. Macrophage NO production is highly affected by LPS or cytokines such as IFN-γ [9]. Since TLRs are present on the surface of macrophages, it is possible that leishmania may also bind TLRs and switch on early genes of the host [10]. *Leishmania*, with its characteristic down modulating effects on monocytes/macrophages, seems to avoid or overcome this immediate response. *L. major* has been shown to induce the transcription of IL-1α through MyD88 [11] and signaling through TLRs are involved in the inflammatory response to leishmania infection [12].

Macrophage derived IL-12 stimulates NK cell cytotoxicity and promotes growth and differentiation of Th1 cells. This is important for the favorable outcome of leishmania infection, which is dependent on a Th1 dominant immune response [13]. IL-10 is a pleiotropic immunomodulatory cytokine, produced by a wide variety of cells, including activated Th2 cells, monocytes and macrophages, B cells, thymocytes and keratinocytes. IL-10 has been shown to play a role in counterbalancing the exacerbated polarized response that may develop following cure from VL [14]. TNF-α has been shown to play an important protective role in leishmaniasis and to synergizes with IFN-γ in mediating killing of *L. major* through the induction of NO [15, 16]. The crude antigenic fraction of *L. major* and its subfractions have been shown to have different effects on macrophages, NK cells and human peripheral blood lymphocytes [17, 18].

We have recently reported that T cell immunostimulatory antigens are localized to high molecular weight fractions (>64.2 Kd) of whole *L. donovani* promastigote antigen using cells from leishmania-exposed individuals [19]. These fractions stimulated equivalent levels of IL-12, IFN-γ but lower levels of IL-10 than the low molecular weight antigens of <64.2 Kd region. Since IL-10 has potent immunomodulatory functions, low IL-10 stimulation by the high MW fractions could be of advantage in exploiting these immunostimulatory fractions as a component of a possible cocktail vaccine. The ability to directly stimulate macrophages and induce IL-12 and TNF-α production would enhance the effectiveness of such antigens as they may then also play the role of a T cell adjuvant. With this background, we have evaluated the ability of whole cell extract and eleven nitrocellulose bound antigenic fractions of a recent clinical isolate of *L. donovani* to stimulate nitric oxide and cytokine production in mononuclear cells using macrophage cell lines, RAW 264.7 and THP-1.

Material and Methods

Culture of parasites

L. donovani strain 2001, isolated from an Indian kala-azar patient, was maintained in golden hamsters as described previously [20]. Amastigotes from spleen of infected animals were cultured in L-15 medium (Invitrogen) at 25°C with 10% heat inactivated fetal calf serum (Invitrogen), 0.3% tryptose phosphate broth (Himedia), and gentamycin (Sigma; 40 mg/litre). Promastigotes were grown to stationary phase, harvested by centrifugation and washed in phosphate-buffered saline before use.

Preparation of antigens from *L. donovani* promastigotes

Whole cell extract (WE)

Stationary-phase promastigotes were harvested by centrifugation and the pellet was dissolved in SDS extraction buffer, vortexed and centrifuged at 1500 × g for 5 min. An equal volume of cold acetone was then added drop by drop to precipitate the protein. The mixture was kept on ice for 15 min and centrifuged at 2000 × g for 15 min at 4°C. Protein concentration was determined and aliquots of antigen were stored at –80°C.

Antigenic fractions

Antigenic fractions were prepared from metacyclic parasites, pelleted by centrifugation at 600 × g for 15 minutes at 4°C. The pellet comprising of approximately 6×10^8 parasites (equivalent to ~3 mg of protein) was suspended in 1X gel loading buffer and proteins were subjected to electrophoresis on 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane, which was dried and cut, into 11 horizontal strips of 1 cm each. These strips were processed to precipitate the antigen bearing particles as described earlier [21]. The eleven antigenic fractions (F1 to F11) with their corresponding molecular weights are shown in Figure 1. Additional strips of blank NC (blotted from a blank portion of the gel) were also processed for use as appropriate antigen controls.

Maintenance of cell lines

The cell lines RAW 264.7 and THP-1 were obtained from the National Center for Cell Science (Pune, Maharashtra, India). Murine macrophage cell line RAW 264.7 was maintained in DMEM medium (Gibco-Invitrogen) supplemented with antibiotic-antimycotic mix and 10% heat inactivated FCS. THP1 cells were cultured in RPMI-1640 medium (Gibco-Invitrogen) supplemented with 25 mM HEPES, L-glutamine (2 mM), antibiotics (penicillin and streptomycin) and an antimycotic agent, 5 μM β-mercaptoethanol and 10% heat-inactivated FCS (Gibco-Invitrogen).

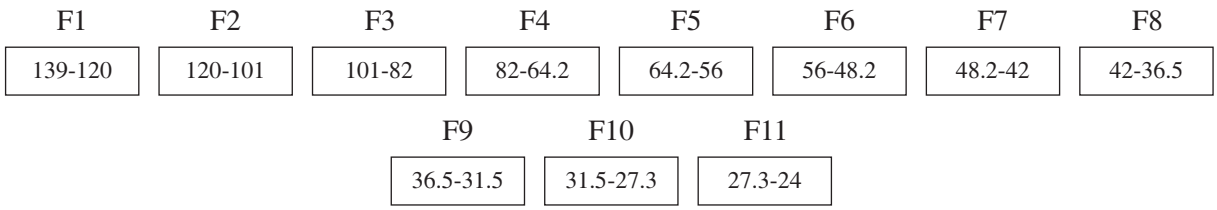

Fig. 1. Antigenic fractions (F1 to F11) prepared from whole parasite of *L. donovani* (2001 isolate) promastigote separated by SDS-PAGE and their corresponding molecular weights

Both the cells were plated at a density of 1×10^6 cells in culture flasks (Tarsons, Kolkata, India) and allowed to grow to confluency prior to use in experiments. All cultures were maintained at 37°C and 5% CO_2.

Effect of WE and antigenic fractions on nitric oxide production by mouse macrophage cell line RAW 264.7

Nitric oxide was measured as nitrite released from mouse macrophage cells, RAW 264.7. RAW 264.7 were plated at a concentration of 25×10^4 cells/well in 96-well culture plate and incubated for 48 hours at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Thereafter, 100 µl of medium was aspirated from each well and replenished with the same amount of fresh medium. Further incubation was done in duplicates with whole extract (1 µg/ml, 10 µg/ml, 100 µg/ml, and 1000 µg/ml), NC bound fractions F1 to F11 (dilutions of 1:5, 1:50, 1:500, 1:5000) and in the presence or absence of lipopolysaccharide (LPS of *Escherichia coli* 005B:5, 0.5 µg/ml). Cells were also stimulated in duplicates with blank NC particles as appropriate antigen/vehicle controls. Supernatants were harvested after 24 hours for determination of NO production by Griess reagent (Sigma) as described elsewhere [22]. NO concentration was determined using standard curve plotted using known quantity of sodium nitrite. Results are presented in µM concentration obtained from mean OD of triplicate wells of each group.

Effect of WE and antigenic fractions on IL-12p40, IL-10 and TNF-α production by human macrophage cell line THP-1

THP-1 cells were plated at a concentration of 2×10^5/well in 96-well culture plate and stimulated with 20 nM PMA for 6 hours. This was done in order to induce the immature THP-1 into a mature phenotype. The cells were washed three times and cultured in fresh medium before stimulation with whole extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 1000 µg/ml) and eleven NC bound fractions F1 to F11 (dilutions of 1:5, 1:50, 1:500, 1:5000) in triplicates. Cells were also stimulated in triplicates with blank NC particles as appropriate antigen/vehicle controls. Supernatants were harvested after 24 hours for determination of cytokines IL-12p40, IL-10 and TNF-α by an OptEIA set ELISA kit (Pharmingen, San Diego, California). The results were expressed as picograms of cytokine/ml, based on the standards provided in the kit. The lower detection limits for various cytokines were as follows: 4.7 pg/ml for TNF-α, 31.3 pg/ml for IL-12, 7.8 pg/ml for IL-10.

Statistical analysis

All statistical analyses were performed using SPSS 10 software, "Statistical package for social sciences" (Windows version 10.0, Chicago, IL, USA). Three sets of experiments were performed. P values less than 0.05 were considered significant. Statistical analysis was performed by Mann-Whitney *U* test.

Results

Effect of WE and antigenic fractions on NO production by RAW 264.7

WE induced comparable levels of NO production by RAW 264.7 cells at 10 µg/ml, 100 µg/ml and 1000 µg/ml, which was significantly higher than the NO produced by unstimulated and blank NC controls ($P<0.0001$). There was no effect on NO production at dose of 1 µg/ml (Fig. 2A).

Four different dilutions of NC bound antigenic fractions (1:5, 1:50, 1:500, 1:5000) were tested for their effect on NO production by RAW 264.7. The eleven antigenic fractions stimulated variable levels of NO; however F1 to F4 (MW range 139-64.2 Kd) showed a trend to higher NO production in comparison to F5 to F11 (MW range <64.2 Kd), though this difference did not reach statistical significance. The levels of NO stimulated by F1 to F11 were significantly higher than that of unstimulated and blank NC controls (for both of which NO concentration was <7.8 µM; $P<0.0001$). Nitric oxide induction at dilutions 1:5, 1:50 and 1:500 were comparable with each other. F1 to F11 did not have any significant effect on NO production at dilution of 1:5000 (Fig. 2B). LPS (at 0.5 µg/ml) stimulated NO production was observed in all experiments (Fig. 2A).

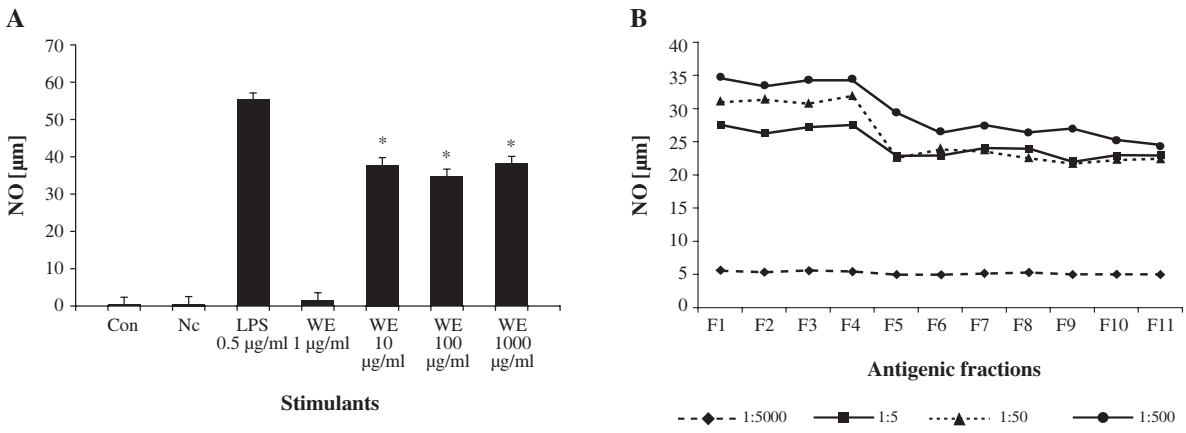

Fig. 2. Nitric oxide levels in mouse macrophage cell line, RAW 264.7 in response to stimulation with (A) LPS & whole cell extract and (B) F1 to F11 antigenic fractions of *L. donovani*. Nitrite concentration is given in µM. In "A" data represents mean ±SD of three different experiments. NC represents blank nitrocellulose control. *Comparison with unstimulated and blank NC controls: $P<0.0001$. In "B" data represents mean of three different experiments. F1 to F4 vs. F5 to F11 and F1 vs. each of the other 10 fractions: P=ns. Statistical analysis was performed by Mann-Whitney U test. ns=not significant

Effect of WE on IL-12p40, IL-10 and TNF-α production by THP-1 cell line

WE stimulated significantly higher levels of IL-12, TNF-α and IL-10 production by THP-1 at all doses (10 mg/ml being optimal) in comparison to unstimulated and blank NC controls ($P<0.0001$; Table 1). There was no significant cytokine production with WE at a dose of 1 µg/ml.

LPS stimulated IL-10 levels were significantly lower ($P<0.05$; Table 1) as compared to whole cell extract (at all doses) stimulated IL-10 levels. In contrast, LPS stimulated IL-12 and TNF-α levels were higher in comparison to that stimulated by WE.

Table 1. IL-12p40, TNF-α and IL-10 levels in human macrophage cell line, THP-1 in response to stimulation with LPS and whole cell extract

Treatment	Cytokines (pg/ml)		
	IL-12	TNF -α	IL-10
control	<31.3	<4.7	<7.8
nitrocellulose	<31.3	<4.7	<7.8
LPS (0.5 µg/ml)	564	1030	76[b]
WE (1 µg/ml)	76	123	42
WE (10 µg/ml)	399*	656*	333*, [a]
WE (100 µg/ml)	401*	666*	376*, [a]
WE (1000 µg/ml)	367*	599*	346*, [a]

*Data represents mean of three different experiments; *Comparison with unstimulated and blank nitrocellulose controls: P<0.0001; [a]comparison with [b]LPS stimulated IL-10 levels: P<0.05. Statistical analysis was performed by Mann-Whitney U test.*

Effect of antigenic fractions on IL-12p40, IL-10 and TNF-α production by THP-1 cell line

Four different dilutions of NC bound antigenic fractions (1:5, 1:50, 1:500, 1:5000) were tested for their effect on cytokine production by THP-1. Fractions F1 to F11 stimulated variable levels of IL-10, IL-12 and TNF-α in all experiments, that were significantly higher in comparison to unstimulated and blank NC controls (for both of which IL-12, IL-10 and TNF-α levels were <31.3 pg/ml, <7.8 pg/ml and <4.7 pg/ml, respectively; $P<0.001$; Fig. 3).

F1 to F4 (MW range 139-64.2 Kd) induced higher levels of IL-12, IL-10 and TNF-α than fractions F5 to F11 (<64.2 Kd) at dilutions of 1:5, 1:50 and 1:500, however, this difference did not reach statistical significance (P=ns). IL-10, IL-12 and TNF-α induction at 1:5, 1:50 and 1:500 dilution of F1 to F11 were comparable with each other (P=ns). F1 to F11 did not have any significant effect on cytokine production at 1:5000 dilution.

LPS (at 0.5 µg/ml) stimulated production of above cytokines was present in all experiments.

Discussion

In the present study, we tested the ability of whole cell extract and NC bound antigenic fractions (F1 to F11; MW range 139-24 Kd) of *L. donovani* to stimulate RAW 264.7 and THP-1 for production of NO and cytokines IL-10, TNF-α and IL-12, respectively. While WE and all eleven antigenic fractions stimulated production of NO and cytokines-IL-12, IL-10 and TNF-α, the effects of fractions F1 to F4 were more pronounced than fractions F5 to F11. However, this difference did not reach statistical significance.

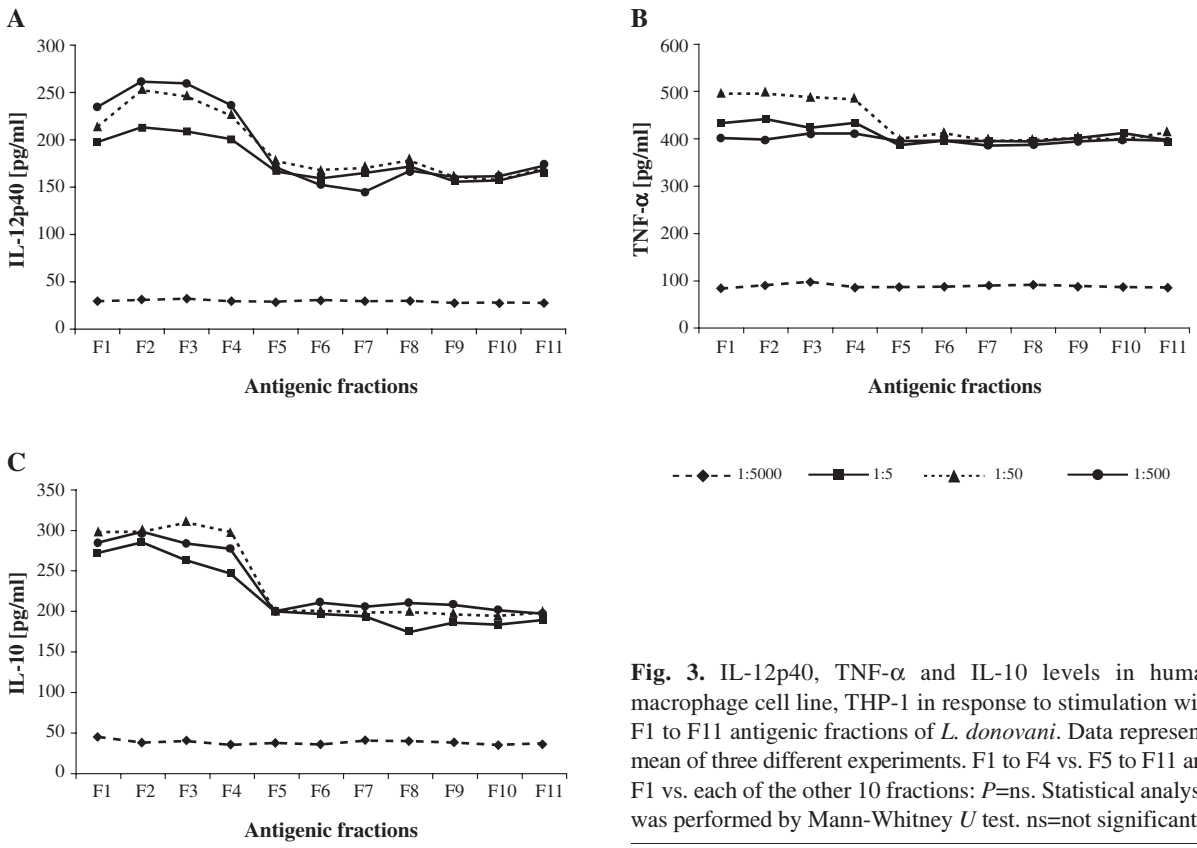

Fig. 3. IL-12p40, TNF-α and IL-10 levels in human macrophage cell line, THP-1 in response to stimulation with F1 to F11 antigenic fractions of *L. donovani*. Data represents mean of three different experiments. F1 to F4 vs. F5 to F11 and F1 vs. each of the other 10 fractions: *P*=ns. Statistical analysis was performed by Mann-Whitney *U* test. ns=not significant

These data suggest that antigens of all MW range have moieties which can directly stimulate monocytic cells.

Ultra structural analysis of leishmanial whole antigen preparations has shown that it is a crude preparation that contains a mixture of membrane, soluble and insoluble proteins [23]. We have also reported that WE of *L. donovani* is a potent stimulator of PBMCs from leishmania exposed individuals [19]. In the present report, we show that antigens present in the WE can activate macrophages directly resulting in production of NO and cytokines IL-12, IL-10 and TNF-α. We found that the NC bound fractions F1 to F11 stimulated THP-1 cell line to produce impressive amounts of proinflammatory cytokines, IL-12, TNF-α and IL-10. These fractions failed to stimulate any cytokine production by normal PBMCs in our previous study, which may be a reflection of the difference due to the milieu. Fractions F1-F11 also stimulated RAW 264.7 to produce NO. These data show that these fractions are potent stimulators of monocytes.

Cells of the monocytes-macrophage lineage are activated through a variety of surface receptors of which TLRs are important for pathogen recognition [24]. Our data suggest that molecules present in WE and its fractions have ligands for one or more cell surface/or internal TLRs. In this context, it should be kept in mind that in addition to binding with surface receptors, F1 to F11 could be efficiently taken up by these cells since these are NC bound particulate antigens. It has been shown that the innate immune response activated by TLRs play an important role in control of leishmania infection [24-26]. The adaptor protein, MyD88 is required for the secretion of IL-1α by mouse peritoneal macrophages following infection by *L. major* promastigotes [11]. MyD88-deficient mice are more susceptible to infection with *L. major* than the wild type mice [27] and LPG has been shown to activate both mouse macrophages and human NK cells through TLR2 [28]. In addition, in vivo studies in mice have revealed an important role for TLR4 in the control of *L. major* infection possibly through the regulation of inducible NO synthase expression [29-30].

The ability of these antigenic fractions to stimulate IL-12, TNF-α and IL-10 by monocytes can be exploited for an adjuvant effect. The fractions stimulated more IL-10 than the known TLR4 ligand, LPS whereas IL-12 and TNF-α levels were less than that stimulated by LPS.

In a study similar to ours, Mirshahidi et al. (1998) investigated the effects of *L. major* promastigotes, crude antigenic fraction (CAF) and its subfractions on NO production and IL-12 secretion by peritoneal macrophages of *L. major* sensitized BALB/c mice. The subfractions 1, 2 and 3 of CAF, in the MW range of 97.4–66, 66–45 and below

45 Kd, respectively, were separated by SDS-PAGE. It was found that NO production was stimulated by promastigotes but not by CAF or its subfractions. IL-12 secretion was stimulated by promastigotes, CAF and fraction 1 (MW range 97.4–66), while fractions 2 and 3 did not have any effect. In our study as well, antigenic fractions F1 to F4 (covering the MW range of >64.2 Kd) induced higher levels of NO and IL-12, IL-10 and TNF-α than fractions F5 to F11 (MW range <64.2 Kd).

WE and fractions F1 to F11 also stimulated impressive NO production, which is a beneficial anti-parasite function. Parasite killing is dependent on NO production [31] and mechanisms for cure or resistance to *Leishmania* infection are associated with TNF-α and IFN-γ induced activation of free radical production by activated macrophages [32]. Studies in mice have outlined NO pathway as a central component of the anti-leishmanial mechanisms of macrophages. However, the outcome of infection is crucially dependent on the Th1:Th2 balance [33]. In the present study, WE and fractions stimulate IL-12, which helps in IFN-γ production by T cells and also polarizing the Th1:Th2 cell balance toward Th1 type, which may be important in effecting cure of leishmanial infections.

Conclusions

Parasite molecules/antigens that can directly activate APCs and through them act as Th1-type adjuvant would be useful component of any vaccine formulation, since the addition of such adjuvants has been shown to be beneficial in various models of leishmaniasis [34, 35]. Our result suggest that high molecular weight fractions should have advantages as components of any immunotherapeutic approach for leishmaniasis since in addition to T cell stimulatory potential, which we have shown before, they also have properties of a Th1 adjuvant.

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