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RESEARCH ARTICLE

Leishmania donovani: Evidence for the role of Kinetoplastid Membrane Protein-11 in cellular immunity and anti-leishmanial macrophage function in human Visceral Leishmaniasis.

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Abstract

Although widely discussed, information related to immunological significance and vaccine prospects of KMP-11 of *L. donovani* is not available in clinical cases of human visceral leishmaniasis. For this reason, after presentation rKMP-11 antigen through macrophages of VL patients to T-cells of healthy control, we examined the immune-stimulatory changes in naive T-cells and simultaneously monitored the influence of withdrawal of parasite inflicted immune-suppression on the function of infected macrophages of VL cases. Macrophage function of VL patients considerably changed in the presence of T-cells from healthy against rKMP-11 antigen, which enhanced HLA-DR expression and instead of a characteristic high level resulted in a sustained low level of IL-10 together with the induction of super-oxide radicals in patients. On the other hand, after presentation of rKMP-11 by infected macrophages from patients, T-cells of healthy control significantly proliferated accompanied with an up-regulated IFN-γ production and a reduced Th2 like cytokine trend (IL-4 and IFN-γ) against rKMP-11 antigen. These *in-vitro* results provide insight into the importance of KMP-11 in VL and support a role for KMP-11 to screen it as a vaccine candidate against VL.

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Introduction

Visceral leishmaniasis (VL, kala-azar) is the systemic and disseminated form of the disease, where the primary target of infection is the bone marrow, spleen and liver. It is typically caused by *L. donovani complex*, which includes three species, *L. donovani* (Indian subcontinent and East Africa), *L. infantum* (Mediterranean basin) and *L. chagasi* (Latin America) (Desjeux, 1992; 1996). Malnutrition, immunosuppressive drugs or immuno-compromised state (HIV infection) can convert subclinical cases into clinical disease (Cerf et al, 1987; Alvar et al, 1997). Therapeutic options for controlling leishmaniasis are limited to a few drugs with inconsistent efficacy and side effects. This has led to increased emphasis to develop a safe and effective *Leishmania* vaccine to help prevent new cases of leishmaniasis worldwide each year (Desjeux, 2004). Attempts were henceforth made to explore major surface antigens, such as the metalloproteinase Leishmanolysin gp63, the promastigote surface antigen (PSA) complex and lipophosphoglycans (LPG) (Chang et al, 1990; Connell et al, 1993). In one study, LPG was observed highly immunogenic with an up-regulated Th1 response in mice and was capable of protecting susceptible mice against *Leishmania* (Russell et al, 1988; Mendonca et al, 1991). A close scrutiny of *L.donovani* LPG complex has revealed that it is non-covalently associated with a protein of molecular size 11 Kda, which is denominated as KMP-11 (Jardim et al, 1995). Further works precisely indicate the significance of KMP-11, since mouse lymphocytes became frequently immuno-reactive in response to KMP-11 antigen suggesting widespread T-cell epitope conservation in this protein (Tolson et al, 1994). We henceforth

examined the macrophage function of VL patients in the presence of immunocompetent T-cells of normal control against rKMP-11 antigen of *L.donovani*. To further explain its prospect as vaccine, changes in immunological response of normal T-cells were examined after presentation of rKMP-11 antigen through *L.donovani* sensitized macrophages from VL cases.

Material and Methods

2.1 Samples from VL patients and control

The present study was approved by the Ethical Committee on Human Subjects at the Rajendra Memorial Research Institute of Medical Science (ICMR) Patna, India. Consents were obtained from all patients and donors for blood sampling. Ten subjects of both sexes aged between 18 and 45 years (10 patients with acute VL in their pre-treatment stage and 10 controls) were studied. Amastigotes of *L. donovani* had been found in bone marrow and/or spleen aspirates from each of the VL cases investigated and, when blood samples were collected, each of these cases had splenomegaly and had been suffering with continuous or intermittent fever for at least two weeks. The control group had no splenomegaly or recent history of illness. Potential subjects who were unwilling to give their informed consent were excluded.

2.2 Parasite culture

Leishmania donovani strain AG83 (MHOM/IN/1983), originally obtained from an Indian VL case, was maintained in golden hamsters (Saha et al., 1995). Triturated spleen from an infected hamster was used to set up a promastigote culture at 24^0C in M199 medium (Sigma, USA) supplemented with 10% foetal calf serum, 200U penicillin and 200 µg gentamycin per 100 ml (Salotra et al., 1995). The soluble leishmania antigen (SLA) was prepared from promastigotes in late log phase, 3-4 days after a few passages in liquid culture. Briefly 200 x 10^6 promastigotes per ml were washed thrice in 5ml of cold PBS. After 5 cycles of freeze and thaw, the suspension was centrifuged at 10,000 x g for 20 min at 4^0C and the supernatant containing soluble leishmania antigens was collected and stored at -30^0C till further use. The protein concentration was measured by Bradford's method.

2.3 Expression, isolation and purification of rKMP-11 protein

To obtain the recombinant protein preparation from *Leishmania,* the plasmid (pQE-30) containing the KMP-11 gene along with pREP4 were introduced into DH5α strain of *Escherichia coli*. The recombinant KMP-11 protein contains 273 bp *L.donovani* specific KMP-11 gene which was 3.4 kb and it was cultured in Luria Bertani medium containing ampicillin (25µg/ml) and Kanamycin (100µg/ml) overnight at 37^0C under 200 RPM in shaker incubator for 4-5 h. The recombinant protein was subsequently induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1mM/ml) overnight in shaker incubator at 22^0C and 200 RPM. The bacterial cells pelleted after harvesting (5000 RPM) were lysed in cell lysing solution (1:4), pH 8.0 (150 mM NaCl, 10 mM Tris HCl, 2% SDS) for 30 min at 4^0C and further lysed by ultra-sonication at 85% amplitude and 0.5 second pulses for 5 min. Following centrifugation (14000 RPM at 4^0C), recombinant protein was purified on a Ni^{+2} NTA super flow column (Qiagen) according to the manufacturer's instructions. The protein was eluted in 8M urea salt buffer with 250 mM imidazole and dialysed in PBS at pH 7.4. The protein expression was analysed by immunoblotting using anti KMP-11 mouse MAb, clone L157 (GenWay, USA). Before use, LPS in the KMP-11 samples was removed by polymyxin B-agarose column (Sigma, USA) according to the manufacturer's instruction.

This KMP-11 protein was now presented to CD14+ macrophages from VL patients co-cultured with T-lymphocytes from healthy control to examine T-cell proliferation, cytokine induction and anti-leishmanial macrophage function in an *in-vitro* set-up.

2.4 Generation of macrophages from peripheral blood mononuclear cells (PBMCs)

Macrophages were obtained following collection of heparinised samples of venous blood and PBMCs isolation from Buffy coats using Ficoll- Hypaque™ (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation (Oliver et al., 1992). Monocytes from PBMCs from KA patients were isolated by adherence of the cells in a plastic petri-dish and its purity was determined by FACS of CD14, CD19, CD45 and CD3 expression (Jayakumar et al., 2008), which were further cultured for another 72h incubation at 37^0C in CO_2 incubator and revealed that morphologically almost all (92%) of the cells left in the dishes appeared to be macrophages. Cells were counted and distributed in different wells, then added different antigens: rKMP-11, SLA at 10 µg/ml concentration and kept for 2 h incubation at 37^0C in CO_2 incubator.

2.5 T – Lymphocytes response in healthy donors after presentation of KMP-11 antigen by infected macrophage of visceral leishmaniasis patients

To examine immunological changes in T-cell proliferation in response to rKMP-11, cells were then treated with 250µl of a solution containing trypsin in

a spermine tetrahydrochloride detergent buffer (BD Pharmingen, San Diego, CA, USA) for 10 min and later treated with 200 µl of a solution containing trypsin inhibitor and RNase buffer (provided with the kit) for 10 min at RT. These cells were incubated with 200 µl of propidium iodide (PI) stain solution for 10 min in the dark on ice to allow PI to bind to isolated nuclei. The stained cells were finally run on a FACS-Calibur (BD, San Diego, CA) with FL-2 detector using a 585/42-band pass filter. Finally, the FL-2-A DNA histogram was analyzed using Modfit software (Becton Dickinson) on the FACS Calibur.
To find out the relation of KMP-11 induced proliferation of T-cells with the effect that KMP-11 had on the production of IFN-γ/IL-4 was determined by flow cytometry. In brief, cells were stimulated with fixed (2% formaldehyde) Ld promastigote antigen in responder (APC) to stimulator (Fixed Ld) ratio of 50:1 for 2h at 37^0C and of them $1x10^6$ cells were cultured in 96 well round bottomed plates in the presence of purified (KMP-11) protein (20µg/ml). Control cultures were set up in medium alone or medium containing PHA 10 µg/ml. Intra cytoplasmic cytokine level was detected on FACS-Calibur as previously described. Cells were cultured for 14h followed by 4h incubation with a protein transport inhibitor, brefeldin-A (1 µg/ml). The harvested cells were consecutively co-incubated with PE conjugated anti- CD-4 antibodies, cytofix/cytoperm solution and FITC conjugated IFN-γ/IL-4 antibodies (BD Pharmingen, USA) before each sample was re-suspended in 500 µl stain buffer. Samples were analysed using the Flow Cytometer using CellQuestPro Software.

2.6 Super-oxide generation and IL-10 production by differentially co-cultured infected macrophages in presence of autologous or T cells from healthy volunteers

Measurement of Reactive oxygen species (ROS) activity in whole blood was done by flow-cytometry as described. Briefly, 100µl blood was triggered by 20 µg/ml of KMP-11 antigen or 10µg/ml SLA and FMLP (5µg/ml) at 37°C in water bath (10 min.). This followed further incubation with 20 µl of 10 µM Dihydrorodamine 1,2,3 (DHR 123) at 37°C in water bath (15min) to allow internalization of the latter into the cell followed by its conversion into the green fluorescent compound and later binding to the oxidative burst produced by stimulated cells. Erythrocytes were then lysed at room temperature with 2 ml FACS lysing reagent (Becton Dickinson), washed (1X PBS, 268g, 5 min.) and re suspended in 450 µl PBS containing 1% formaldehyde. The ROS produced by the stimulated cells were measured on the basis of mean fluorescence Intensity (MFI) detected on FACS.
Further investigation was carried out to find out the effect of KMP-11 on the activation of macrophages in relation to cytokine (IL-10) release after a support through immunocompetent T-cells. In brief, cells were cultured for 14h followed by 4h incubation with a protein transport inhibitor, brefeldin-A (1 µg/ml). The harvested cells were consecutively co-incubated with FITC conjugated anti-CD14APC antibodies, cytofix/cytoperm solution and PE conjugated IL-10 (BD Pharmingen, USA) before each sample was re-suspended in 500µl stain buffer. Samples were analysed using the Flow cytometry.

2.7 Statistical Analysis

All data were expressed as mean ± SE (standard error of the mean). Statistical analysis was carried out using GraphPad Prism5, USA software, by one way analysis of variance with a post-test, only perform a post-test if $p<0.05$ (Tukey test compared all pairs of columns). A value of significance $p<0.05$ was considered statistically significant.

Results

3.1 Characterisation of KMP-11

Results on SDS polyacrylamide gel electrophoresis of KMP-11 purified recombinant protein and the localization of KMP-11 protein after immunoblotting with anti-KMP-11 anitbody is shown in Figure 1. The extraction of KMP-11 protein was achieved by Ni^{2+}-NTA immobilized column chromatography. The bound protein was eluted in the presence of imidazole and later subjected to 12.5% SDS-PAGE (Figure 1A). The anti-KMP-11 mAbs were later used in immunoblots on SDS-PAGE separated KMP-11 proteins from *Leishmania* spp. which were previously purified by Ni^{2+}-NTA affinity column. The mAbs detected bands of approximately 11 KDa (Figure 1B).

3.2 Leishmania KMP-11 antigen induces HLA-DR expression in a major extent in macrophages than with other stimulants

Since the protective immunity is T-cell mediated, the antigen of *Leishmania* is mostly among those presented in context to MHC class II molecule, we initially examined the modulation in the response of antigen presenting cells for HLA-DR expression required for activation of cellular immune response in KMP-11 treated macrophage cells. As illustrated in Table-1, infected macrophages from VL patients when co-incubated in the presence of T-cells from healthy, demonstrated an up-regulated HLA-DR expression against rKMP-11 antigen administered

either alone ($p<0.001$) or in combination with SLA ($p<0.001$).

Figure 1

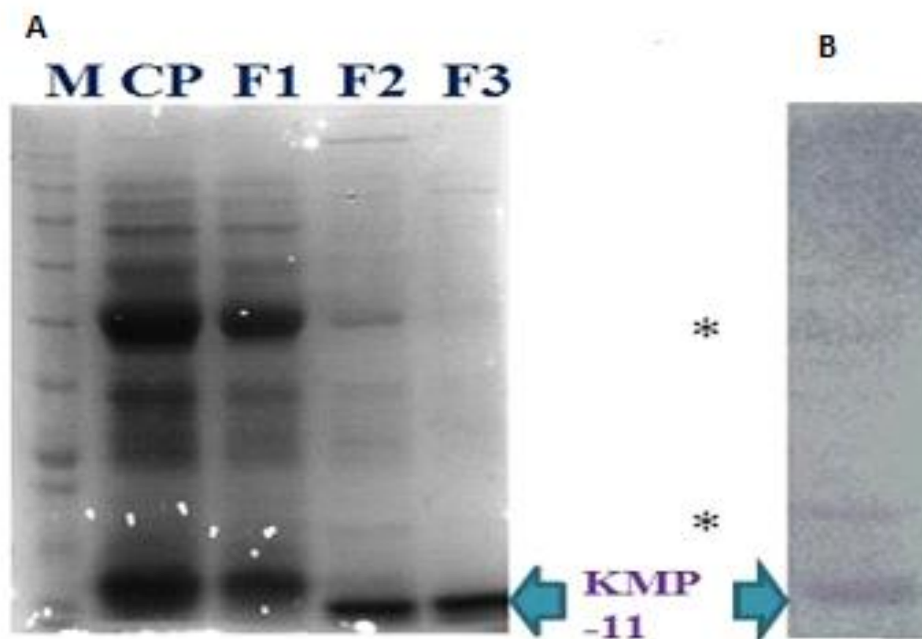

Figure 1:- SDS-PAGE and western blot of KMP-11. (A) SDS Polyacryamide gel electrophoresis of *L.donovani* of KMP-11 purified recombinant protein. Lane 1 Marker, Lane 2 CP- Crude Protein, Lane 3 & 4 protein fractions of KMP-11 and Lane 5 shows the purified KMP-11 recombinant protein. (B) KMP-11 recombinant protein purified by affinity chromatography, transferred on nitrocellulose paper and probed with anti-KMP-11 antibody. The arrow indicates the localization of KMP-11 protein. The asterisks show the dimeric and trimeric forms of the KMP-11 protein.

Table 1:- Immunological changes after presentation of KMP-11 antigen by infected macrophage of visceral leishmaniasis patients to T-Lymphocytes of healthy donors

Parameters		% Cell after stimulation with			
		Saline	SLA	KMP-11	KMP-11+ SLA
HLA- DR	Macrophage	**6.19**±0.78	**10.18**±0.36	**26.92**±0.56	**49.55**±0.91
Proliferating T-Cells in S- phase	After 2h	**0.02**±0.01	**1.32**±0.71	**0.97**±0.13	**1.54**±0.33
	After 16h	**0.78**±0.02	**1.33**±0.03	**3.57**±0.91	**3.62**±0.01
Cytokines	CD4+IFN-γ	**7.98**±0.91	**9.12** ±0.09	**10.34**±0.05	**15.36**±0.17
	CD4+IL-4	**13.19**±0.97	**18.10**±0.05	**11.10**±0.05	**4.98** ±0.08
	CD14+IL-10	**4.31**±0.20	**5.70** ±0.05	**3.77** ±0.01	**2.64** ±0.02

$^{\$}$Macrophages from VL patients stimulated with 20 μg/ml of KMP-11 antigen or 10 μg/ml SLA and co-cultured in the presence of T-cells of healthy control. For HLA-DR, cultured cells were stained with anti-HLA-DR PE. For cell cycle study the cultured cells were stained with BD-Cycle Test plus Kit. For intracellular cytokine study, the cultured cells were stained using BD cytofix/cytoperm kit as per instruction manual. All stained cells were acquired and analysed on flow cytometer. Data represents mean ± SE.

Abbreviations: SLA= Soluble leishmania antigen, KMP-11= Kinetoplastid membrane protein-11, S-phase= synthesis phase of cell cycle.

3.3 Modulation in T-cell response during inductive phase in healthy after presentation of KMP-11 antigen by infected macrophages from VL patients

When the progression of T-cells of healthy control were examined against rKMP-11 antigen in the cell cycle (Table-1) it was demonstrated that the antigen induced a significant proliferation of T-cells in S-phase. The ability of T-cells to enter in S-phase of cell cycle became higher than other stimulation group at 16 h incubation with rKMP-11 antigen primed macrophages of VL patients ($p<0.001$ with saline; $p<0.005$ with SLA). The corresponding value for % of T-cells in S-phase induced by these antigens at 2 h incubation in group stimulated with SLA linked to KMP-11 antigen was less ($p<0.005$) which went further low when KMP-11 antigen was used alone.

3.4 Modulation in cytokine response

As mentioned in Table-1, the response of the same T-cells to produce IFN-γ was observed low (9.12%) against SLA. Considerable immunological changes were observed in T-cells of healthy control, which generated 15.36% IFN-γ in response to Ld-sensitized macrophage presented rKMP-11 antigen, indicating the T-cells immunogenic characteristic of this antigen. Subsequently it was observed that CD4 T-cells produced a high level of IL-4 with SLA (18%) but it produced relatively less IL-4 about 4.98% against rKMP-11 antigen which was delivered to these cells by infected macrophage. We went further to examine the IL-10 response from where a direct evidence for a positive transformation in the functioning of *Leishmania* infected macrophages was obtained. As demonstrated, instead of a characteristic high IL-10 reportedly produced in VL patients (Bimal et al, 2008; Narayan et al, 2009) a sustained low level of the IL-10 level was obtained against all

stimuli including the KMP-11 protein (2.64%) during a co-culture of patients macrophages with T-cells of healthy control.

3.5 Super-oxide generation by differentially co-cultured infected macrophages in presence of autologous or T cells from healthy volunteers

We also examined the yield of super-oxide generated in patient's macrophages after the co-culture to address whether the activated healthy T-cells could mount an effect on the functioning of the infected macrophages (Fig2A, 2B). Macrophages showed a lower mean fluorescence intensity for super-oxide generation *ex-vivo* with both autologous and T-cells of healthy control ($p>0.001$); this trend was slightly lower with autologous T cells. A co-culture with previously activated T-cells of healthy control always yielded a higher mean fluorescence intensity for super-oxide generation in the macrophages of the patients compared to the values shown by these cells in the presence of their autologous T-cell counterparts. Among stimulated groups, the effect of KMP-11 was observed much higher along with SLA ($p<0.001$) rather than with purified protein alone in which the difference was not more significant than soluble leishmania antigen with both autologous or T-cells of healthy control ($p<0.05$), when compared with *ex-vivo*.

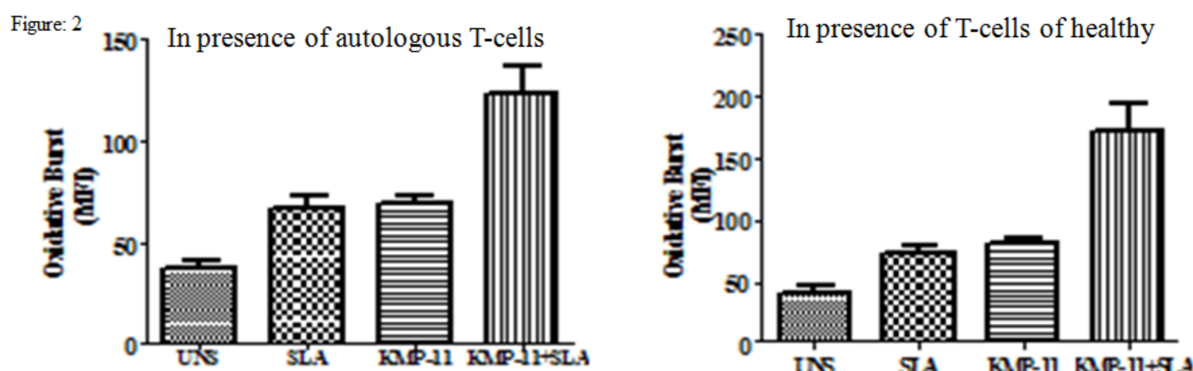

Figure 2:- The ROS produced by the stimulated cells. The Reactive oxygen species produced by the stimulated cells were measured on the basis of mean fluorescence Intensity(MFI) detected on FACS- (A) In presnce of Autologous T-cells, (B) In presence of T-cells of healthy control.

Discussion

The pentavalent antimonials (Sbv) meglumine antimoniate and sodium stibogluconate (SSG) were the most successfully used drugs for treatment of all forms of leishmaniasis including VL since 1940 (Jha, 2006). This drug has however failed to ensure complete protection against VL with increased unresponsiveness in VL patients reported using WHO regimens in Bihar (India). To respond to this problem of drug failure in kala-azar, attempt to identify the appropriate vaccine target may be an option. The KMP-11 antigen has been shown to be highly immunogenic in previous studies (Tolson et al, 1994). This background together with the failure of T-cells from patients with visceral infection to secrete IFN-γ has given a strong argument for researchers to test this antigen as a vaccine candidate (Bimal et al 2008; Narayan et al 2009). Unfortunately, the most of the vaccine studies aiming to test the potential of leishmanial antigens as vaccine targets have failed with inconsistent results as studied in experimental animals. Certain responses in case of humans may not be apparent from studies in animal models.

Therefore we have tested the potential of KMP-11 antigen in a criss-cross experimental set up, whereby, the immuno-modulator, the KMP-11 antigen exerts wider immuno-stimulatory effects in non-sensitized T-cells of apparently healthy individual following the presentation of this antigen by *L. donovani* sensitized infected macrophages from VL patients and simultaneously, the antigen sensitized macrophages of patients might develop strategies, withdrawing the parasite inflicted immuno-suppression.

The response magnitude which we observe in T-cells of healthy individuals following KMP-11 presentation through infected macrophage of patients yielded useful information. Given that the proliferation of T-cells in response to an antigen is instrumental for activation, we monitored the progression of T-cells of healthy control in the cell cycle in the presence of KMP-11 antigen which was presented to them by infected macrophages. A

majority of T-cells were observed in G0/G1 phase after 16 h culture with Ld antigen, which suggests a reduced proliferative stage in T-cells due to lack of immunological memory against the *L. donovani* parasite. As such, to validate the significance of KMP-11 antigen in T-cell function, it was important to establish that after stimulation of KMP-11 by infected macrophages, normal T-cells could proliferate in response to *L.donovani.* On the other hand, after stimulation of *Leishmania* un-sensitized T-cells in control with KMP-11 antigen, the cells behaved differently and progressed more in terms of the proportion of T-cells in G2/M and in S-phase. These results are a strong indication that KMP-11 antigen of *L.donovani* can enhance the adhesive forces to optimize antigen recognition and subsequent T-cells proliferation.

At this point, given the fact that KMP-11 was a key candidate in driving a pronounced change in cell cycle behaviour, we attempted to relate this with the potential of KMP-11 antigen in un-sensitized healthy individuals to induce a protective response in CD4+ cells. That the stimulation of un-sensitized T-lymphocytes in healthy control by KMP-11 antigen induced a marked increase in IFN-γ production illustrates how the actual activation of T-cells by this antigen can help to improve the *L.donovani* recognition process of T-cells. It was also shown that T-lymphocytes suppress their activation for the secretion of IL-4 and as such we assume that KMP-11 antigen, if taken as vaccine candidate can help transform the T-cells in immunocompetent stage in order to combat VL, if exposed in the future.

The long term adaptive immune response against phagosomal *Leishmania donovani* initiates through binding of TCR-CD3 complex with peptide-MHC class II complex on the surface of APCs (Bimal et al., 2005).In this context, it has also been shown that, HLA-DR has an important role in the processing of *L. donovani* peptide antigens for presentation of Th-cells (Bimal et al 2001). Our data showing the enhanced HLA-DR expression in response to KMP-11 antigen on macrophages of VL cases in the presence of T cells of control corroborate with earlier encouraging findings obtained in response to this antigen in the activation of T-cells and suggests that KMP-11 can enhance antigen presentation during the initial encounter of antigen specific T-cell receptor on the surface of *Leishmania* infected macrophages. This effect signifies the importance of KMP-11 antigen as future vaccine prospect since expression of class-II MHC is largely brought about due to IFN-γ produced by the T-lymphocytes following the antigen presentation, which eventually has been shown to induce class II trans-activated (CIITA), a factor better known for the promotion of class II MHC molecules such as HLA-DR on the macrophages.

The ability of Leishmanial antigen to survive in the macrophages depends on the degree of the magnitude through which parasite impairs free radicals (super-oxide and nitric oxide) generation and facilitate disease progression by increasing the amount of IL-10 in the macrophages (Bimal et al 2012). Therefore, we also examined the potential of KMP-11 antigen to stimulate the infected macrophages, particularly in a situation when there was no suppression and it was supported through immunocompetent T-lymphocytes. The results suggested that in combination with a protective T-cell support against rKMP-11 antigen, there was induced a significantly higher super oxide free radicals, which possibly worked in tandem with T-cells from which a very high production of IFN-γ was produced. As shown in the present study KMP-11 antigen of *L.donovani* can be a strong inducer of IFN- γ con-currently with minimizing the impairment due to *L.donovani* parasite in the generation of free radicals. Therefore we demonstrate that a novel vaccine approach can be implemented with the use of KMP-11 antigen eliminating the parasite induced immuno-suppression and inducing collateral host protective changes in macrophages.

Conflict of interest

The authors declare that there is no conflict of interest.

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