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A New Vaccine Strategy of Dendritic Cell Presented Kinetoplastid Membrane (KMP-11) as Immunogen for Control against Experimental Visceral Leishmaniasis

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Abstract

Available reports suggest that, *Leishmania donovani* antigen KMP-11 may be significant in the modulation of immune responses in visceral leishmaniasis (VL). This study evaluated vaccine prospect of presentation of KMP-11 antigen through murine dendritic cells against VL in infected BALB/c mice. We report here that immunization with KMP-11 delivered through bone marrow derived dendritic cells can lead to killing of *L. donovani* in infected BALB/c mice. Furthermore, the strategy to use KMP-11 as vaccine delivered through DCs can stimulate the production of IFN-γ, IL-12, IL-2R and TNF-α with concomitant down-regulation of IL-10 and IL-4. Furthermore, anti-leishmanial defence function (ROS) of splenocytes was observed increased in the presence of DC-delivered KMP-11 vaccination accompanied with an increased p38-MAPK signalling in vaccinated splenocytes. We summarized from our data that KMP-11 delivered through DCs has potential for eliciting protective immunity through pro-inflammatory cytokines (IFN-γ, IL-12, IL-2, TNF-α) following an up-regulation in signalling event of p38-MAPK. Therefore the study suggests a new control strategy against VL in future.

Keywords

Visceral Leishmaniasis, Kinetoplastid Membrane Protein 11, Soluble Leishmania Antigen, Interferon-γ, Interleukin-12, Interleukin-10, Dendritic Cell Primed KMP-11

1. Introduction

Leishmania, a protozoan pathogen, is a causative agent of various forms of

Leishmaniasis like cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL), of which VL is almost fatal if untreated [1] [2] [3]. Drugs used for chemotherapy of leishmaniasis, such as antimonials, miltefosin, paromomycin, and amphotericin-B, are toxic and expensive, and frequent resistant occurs against these drugs in endemic areas [4]. Previous studies have shown that clinical resolution of VL depends on protective Th1 type of immune-response. This remains suppressed in VL cases as evidenced by decreasing production of IFN-γ and IL-12. Such responses are critically regulated by IL-10, a pleiotropic cytokine secreted by many cell types including macrophages. In addition, rIL-10 is reported to have inhibitory action on NO-mediated leishmanicidal activities in macrophages (MΦs). A third front exists, which suggests that in endemic areas, many individuals reside which do not manifest clinical symptom, but they show elevated level of Th1 type of immune response. This might be due to stimulation of protective immune response by some leishmanial antigens. Therefore, any intervention that helps to shift the Th2 type of immune response towards Th1 may be beneficial for control against VL.

Infection of *Leishmania* parasite in dendritic cells has been reported in several studies [5] [6]. Antigen presenting cells (APCs) help in establishment of immunological memory as well as induction of a primary immune response [7]. In the light of these findings, it appears that macrophages through the preferred host for *Leishmania* may synergise with *Leishmania* to facilitate infection [8] [9] and as such secreting dendritic cells for parasite delivery to provoke host protective immune response is necessity.

The outer membrane of *Leishmania* is covered by a dense glycocalyx consisting mostly lipophosphoglycan (LPG) [10]. Kinetoplastid Membrane Protein 11 (KMP-11) is a major surface protein of *L. donovani* species which remains non-covalently attached with LPG [11]. KMP-11 plays important role in reversal of T-cell anergy and synthesis of interleukin-12 as well as up-regulation of inducible nitric oxide synthase (iNOS) in animal model [12] [13]. We reported earlier, immunomodulation in monocyte derived dendritic cells leads to induction of IFN-γ by rLdKMP-11 antigen [14].

2. Material and Methods

2.1. Dendritic Cells Generation from Bone Marrow of BALB/c Mice

Bone marrow cells from BALB/c mice were taken and cultured for 8 - 9 days in 6 well plates in the presence of murine recombinant GM-CSF (20 ng/ml) (BD Biosciences, USA) and antibiotics (Sigma, USA) in complete RPMI-5 media (supplemented with FSC 5%) [15]. 1×10^6 cells/ml were added in each well. Cells were washed and fed every 2 days. Between day 5 and day 8, many aggregates of immature DCs were found which were dislodged and centrifuged and re- suspended the pellet in RPMI-5. During the 24 to 48 h following transfer, we collected the non-adherent, non-proliferating, maturing DCs at the interval of 24 h and counted the viable cells by microscope and assessed the yield of DCs by

Flow Cytometry. DCs were positive for CD86 and DEC 205.

2.2. Effect of Doses and Concentration of Immunogen for Tolerance of Splenocytes in BALB/c Mice

Briefly, splenocytes were subjected to immunogen at 3 different concentrations (5, 10 & 20 μg/mL) for 48 h incubation followed by RPMI-MTT treatment (1 mg/mL) at 37˚C for 3 h then it was kept in isopropanolic solution for another 15 min and finally absorbance was taken at 570 nm [16].

2.3. Detection of Anti-KMP-11 Antibody in VL Patients

The recombinant KMP-11 protein was obtained as described previously [14]. Serum samples were pooled from 10 treated VL patients (3 ml each). Then serum (0.1 ml) was mixed with 0.1 ml of 6% polyethylene glycol (PEG) in PBS and incubated for 1h at 4˚C. Mixtures were centrifuged at 1000 g for 60 minutes at 4˚C and the pellet was washed twice in a final concentration of 3% PEG and resuspended this pellet in 0.1 ml PBS. Now this resuspended pellet in PBS were run on SDS-PAGE (12.5%) by following Laemmli method [17], transferred this gel onto NCP membrane (Sigma, USA) using trans blotting chamber at 50 V for 1 h then at 100 V for 1 h. This membrane were kept overnight at 4˚C in blocking buffer (5% BSA in Tris-buffered saline) then washed 3× TBS-T (0.1% tween), added primary antibody anti-KMP-11 (GenWay Biotech, Inc, San Diego, CA, USA) at 1:1000 dilution and incubated for 2 h at room temperature, again washed 3× and added secondary antibody anti-IgG HRP in different dilutions (1:500 & 1:1000). Finally the enzyme activity was visualized by the use of DAB (Diaminobenzidine) as chromogen in the presence of H_2O_2 [18].

2.4. Immunogen, Immunization and Challenge

BALB/c mice were divided into 5 groups: (a) uninfected control, (b) infected control, (c) immunized with DC-KMP-11 and infected, (d) immunized with SLA and infected and (e) immunized with Con-A and infected. Mice were immunized by intramuscular injection in the hind thigh with sterile saline on days 1 and 15. On day 27, immunized mice were challenged with intra-cardial injection of 1×10^7 virulent strain of freshly transformed *L. donovani* (AG83) promastigotes. Finally, animals were sacrificed on 60 days after infection, and spleen from different groups were isolated to determine splenic parasite burden. The number of parasites present in the spleen was determined by microscopic evaluation of Giemsa-stained tissue imprints as described previously [19]. The parasite burden in the respective organs was expressed as LDU [20].

2.5. Determination of Live Parasite Burden

Briefly, a weighed portion of spleen from different groups of experimental mice 60 days after infection was dissected out and mildly homogenized in complete medium 199 (M199, Sigma USA) and re-suspended at a final concentration of

1 mg/mL in the same medium. Cells were washed, fixed in methanol and giemsa stained for enumeration and expression of parasite load as amastigotes/100 macrophage. To further determine the efficacy of DC-primed KMP-11 as vaccine, splenocytes were washed and stained with popidium iodide (PI) for FACS determination of percentage of killing in different groups of animals. The mean of fluorescence intensity of the cells were analyzed by flow cytometry FACS Calibur and CellQuest software.

2.6. Assessment of T-Cell Proliferation

Proliferation of splenocytes isolated on day 60 after infection from the infected groups of mice (n = 10) was measured in response to KMP-11 (10 μg/mL). Briefly, single cell suspensions of splenocytes were plated at a concentration of 0.1×10^6 cells in 96 well plates and were allowed to proliferate for 3 days at 37˚C in a 5% CO_2 incubator in the presence or absence of KMP-11 antigen (10 μg/mL). Cell proliferation was determined by BrdU (5-bromo-2' deoxyuridine) incorporation on FACS Calibur. Soluble leishmania antigen (SLA) was used as control.

2.7. Analysis of Th1 and Th2 Cytokines

Subsequent experiments were performed to look at the potential of vaccination to drive CD4 T-cell cytokine response in splenocytes of vaccinated mice. Splenocytes were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). Cells (2×10^6/mL) were cultured in 12-well plates for 24 h with purified *L. donovani* protein (KMP-11). The culture conditions involved humidified 5% CO_2 incubation at 35˚C ± 1˚C, 6 h prior to completion of incubation, the plate was supplemented with monensin containing golgi stop (2 μM/mL final concentration, Pharmingen). The cells were then harvested using ice-cold PBS plus azide, cytofixed using 2% formaldehyde. The cells were then permeabilized with a solution of saponin and stained for 30 min using antimouse cytokine antibodies directly conjugated with FITC (IFN-γ, IL-12 & IL-2R), APC (IL-4 and IL-10). PE and FITC labeled immunoglobulin control antibodies as well as a control of unstimulated peripheral blood mononuclear cells (PBMCs) were included in these experiments. The cells were acquired and analyzed within 24 h through FACS Calibur (Flow Cytometer, Becton Dickinson, USA) [21]. The results were analyzed by using BD Cell Quest software (Becton Dickinson, USA).

2.8. Comparison of Anti-Leishmanial Macrophage Function in Un-Immunized and Immunized Group of Mice

Measurements of reactive oxygen species (ROS) activity in splenocytes were accomplished with flow cytometry. Briefly, 100 μL of splenocyte blood was triggered with N-Formylmethionine-leucyl-phenylalanine (FMLP; 5 mg/mL) at 37˚C in a water bath for 10 min, followed by further incubation with 20 μL of 10 mm dihydrorhodamine-123 (DHR-123) at 37˚C in a water bath for 15 min to allow for the internalization of the later into the cell and converted into green

fluorescent compound which binds to oxidative bursts produced by stimulated cells. Further incubation, erythrocytes were lysed at room temperature with 2 mL FACS lysing reagent, washed (1×PBS, 268 g, 5 min) and re-suspended in 450 mL PBS containing 1% paraformaldehyde. The ROS produced by the stimulated cells were measured as mean fluorescence intensity (MFI) and detected by flow cytometry (FACS Calibur).

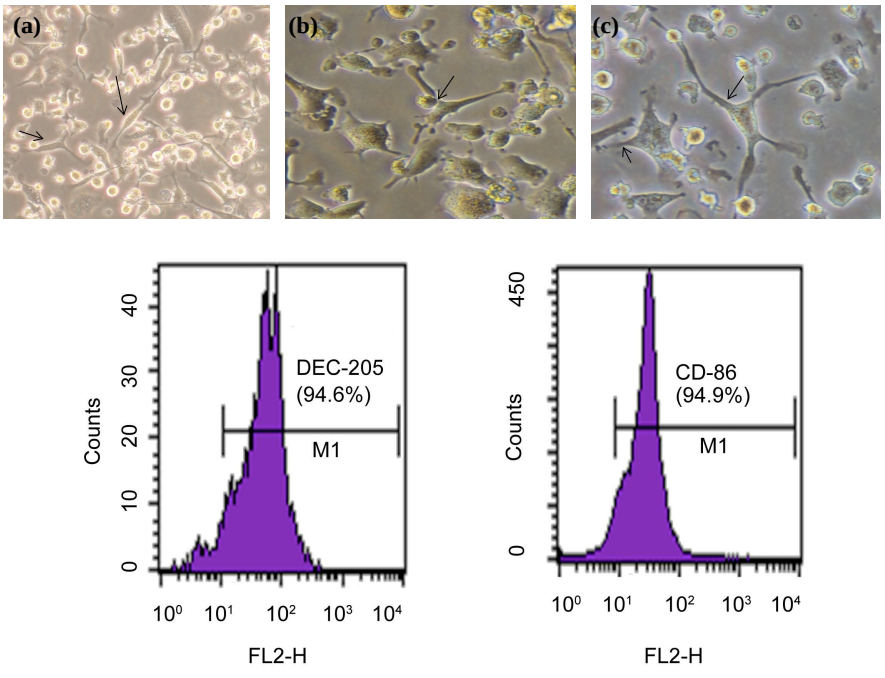
2.9. Statistical Analysis

All data were expressed as mean ± SE (standard error of the mean). One way analysis of variance with Tukey post-hoc test was carried out using Graph Pad Prism 5, USA software. A value of significance $p < 0.05$ was considered statistically significant.

3. Results

3.1. Dendritic Cells Generation from Bone Marrow of BALB/c Mice

DCs from bone marrow of BALB/c mice were obtained as described earlier. Finally, viable cells were counted by inverted microscopic analysis (Figures 1(a)-(c)) and assessed the yield of DCs by Flow Cytometry. DCs were positive for CD86 and DEC 205 (Figure 1(d)).



(d)

Figure 1. (a)-(c): Murine Dendritic Cells from bone marrow cells. The Morphology of murine bone marrow derived dendritic cells cultured for 8 - 9 days in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) under Inverted Microscope; (d) Flow cytometric analysis of sorted murine bone marrow derived DC demonstrates that high level of DEC205+ (94.6%) & CD86+ (94.9%) cells. Data are representative of three separate experiments.

3.2. Effect of Doses and Concentration of Immunogen on BALB/c Mice

It was observed that 10 μg/ml KMP-11 given with 1×10^6 DC resulted in highest viability of splenocytes (Table 1).

3.3. KMP-11 Is Naturally Immunogenic in VL Patients which Possesses Abilities to Overcome Impaired T-Cell Proliferation Associated with VL

The presence of antibodies (1:500 & 1:1000) against KMP-11 in successful treated VL patients sera was detected in western blot analysis with purified protein. We found that treated VL patients sera had antibody against KMP-11 antigen (Figure 2). These findings suggest that KMP-11 antigen is immunogenic in VL patients and hence observed to be appropriate for vaccine testing in animals.

3.4. The Ability of DC-Primed KMP-11 Vaccination in Protection against Virulent *L. donovani*

DC primed rKMP-11 immunization protects against VL. DC primed KMP-11 immunized animals were challenged with *L. donovani* and no. of intracellular parasites were compared on day 60 post infection in spleen from different groups of animals (Figure 3(a)). We observed that following SLA immunization, the splenic percent killing were reduced by 82.5% whereas DC-primed KMP-11 immunization reduced 90.58% parasites from spleen (Table 2). Splenocytes staining with propidium iodide and subsequent FACS data corroborated the preceding finding, ensuring that considerable degree of protection was afforded after DC immunization (Figure 3(b)).

Table 1. Effect of doses and concentration of immunogen on BALB/c mice (O.D 570 nm).

	DCs count		
KMP-11 (Concentration)	**0.5×10^6**	**1.0×10^6**	**2.0×10^6**
5 μg/mL	0.410	0.604	0.712
10 μg/mL	0.414	0.812	0.452
20 μg/mL	0.612	0.680	0.521

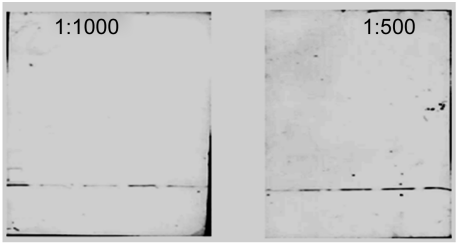


Figure 2. Western blot analysis of sera from VL patients at different dilutions (1:1000/1:500).

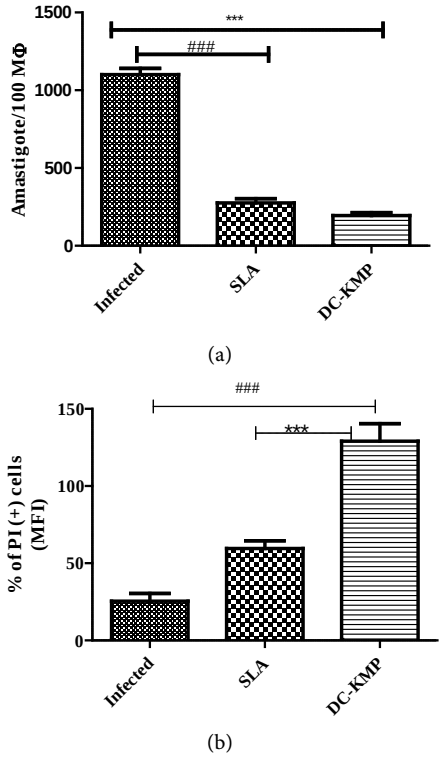


Figure 3. (a) Live parasite burden after DC-KMP immunization (Day 60 post infection). DC primed KMP-11 immunized animals were challenged with *L.d* and no. of intracellular parasites were compared on day 60 post infection in spleen from different groups (SLA/DC-KMP) of animals ### and *** ($p < 0.001$) indicates statistically highly significant between Infected groups vs SLA/DCs-KMP-11. (b) Splenocytes of BALB/c mice were stained with propidium iodide (PI) for FACS determination of percentage of killing in different groups (Infected/SLA/DC-KMP) of animals. Results were measured in mean fluorescent intensity (MFI) by FACS Calibur using CellQuest software.

Table 2. Percent killing of *L. donovani* in spleen following infection at different time kinetics in immunized and un-immunized groups.

Immunization days	Immunized groups (% Killing)		Infected groups
	SLA	DC-KMP	
Day 10	100	400	35
Day 21	50	125	40
Day 42	55	115	25
Day 60	60	132	22

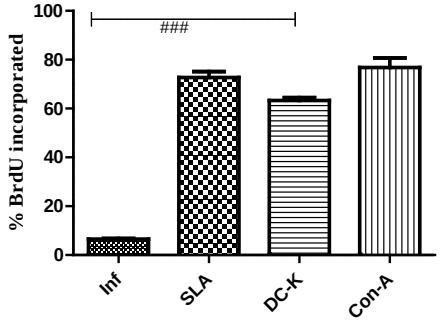
3.5. Dendritic Cells Primed KMP-11 Immunization Reverts Dysfunction of T-Cells Proliferation in Infected BALB/c Mice

We observed enhanced T-cell proliferation from splenocytes in response to KMP-11 antigen in DC-KMP-immunized group compared to infected group ($p < 0.001$). Functional significance of splenocyte proliferation upon re-stimulation to KMP-11 antigen was determined by production of BrdU and IL-2R. Furthermore, the higher trend of proliferation triggered by KMP-11 was corrobo-

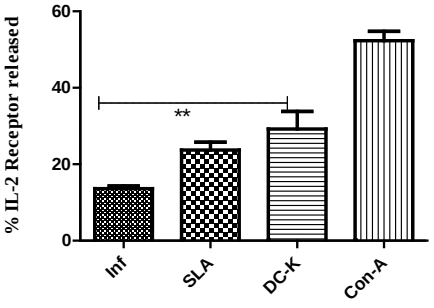
rated by higher production of BrdU (~9.7 fold, $p < 0.001$) andIL-2R (~2.1 fold, $p < 0.01$) compared to untreated infected control (Figure 4(a), Figure 4(b)).

3.6. Evaluation of Ability of Vaccine to Trigger Th1 and Th2 Response in Immunized Mice

As functionally more splenocytes proliferated upon antigenic re-stimulation to KMP-11 antigen, we further extended our investigation to understand nature of immune response generated by vaccination. A total of 10 mice each was tested for this investigation from different study group. As shown in Figures 5(a)-(e) significant increase was observed in IFN-γ production in DC-KMP-11 immunized mice in comparison to un-immunized infected mice ($p < 0.001$). DC–KMP-11 immunization in response to rKMP-11 protein showed about 6.38 fold ($p < 0.001$) more IFN-γ, 4.40-fold ($p < 0.001$) more IL-12 and 3.26 fold ($p < 0.001$) more TNF-α, whereas cells from SLA vaccinated mice produced about 1.57, 2.36 ($p < 0.05$), and 1.79 ($p < 0.01$) fold more IFN-γ, IL-12 and TNF-α, respectively in comparison to controls (Figures 5(a)-(c)). In contrast, vaccination with DC primed KMP-11 inhibited the production of IL-10 by 5.81 ($p < 0.001$) and 1.08 fold (Figure 5(d)) and reduced the production of IL-4 by 2.47 ($p < 0.01$) and 1.16 fold, respectively, compared to controls (Figure 5(e)).



(a) BrdU



(b) IL-2R

Figure 4. T-cells proliferation measurement by 5-Bromo-2'-deoxyuridine (BrdU) incorporation and IL-2 receptor expression from splenocytes of different groups (Infected/SLA/DC-K/Con-A) of mice. Results were measured in mean fluorescent intensity (MFI) by FACS Calibur using CellQuest software (BD Bioscience). (###$p < 0.001$ for infected vs DC-KMP-11 in % BrdU incorporation) and **, $p < 0.01$ for infected vs DC-KMP-11 in % IL-2 receptor released).

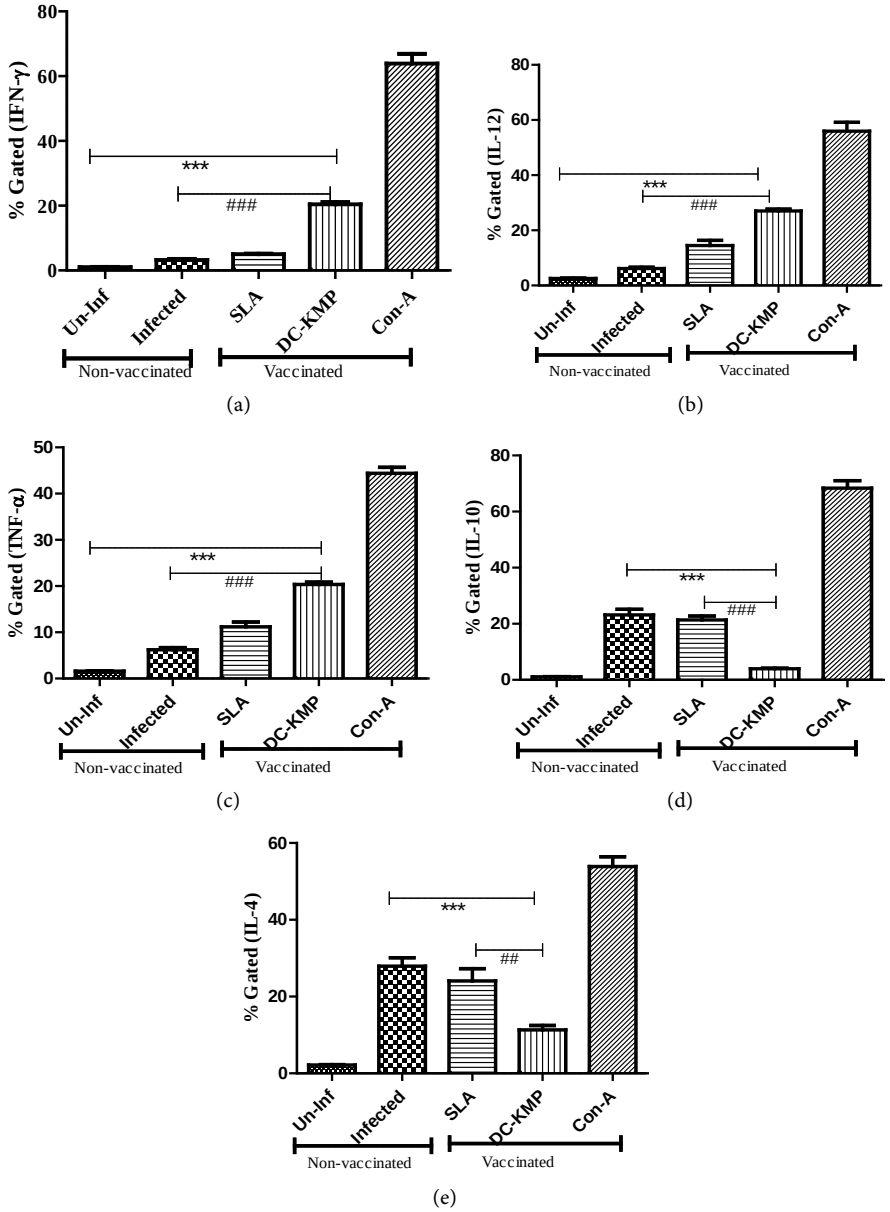


Figure 5. Evaluation of ability of vaccine to trigger Th1 and Th2 response in immunized Mice. Production of different cytokines upon immunization with different groups: Non-vaccinated groups (un-infected/infected) and vaccinated groups (SLA/ DC-KMP/Con-A) by percentage gated population of (a) IFN-γ, (b) IL-12, (c) TNF-α, (d) IL-10 & (e) IL-4 by FACS Calibur (BD Bioscience). (###, ***$p < 0.001$ highly significant among the groups and ##, $p < 0.01$ significant among groups).

3.7. Vaccination with DC-KMP Promoted ROS Production in Macrophages Which Is p38MAP Kinase

Because reactive oxygen species are important leishmanicidal molecules, we examined if KMP-11 immunization contributed to *L. donovani* elimination by inducing ROS. It was observed that DC-KMP vaccination increased ROS production by about 3.54 fold in comparison with infected control ($p < 0.001$). These results suggest the requirement of ROS in KMP-11 mediated *Leishmania*

killing. Because *Leishmania* suppresses the host protective response such as expression of ROS by modulating p38MAP kinase, we examined whether DC-KMP-11 immunization modulated p38MAP kinase in infected macrophages. We observed that p38MAPK which is associated with IL-12 production and development of protective immunity was significantly up-regulated in DC-KMP immunized groups of infected mice compared to non-vaccinated infected groups ($p < 0.001$). These results indicate that DC-KMP immunization enhances the protective responses by modulating the p38MAP-Kinase phosphorylation (**Figure 6(a)**, **Figure 6(b)**).

3.8. Discussion

This study gives a new vaccine strategy of dendritic cell presented KMP-11 as immunogen for control against experimental VL. It was also apparent that KMP-11 antigen promoted the activation of DCs in particular compared to *L. donovani* infected mice that produced IL-12.

LPG linked KMP-11 is the major protein on the promastigote surface which plays a key role in immunity against *L. donovani* [22] [23]. We have earlier reported immunomodulation in human DCs leads to induction in IFN-γ production by rLd-KMP-11 antigen in VL [14]. Basu *et al.* reported earlier that 30 peptides of KMP-11 antigens triggers the secretion of IFN-γ [24]. It is well known that strong immune-suppression results during *L. donovani* infection which encourages the parasite to impair the generation of free radical like super oxide and nitric oxide [25]. IL-12 is a host protective cytokine production, which is also required for the generation of Th1-related chemokines like XCL1 [26]. In experimental *Leishmania* infection, CXCL10 or IP-10 (Inferferon inducible protein)

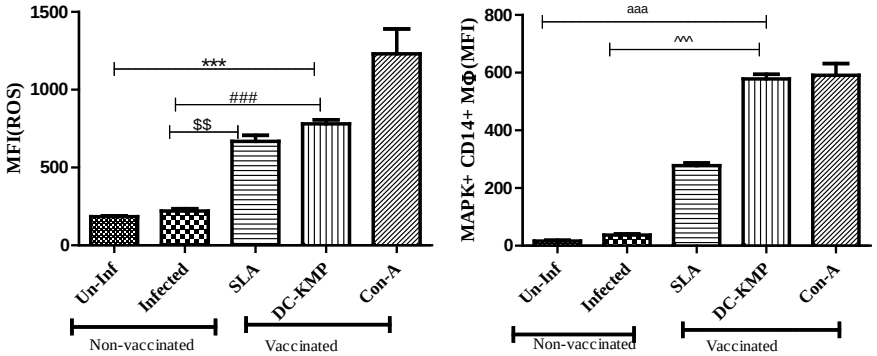


Figure 6. (a) DC-KMP-11 immunization contributed to *L. donovani* elimination by inducing Reactive oxygen species (ROS). (b) p38MAP Kinase associated with IL-12 production & development of protective immunity was significantly up-regulated in DC-K immunized groups of infected mice compared to non-vaccinated infected groups. Results were measured in mean fluorescent intensity (MFI) by FACS Calibur using CellQuest software. [In ROS: ### $p<0.001$ for infected (NV) vs DC-KMP vaccinated and ***, $p < 0.001$ for Uninfected (NV) vs DC-KMP vaccinated & $$ infected (Non-vaccinated) vs DC-KMP vaccinated]. [In p38MAPK: aaa, $p < 0.001$ for uninfected (NV) vs DC-KMP vaccinated and ^^^, $p < 0.001$ for infected (Non-vaccinated) vs DC-KMP vaccinated].

are linked with immune protection [27].

We earlier reported the cytokine response produced by KMP-11 antigen in DCs had an upliftment of IFN-γ and IL-12 production with down-regulation in IL-4 and IL-10 production. The information became the initial breakthrough in testing the efficiencies of KMP-11 antigen through dendritic cell.

There was ample evidence on the abilities of KMP-11 immunomodulation through DCs which clear *Leishmania* amastigotes in infected animals more effectively than un-immunized groups. The above encouraging findings on immunization and vaccine prospect of DC primed KMP-11 in the study prompted to look at the infected splenocytes. Previously, Padigel *et al.*, and Jones *et al.* found the outcome of experimental leishmaniasis in mice is determined by a balance Th1 and Th2 cells [28] [29]. The finding obtained in this regard assumes significance and pinpoint relevance of KMP-11 for its vaccine potential. This was well reflected through an up-regulated (IFN-γ, IL-12, TNF-α and IL-2) and down- regulated (IL-10 and IL-4) cytokines in FACS analysis in host cells compared to infected controls.

KMP-11 primed in DCs triggered the release of IL-2 and IL-2R on day 60 after infection ensuring a higher durability for proliferation in T-cells. DC–KMP-11 immunization in response to rKMP-11 protein showed about 6.38 fold more IFN-γ, 4.40-fold more IL-12 and 3.26 fold more TNF-α, whereas cells from SLA vaccinated mice produced about 1.57, 2.36 and 1.79 fold more IFN-γ, IL-12 and TNF-α, respectively in comparison to controls. In contrast, vaccination with DC primed KMP-11 inhibited the production of IL-10 by 5.81 and 1.08 fold and reduced the production of IL-4 by 2.47 and 1.16 fold, respectively, compared to controls. The disease promoting cytokines such as IL-10 were down-regulated following immunization. Pro-inflammatory cytokines such as IFN-γ and TNF-α play a critical role in the induction of ROS during VL [30], whereby IL-10 inhibits ROS generation. ROS generation was significantly elevated in KMP-11 vaccination after priming through DCs in vaccinated BALB/c mice. This ROS mediated anti-leishmanial defence was abrogated in infected animals confirming a role for ROS in KMP-11 mediate intracellular parasite clearance.

We also investigated about KMP-11 mediated alteration in signaling pathways that result in the production of pro-inflammatory cytokines such as IL-12. We have shown that the effector response in form of ROS and IL-12 was mainly because following priming DCs with KMP-11, the defective p38MAPK was restored in splenocytes. The p38MAPK inhibition increased parasite load, but impaired ROS production and IL-2 secretion. In conclusion, we report a new vaccine approach for control against VL.

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Ethical Approval

Ethical approval was taken from Ethical Committee of this Institute (RMRIMS, Patna).

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