Vaccine 31 (2013) 1905–1915

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Heterologous priming–boosting with DNA and vaccinia virus expressing kinetoplastid membrane protein-11 induces potent cellular immune response and confers protection against infection with antimony resistant and sensitive strains of *Leishmania* (*Leishmania*) *donovani*

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ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form 8 February 2013
Accepted 11 February 2013
Available online 26 February 2013

Keywords:
Visceral leishmaniasis
Leishmania (*Leishmania*) *donovani*
Drug resistance
Heterologous prime-boost immunization
Kinetoplastid membrane protein-11
Recombinant vaccinia virus

ABSTRACT

Background: Emergence of resistance against commonly available drugs poses a major threat in the treatment of visceral leishmaniasis (VL), particularly in the Indian subcontinent. Absence of any licensed vaccine against VL emphasizes the urgent need to develop an effective alternative vaccination strategy.
Methodology: We developed a novel heterologous prime boost immunization strategy using kinetoplastid membrane protein-11 (KMP-11) DNA priming followed by boosting with recombinant vaccinia virus (rVV) expressing the same antigen. The efficacy of this vaccination regimen in a murine and hamster model of visceral leishmaniasis caused by both antimony resistant (Sb-R) and sensitive (Sb-S) *Leishmania (L.) donovani* is examined.
Result: Heterologous prime-boost (KMP-11 DNA/rVV) vaccination was able to protect mice and hamsters from experimental VL induced by both Sb-S and Sb-R-*L. (L.) donovani* isolates. Parasite burden is kept significantly low in the vaccinated groups even after 60 days post-infection in hamsters, which are extremely susceptible to VL. Protection in mice is correlated with strong cellular and humoral immune responses. Generation of polyfunctional $CD8^+$ T cell was observed in vaccinated groups, which is one of the most important prerequisite for successful vaccination against VL. Protection was accompanied with generation of antigen specific $CD4^+$ and $CD8^+$ cells that produced effector cytokines such as IFN-γ, IL-2 and TNF-α. KMP-11-DNA/rVV vaccination also developed strong cytotoxic response and reversed T-cell impairment to induce antigen specific T cell proliferation.
Conclusion: KMP-11 is a unique antigen with high epitope density. Heterologous prime boost vaccination activates $CD4^+$ and $CD8^+$ T-cell mediated immunity to confer resistance to VL. This immunization method also produces high quality T-cells secreting multiple effector cytokines thus enhancing durability of the immune response. Thus the vaccination regime as described in the present study could provide a potent strategy for future anti-leishmanial vaccine development.

1. Introduction

Leishmaniasis is a parasitic disease caused by the genus *Leishmania* and ranges from chronic visceral disease to localized cutaneous lesions [1]. Pentavalent antimonial (Sb) compounds were the first line drugs for chemotherapy but rapid emergence of Sb-resistance in endemic areas [1] has paved path for alternative compounds. Amphotericin B, paromomycin, and miltefosine are gradually replacing pentavalent antimonials but these are prohibitively expensive. Reports of resistance to these new compounds emphasize the need for the development of a vaccine unequivocally [2,3]. Till date there are no vaccines licensed against this disease [4].

Abbreviations: VL, visceral leishmaniasis; LD, *Leishmania* (*Leishmania*) *donovani*; p.i., post-infection; CL, cutaneous leishmaniasis; Sb-S, pentavalent antimonial sensitive; Sb-R, pentavalent antimonial resistant; CMI, cell mediated immunity; rVV, recombinant vaccinia virus; KMP-11, kinetoplastid membrane protein-11; WR-VV, Western Reserve strain of vaccinia virus; HPB, heterologous prime-boost; i.d., intradermal; i.v., intravenous; BrdU, bromodeoxyuridine.

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http://dx.doi.org/10.1016/j.vaccine.2013.02.025

DNA vaccination can be used against intracellular pathogens as it stimulates both cellular and humoral response [5]. Efficacy of different candidate leishmanial antigens such as LACK [6], NH36 [7], cysteine proteinases [8], HASPB [9], A2 [10], and histones has been tested in various forms of DNA vaccines [11,12].

Kinetoplastid membrane protein-11 (KMP-11), a highly conserved membrane surface protein in the genus *Leishmania*, is differentially expressed in promastigote and amastigote forms [13]. Earlier studies from our lab have shown that vaccination with plasmid encoding KMP-11 confers protection in experimental visceral leishmaniasis (VL) [13,14] and is also partially effective against cutaneous leishmaniasis (CL) [14]. KMP-11 is also a component of other vaccination studies, e.g., in a nanoparticle delivery system, as a component of multivalent therapeutic DNA vaccine [15,16]. Very high HLA-I epitope density and their ability to be recognized by $CD8^+$ T-cells make it suitable for T-cell directed vaccine development [17].

The antigen delivery system plays a crucial role in the success or failure of a vaccine. Naked DNA delivery systems have shown poor immunogenicity in humans [18,19]. Vaccinia virus vectors are found to be efficient antigen delivery system in various infectious diseases [20,21]. DNA priming followed by a booster with a viral vector encoding the same antigen [21,22], is a probable alternative and is a form of heterologous prime-boost (henceforth abbreviated as HPB) vaccination. In experimental animal models this approach has given robust protection which is correlated with induction of potent CD8 response [23,24]. Priming with DNA encoding LACK and boosting with replication competent recombinant WR-VV (Western Reserve-vaccinia virus) expressing the same antigen confers protection against CL [25]. Studies with TRYP antigen or polytope approach employing DNA/MVA for immunization have yielded promising results [18,26].

In the present study, plasmid DNA encoding KMP-11 was used for priming and replication competent WR-VV expressing the same antigen for boosting to generate strong immunogenic response. This was followed by challenge with Sb-sensitive (Sb-S) and Sb-resistant (Sb-R) strains of *L.* (*L.*) *donovani* (LD) to assess the efficacy of the immunization strategy in eliciting an effective immune response and providing protection.

2. Materials and methods

2.1. Mice

Female BALB/c mice 4–6 weeks old were reared in our animal facilities and used in the experiments. All animal experiments were reviewed and approved by the Institutional Animal Care and Ethics Committee and were performed in accordance with relevant guidelines and regulations.

2.2. Plasmids and recombinant vaccinia virus

pCMV-LIC-KMP-11 plasmid was generated as previously described [13] and purified using EndoFree Plasmid Mega kits. Recombinant vaccinia virus expressing KMP-11 gene (rVV) was developed using Western Reserve strain of vaccinia virus (WR-VV) by a standard procedure [25,27,28] described in supplementary section.

2.3. Immunization

Mice were inoculated intradermally (i.d.) with 100 μg of KMP-11 or vector DNA. After 2 weeks, animals were boosted i.d. with 5×10^7 PFU rVV (referred to as KMP-11/rVV below). Control mice received vector DNA followed by vector VV (referred to as Vector/VV below). Few immunized mice were sacrificed 10 days post-boosting for immunogenicity analysis.

2.4. Parasites and infection

In this study two strains of *L.* (*L.*) *donovani*, originally isolated from Indian kala-azar patients, were used – the pentavalent antimony sensitive AG83 (MHOM/IN/83/AG83) [13] and resistant BHU575 (MHOM/IN/09/BHU575/0) [29]. For convenience they have been abbreviated as Sb-S and Sb-R henceforth. Twelve days after the boost BALB/c mice were challenged with freshly isolated 1×10^7 amastigotes (described in supplementary section) of Sb-S and Sb-R. At 21 days post-infection (p.i.), animals were sacrificed for assessment of splenic and liver parasite burden. For impression smear count, parasite burden was expressed as Leishman Donovan units (LDU) [9]. Parasite burden calculation by serial dilution method was done as previously described [13] with 4-fold dilutions. Few mice were sacrificed 60 days p.i. to check the durability of protection.

2.5. Measurement of anti-KMP-11 antibody responses

Serum samples from different groups of BALB/c mice were obtained 10 days after boosting and 21 days after parasite challenge and were used to estimate the respective anti-KMP-11-specific, IgG1and IgG2a Ab as described previously [14].

2.6. IFN-γ ELISPOT assays

The frequency of IFN-γ producing KMP-11 specific T cells was measured by ELISPOT assay. From individual animals, 2.5×10^5 splenocytes or 10^5 purified $CD8^+$ cells (with 2×10^5 naïve splenocytes) were seeded per well with rKMP-11 protein (10 μg/ml) or H2-D^d restricted KMP-$11_{HYEKFERMI}$ Peptide (5 μg/ml) for 24 h at 37 °C [16,30]. Developed plates were analyzed for IFN-γ spots (ImmunoSpot, C.T.L) and expressed as spot-forming cells (S.F.Cs) per 10^6 spleen cells.

2.7. T cell proliferation assay

Splenocytes from different groups of mice were plated in triplicate at a concentration of 2×10^5 cells/well in 96-well plates and allowed to proliferate for 3 days at 37 °C in a 5% CO_2 incubator both in presence and absence of rKMP-11 (10 μg/ml) [14]. At 3 days post-stimulation, BrdU solution was added for last 2 h. The level of BrdU incorporation was measured according to manufacturer's protocol (Millipore) and the absorbance was measured at 450 nm using ELISA plate reader (DTX 800 multimode detector: Beckman Coulter).

2.8. Cytometric bead array for the analysis of cytokines in the culture supernatant

Splenocytes from mice were plated in 96 well plates at 2.5×10^5 cell/well concentrations, and kept in the presence or absence of 10 μg/ml rKMP-11 for 48 h at 37 °C in 5% CO_2 incubator. After 48 h culture supernatant was collected, cytokines were measured using Mouse Th1/Th2 and Mouse Inflammatory kit by cytometric bead array (CBA) [31]. All the data were analyzed using FCAP Array in a FACS ARIAII Flowcytometer (BD).

2.9. In vitro CTL assay

In vitro CTL assay was performed as described earlier [26] with some modifications. The parasitized J774A.1 cells were used as target cells and in vitro rKMP-11 stimulated splenocytes, isolated from different groups of mice were used as effector cells.

Cytolytic activity of splenocytes was evaluated by measuring lactate dehydrogenase (LDH) activity released into the medium, using the CytoTox96 (Promega) nonradioactive assay according to manufacturer instructions. The Effector (E) and Target (T) cells were incubated for 4 h at E/T ratio of 1:10, and the cell lysis was quantified by measuring the absorbance at 490 nm. The percentage of specific lysis was calculated as follows: [(Experimental – Spontaneous release)/(Total maximum release – Spontaneous release)] × 100.

2.10. Flow cytometric analysis of intracellular cytokines

Splenocytes (2×10^6 cells/well) were restimulated in vitro for 24 h at 37 °C in the presence of rKMP-11 (10 μg/mL). Brefeldin-A (10 μg/mL) was added for the last 4 h. The cells were then washed, stained with the LIVE/DEAD Kit, and Fc receptors were blocked using anti-CD16/CD32 antibodies. The cells were then stained with the anti-mouse antibodies CD4-PerCP, CD3-PE-Cy™7 and CD8-APC-Cy™7. Cells were permeabilized using the BD-Cytofix/Cytoperm Kit and were stained for the intracellular cytokines with, IFNγ-PB, IL2-PE and TNFα-FITC. 100,000 lymphocytes were acquired on FACSARIA II Flowcytometer and analyzed with FlowJo (Tree Star).

2.11. Statistical analysis

Statistical differences ($P < 0.05$) between the immunization groups were determined using the unpaired, two-tailed Student's *t* test. The data are representative of at least three replicate experiments.

3. Results

3.1. Strong cellular and humoral immune responses were induced upon heterologous prime-boost vaccination

Both cellular and humoral immune responses in mice were examined 10 days after boosting to assess the immunogenic potential of our heterologous prime-boost immunization strategy (Fig. 1A). Antigen recall with the r-KMP-11 protein shows enhanced IFN-γ (20 times) and TNF-α (6 times) production by splenocytes

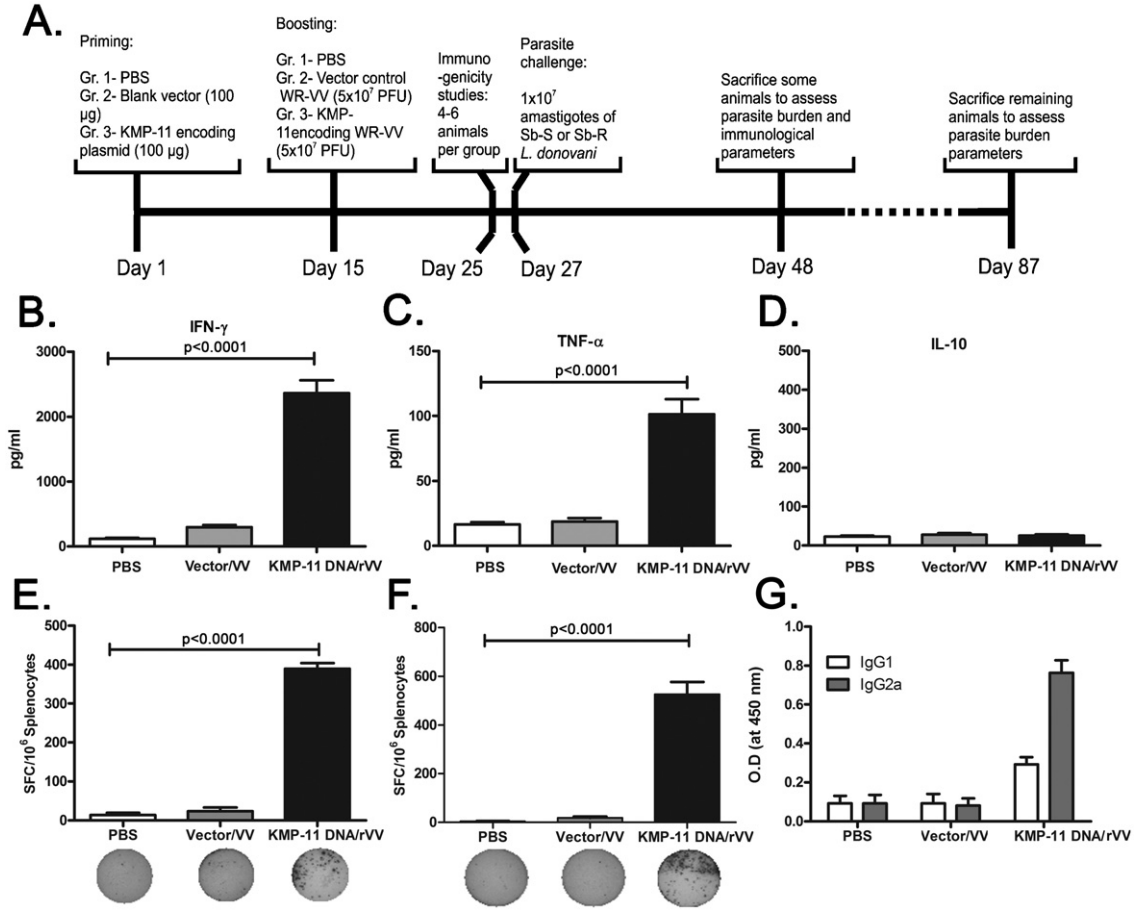

Fig. 1. Generation of strong cellular and humoral response in KMP-11-DNA/rVV vaccinated group. (A) Immunization schedule. BALB/c mice were primed with 100 μg of DNA (either 100 μg KMP-11 DNA or empty vector, or PBS) intradermally (i.d.) at the start of the vaccination protocol. Two weeks later, the mice were boosted by intradermal (i.d.) injection with 5×10^7 PFU of rVV, Control VV, or PBS. Ten days post-boost, six to eight mice were sacrificed to analyze the adaptive immune response. The remaining mice were challenged with antimonial sensitive (Sb-S) or antimonial resistant (Sb-R) *Leishmania donovani* isolates. (B–D) Pattern of cytokine secretion in supernatants of splenocytes after rKMP-11 (10 μg/ml) restimulation 10 days post-boosting, measured by cytometric bead array (CBA); (B) IFN-γ, (C) TNF-α and (D) IL-10. (E) Vaccine induced KMP-11 specific IFN-γ producing cells measured by IFN-γ ELISPOT assay, after stimulating the isolated whole splenocytes from immunized and control groups with rKMP-11 protein. A representative ELISPOT well from different groups are depicted at bottom of the figure. (F) The number of IFN-γ producing $CD8^+$ T cells was determined by IFN-γ ELISPOT assay. Briefly 10^5 purified $CD8^+$ cells (with 2×10^5 naïve splenocytes) from immunized and control groups were seeded per well and stimulated with H2-D^d restricted $KMP11_{HYEKFERMI}$ peptide and number of IFN-γ producing cells were determined. Data represented as SFCs per 10^6 splenocytes. A representative ELISPOT well from different groups are depicted at bottom of the figure. No IFN-γ producing cells were detected after stimulation with non-specific H2-D^d restricted HASPB peptide GGPKEGENL used as negative control (data not shown). (G) Level of KMP-11 specific IgG1 and IgG2a post-vaccination in immunized and control groups. All the data were analyzed by *t* test ($n = 6$–8 per group), and level of significance are indicated by *p* values. Level of significance between vaccinated and PBS controls groups are represented, similar level of significance was also found with Vector/VV control groups.

of vaccinated animals compared to the control groups (Fig. 1B and C). No significant change was observed in the disease promoting IL-10 level between the groups (Fig. 1D). Immunization also led to a ~17-fold increase in antigen-specific IFN-γ producing cells as compared to controls in whole splenocytes populations (Fig. 1E). ELISPOT assay was performed with isolated $CD8^+$ cells to detect the IFN-γ producing $CD8^+$ cells in response to the immunodominant H2-D^d restricted KMP-11 peptide HYEKFERMI [16]. Significantly high response against KMP-$11_{HYEKFERMI}$ was detected in our vaccinated group, indicating the generation of high frequency KMP-$11_{HYEKFERMI}$ specific $CD8^+$ T cell response (Fig. 1F).

Strong Th1 biased immune response was also reflected by the presence of higher KMP-11 specific IgG2a in the prime-boost immunized group (Fig. 1G).

3.2. Heterologous prime-boost immunization strategy confers protection against experimental challenge with Sb-sensitive and Sb-resistant-LD parasites

In order to assess the efficacy of our vaccine constructs animals were primed with KMP-11-DNA and then boosted with rVV as per the schedule mentioned (Fig. 1A). Twelve days after rVV boosting, animals were infected with 1×10^7 amastigotes of Sb-S and Sb-R-LD isolates. 21 days and 60 days post-parasite challenge, liver and spleen parasite burdens have indicated that the KMP-11-DNA/rVV receiving mice were protected up to 90–95% ($P < 0.0001$) against the experimental challenge with both Sb-S and Sb-R parasites, in contrast to the PBS control group (Fig. 2A–D). Vector/VV group also exhibited slight reduction in the liver parasite burden (43% and 46% in Sb-S and Sb-R challenged groups) but no significant reduction was observed in the splenic parasite burden. Similar levels of protection were also observed in the vaccinated groups in hamster model (Fig. 6A–D). IgG2a:IgG1 ratio was found to be higher in the vaccinated groups associated with protection (Fig. 2E).

3.3. Enhanced lymphoproliferation along with IL-2 generation in the vaccinated animals following infection

Splenocytes from vaccinated Sb-S and Sb-R-LD infected mice of different groups were stimulated with rKMP-11 protein and T-cell proliferation was measured after 72 h. The splenocytes of KMP-11 DNA/rVV group displayed 3.5- and 3-fold higher T-cell proliferation compared to the PBS control following infection with Sb-S and Sb-R parasites, respectively (Fig. 3A).

As a functional read out of T-cell activation IL-2 was measured in the rKMP-11 protein stimulated cell culture supernatant. Significantly higher level of IL-2 was present in culture supernatants from splenocytes of KMP-11 DNA/rVV immunized mice compared to the control groups after Sb-S and Sb-R challenge (Fig. 3B).

3.4. Development of potent cytotoxic activity against infected macrophages in the vaccinated animals

In order to detect the cytolytic activity of splenocytes isolated from all the vaccinated groups of mice 21 days p.i., we tested their capacity to lyse the LD-infected macrophage cells. Splenocytes from Sb-S and Sb-R infected KMP-11 DNA/rVV groups were found to be having 62.6% and 61% specific lytic ability, which was considerably higher than the control groups (Fig. 3C).

3.5. Ex vivo antigen recall of the KMP-11-DNA/rVV splenocytes from infected animals displays significant production of pro-inflammatory cytokines

IFN-γ and TNF-α are associated with host protection in VL [32,33]. Significantly higher level of IFN-γ and TNF-α were observed in KMP-11-DNA/rVV group at 21 days p.i. (Fig. 3D and E). Following infection with Sb-S and Sb-R parasites, IFN-γ level increased by 39 and 33 times, respectively, among the vaccinated animals compared to PBS control, while 6-fold increase in TNF-α was observed with both Sb-S and Sb-R infection.

In contrast, IL-10 was found to be significantly lower in the KMP-11-DNA/rVV group than the two control groups (Fig. 3F).

3.6. Generation of antigen specific $CD4^+$ and $CD8^+$ T-cell responses in vaccinated groups correlates with protection

Splenocytes from different groups of mice were stimulated with rKMP-11 protein and multi-parameter flowcytometry was utilized to check the production of IFN-γ, IL-2 and TNF-α from $CD4^+$ and $CD8^+$ subsets.

The vaccinated animals show a steady increase in the frequency of KMP-11 specific IFN-γ, IL-2 and TNF-α producing $CD4^+$ cells (Fig. 4A and B). Furthermore, Boolean gating with these three cell populations was performed to address the issue of polyfunctional T cells [30]. It was observed that majority of cytokine secreting $CD4^+$ cells are single positive for the three cytokines (Fig. 4C). There was an increase in IFN-γ^-IL-2^+TNF-α^+, IFN-γ^+IL-2^-TNF-α^+ and IFNγ^+IL-2^+TNFα^- double positive $CD4^+$ cells in the vaccinated group (Fig. 4C). The increase in IFN-γ, IL-2 and TNF-α producing $CD8^+$ cells were also analyzed by combination gating to find out the quality of the CD8 response. Apart from an increase in IFN-γ, IL-2, TNF-α single positive cells in the vaccinated group, an enhanced number of IFN-γ^+ IL2^+TNF-α^-, IFN-γ^+IL-2^-TNF-α^+ and IFN-γ^-IL-2^+ TNF-α^+ double positive $CD8^+$ cells (Fig. 5A–C) is seen. Triple positive $CD8^+$ cells are exclusively detected in the vaccinated group, although they constitute a very small fraction of total cytokine secreting cells.

IL-10 from subsets of $CD4^+$ cells has been implicated in the failure of vaccines against *L. major* infection [28]. A steady decrease in the frequency of IL-10 producing $CD4^+$ cells was observed among the vaccinated groups infected with either Sb-sensitive or resistant parasite strains (Fig. 4D and E).

4. Discussion

Theoretically, a prophylactic vaccine against VL is possible as recovered patients gain lifelong immunity against re-infection [34]. In the present study we evaluate the efficacy of a prophylactic vaccination strategy by utilizing the heterologous prime-boost approach against both Sb-sensitive and resistant parasites.

We have earlier reported that the protection against VL is achieved by the administration of KMP-11 DNA vaccine [13,14]. But intramuscular administration of DNA vaccines proved to be less immunogenic in primates and human trials than they are in rodents [35,36]. Therefore, substantial improvement in the immunogenicity of DNA vaccine in rodent models is a prerequisite for immune response of sufficient magnitude in primates and human [37]. Various reports suggest that heterologous prime-boost immunization with DNA priming followed by boosting with viral vectors elicits greater quality and magnitude of $CD4^+$ and $CD8^+$ T-cell responses [37,38].

Here, using HPB vaccination strategy, more than 90% reduction was observed in splenic and hepatic parasite load with both Sb-S and Sb-R-LD infection in vaccinated groups in both mice and hamster model (Figs. 2A–D and 6A–D). In our study we used very high (1×10^7) dose of amastigotes for i.v. challenge as this leads to a rapid and profuse infection. Considering the higher infectious dose of parasites the level of protection achieved in murine and hamster model is of exceptional significance. The level of protection may vary considerably in natural sand fly bite from needle injection

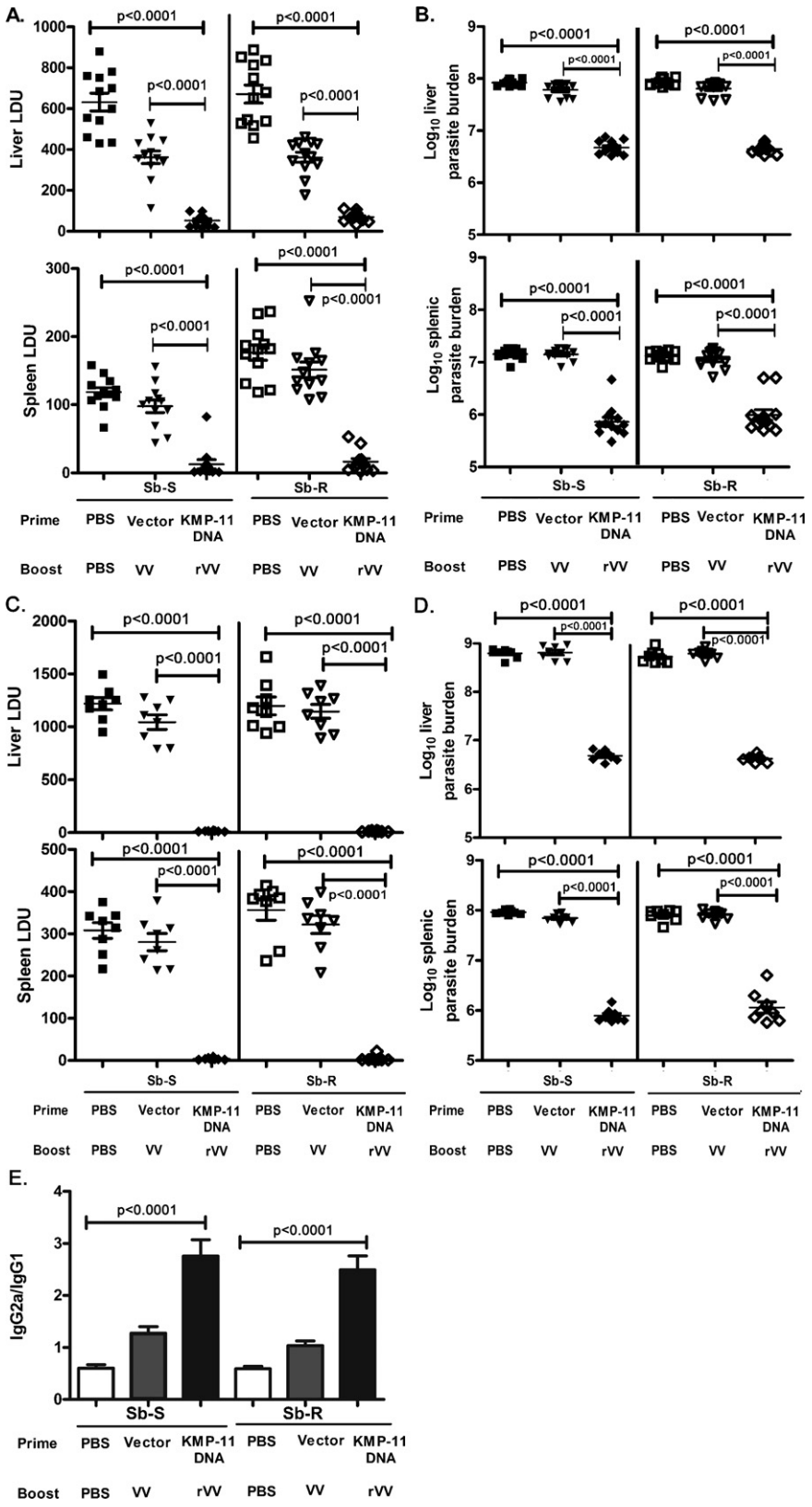

Fig. 2. Heterologous prime-boost immunization (KMP-11 DNA/rVV) protects against both Sb-S and Sb-R-LD challenge and generates higher IgG2a/IgG1 ratio. Parasite load in spleen and liver from respective animals were determined by impression smear and serial dilution method 21 days and 60 days post-parasites challenge. Parasite load is expressed in terms of LDU and Log_{10} organ parasite burden. Hepatic and splenic parasite burden at 21 days post-infection calculated from (A) impression smear method and (B) serial dilution method. Hepatic and splenic parasite burden at 60 days post-infection calculated from (C) impression smear method and (D) serial dilution method. (E) Level of KMP-11 specific IgG1 and IgG2a were measured in the serum of different groups at 21 days p.i. and IgG2a/IgG1 ratio is represented. Data were analyzed by *t* test, and levels of significance are indicated by *p* values. $n = 4$ per group. Experiment performed at least twice independently and mean ± S.E.M. are represented.

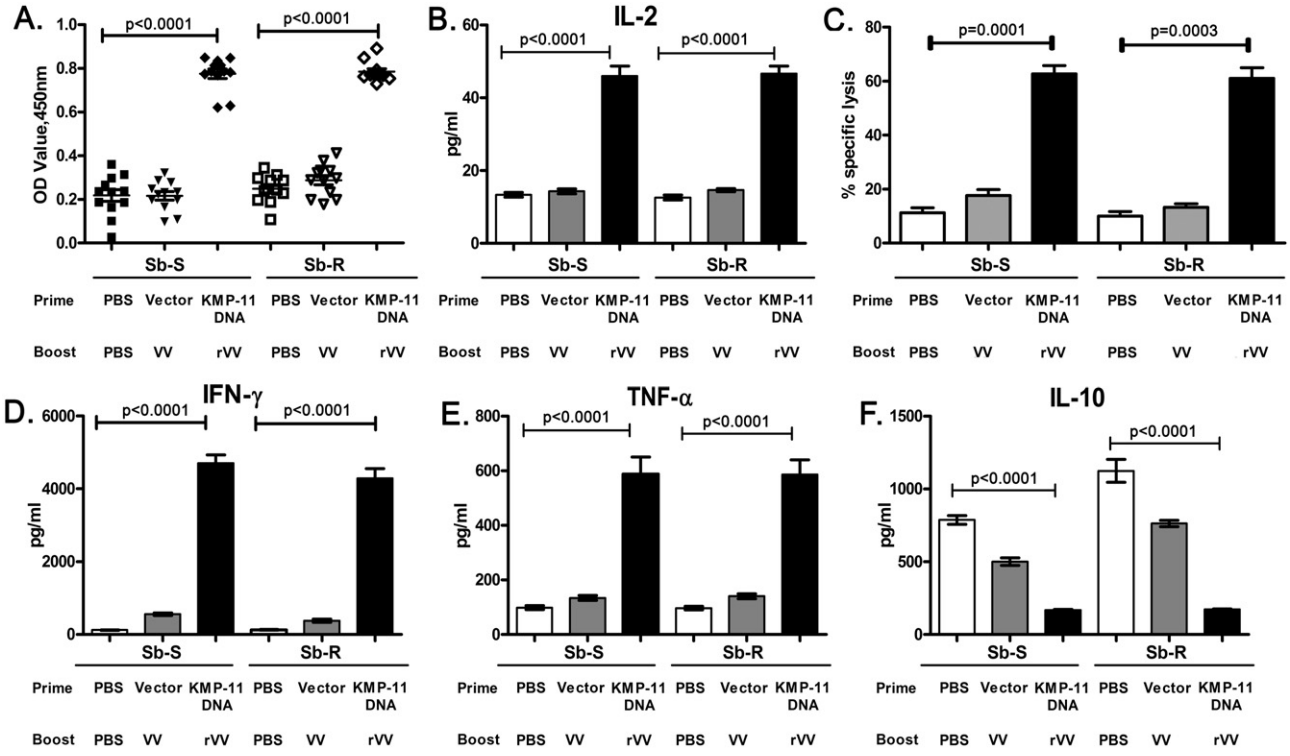

Fig. 3. Prime-boost vaccination prevents impaired T cell response and generates cytotoxic response in vaccinated groups with concomitant generation of Th1 skewed cytokines. (A) Splenocytes isolated 21 days post-infection from different group of mice were allowed to proliferate for 3 days at 37 °C in response to rKMP-11 (10 μg/ml). Finally, cell proliferation was measured by BrdU incorporation. Splenocytes isolated from untreated and infected animals were used as control. Results are expressed as mean unit of absorption ± S.E.M. from three independent experiments ($n = 4$ per group). (B) Functional significance of splenocytes proliferation in response to rKMP11 was determined by measuring the secretion of IL-2 in the culture supernatant by splenocytes isolated from different groups of mice after 48 h of incubation at 37 °C by cytometric bead array (CBA). Similarly, levels of different cytokine in the culture supernatant were also measured after 48 h of incubation with rKMP-11. (D) IFN-γ, (E) TNF-α and (F) IL-10. (C) Parasitized J774A.1 cells were used as target cells and in vitro rKMP-11 stimulated splenocytes, isolated from different groups were used as effector cells. Cytolytic activity of splenocytes from different groups were measured and expressed as % specific lysis. Data were analyzed by *t* test, and levels of significance are indicated by *p* values. $n = 4$ per group. Experiment performed three times independently and mean ± S.E.M. are represented.

[39]. Studies with sand flies are gaining momentum and future studies should rely more on this challenge model. Another interesting alternative could be subcutaneous/intra-dermal challenge routes, as it closely approximates the natural course of infection and increases the efficacy of vaccines against VL in murine model which may have failed due to high dose i.v. parasite challenge [40].

Chronic VL is associated with increased membrane fluidity leading to impaired antigen presentation which in turn results in a compromised T cell response [41]. Following infection, the splenocytes from KMP-11-DNA/rVV responded well to antigen stimulation by proliferating and producing IL-2, while the control groups failed to do so (Fig. 3A and B). Other proinflammatory cytokines like IFN-γ and TNF-α also display a remarkable increase in the vaccinated groups (Fig. 3D–F), which may be associated with host protection.

Recently, there has been interest in measuring polyfunctional T-cell response to characterize the quality of T-cell response following immunization with various vaccines or in different diseases [30,38]. Darrah et al. demonstrated that generation of polyfunctional $CD4^+$ T-cells strongly co-relates with protection against CL [30], similar scenario was observed in tuberculosis [42]. Polyfunctional $CD4^+$ and $CD8^+$ T-cell generation has been reported by Sanchez-Sampedro et al. using prime-boost regime against *L.* (*L.*) *major* infection [43], but to our knowledge generation of polyfunctional $CD4^+$ and $CD8^+$T-cell response using any vaccination regime against VL has been lacking. Here, using our vaccination regime we demonstrated the generation of polyfunctional $CD4^+$ and $CD8^+$ T-cell responses against VL. Significant increase in the IFN-γ^+IL-2^+TNF-α^-, IFN-γ^+IL-2^-TNF-α^+ and IFN-γ^-IL-2^+TNF-α^+ double positive $CD4^+$ cells among the vaccinated animals in our regime supports the significance of our findings (Fig. 4A–C). In fact triple positive (IFN-γ^+IL-2^+TNF-α^+) $CD4^+$ cells also increased in vaccinated groups (Fig. 4C). Though the frequency of these polyfunctional T-cells are low, it reflects that each single cell is capable of broader range of functions [38,43]. In addition, IFN-γ^+IL-2^+TNF-α^-, IFN-γ^+IL-2^-TNF-α^+ and IFN-γ^-IL-2^+TNF-α^+ double positive $CD8^+$ cells were significantly higher in vaccinated animals, corresponding to immunity (Fig. 5A–C). IL-2 producing double positive $CD8^+$ cells were supposed to be better in generating long-term memory response [30]. Triple positive $CD8^+$ cells were detected in vaccinated groups (Fig. 5C). Generation of only IFN-γ or TNF-α producing T-cells is not always sufficient for protection against infection [38,44,45], however, production of IFN-γ and TNF-α together lead to enhanced killing of different intracellular pathogens [38,43,46], compared to each cytokine alone [38,47]. Similarly, production of IL-2 promotes the expansion of $CD4^+$ and $CD8^+$ T-cells, thereby amplifying effector T-cell response [38]. Recently, presence of high quality polyfunctional $CD4^+$ T-cells was reported in PBMCs from patients that healed American CL [48]. Thus if a polyfunctional $CD4^+$ or $CD8^+$ T-cell is capable of producing these cytokines together they can offer a broader repertoire to fight against pathogens with a simultaneous generation of strong immunogenicity.

Fig. 6.

Our vaccination regime also generates strong cytolytic activity (Fig. 3C) which usually decreases during chronic infection [49]. This cytolytic activity may be due to generation of potent CTLs in vaccinated protected groups.

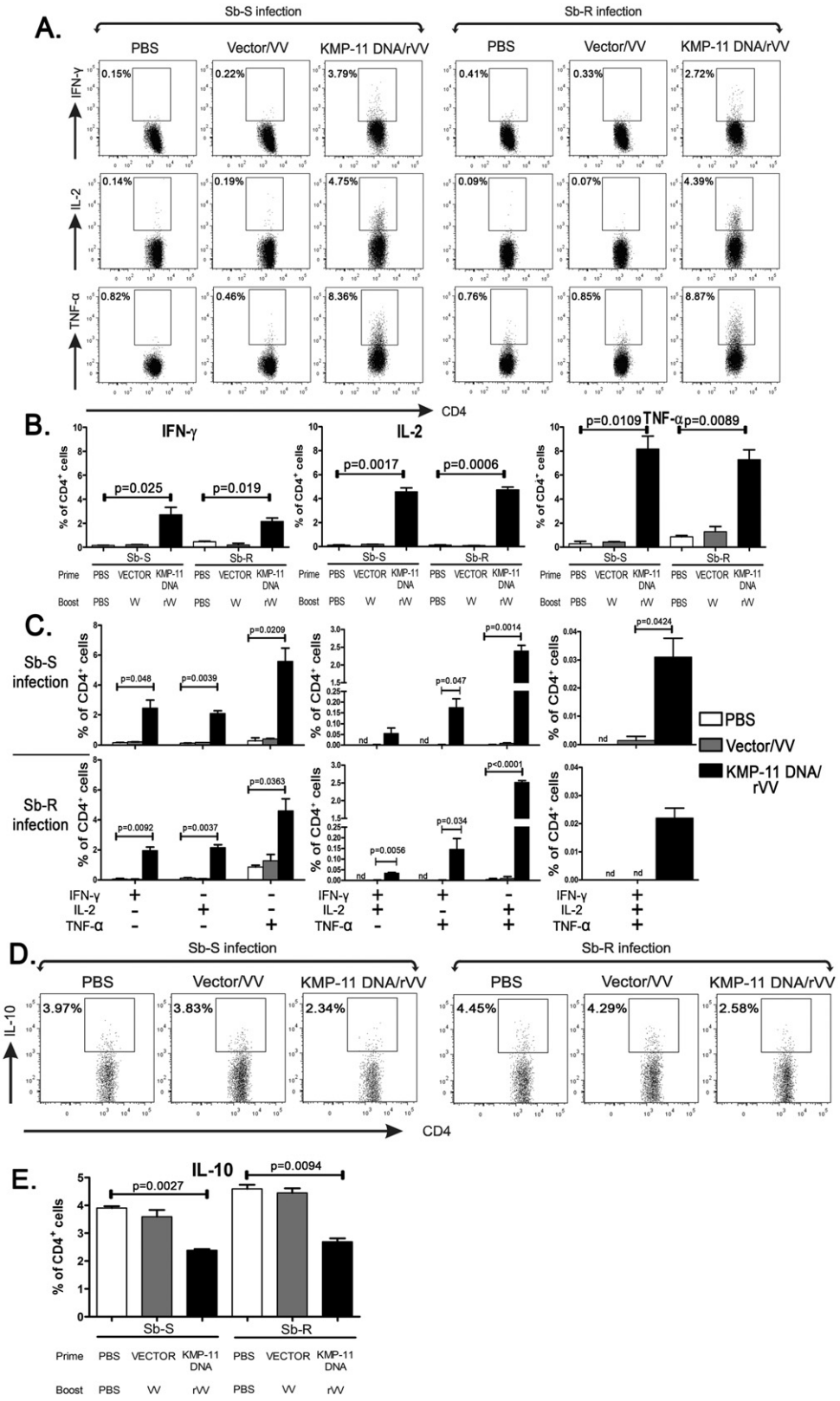

Fig. 4. Antigen specific CD4$^+$ T cell response and analysis of polyfunctionality. Analysis of KMP-11 specific CD4$^+$ T cell responses 21 days post-infection. Splenocytes prepared 21 days post-infections from different groups of immunized mice were stimulated with rKMP-11 for 24 h and cytokine production was detected by intracellular staining. (A) Representative dot-plots from one individual animal per group showing production of IFN-γ, IL-2 and TNF-α from CD4$^+$ cells. (B) Represents mean number of CD4$^+$ cells secreting IFN-γ, IL-2 and TNF-α in each group using three biological replicates ± S.E.M. (C) Boolean gating of IFN-γ, IL-2 and TNF-α positive CD4$^+$ cells to reveal the percentages of single, double and triple positive cells. (D) Representative dot-plots from one individual animal per group illustrating IL-10 production from CD4$^+$ cells following 48 h antigen stimulation, as detected by IL-10 secretion assay (detailed description in supplemental file). (E) Represents mean number of CD4$^+$ cells secreting IL-10 in each group using three biological replicates ± S.E.M.

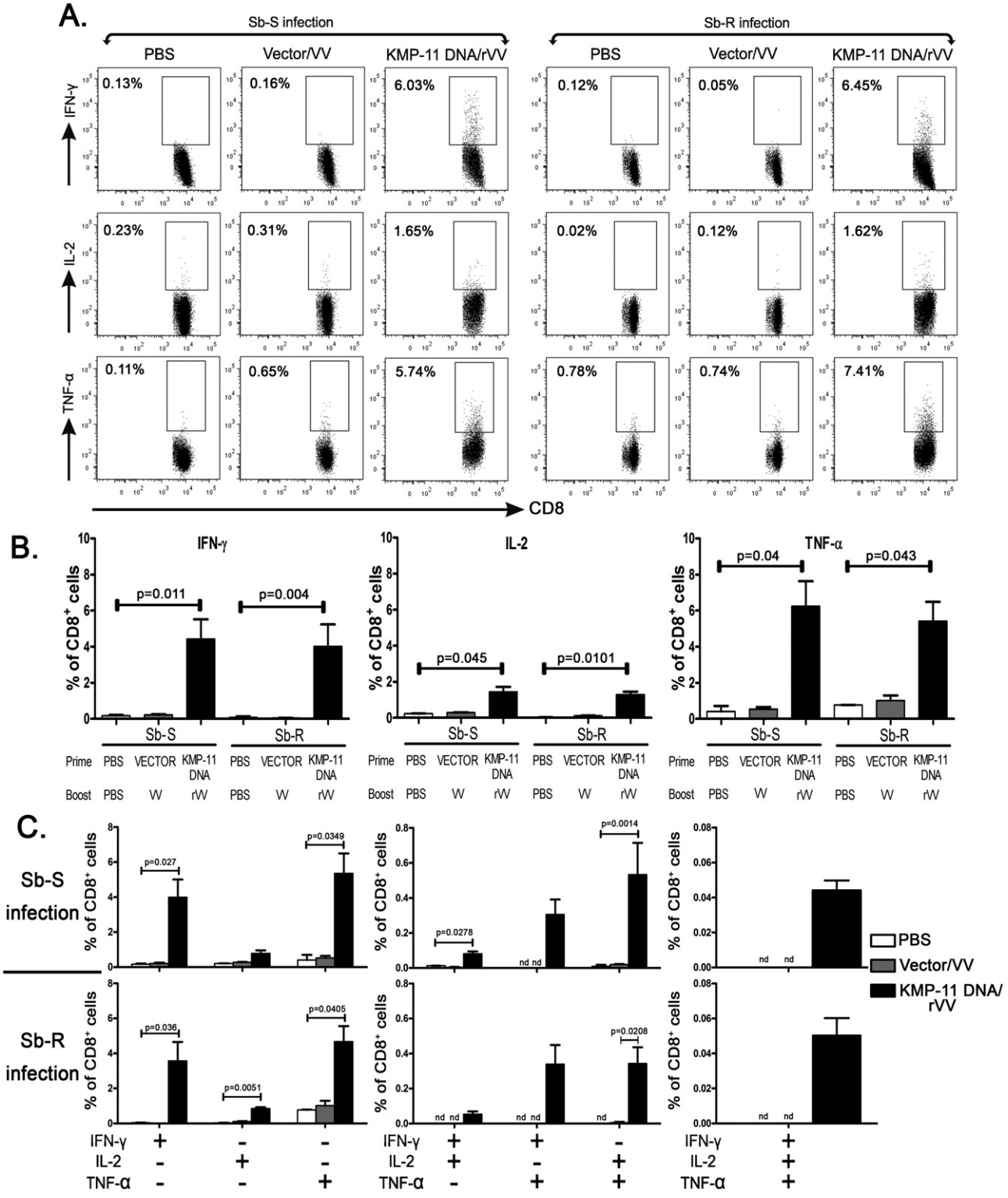

Fig. 5. Antigen specific CD8$^+$ T cell response and analysis of polyfunctionality. Analysis of KMP-11 specific CD8$^+$ T cell responses 21 days post-infection. Splenocytes prepared 21 days post-infection from different groups of immunized mice was stimulated with rKMP-11 for 24 h and cytokine production was detected by intracellular staining. (A) Representative dot-plots from one individual animal per group showing production of IFN-γ, IL-2 and TNF-α from CD8$^+$ cells. (B) Represents mean number of CD8$^+$ cells secreting IFN-γ, IL-2 and TNF-α in each group using three biological replicates ± S.E.M. (C) Boolean gating of IFN-γ, IL-2 and TNF-α positive CD8$^+$ cells to reveal the percentages of single, double and triple positive cells.

The drug resistant parasites have higher virulence and generate higher parasitemia during infection; more over they also generate a distinct cytokine milieu [29,50]. The fact that HPB – vaccination with KMP-11 as an antigen conferring protection against recent drug resistant isolate, highlights the importance of this strategy for future vaccine development against Leishmaniasis.

In conclusion it could be inferred that heterologous prime boost immunization with KMP-11 DNA priming followed by boosting

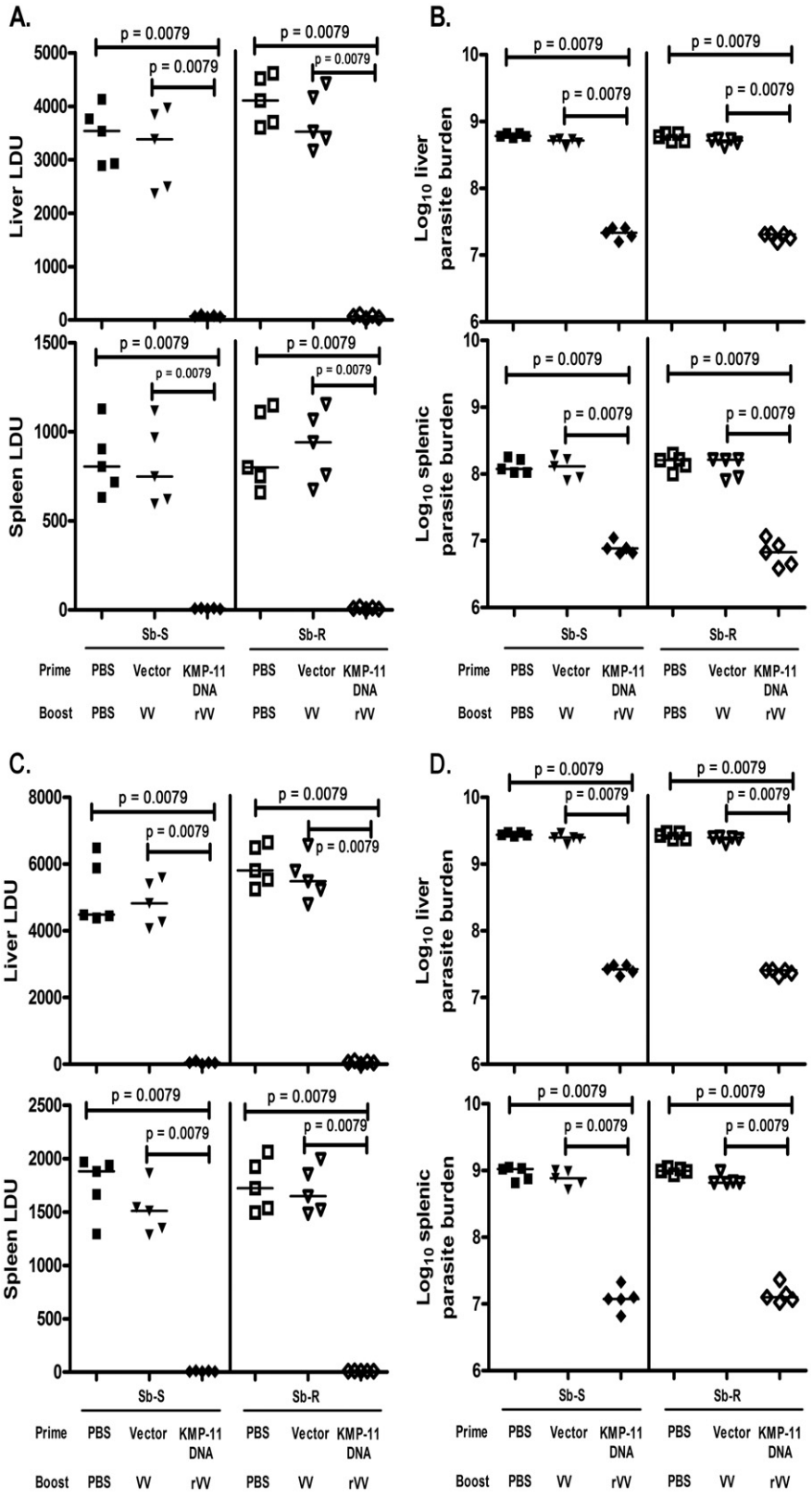

Fig. 6. Hamsters immunized by KMP-11 DNA/rVV are protected at 21 and 60 days following Sb-S and Sb-R-LD challenge. Parasite load in spleen and liver from respective animals were determined by impression smear and serial dilution method 21 and 60 days post-parasites challenge. Parasite load is expressed in terms of LDU and Log_{10} organ parasite burden. Hepatic and splenic parasite burden at 21 days post-infection calculated from (A) impression smear method and (B) serial dilution method. Hepatic and splenic parasite burden at 60 days post-infection calculated from (C) impression smear method and (D) serial dilution method. Data were analyzed by Mann–Whitney test and levels of significance are indicated by *p* values. Data from one representative experiment where $n = 5$. Median value is indicated on the graph.

with recombinant Vaccinia virus expressing KMP-11 gene results in protection against experimental VL in both pentavalent antimonial resistant and sensitive cases. This approach of heterologous prime boost immunization clearly demonstrates the importance of DNA vaccines as priming vehicles and attenuated viruses as boosters, and could serve as a potent strategy for anti-leishmanial vaccine development.

Acknowledgements

We thank Dr. Bernard Moss (NIAD, Bethesda) and Dr. Sekhar Chakrabarti of NICED for the Western Reserve strain of Vaccinia virus and pSC65 shuttle vector. We thank Prof. Paul M. Kaye, Professor of Immunology and Director of the Centre for Immunology and Infection, University of York for his advice about different immunodominant peptides used here for ELISPOT assay. We would also like to thank Dr. Ranjan Kumar Nandy of NICED for allowing us to use their ELISPOT reader. We thank Dr. Sandip. K. Bandhopadhyay, IPGMER for his effort in bringing the two cell lines used in this work.

Funding and Fellowship: This work was supported by the European Commission's 7th Framework project Kaladrug-R (Health-F3-2008-222895) and the Council of Scientific and Industrial Research, New Delhi, India. R.G. and J.G. are recipients of University Grant Commission (UGC) fellowships and S.D is recipient of Council of Scientific and Industrial Research (CSIR) fellowships.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.02.025.

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