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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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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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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.