SUMMARY AND CONCLUSIONS

Malaria, caused by Plasmodium species, remains a major public health problem, even though the implementation of control measures has significantly reduced the overall transmission in the past few years. The spread of multidrug-resistant strains of parasites and insecticide-resistant vectors has emphasized the need for developing novel intervention measures. Several vaccine candidates mainly focused on P. falciparum are in different phases of clinical development. The multistage life cycle of Plasmodium and the intricate host parasite interactions during the course of malaria infection support the idea of targeting several antigens simultaneously for vaccine development. In present study, immunogenic potential and protective efficacy of 66 kDa and 79 kDa antigens of P. berghei NK-65 were evaluated in mouse model. Various parameters in the study were checked on day 0 before challenge, and on days 4, 8, 12 and 30 post-challenge.

6.1. Parasite

Plasmodium berghei (NK-65 strain), a rodent malaria parasite, was used as experimental model. Asexual erythrocytic stages of P. berghei were maintained in vivo by weekly passage in BALB/c mice. The course of parasitemia in mice was monitored by preparing thin blood smears through tail vein incision. It has been observed that the BALB/c mice were susceptible to P. berghei and parasite was highly lethal to these
animals. Most of the animals had died because of high infection by the day 7 or 8 post-inoculation, except those in which parasite migrated to reticulocytes followed by increase in parasitemia till death of mice within two weeks. Maximum parasitemia of 49.35±2.89% was detected on day 10 post-inoculation in these animals.

6.2. Identification of antigens

Cell free parasites were isolated from infected blood by lysis of infected blood cells using saponin. Isolated parasites were homogenized and parasite homogenate was subjected to SDS-PAGE to identify various proteins present in it. The desired bands of antigens, i.e. 66 kDa and 79 kDa, were electro-eluted from the gels and suspended in PBS. Protein concentration was found to be 300 µg/ml in 66 kDa solution and 250 µg/ml in 79 kDa solution after electro-elution.

6.3. Preparation of vaccine formulations

Three types of vaccine formulations were prepared using 66 and 79 kDa antigens individually as well as in combination. FCA and FIA were used as adjuvant in formulations of prime and booster doses respectively. Vaccine formulations were prepared by thorough mixing of antigen solution with equal volume of adjuvant till the formation of a viscous suspension.

6.4. Immunization and challenge of mice

Three groups of BALB/c mice were immunized with various vaccine formulations. Each mouse was injected s.c. with 15 µg of protein and FCA on day 0. Two booster doses were given at an interval of two weeks using FIA. Control mice were injected with PBS emulsified in Freund's adjuvant on respective days. After 7 days of third immunization, animals were challenged by i.p. injection of 1×10^6 P. berghei infected erythrocytes per mouse. Protective efficacy of vaccination was assessed in terms of parasitemia, patent period and percentage of survived animals. Significantly lower parasitemia (p<0.05) was observed in all the
immunized animals as compared to controls. 66 kDa immunized mice had shortest patency period of 15 days and maximum parasitemia of (20.79±14.56%) was recorded on day 13 post-challenge. 79 kDa immunized animals had longest patency period of 23 days, and maximum infection (29.66±10.38%) was recorded on day 15 post-challenge. Whereas, in co-immunized (66+79 kDa) mice, patency period was of 18 days and highest infection (24.2±15.41%) was noted on day 13 post-challenge. On the other hand, all the control animals succumbed to the parasite by day 11 post-challenge due to high infection (44.61±3.7%). Present study revealed that maximum protection was conferred by 66 kDa immunized mice as 67% animals survived the challenge, followed by 66+79 kDa immunized mice (50% animals). Least but significant protection was achieved in case of 79 kDa immunized animals as only 33% animals survived the challenge infection.

6.5. **Immunofluorescence assay**

Specificity of antigen-antibody reaction and antibody titers were analyzed by IFA test in serum samples of immunized mice. The parasite-specific fluorescence obtained in present study revealed that antibodies produced by 66 kDa and 79 kDa antigens in all three vaccine formulations reacted specifically to merozoite proteins. Immunization induced antibodies were observed in pre- as well as post-challenge sera. Maximum antibody response was observed on day 8 post-challenge in all the immunized animals. On this day, 66 kDa immunized and co-immunized groups had antibody titers of 1:4096 whereas, 79 kDa immunized animals had antibody titer of 1:2048. Among immunized groups, highest antibody titers were observed in 66 kDa immunized mice. Antibody responses in 79 kDa immunized and co-immunized groups were comparable except day 8 post-challenge.

6.6. **Parasite-specific antibody responses**

Parasite-specific IgG, IgG1 and IgG2a antibodies were analyzed in serum samples of immunized as well as control animals. Data of present study revealed that serum samples of immunized animals had significantly higher (p<0.001) levels of IgG antibody as compared to controls. Maximum
antibody response was detected in 66 kDa immunized group followed by animals immunized with 66+79 kDa. In both of these groups, IgG response was maximum on day 30 post-challenge, and it was significantly higher (p<0.05) than that of 79 kDa immunized mice. Maximum IgG response in 79 kDa immunized group was detected on day 8 post-challenge.

The IgG1 isotype antibody is the signature antibody of Th2 type of immune response. Pre-challenge sera of immunized animals had significantly higher (p<0.05) IgG1 response as compared to controls except 79 kDa immunized mice. Challenge of animals induced significant (p<0.05) IgG1 isotype particularly on day 12 post-challenge. Animals immunized with 66 kDa had highest isotype levels followed by 66+79 kDa immunized mice. A considerable IgG1 response was also induced by 79 kDa antigen. Decreased antibody levels were observed when checked after clearance of infection from immunized animals.

IgG2a isotype antibody is the marker antibody of Th1 type immunity. Significantly higher (p<0.001) production of IgG2a was detected in pre- as well as post-challenge serum samples of immunized animals except 79 kDa immunized group on day 8. 66 kDa antigen induced the maximum antibody response. IgG2a antibody produced by 66+79 kDa antigen was comparable to that produced by 66 kDa antigen. 79 kDa antigen induced significantly lower (p<0.05) IgG2a response as compared to other immunized groups.

6.7. Cytokine responses

Quantification of different cytokines was done in culture supernatants of splenocytes of immunized animals using commercial cytokine ELISA kits. Interferon-gamma (IFN-γ) is an indicator cytokine of Th1 type of immune response. Significantly higher (p<0.01) production of IFN-γ was observed in immunized animals as compared to controls. Maximum production of the cytokine was observed on day 12 post-challenge. Among immunized groups, maximum induction of the IFN-γ was detected in 66 kDa immunized animals followed by co-immunized animals (66+79 kDa). 79 kDa immunized animals had significantly lower
(p<0.05) cytokine production on all post-challenge days as compared to other immunized groups. Decreased levels of IFN-γ were observed after parasite clearance.

Interleukin-2 (IL-2), also a Th1 cytokine, was also significantly (p<0.001) induced by all the immunized animals in comparison to controls. In immunized animals, maximum IL-2 response was detected on day 8 post-challenge. Among immunized groups, splenocytes of 66 kDa immunized animals induced highest IL-2 except days 0 and 4 post-challenge. On these days, IL-2 production was maximum in 66+79 kDa immunized animals. Mice immunized with 79 kDa antigen induced significantly lower (p<0.05) IL-2 concentration as compared to other immunized mice except on day 30 post-challenge, when IL-2 production in these animals was comparable to that of co-immunized mice.

Interleukin-4 (IL-4) cytokine production was checked as an indicator of Th2 immune response. Significantly higher (p<0.01) expression of IL-4 was observed in immunized animals as compared to controls except 79 kDa immunized mice. IL-4 production in 79 kDa immunized and controls was comparable on all experimental days. Maximum IL-4 response was observed on day 12 in the immunized animals, and on day 4 post-challenge in control animals. When checked after parasite clearance, decreased levels of cytokine were obtained.

6.8. Western blotting

Expression of the immunizing as well as other antigens was checked by western blotting using immune sera from various groups. It has been observed that sera obtained from immunized as well as control animals reacted with a number of antigens in addition to 66 kDa and 79 kDa antigens. Proteins of molecular masses 46, 48, 66, 79 and 116 kDa showed reactivity with sera of all the groups, whereas, 133 kDa was expressed only in immunized groups. Among immunized groups, sera of co-immunized group recognized maximum number of antigens. Bands recognized by sera of immunized animals were comparatively more prominent as compared to
control sera. No reactivity was observed with serum samples of naïve mice.

6.9. **Delayed type hypersensitivity (DTH) response**

DTH is an index of cell-mediated immunity. It was checked in the immunized mice in terms of percentage of increase in footpad thickness after intradermal inoculation of 40 µg of antigen and PBS in left and right hind footpad respectively. After 24 h, it was observed that immunized animals had significantly higher (p<0.05) DTH response as compared to controls. Maximum hypersensitivity was detected in co-immunized animals, followed by 66 kDa immunized mice. Lower but significant (p<0.05) DTH response was observed in 79 kDa immunized animals.

6.10. **Parasite invasion inhibition assay**

The ability of immune sera to inhibit parasite invasion into erythrocytes was evaluated in short term *in vitro* culture of *P. berghei*. 5% (v/v) immune sera were added to each culture well at 0 h. After 21 h of incubation, invasion inhibition was studied from culture smears. Sera of 66 kDa immunized mice exhibited maximum inhibition activity of 72.5% on day 8, whereas, maximum inhibition in 79 kDa (37.5%) and co-immunized groups (57.5%) was observed with serum samples of day 12 post-challenge. In culture smears, the number of trophozoites and schizonts was less as compared to controls in presence of immune sera. Clumping of free merozoites was also seen in smears of experimental cultures and not in the control smears.

6.11. **Analysis of cross reactivity**

The presence of homologous antigens in different plasmodial strains was checked by cross reactivity. For this, reactivity of immune sera raised against *P. berghei* NK 65 antigens was checked against *P. falciparum*, *P. knowlesi* and *P. yoelii* and *P. berghei* K173 merozoites. No cross reactivity was detected with any of the parasite strains, except *P. berghei* K173. This points towards the fact that the cross-reactivity in present study is species-specific.
From the results of present study, the following conclusions can be drawn:

- 66 kDa protein is highly immunogenic and imparted maximum protective efficacy in vivo.

- Both, Th1 (IFN-γ, IL-2 and IgG2a) as well as Th2 (IL-4 and IgG1) type of immune responses were involved in protective mechanisms, particularly dominated by Th1 response.

- Challenge of animals had considerable impact on enhancement of parasite-specific antibodies as well as on cytokine expression.

- Studies on different post-challenge days revealed that maximum immunogenicity was observed on days 8 and 12 post-challenge, the time around which parasitemia was almost at its peak level in controls and immunized mice respectively.

- In vitro, maximum merozoite invasion inhibition was observed with sera of 66 kDa immunized mice, followed by sera of co-immunized animals.

- Immunizing antigens, i.e. 66 kDa and 79 kDa, were well recognized by the host immune system, as sera generated showed immunoreactivity with parasite proteins in immunofluorescence assay and western blots.

- Cross-reactivity in present study is species-specific as immune sera raised against *P. berghei* NK-65 proteins showed reactivity only with *P. berghei* K173, and no reactivity was observed with *P. falciparum*, *P. knowlesi* and *P. yoelii*.

- Profound DTH response was observed in all the immunized animals, particularly in animals of co-immunized group.

- Immunogenicity as well as protective efficacy of co-immunization using 66+79 kDa was intermediate to that exhibited by 66 kDa and 79 kDa antigens individually. From this, it can be concluded that immune response generated in present study is antigen-specific and combination has not affected the immunogenicity of individual antigens, and hence no evidence of vaccine interference.
Further studies are required to check the immunogenicity and protective efficacy of 66 kDa and 79 kDa antigens using different adjuvant systems.

Data of this study are encouraging for the development of multivalent subunit malaria vaccines.