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Kix domain specific Immunoglobulin A can protect from adverse lung and cerebral pathology induced by *Plasmodium berghei* ANKA

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ABSTRACT

Plasmodium specific IgA has been detected in serum and breast milk among the endemic population but the role it can play *in vivo* is not clear. In this report, we demonstrate the utility of Malaria specific IgA, elicited by peptide sequences (referred as Mpep3 and Mpep4) of region VI of EBA-175 (PfrVI). Immunization of mice with KLH tagged or untagged peptides of Mpep3, Mpep4 or with PfrVI have resulted in specific IgA response that inhibits the *in vitro* invasion of *Plasmodium falciparum* merozoites. Mice having the IgA specific to Mpep4 have exhibited higher tolerance to *Plasmodium berghei* ANKA parasitemia, exhibited several fold lesser sequestration of infected RBC, lesser damage to microvasculature with no signs of perivascular haemorrhage and lesser lung inflammation in comparison to unimmunized mice. In addition, the immunized mice have B-cell population that secrete the IgA specific to PfrVI. These results suggest that the IgA specific to these malarial antigens can confer significant advantage to hosts and it may also reduce the severity of malaria infection.

1. Introduction

Plasmodium falciparum, which causes fatal and life-threatening complications like cerebral and placental malaria, utilizes several redundant ligands to invade erythrocyte membrane to evade immune surveillance [1]. Among the ligands, EBA-175 is the primary ligand for invasion by merozoites. The EBA-175, comprises several regions *viz*. Regions I-VI, is secreted by microneme onto the surface of merozoites and is shed at or around the point of invasion [2,3]. While the regions I and II have the Duffy Binding Domains, the regions III-V have variable functions and the C-terminal Cysteine rich region VI is followed by a transmembrane domain (TMD) with a cytoplasmic tail (Fig. 1A). It is reported that the region VI of EBA-175 (PfrVI) participates in the formation of tight junction complex with host membrane, dimerization of the EBA-175 and also for trafficking to microneme. Moreover, the PfrVI has sequences that are conserved across the DBL family proteins. For example, it contains eight highly conserved Cys residues (Fig. 1B, marked in bold) and a ROM4 cleavage site needed for shedding of EBA-175 form the junctional complex for completion of the invasion cycle. Shedding of this junctional complex occurs irrespective of EBA-175 being used as a primary ligand or not [3]. However, the nature of immune response elicited by this domain and its *in vivo* role has not been investigated in detail.

Antibody response against region II and EBA-peptide 44 (42 aa within the region V) can block the binding of native EBA-175 to human erythrocytes and also inhibits the merozoite invasion *in vitro* [2,4]. Importantly, antibodies against EBLs and RBLs are elicited in a kinetic manner i.e. initially to EBA175, EBA181, EBA140 (involved in sialic acid-dependent pathway), and later to PfRh2 and PfRh4 (involved in sialic acid -independent pathway) [1]. The success of passive immunization also highlights the role of Immunoglobulin (Ig) based therapies since anti-EBA-175 IgG has been able to block the invasion by ~90% [5]. Further, animal models based on passive immunization with mono specific antibodies (raised against synthetic peptides) or adoptive transfer of B-cells (specific to malarial antigens) have also highlighted the role of adaptive immune response against malaria infection [6–9]. In addition, susceptibility of B-cell-deficient hosts has, once again, underlined the role of antibodies in fighting the infection [9].

Hence, it is curious whether or not antibody response, exclusively directed towards the membrane proximal region of PfrVI, can have any role during the erythrocyte invasion step. It appears that

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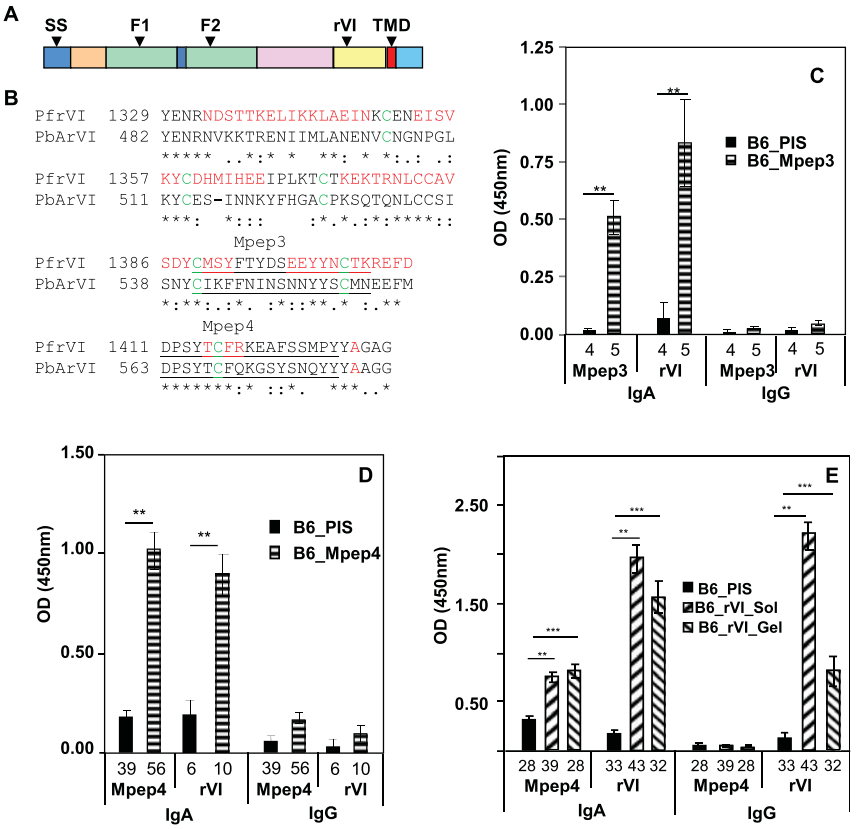

Fig. 1. Immune responses of Mpep3, Mpep4 and PfrVI in mice: **A: Diagrammatic presentation of domains of EBA-175 of *Plasmodium falciparum*.** The markings SS, F1, F2, rVI, TMD respectively represent signal sequence, duffy binding domains 1 and 2, region VI and transmembrane domains. **(B) Homology between PfrVI and PbArVI**: The PfrVI and PbArVI have 44% homology between them and the PfrVI contains a cleavage site (A'G) of PfROM4 protease. The peptides sequences, Mpep3 (17mer) and Mpep4 (18mer), used in the study are underlined. **(C–E)** Immunoglobulin profile elicited by the antigens Mpep3 (C), Mpep4 (D) and PfrVI(E) in C57BL/6 mice: The immunoglobulin isotype was determined by ELISA. The antigen coated on the plate is shown below the group of bars and the isotype detected is shown below for each group. A sample with an OD value which were at least two standard deviations (SD) higher from that of the negative controls (described in methods sections) were considered positive. (**C**) Represents the average data of one of the five independent Mpep3 immunizations of C57BL/6 mice. (**D**) Represents the average data of seven independent immunizations of Mpep4 of C57BL/6 mice. (**E**) Represents the average data of five independent PfrVI immunizations of C57BL/6 mice. The 'sol' and 'gel' form respectively represent the antigen, PfrVI, in solution (purified through Ni-NTA column) and isolated from SDS-PAGE gel specific to the PfrVI band respectively. The number of mice used for obtaining the average for each bar is given below.

the rVI of EBA-175 can play an important role during the erythrocyte invasion step. In this regard, we have observed that the rVI derived peptide sequences i.e. Mpep3 and Mpep4 elicit predominant IgA isotype in Balb/C background. Interestingly, endemic patient sera have also shown positive reactivity towards these sequences. We have also detected the presence of B-cells specific to Mpep3 and Mpep4 in immunized mice and the B-cells secrete IgA in *in vitro* culture (under review else where). In view of these observations, we have attempted to understand the utility of this IgA in the context of experimental animal infection. Since, the Mpep4 sequence has good homology with the rVI domain of *Plasmodium berghei* ANKA (PbA), we have attempted to understand the role of IgA, if any, during the onset of infection. Our results show that the IgA can substantially improve the cerebral pathology of C57BL/6 mice. This can pave the way for understanding the role of IgA in malarial infections.

2. Materials and methods

2.1. Animal experimentation

All protocols were approved by the Institutional Animal Care Committee of National Centre for Cell Science, Pune. All experiments on mice were conducted in pathogen free, approved environment.

2.2. Peptide synthesis

All the peptides used were obtained from commercial sources which were HPLC purified with >99% purity and stored at −80C in dry power form in air tight container. All peptides have been verified by MALDI and MS–MS analysis.

2.3. Cloning of EBA-175 (PfrVI)

The PfrVI construct (coding sequence shown in Fig. 1B), was cloned into pET32a+ using NcoI and HindIII restriction sites and it has 109 amino acid Thioredoxin protein at C-terminus to kill the cytotoxicity associated with PfrVI expression alone. Removal of Trx tag has resulted in precipitation and/or loss of yield. The construct was verified by DNA sequencing.

2.4. Expression and purification of PfrVI by Ni-NTA column

Purification of recombinant PfrVI was essentially carried out using Ni-NTA matrix. *E. coli* BL21(DE3) codon$^+$ strain, transformed

with pET32/PfrVI, was cultured in LB medium in the presence of amp (100 μg/ml) at 37 °C and induced with 0.5 mM IPTG (OD~ 0.6). The bacterial pellet was sonicated in 10 mM Tris-phosphate buffer (pH-8.0) containing 6M urea and 0.1% Triton-X100 and the resultant supernatant was purified by Ni-NTA chromatography. The urea was gradually decreased from 6M to 0M by washing the matrix with buffer containing 20 mM imidazole and the protein was eluted with 250 mM imidazole containing 10% glycerol. The PfrVI was confirmed by MS–MS analysis.

2.5. Preparation of emulsion

Emulsion was prepared by adding 1:1 ratio of antigen and Fruend's complete for 1st and incomplete adjuvants for all subsequent immunizations.

2.6. Immunization of mice

Female C57BL/6 (4–6 weeks), after collecting pre-immune sera (PIS), were immunized with 5–10 μg of respective antigen in 40–50 μl of emulsion for 3 times on 0, 21 and 42 days through subcutaneous route. After 7 days of each immunization, tail vein blood (10 μl) was collected in PBS for the estimation of immune response. We have observed that the IgA response is independent of peptide conjugation to carrier agent such as KLH.

2.7. Preparation of antigen coated plates

The 96-well plates were coated with Mpep3 (1 μg/well) Mpep4 (1 μg/well) and PfrVI (0.3 μg/well) in bicarbonate buffer (pH 9.6) at 4 °C by over-night incubation.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The ELISA for detection of IgA has been carried out as described earlier. Briefly, antigen coated plates, were washed with 0.05% Tween-20 in Tris-buffered saline (TBST, pH-8.0) thrice, blocked for 2 h with 1% gelatin (Bio-Rad) in TBST at RT. Plates were incubated with 100 μl of appropriately diluted anti-sera after washing. The bound Ig isotype was detected with 100 μl of HRP-conjugated secondary antibodies viz. goat-anti-mouse IgA-HRP (Invitrogen; Cat # 62-6720; Lot 575981A), rat anti-mouse IgG-HRP (Invitrogen; Cat # 04-6020; Lot 692174A). The values of all controls were deducted from each sample. A sample with an OD value with at least two standard deviations (SD) higher from that of the negative controls was considered positive. Data from the different groups were compared with One Way ANOVA test using Sigma Plot.12 software and statistical significance was determined.

2.9. In vitro invasion assay

In vitro invasion assays were performed exactly as described earlier by using synchronised *P. falciparum* 3D7 cultures obtained by sorbital treatment [4]. The plates were incubated in a humidified gas chamber (~95% nitrogen, 4% CO_2, 1%O_2) for 16–24 h at 37 °C. After 24 h, Blood smear was examined under fluorescence microscope with Acridine-orange staining to assess the ring stage (fresh invasion). Rate of inhibition of erythrocyte invasion was estimated by following formula: Inhibition of Invasion (%) = [1-parasitemia (%) in treated wells/parasitemia (%) in control wells] x100 [10–12].

2.10. Mice infections

P. berghei ANKA-1 (PbA-1) was kindly gifted by Prof. Namita Surolia, JNCASR, Bangalore, India. *P. berghei* ANKA (MRA-671) and *P. berghei* ANKA (MRA-311) were obtained from MR4, ATCC, USA. All strains of *P. berghei* ANKA were maintained exclusively either in C57BL/6 or Balb/C female mice. None of the parasite strains used in the study was interchanged between mice strains. All infection experiments were conducted after 2–3 passages from a frozen vial. All mice were infected with 5×10^4 infected red blood cells (iRBC) through intra-peritoneal injection.

2.11. Estimation of parasitaemia

Parasitaemia was estimated by microscopic observation of thin blood smears on indicated days by staining with 50 mM Acridine Orange (Invitrogen) after they were fixed in absolute methenol for 30–60 s. Parasitaemia was estimated by calculating the percentage of red blood cells infected in at least 10 microscopic fields by counting a minimum of 1000 or more RBCs.

2.12. Histopathology of brain tissue

Infected mice were monitored daily and brain, lung, liver, kidney and spleen were harvested from mice immediately after death. Harvested organs were fixed in 10% (v/v) formaldehyde in distilled

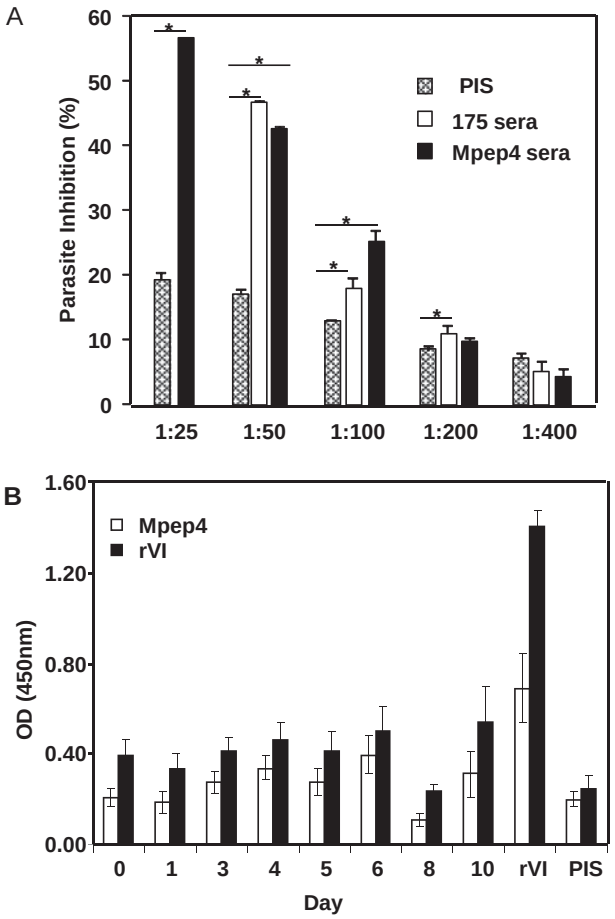

Fig. 2. In vitro invasion Assay: (**A**) **Inhibition of Erythrocyte Invasion by immune sera: (A)** Inhibition of erythrocyte invasion at different dilutions of the indicated immune sera. The data shown are an average of 3 independent experiments. **(B) Absence of IgA in first infection by MRA-671**: Post infection immunoglobulin profile of unimmunized control C57BL/6 mice. The sera of mice infected with MRA-671, collected on day 0, 1, 3, 4, 5, 6, 8 and 10 after infection were examined by ELISA. A sample with an OD value which was at least two standard deviations (SD) away from that of the negative controls was considered positive.

water. Fixed brain tissues were cut into two half through sagittal axis and embedded in wax block. We have examined various parameters of the sagittal sections of the mice brain *viz*. sequestration of infected RBC, cerebral hemorrhage, and damage of endothelial lining in brain tissue by scoring the haematoxylin-eosin stained section of the tissue at 20X magnification. A minimum of three sagittal sections of each of the brain tissue with more than 50 images from each group were independently examined by pathologist for deviations in cerebral pathology.

2.13. Quantification of histopathology of brain section

Haematoxylin-Eosin stained thin (1μ) sections of brain and other tissues were examined by light microscopy. At least 50 images of sagittal sections containing blood vessels (or all vessels on small or sparsely vascularized specimens) from each of the groups were identified and scored for a variety of characteristics. These included sequestration of infected RBCs (scored as 0 for no infected RBC, 1 = loosely packed with infected RBCs, 2 = moderate packing with infected RBCs and 3 = highly packed infected RBCs with obvious distortion). Similarly the states of endothelium lining of brain vasculature and perivascular hemorrhage were scored as 0 if intact and 1 for damaged areas. The degree of sequestration of blood vessels, damage to the endothelial lining and perivascular hemorrhage were expressed as percentage score (ratio of score obtained/highest score for the characteristic per 100 observations. One way ANNOVA was carried as described above.

3. Results and discussion

3.1. IgA response is centered on the N-terminal side of the Mpep3 and Mpep4

The antisera of C57BL/6 mice, immunized as described in methods section, have exhibited IgA specific to Mpep3 (Fig. 1C), Mpep4 (Fig. 1D) and PfrVI (Fig. 1E). It should be noted that the recombinant PfrVI is useful for verification of the immune response since it contains both Mpep3 and Mpep4 sequences and hence, acts as an independent antigen. We have also ascertained that the goat anti-mouse IgA-HRP, used here, did not bind to mouse-IgG (data not shown). The panels Fig. 1C–E indicate that both Mpep3, Mpep4 elicit IgA but not IgG response in about 30 independent immunizations performed over 3 years with different antigen preparations. However, the PfrVI elicits IgG in addition to the IgA (Fig. 1E). In view of the non-requirement of conjugation we have used the HPLC purified peptides (>99% purity) in all our immunizations. Although, not dramatic, IgA response in Balb/C has been found to be always stronger than that of C57BL/6 mice (data not shown). Moreover, there is also no cross-reactivity by the observed IgA i.e. the IgA specific to Mpep3 does not cross react with Mpep4 and *vice versa* (data not shown). The panel E also illustrates that the IgA response elicited by the PfrVI (for both in-solution and in-gel form of antigens) is certainly centred on the Mpep4 sequence since Mpep4 coated wells showed strong IgA than IgG. Similar IgA response against Mpep3 is also seen upon PfrVI immunization (data not shown). Thus, both Mpep3 and Mpep4 have elicited only IgA response, the PfrVI has elicited both IgA and IgG upon immunization.

3.2. Mpep3 and Mpep4 antisera inhibit erythrocyte invasion in vitro

We next examined whether or not the observed IgA can interfere in *in vitro* invasion of *Plasmodium falciparum* 3D7 (Pf) merozoites. The *in vitro* invasion assay, in the presence of mice serum, was done exact as described earlier [10–12]. As seen in Fig. 2A, even with 50 fold diluted peptide anti-sera or anti-PfrVI anti-sera, we have seen about ~50% inhibition of invasion. We have consistently observed best inhibition with anti-Mpep4 antiserum in all our *in vitro* inhibition assays. This result suggests that the IgA, specific to PfrVI and Mpep4, can inhibit *in vitro* invasion of Pf merozoites.

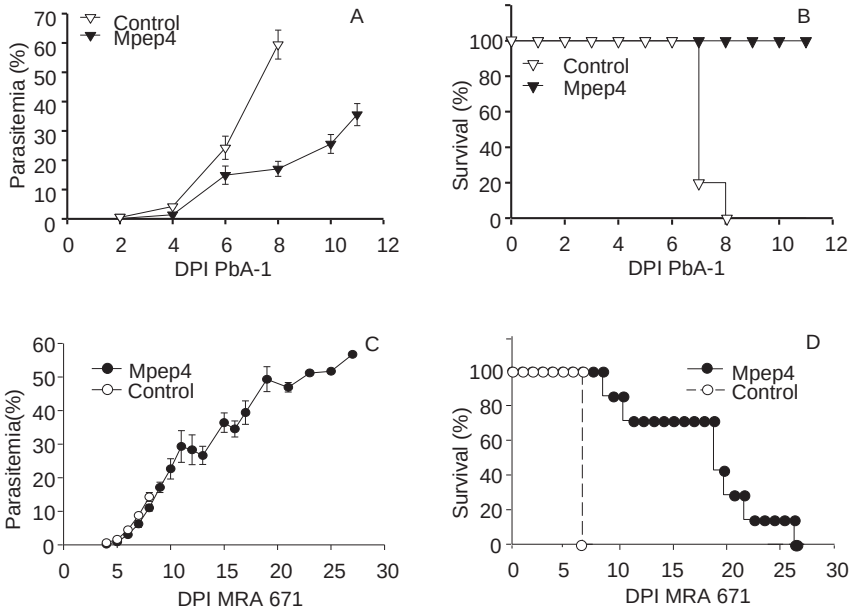

Fig. 3. Progression and Survival of PbA infection: Progression (A and C) and survival (B and D) of Mpep4 immunized mice was monitored as described in methods section. C57BL/6 mice were immunized with Mpep4 and after 2 boosters on day 21 and day 42, the mice were challenged with either PbA-1 or MRA-671 strains on day 47 and the progression of parasitemia was monitored every 36–48 h. The panels B (for control and immunized, n = 05) and D (for control and immunized, n = 6 and 7 respectively) show the survival pattern of the Mpep4 immunized and the control mice. Panels **A-D** represents the data of one of three independent experiments.

3.3. First infection with PbA does not elicit IgA response

To know the IgA isotype response after a fresh infection we have examined the mice sera collected on day 0, 1, 3, 4, 5, 6, 8 and 10 after infection with MRA-671. This is important because IgA in humans was detected only among the individuals living in endemic areas who have had multiple episodes of malarial infection (3 or more). As shown in Fig. 2B, first time infection of mice with PbA has not elicited IgA response specific towards Mpep3 (although not expected due to lack of homology) or Mpep4 or PfrVI based on the data collected over several independent infections even after day 12. This clearly indicates that first infection doesn't lead to elicitation of IgA against the sequences identified by us.

3.4. Mpep4 specific IgA improves survival and overall cerebral pathology

In view of the above results, we have next examined the role of IgA in mice during infection. The homology between PfrVI and PbArVI (Fig. 1A) gave us the opportunity to probe the protective role, if any, by the IgA. Two parasite strains were used in the study *viz.* PbA-1, MRA-671 (MR4, USA). The characteristics of PbA-1 are similar to the MRA-311 (data not shown), which usually exhibited average parasitemia 30–50% and the average life span of infected mice is 8–13 days post infection (dpi) and the parasitemia and cerebral pathology were similar to several published reports [13,14]. On the other hand, the MRA-671 always exhibited typical parasitemia in the range 12–18% and an average life span of 8–13 days post infection (dpi) to that of control mice.

Immunization of mice with three different doses 5 μg, 10 μg and 20 μg of Mpep4 have elicited strong IgA immune response and the titres of the individual subjects were similar among the group of mice studied. In mice immunized with 5 μg of Mpep4, the progression of PbA-1 was slower initially but reached ~40% in 11 days dpi in comparison to the control mice for which the parasitemia reached over 55% by day 8 (Fig. 3A). Similarly for the MRA-671 strain,

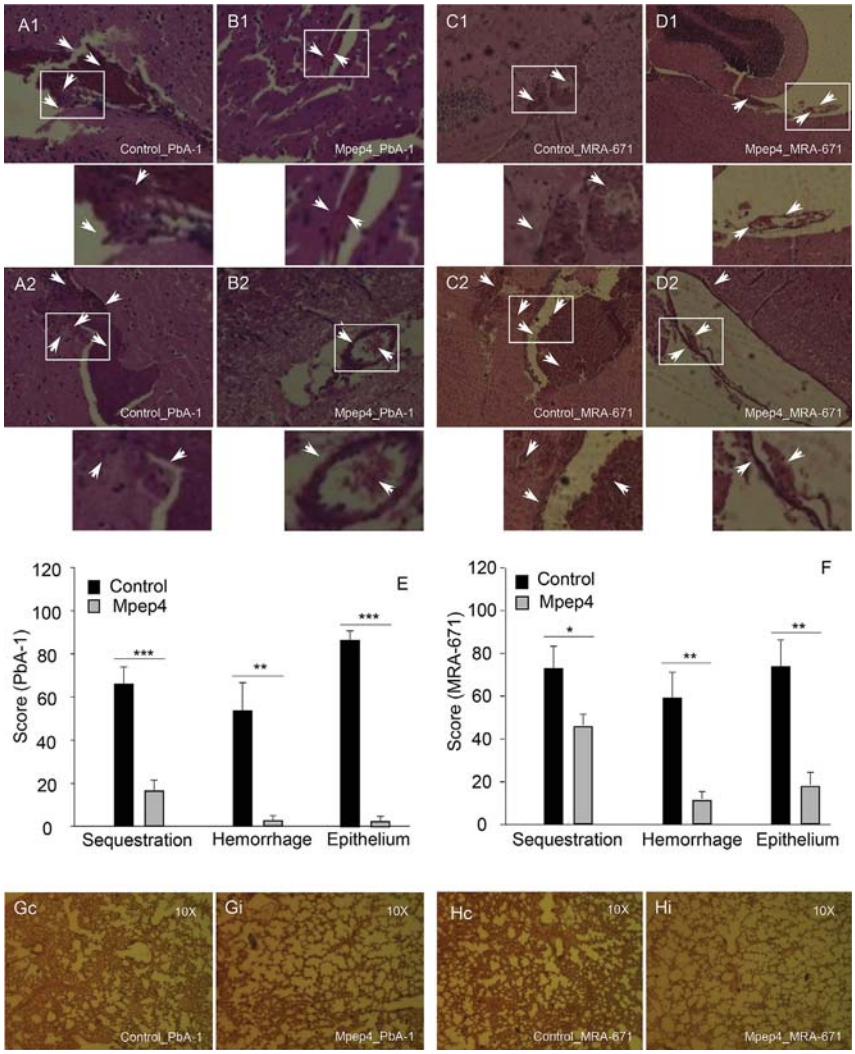

Fig. 4. IgA improves cerebral pathology of mice challenged with PbA: **(A–H): Histology of brain sections of the infected Mpep4 immunized C57 BL/6 mice**: The controls and immunized mice were sacrificed on the last day of the curve (Fig. 3A and C) for quantitative histopathological examination of the brain sections as described in methods. The panels A1, A2 (PbA-1) and C1 and C2 (MRA-671) are representative of brain sections of unimmunized, infected controls and the B1, B2 (PbA-1) D1, D2 (MRA-671) were of Mpep4 immunized mice. The panels E (PbA-1) and F(MRA-671) represent the quantitation of sequestration, hemorrhages and damage to epithelial lining observed in various sections of controls (black bars) and Mpep4 immunized mice (grey bars). The Mpep4 immunized mice consistently showed far less sequestration and no damage to the microvasculature or sign of perivascular hemorrhages as compare to that of unimmunized mice while the control mice showed higher iRBC sequestration, damaged epithelial lining of microvasculature and perivascular hemorrhages despite of similar peripheral parasitemia scored. The panels shown were a representative of three repetitions. The panels labeled with Gc, Hc and Gi and Hi are representative lung sections of mice infected with PbA-1 and MRA-671 infected mice. This data shown is a representative of three independent experiments which showed similar results.

mice immunized with 5 μg of Mpep4, the progression of parasitemia was slower (Fig. 3C) and reached parasitemia upto 60% and survived at least 15 days longer (Fig. 3B–D) whereas the unimmunized control mice died with 12–15 % parasitemia. For all the mice that survived, the brains sections of the mice were examined by histopathology. We have specifically looked for sequestration of infected RBC (iRBC) in the brain tissue, perivascular hemorrhage and damage to endothelial lining in the H&E stained sections of the brains. Histopathology of brain sections of mice immunized with Mpep4 (for various immunization doses) in both strains (PbA-1 and MRA-671) clearly showed several fold lesser sequestration of iRBC, little or no damage to microvasculature and no sign of perivascular hemorrhage (Fig. 4, panels B1, B2 and D1 and D2) in comparison to the brain sections of unimmunized C57 mice which showed higher sequestration of iRBC, damaged epithelial lining of microvasculature and frequent perivascular hemorrhages (Fig. 4, panels A1, A2, C1, and C2). About >75% tissue sections examined, we have noted that the small blood vessels have remained intact and the damage to endothelial lining is observed only in case of large blood vessels as well as hemorrhages (Fig. 4E and F). A similar, markedly improved pathology is also seen in case of lungs of PbA infected mice upon immunization. The panels, Fig. 4Gc and Hc, show extreme congestion of the alveolar space while the immunized mice have a normal like appearance (Fig. 4Gi and Hi) despite the high parasitemia. These results suggest us that the IgA can offer protection against parasite mediated sequestration and damage of endothelial lining. Overall, the immunized mice did not exhibit any symptoms of experimental cerebral malaria described in the literature.

Our study, for the first time, demonstrates the utility of anti-malarial IgA. Based on the literature, it is clear that malaria induces high level of IgG and the resultant response, in principle, can compete with the FcR occupancy. In this regard, the IgA described here can have an obvious advantage. In addition to this, antibody based therapies involving both IgG_1 and IgG_3 subtype may be disadvantageous since they can bind FcγRs present on cells (platelets, B-cells, endothelial cells and placental tissue) that do not eliminate *P. falciparum* and may lead to triggering of inhibitory FcγRIIb as observed among endemic patients. It is apparent form the literature that the immunoglobulin subtypes IgM, IgG and IgE were all implicated in various pathologies i.e. the pathology associated with rosette formation, placental malaria and severe malaria [9]. Interestingly, the IgA isotype has not been shown to be responsible for any undesired pathology in malarial infection. This possibility opens-up the debate on IgA based anti-malarial therapy. In the study involving western Kenyan children samples, it was observed that the IgA associated immune complex has a protective effect to cerebral pathology, unlike IgG_1, IgG_3 and IgE associated immune complexes [15]. In another study, the retinoic acid, a major oxidative metabolite of Vitamin A, induces IgA isotype switching mainly through RARα in human B cells and Vitamin A deficiency increases the mortality in African children, and malaria being an all-important cause of death of children of this age [16–18]. This is because human FcαR is constitutively expressed on monocytes, neutrophils, eosinophils, Kupffer cells and dendritic cells, which can capture and process the IgA opsonized parasite. It is relevant to note that the receptor for IgA in mice (human CD89 equivalent) has not been confirmed. Moreover, IgA is the second most abundant antibody (~1–4% of total serum protein) in humans and mice. Moreover, the malaria associated IgA i.e. positive serum of endemic patients, also appears to inhibit the *in vitro* invasion of *P. falciparum* merozoites [19]. Notably, the IgA, detected among the endemic Indian population, was significant only among individuals who had multiple clinical attacks of plasmodium infection. It was also reported that the IgA titer were found to increase with the age among the seropositive individuals.

Summarily, our study will serve as an important starting point for the development of IgA based therapy against malaria infection. Monoclonal antibodies against Mpep4 sequence may serve as a potent antibody mediated therapy to provide an alternative approach to combat malaria successfully.

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