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The utility of *Plasmodium berghei* as a rodent model for anti-merozoite malaria vaccine assessment

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Rodent malaria species *Plasmodium yoelii* and *P. chabaudi* have been widely used to validate vaccine approaches targeting blood-stage merozoite antigens. However, increasing data suggest the *P. berghei* rodent malaria may be able to circumvent vaccine-induced anti-merozoite responses. Here we confirm a failure to protect against *P. berghei*, despite successful antibody induction against leading merozoite antigens using protein-in-adjuvant or viral vectored vaccine delivery. No subunit vaccine approach showed efficacy in mice following immunization and challenge with the wild-type *P. berghei* strains ANKA or NK65, or against a chimeric parasite line encoding a merozoite antigen from *P. falciparum*. Protection was not improved in knockout mice lacking the inhibitory Fc receptor CD32b, nor against a Δ*smac P. berghei* parasite line with a non-sequestering phenotype. An improved understanding of the mechanisms responsible for protection, or failure of protection, against *P. berghei* merozoites could guide the development of an efficacious vaccine against *P. falciparum*.

Despite the deployment of effective control and prevention strategies, *Plasmodium falciparum* malaria remains a huge burden on global public health[1]. The development of a highly effective vaccine is still necessary if ambitions for local elimination, and ultimate eradication, are to be realized. Promising results in Phase IIb field trials with the leading candidate vaccine, RTS,S, raised hopes that this formulation may be an effective contributor to malaria control public health measures[2]. However, more recent results in a Phase III trial, especially in the target infant age group, have revealed relatively low levels of efficacy against clinical and severe malaria[3]. Further work is vital in order to develop and establish a highly effective vaccine for use in the field[4], and the continuing deaths of nearly a million children each year demonstrates the urgency with which this work needs to take place[1].

Numerous vaccine strategies have been proposed to tackle the complex lifecycle of the *Plasmodium* parasite. Leading vaccines in clinical development primarily target the sporozoite and/or liver-stages of parasite development[5]. However significant efforts have also been made to develop vaccines against the pathogenic blood-stage infection[6] – especially given this is the lifecycle stage of the parasite against which natural immunity is acquired following repeated exposure[7]. Numerous blood-stage candidate vaccines have now been tested in over 40 Phase I/II clinical trials[6], but the field has faced much disappointment without a single formulation demonstrating significant efficacy in the primary endpoint of a Phase IIa/b clinical trial. A substantial number of these vaccines have targeted the leading merozoite antigens merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1)[8–11] – targets historically identified as associated with naturally-acquired immunity, and important for the invasion process of new host red blood cells[12,13]. Most recently, a Phase IIb field trial of a protein vaccine, based on the 42 kDa C-terminus of MSP1 ($MSP1_{42}$) in Kenya showed no efficacy[9], but encouragingly strain-specific efficacy was reported in the secondary analysis of a mono-valent vaccine based on the 3D7 allele of AMA1 tested in Malian children[10]. Intriguingly, a previous Phase IIa controlled human malaria infection (CHMI) study of the same vaccine in US adult volunteers showed no significant efficacy against challenge with the homologous 3D7 parasite clone[14]. It remains to be established whether future AMA1 vaccine candidates can overcome the significant hurdles surrounding the problems of antigen polymorphism[15].

Vaccine research relies heavily on animal models to screen putative candidates, and the MSP1 and AMA1 targets have been previously validated in this manner as protective determinants of vaccine-induced antibodies[12,13]. In particular, rodent malaria species and some chimeric rodent/human malaria models have been widely used to assess the merits of various vaccine delivery platforms and candidate antigens[16,17]. The *P. yoelii* and *P. chabaudi* rodent malaria species have both been commonly utilized to demonstrate protection against blood-stage parasitemia by vaccines targeting the orthologous MSP1 and AMA1 antigens[18–22]. In the case of MSP1, the 19 kDa C-terminus of MSP1 ($MSP1_{19}$) has been found to be critical for the induction of antibody-mediated protection[23], whilst the neighbouring 33 kDa region ($MSP1_{33}$) appears important for T cell help[24,25]. Numerous subunit vaccines therefore rely on the use of the entire C-terminal $MSP1_{42}$ region[24], whilst some protein-in-adjuvant vaccines can induce antibody-mediated protection when based on $MSP1_{19}$ alone[20].

P. berghei represents a third rodent malaria species that has been widely used for the assessment of pre-erythrocytic[26,27] and transmission-blocking[28,29] subunit vaccine efficacy. However, despite the widespread use of this model, a search of the literature revealed only three English-language publications that have demonstrated protection against blood-stage *P. berghei* by subunit vaccination[30–32]. Subunit vaccines that are reported to have been successful in protecting against blood-stage *P. berghei* strain NK65 in BALB/c mice include *P. berghei* NK65 merozoite surface protein 9 (PbMSP9) N-terminus recombinant protein formulated in alum[30]; a recombinant *Salmonella* vaccine encoding regions of blocks 3 and 4 of PbMSP1 ANKA strain[31]; and a recombinant protein against $PbMSP1_{19}$ in alum[32]. The recombinant *Salmonella* vaccine additionally demonstrated limited protection against *P. berghei* NK65 strain in outbred CD1 mice[31]. A further paper published in Chinese describes induction of partial protection against blood-stage *P. berghei* following immunization with recombinant PbAMA1 in Freund's adjuvant[33]. Despite the limited number of studies demonstrating protection against blood-stage *P. berghei* malaria following subunit vaccination, it is this species of rodent malaria that forms the basis of currently available chimeric parasites, designed for testing and optimizing *P. falciparum* vaccines against the blood-stage in mice[34,35].

It is possible that additional laboratories have tested blood-stage subunit vaccines targeting *P. berghei* but publication bias has resulted in a lack of published descriptions of failures to achieve protection. However, more recently, two publications have reported failed protection by subunit vaccines targeting PbMSP1 and PbAMA1[36,37]. A head-to-head comparison of blood-stage vaccine efficacy against *P. berghei* ANKA or *P. yoelii* 17XL using baculoviral-based vaccines (BBV) targeting *P. berghei* or *P. yoelii* $MSP1_{19}$ and AMA1 found that BBV vaccines could induce complete or partial protection against *P. yoelii* but not against *P. berghei* in BALB/c mice[36]. In the second study, DNA vaccines expressing $PbMSP1_{42}$ were tested with and without fusion to a putative molecular adjuvant (complement protein C3d) in BALB/c mice[37]. In this study both adjuvanted and control vaccines induced anti-$PbMSP1_{42}$ antibody responses, but neither of the two vaccines protected against *P. berghei* blood-stage challenge.

In agreement with these studies, here we report on similar difficulties relating to the induction of protective immunity against *P. berghei* using viral vectored vaccines, as well as recombinant protein-in-adjuvant, targeting well-studied merozoite antigens which have previously been successful in protecting against *P. yoelii* and *P. chabaudi*[18–21,24,38,39]. Protection was not achieved against either the ANKA or NK65 *P. berghei* strains, nor in a range of inbred mouse strains following active subunit immunization. Similarly, neither active immunization with vaccines against PfMSP1 nor passive transfer of anti-$PfMSP1_{19}$ IgG from rabbits afforded protection in mice against a *P. berghei* parasite line chimeric for *P. falciparum* $MSP1_{19}$. An improved understanding of protective immune mechanisms against *P. berghei* merozoites is therefore still needed, and could help to guide the development of an effective vaccine against *P. falciparum*.

Results

***P. berghei* $MSP1_{19}$ protein vaccine production and immunogenicity.** Recombinant $PyMSP1_{19}$ protein-in-adjuvant vaccines are known to provide high levels of protection against *P. yoelii* blood-stage parasite challenge[20,39] and protection against *P. chabaudi* can be similarly induced using vaccines encoding the orthologous antigen ($PcMSP1_{19}$)[21]. In order to assess whether similar vaccines could protect against *P. berghei*, we generated a comparable protein subunit vaccine. Recombinant $PbMSP1_{19}$ protein from the ANKA strain was produced and purified from *E. coli* as a soluble GST-fusion protein as described in Methods.

To test the immunogenicity of GST-$PbMSP1_{19}$ protein, BALB/c mice were immunized using a regime that has been shown to induce extremely high levels of protective efficacy against *P. yoelii* pRBC challenge following $PyMSP1_{19}$ immunization[20]. Five doses of 20 μg GST-$PbMSP1_{19}$ protein were administered using CFA (at week 0), IFA (weeks 3, 6, 8) and PBS (week 9). Sera were collected at regular intervals (weeks 5, 9, 11) and assessed by ELISA. The total IgG titer against GST-$PbMSP1_{19}$ increased significantly over time, as further doses of protein were administered (Figure 1a). As the ELISA assay was unable to distinguish between anti-GST and anti-$PbMSP1_{19}$ responses, *P. berghei* immunofluorescence assays (IFA) were also performed using sera collected from mice two weeks following the final immunization of five doses of GST-$PbMSP1_{19}$ protein (week 11). The use of these immunized sera in IFA against *P. berghei* ANKA thin-blood smears confirmed the presence of IgG capable of binding to native $PbMSP1_{19}$ protein (Figure 1b).

***P. berghei* blood-stage viral vector vaccine production and immunogenicity.** Similar to protein vaccines, AdHu5-MVA viral vector prime-boost regimes targeting $PyMSP1_{42}$ have been demonstrated to induce protection against blood-stage *P. yoelii* challenge[18]. Recombinant AdHu5 and MVA viruses encoding $PbMSP1_{42}$ from the ANKA strain were produced in order to determine the immunogenicity and protective efficacy of viral vectors against *P. berghei*. Mice were immunized with AdHu5 expressing $PbMSP1_{42}$ and eight weeks later were boosted with MVA expressing the same antigen. This regime induced $PbMSP1_{19}$-specific total IgG, and titers significantly increased following the MVA boost (Figure 2a). Although this ELISA used the same GST-$PbMSP1_{19}$ protein as above, the entirety of the measured response following viral vector immunization will be directed to the $PbMSP1_{19}$ moiety. Moreover, similar to the protein vaccine, endpoint titers here as determined by ELISA were also high, thus confirming the strong immunogenicity of the viral subunit vaccine delivery platform.

The viral vectored vaccine platform is suited to the purposes of antigen screening[40], and thus whilst generating vectors encoding $PbMSP1_{42}$, similar recombinant AdHu5 and MVA vaccines were also generated encoding the PbAMA1 and PbMSP9 antigens, for which protection data have previously been reported in the *P. berghei* model[30,33]. These vectors were also used to immunize groups of BALB/c mice in a similar manner to those encoding $PbMSP1_{42}$. Recombinant PbAMA1 or PbMSP9 antigens were not available to test the induction of antibodies by ELISA, and therefore the induction of antibodies capable of recognizing native parasite antigen by vaccination was instead confirmed by IFA. Sera from AdHu5-MVA immunized BALB/c mice were assessed for binding to *P. berghei* ANKA mature schizonts (Figure 2b), which were the homologous parasite for $PbMSP1_{42}$ and PbAMA1, but heterologous for PbMSP9 (where the antigen sequence was based on the NK65 strain of *P. berghei*). However, these data demonstrated that the vaccines against

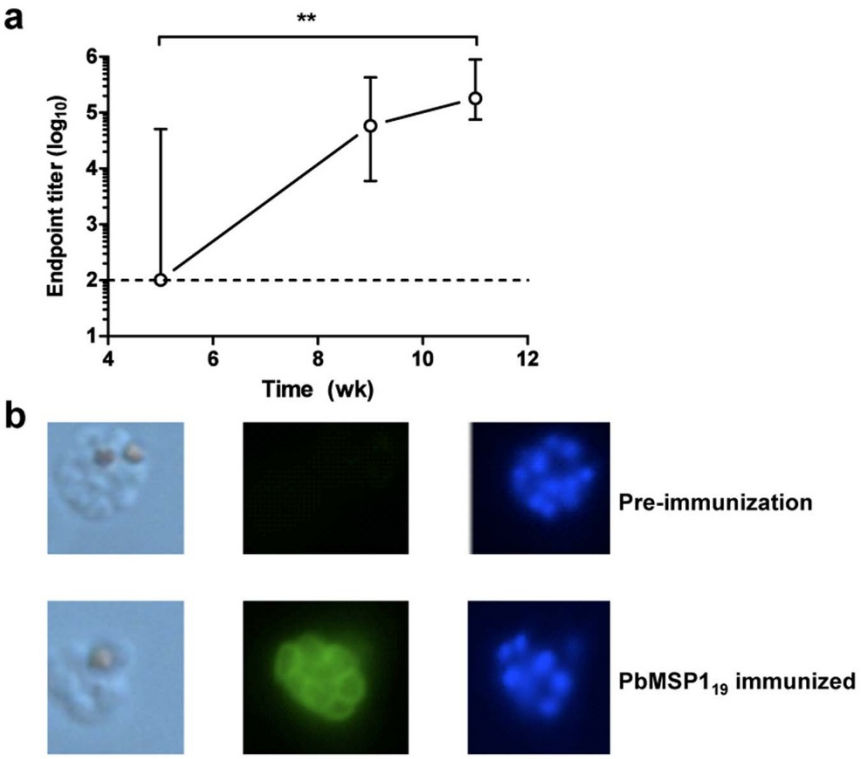

Figure 1 | Immunogenicity of GST-PbMSP1$_{19}$ immunization. BALB/c mice ($n = 5$) were immunized with five doses of 20 μg GST-PbMSP1$_{19}$ protein. Doses were administered at week 0 s.c. in CFA, at week 3 s.c. in IFA, at weeks 6 and 8 i.p. in IFA, and at week 9 i.p. in PBS. (a) Total IgG responses against recombinant GST-PbMSP1$_{19}$ protein were measured by ELISA in the serum of mice taken at weeks 5, 9 and 11. Median and range of the titers are shown. The dashed line indicates the lower limit of detection. ** $P < 0.01$ by Friedman test with Dunn's correction for comparison of all time-points. (b) *P. berghei* ANKA schizonts were fixed with 4% formaldehyde and permeabilized with 0.1% NP40. Pooled sera from week 11 in (A) were tested at a dilution of 1 : 100. Naïve pre-immunization serum was used as a negative control. Bound antibodies were detected using goat anti-mouse IgG conjugated to Alexa Fluor 488. Images show left to right the visible schizonts, followed by fluorescence (green) and DAPI staining (blue).

all three antigens were immunogenic, and confirmed that IgG antibodies capable of binding to native antigen in schizonts were produced following AdHu5-MVA immunization with PbAMA1 and PbMSP9, but not following immunization with vectors expressing a control antigen (OVA).

Protective efficacy of MSP1 vaccines against blood-stage *P. berghei* parasites. Mice can be sterilely protected against blood-stage *P. yoelii* challenge using a schedule of five immunizations of PyMSP1$_{19}$ (His$_6$-tagged protein produced in *Saccharomyces cerevisiae* in Freund's adjuvant[20]). In order to determine if similar

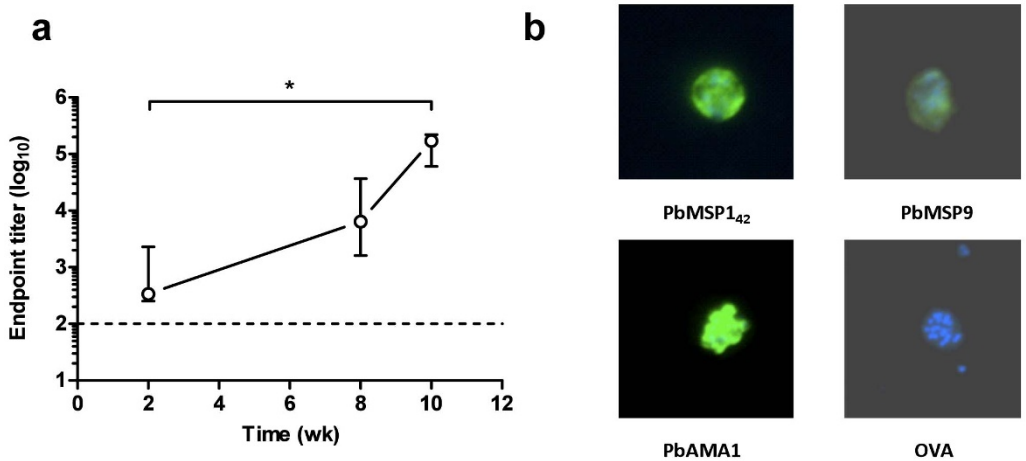

Figure 2 | Immunogenicity of AdHu5-MVA viral vector vaccines. (a) BALB/c mice ($n = 5$) were immunized i.d. with 1×10^{10} vp AdHu5 and boosted eight weeks later i.d. with 1×10^{7} pfu MVA expressing PbMSP1$_{42}$. Total IgG responses against recombinant GST-PbMSP1$_{19}$ protein were measured by ELISA in the serum of mice taken at weeks 2, 8 and 10. Median and range of the titers are shown. The dashed line indicates the lower limit of detection. * $P < 0.05$ by Friedman test with Dunn's correction for comparison of all time-points. (b) *P. berghei* ANKA schizonts were fixed with 4% formaldehyde and permeabilized with 0.1% NP40. Sera from mice immunized with 1×10^{10} vp AdHu5 and boosted with 1×10^{7} pfu MVA expressing the indicated antigens were used at a dilution of 1 : 100. Bound antibodies were detected using goat anti-mouse IgG conjugated to Alexa Fluor 488. Images show fluorescence (green) and DAPI staining (blue).

protection could be seen in the *P. berghei* model, mice were immunized as above with either the GST-PbMSP1$_{19}$ protein vaccine or a previously described PyMSP1$_{19}$ vaccine, termed PyMSP1$_{19}$-IMX108 [41]. Subsequently, in a head-to-head experiment, the mice were challenged with homologous strain blood-stage parasites (*P. berghei* ANKA or *P. yoelii* YM respectively). However, only PyMSP1$_{19}$ immunized mice were protected against a corresponding blood-stage *Plasmodium* infection. Sterile protection against *P. yoelii* was seen in all immunized mice (Figure 3a) whilst PbMSP1$_{19}$ immunized mice showed no protection against *P. berghei* either at the onset of patent infection (Figure 3b), or throughout the course of the infection (Figure 3c,d). As before (Figure 1a), the PbMSP1$_{19}$ immunized mice achieved high antibody titres to their respective MSP1$_{19}$ protein, and the PyMSP1$_{19}$ immunized mice also developed high endpoint titers prior to challenge [ELISA assay against GST-PyMSP1$_{19}$ protein showed a median endpoint of 1.3 $\times$ 10^6 with a range of 0.42 $\times$ 10^6–1.9 $\times$ 10^6]. Thus despite induction of IgG antibodies against both MSP1$_{19}$ antigens by the protein–in-adjuvant vaccines, protective efficacy was only achieved against *P. yoelii*.

Viral vectored vaccines are capable of inducing antibodies against encoded transgenes, but induce qualitatively different types of responses as well as cellular immunity[18,39]. We have previously reported that significant protection against *P. yoelii* pRBC challenge can be achieved using a schedule of just two immunizations in an AdHu5-MVA PyMSP1$_{42}$ heterologous prime-boost regime, without a need for additional adjuvant[18]. Protection following pRBC challenge is dependent on the induction of high-titer anti-PyMSP1$_{19}$ antibody responses[18,20]. In order to determine if similar protection could be seen with *P. berghei* MSP1$_{42}$, BALB/c mice were immunized with AdHu5-MVA vaccines as described earlier. As expected, both AdHu5-MVA PbMSP1$_{42}$ and AdHu5-MVA PyMSP1$_{42}$ immunized mice achieved high antibody titres to their respective MSP1$_{19}$ proteins (data not shown), however only AdHu5-MVA PyMSP1$_{42}$ immunized mice were protected against a homologous blood-stage *Plasmodium* infection. Protection against *P. yoelii* was seen in AdHu5-MVA PyMSP1$_{42}$ immunized mice (Figure 4a), with delayed onset parasitemia and 83% survival – comparable to previously published data for the same regime using slightly higher viral vaccine doses[18]. In contrast, AdHu5-MVA PbMSP1$_{42}$ immunized mice showed no protection against *P. berghei* ANKA infection (Figure 4b,c), identical to the results obtained with protein-in-adjuvant immunized animals (Figure 3c,d).

Protective efficacy of AMA1 and MSP9 vaccines against blood-stage *P. berghei* parasites. Despite the lack of efficacy with vaccines encoding the C-terminal regions of PbMSP1, there have been reports that immunization against PbAMA1 and PbMSP9 can elicit

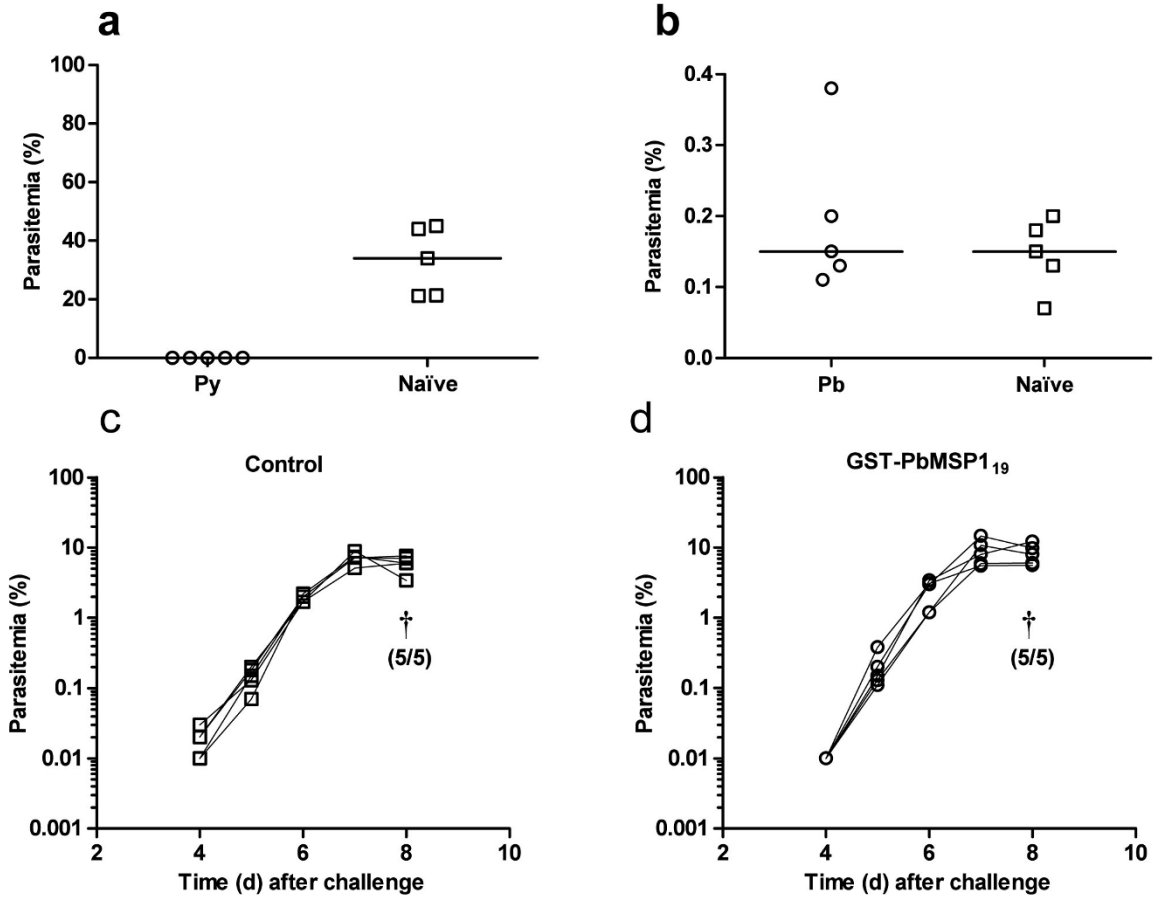

Figure 3 | Efficacy of *P. yoelii* and *P. berghei* MSP1$_{19}$ protein immunization. BALB/c mice (n = 5/group) were immunized with five doses of 20 μg GST-PbMSP1$_{19}$ protein (Pb) or a recombinant PyMSP1$_{19}$ protein fused to IMX108 (Py) as described in Figure 1. Ten days following the final immunization Pb vaccinated mice and naïve non-immunized controls were challenged i.v. with 5 $\times$ 10^2 *P. berghei* ANKA pRBC. Similarly Py vaccinated mice and naïve non-immunized controls were challenged i.v. with 1 $\times$ 10^4 *P. yoelii* YM pRBC, followed by daily monitoring for parasitemia. (a) Py vaccinated mice were sterilely protected (monitored out to day 21). Day 4 parasitemias for individual control and vaccinated mice with medians are shown. (b) Day 5 parasitemias for individual Pb vaccinated mice and controls are shown with medians, as well as time courses of parasitemia for individual (c) control and (d) Pb vaccinated mice. † indicates that mice were culled, and the number culled from the total number in the group is shown in brackets.

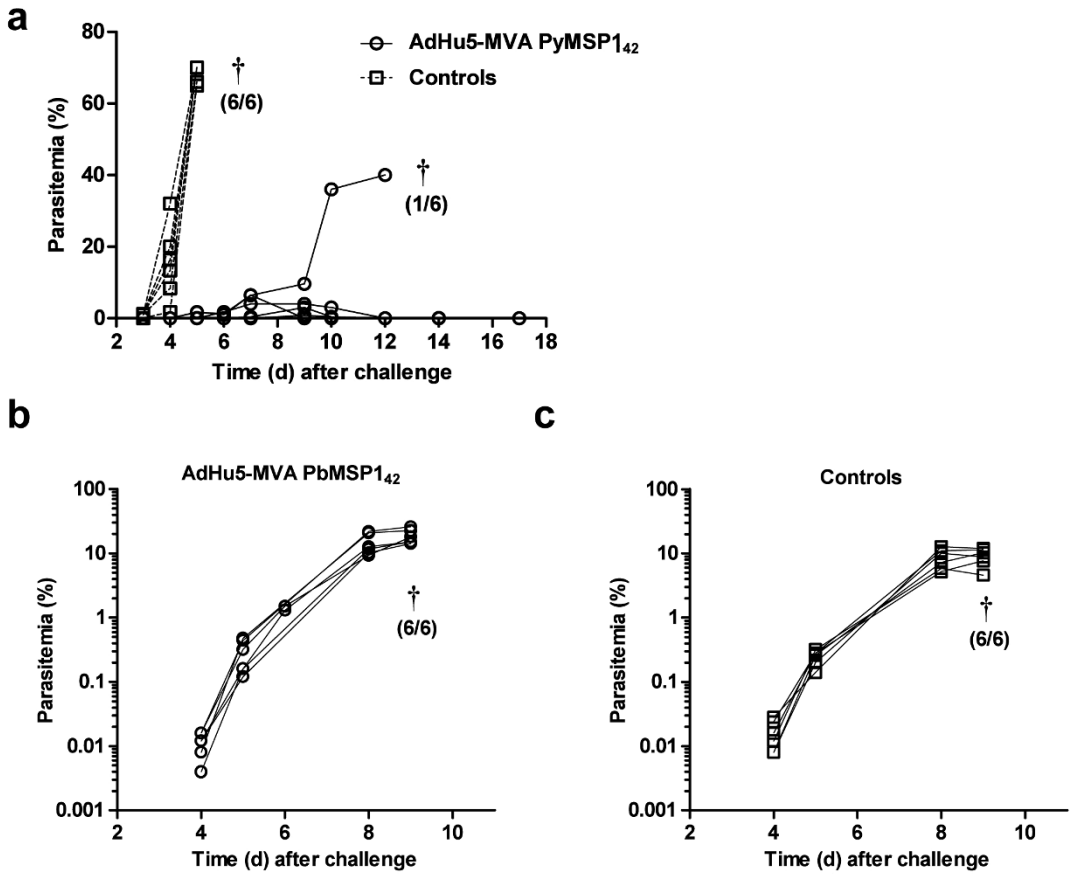

Figure 4 | Efficacy of AdHu5-MVA PbMSP1$_{42}$ and PyMSP1$_{42}$ immunization. BALB/c mice (n = 6/group) were immunized i.d. with 1×10^{10} vp AdHu5 and boosted eight weeks later i.d. with 1×10^{7} pfu MVA expressing (a) PyMSP1$_{42}$ or (b) PbMSP1$_{42}$. Two weeks following the final immunization vaccinated mice and non-immunized controls were challenged with (a) 1×10^{4} *P. yoelii* YM or (b,c) 5×10^{2} *P. berghei* ANKA pRBC and monitored for parasitemia. Lines represent individual mice. † indicates that mice were culled and the number culled from the total number in the group is shown in brackets.

protection against *P. berghei* at the blood-stage[30,33]. We therefore assessed the efficacy of the viral vector vaccines targeting PbAMA1 and PbMSP9 in BALB/c mice. Mice were immunized with AdHu5 expressing PbAMA1, PbMSP9 or no malaria antigen and then boosted with MVA expressing the same antigen. Two weeks later mice were challenged with 5×10^{2} *P. berghei* ANKA pRBC and monitored daily. Similar to PbMSP1$_{42}$, no significant efficacy was observed with either of these vaccines compared to control immunized mice (Figure 5). By day 7 post-challenge, 8/8 PbAMA1 immunized mice were culled (Figure 5a), compared to 3/8 PbMSP9 (Figure 5b) immunized and 1/8 control (Figure 5c) immunized mice. This was not due to a difference in parasitemia between groups, but rather reflected symptomatic illness which was pre-defined as the humane endpoint. All remaining mice reached this point by day 13 post-infection. Overall, these data show an absence of efficacy of both the PbMSP9 and PbAMA1 antigens, delivered by AdHu5-MVA, against blood-stage *P. berghei* ANKA infection.

Published studies demonstrating protection at the blood-stage against *P. berghei* were all noted to have employed an NK65 strain challenge[30–32]. Biological differences between *P. berghei* strains have been observed, such as differences in virulence, sensitivity to drugs, and preference for invasion of normocytes or reticulocytes[42,43]. The AdHu5-MVA vaccines encoding PbMSP1$_{42}$, PbAMA1 and PbAMA1, or a co-administered mixture of all three, were therefore additionally tested against challenge with *P. berghei* NK65 pRBC. Also, given the PbMSP9 immunogen was originally based on the NK65 strain sequence, this experiment allowed for a homologous parasite challenge, in comparison to heterologous challenge as in the previous experiment. However, as seen with *P. berghei* ANKA, no protective efficacy was observed in any of the vaccinated mice (Supplemental Figure 1).

Protective efficacy of MSP1 and AMA1 vaccines against *P. berghei* sporozoite challenge. In previous studies with the AdHu5-MVA vectors encoding PyMSP1$_{42}$, we reported improved efficacy and survival outcome against *P. yoelii* strain YM sporozoite challenge in comparison to pRBC challenge. In these studies, vaccinated mice showed complete survival against a challenge dose of both 50 and 250 sporozoites[24].

In the case of PbMSP1$_{42}$, BALB/c mice, immunized as before, were challenged with 500 sporozoites 14 days following the MVA boost. However, similar to the pRBC challenge studies, no efficacy was observed in vaccinees as compared to non-immunized controls (Figure 6a). To confirm that this lack of efficacy was not specific to the BALB/c strain, we also tested this regime in C3H and C57BL/6 mice. Despite achieving higher anti-PbMSP1$_{19}$ antibody titers in C3H mice (Figure 6b) we still observed no efficacy following sporozoite challenge (Figure 6a).

In addition to its well-known blood-stage role, AMA1 is also known to be expressed on sporozoites[44], and DNA and viral vectored vaccines encoding *P. falciparum* AMA1 have shown some potential pre-erythrocytic efficacy in humans against controlled infection delivered by mosquito bites[8,45]. We therefore additionally tested AdHu5-MVA PbAMA1 vaccines against sporozoite challenge in C57BL/6

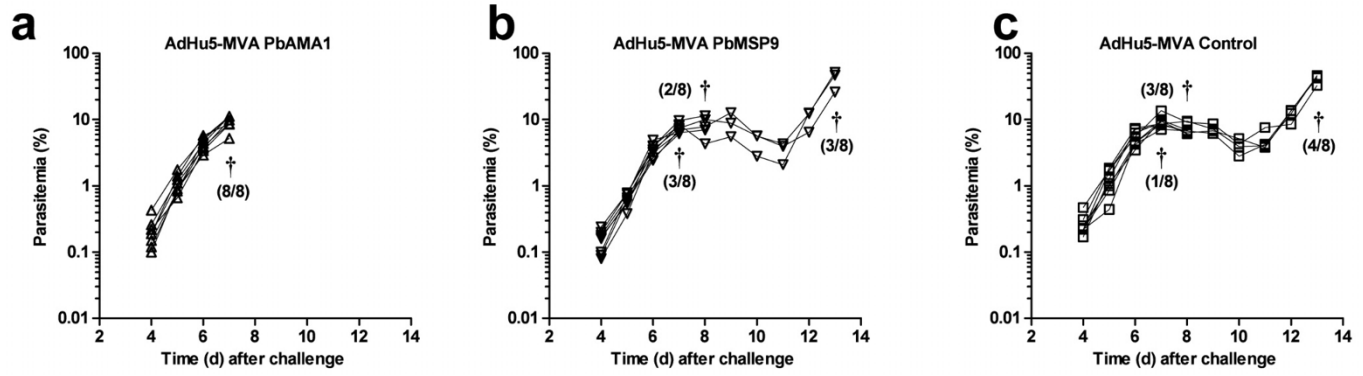

Figure 5 | Efficacy of AdHu5-MVA PbAMA1 and PbMSP9 immunization against *P. berghei* ANKA pRBC infection. BALB/c mice ($n = 8$/group) were immunized i.d. with 1×10^{10} vp AdHu5 and boosted thirteen weeks later with 1×10^{7} pfu MVA encoding either PbAMA1, PbMSP9 or no malaria antigen (Control). Two weeks post-boost mice were challenged with 5×10^{2} *P. berghei* ANKA pRBC and monitored for parasitemia. Lines represent individual mice. † indicates that mice were culled and the number culled from the total number in the group is shown in brackets.

(Figure 6c) as well as outbred CD1 mice (Figure 6d). Following challenge with 2000 sporozoites, there was no significant protection observed in either of the mouse strains, although there was a single PbAMA1 vaccinated animal on the outbred CD1 background that did not become parasitemic, indicating that on rare occasions PbAMA1 may be able to impart some protective efficacy in this model. However, given the extremely low level of efficacy afforded, the potential mechanism(s) of protection were not further explored.

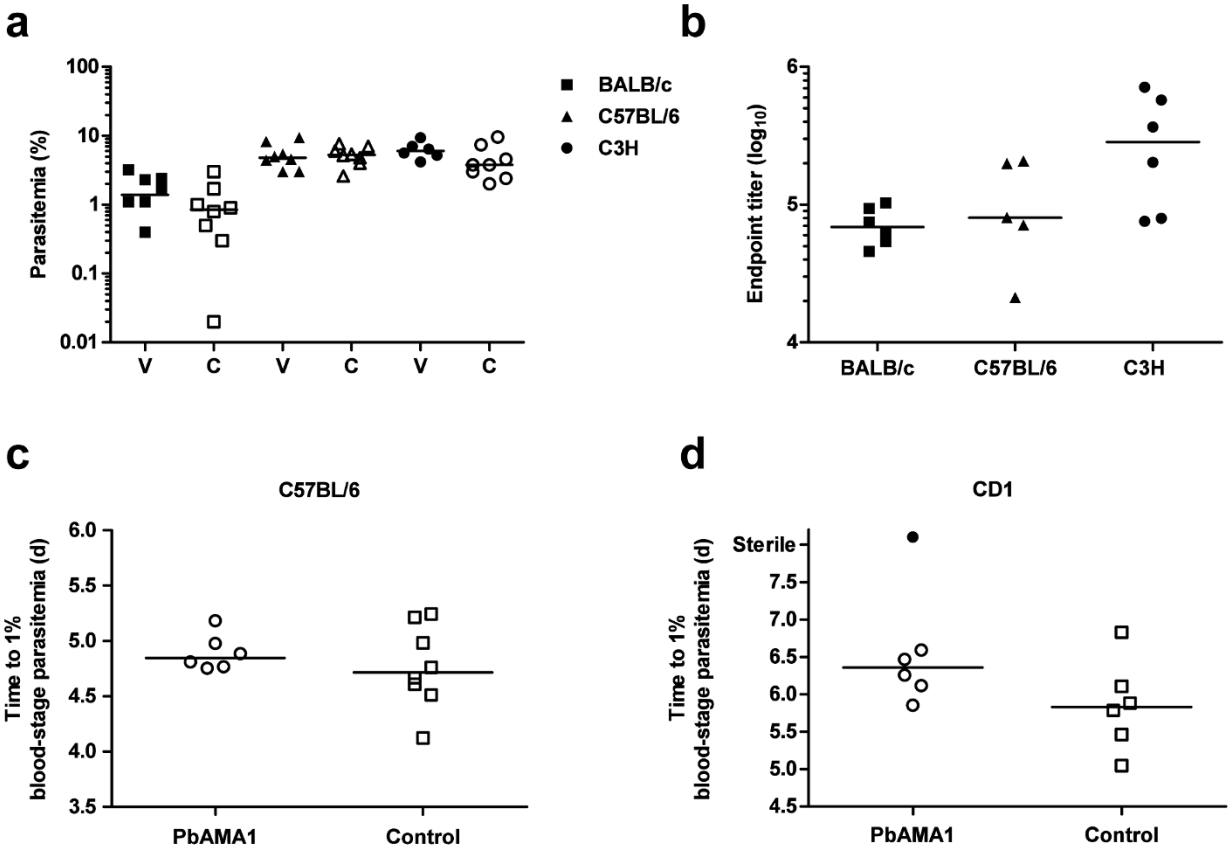

Figure 6 | Efficacy of AdHu5-MVA PbMSP1$_{42}$ and PbAMA1 immunization against *P. berghei* ANKA sporozoite infection. (a) Mice ($n = 6$-8/group) were immunized i.d. with 1×10^{10} vp AdHu5 and boosted eight weeks later i.d. with 1×10^{7} pfu MVA expressing PbMSP1$_{42}$. Two weeks following the final immunization vaccinated mice and naïve non-immunized controls were challenged with 5×10^{2} *P. berghei* ANKA sporozoites. Each mouse was monitored for the development of blood-stage infection. Parasitemia for individual vaccinated (V) and non-immunized control (C) mice and medians are shown at day 7 following challenge of three different inbred mouse strains (BALB/c, C57BL/6 and C3H). (b) Total IgG responses against recombinant GST-PbMSP-1$_{19}$ protein were measured by ELISA in the serum of mice taken pre-challenge at week 2 post-boost immunization. Individual titers and medians are shown. (c) C57BL/6 and (d) CD1 mice ($n = 6$–8/group) were immunized i.m. with 8×10^{9} vp AdHu5 and boosted eight weeks later i.m. with 1×10^{7} pfu MVA expressing PbAMA1. Two weeks following the final immunization vaccinated mice and non-malaria antigen vector-immunized controls were challenged with 2000 *P. berghei* ANKA sporozoites. Median and individual time to 1% parasitemia is shown. One CD1 mouse showed sterile protection and did not develop patent blood-stage infection.

Protective efficacy of *P. falciparum* MSP1 vaccines against a chimeric *P. berghei* parasite. Novel chimeric parasite lines have also been developed to enable functional assessments of immune responses to *P. falciparum* antigens in small mammals[16]. The chimeric *P. berghei* parasite model Pb-PfM19 was developed to enable the testing of vaccines targeting the *P. falciparum* $MSP1_{19}$ antigen in mice[34]. Antibody-mediated protection against Pb-PfM19 in mice has been demonstrated following passive transfer of monoclonal or polyclonal antibody into mice or following infection with Pb-PfM19 and drug-cure[34,46,47]. We have previously reported the preclinical development of human and simian adenoviral as well as MVA vectored vaccines encoding PfMSP1-based transgenes termed PfM115 and PfM128[48]. These vectors induced anti-$PfMSP1_{19}$ IgG responses in mice and rabbits that showed functional growth inhibitory activity (GIA) against *P. falciparum* parasites *in vitro*[18,40,48,49]. A chimpanzee adenovirus 63 (ChAd63) and MVA encoding PfM128 have also been shown to immunogenic for $PfMSP1_{19}$ antibody induction in Phase I/IIa clinical trials[8,50]. In a parallel series of experiments to those reported above using wild-type *P. berghei* parasites, we also tested the protective efficacy of PfMSP1-based vaccines following active immunization of mice. In agreement with data using the PbMSP1-based vaccines, no protective efficacy or effect of the vaccine on blood-stage parasite growth was conferred following AdHu5-MVA immunization with the PfM115 immunogen, following challenge with 10^4 pRBCs of the chimeric parasite (Table 1). We observed the same results in both BALB/c and C57BL/6 mice when the experiment was repeated using a lower challenge dose of 500 pRBCs as employed in another published study[47]. We also tested a recombinant $PfMSP1_{19}$-based protein vaccine, previously reported[41], administered in Freund's adjuvant and similarly observed no efficacy in BALB/c mice. Overall, these data confirmed that active immunization of inbred mouse strains with subunit vaccines encoding the $PfMSP1_{19}$ antigen failed to elicit efficacy against a pRBC challenge with the chimeric Pb-PfM19 parasite.

Protection against the chimeric parasite Pb-PfM19 by active subunit PfMSP1 vaccine immunization was not possible in our hands and has not been reported elsewhere. However, sterile protection of BALB/c mice following i.v. transfer of 900 μg purified total IgG from $PfMSP1_{42}$-immunized rabbits has been reported in one study[47], and also in a second study using 1.5 mg purified total IgG from $PfMSP1_{19}$-immunized rabbits and an alternative but highly similar chimeric parasite[35]. We thus also investigated here whether passive transfer could protect against challenge with the Pb-PfM19 parasite. Total IgG was purified from the serum of New Zealand white rabbits that had previously been immunized with an AdHu5-MVA or ChAd63-MVA regime and vectors encoding the PfM128 antigen. These rabbits had raised antibody responses against the 3D7/MAD20/ETSR allele of $PfMSP1_{19}$ that were comparable to those reported in our previous studies (Supplemental Figure 2A) and homologous to the $PfMSP1_{19}$ strain D10 sequence in the chimeric parasite[34]. Rabbits were primed i.m. with 5×10^{10} vp ChAd63 or AdHu5 expressing PfM128 on day 0 and boosted i.m. with 1×10^8 pfu MVA-PfM128 on day 56. Serum was harvested two weeks later on day 70. Day 0 pre-immune IgG from the same rabbits and from a control viral vector immunized rabbit were also purified. 0.5mg IgG was transferred i.v. to BALB/c mice on days −1, 0 and +1, and on day 0 mice were challenged with 5×10^2 PbPfM19 pRBC by i.v. injection. However, no significant differences in parasitaemia were observed between any groups following challenge (Supplemental Figure 2B).

The role of FcγRIIb (CD32b) in antibody-mediated protection against *P. berghei*. The data so far have demonstrated an inability of vaccines targeting three different merozoite antigens to protect against blood-stage *P. berghei* infection. Data from natural infection studies have suggested antibody Fc-mediated mechanisms are important in protection against blood-stage *P. berghei*. This is supported by the findings that mice lacking the common γ signalling chain, required for activatory signalling through Fc-gamma receptors (FcγR), have increased susceptibility to blood-stage *P. berghei* XAT infection[51]. Additional support is observed as human antibodies against $PfMSP1_{19}$ were able to protect against the chimeric Pb-PfM19 parasite only in mice transgenic for human FcγRI (CD64)[46]. The inhibitory effect of IgG1 binding to the inhibitory Fc receptor (CD32b/FcγRIIb) may in fact act to prevent activatory signals induced by IgG2a through other FcRs[52,53]. Given viral vectored vaccines are known to induce both IgG1 and IgG2a[18,48,54], FcγRIIb knockout mice ($CD32b^{-/-}$) were therefore used to investigate whether removing the inhibitory signals induced through FcγRIIb allowed protection against blood-stage *P. berghei* infection to be observed following subunit vaccination.

$CD32b^{-/-}$ mice on a C57BL/6 background were immunized with AdHu5-MVA vectors expressing either $PbMSP1_{42}$ or no antigen (vector control). As before, anti-$PbMSP1_{19}$ IgG responses were induced by immunization (Figure 7a). Two weeks post-boost, mice were challenged with *P. berghei* ANKA pRBC and monitored for parasitemia. No significant differences in parasitemia were observed in the $PbMSP1_{42}$ immunized mice (Figure 7b) in comparison to control immunized (Figure 7c) or naïve non-immunized mice (Figure 7d). The lack of the FcγRIIb also did not appear to influence the kinetics of the infection in naïve mice, with no significant differences observed in parasitemia between naïve non-immunized $CD32b^{-/-}$ (Figure 7d) and naïve non-immunized wild-type C57BL/6 mice (Figure 7e). This result suggests that the lack of

Table 1 | Chimeric parasite challenge experiments. Protein or viral vectored vaccines were administered as shown. Regime lists dose, route and day (d) of immunizations. Survival outcome is reported for the Pb-PfM19 chimeric parasite, and wild-type Pb-PbM19 control. n.d. = not done. N/A = not applicable

Vaccine	Regime	Challenge	Antigen	Mouse strain	Pb-PfM19 survival	Pb-PbM19 survival
$PfMSP1_{19}$-IMX108	3×40 μg s.c. d0, d14, d28 CFA/IFA/IFA	d42 10^4 pRBC i.v.	$PfMSP1_{19}$	BALB/c	0/6	n.d.
AdHu5-MVA	5×10^{10} vp i.d. d0 5×10^7 pfu i.d. d56	d70 10^4 pRBC i.v.	PfM115	BALB/c	0/6	0/6
Naïve	N/A	10^4 pRBC i.v.	N/A	BALB/c	0/3 + 0/6	0/6
AdHu5-MVA	5×10^{10} vp i.d. d0 5×10^7 pfu i.d. d56	d70 5×10^2 pRBC i.v.	PfM115	BALB/c	0/6	n.d.
AdHu5-MVA	5×10^{10} vp i.d. d0 5×10^7 pfu i.d. d56	d70 5×10^2 pRBC i.v.	PfM115	C57BL/6	0/6	n.d.
Naïve	N/A	5×10^2 pRBC i.v.	N/A	BALB/c	0/6	n.d.
Naïve	N/A	5×10^2 pRBC i.v.	N/A	C57BL/6	0/6	n.d.

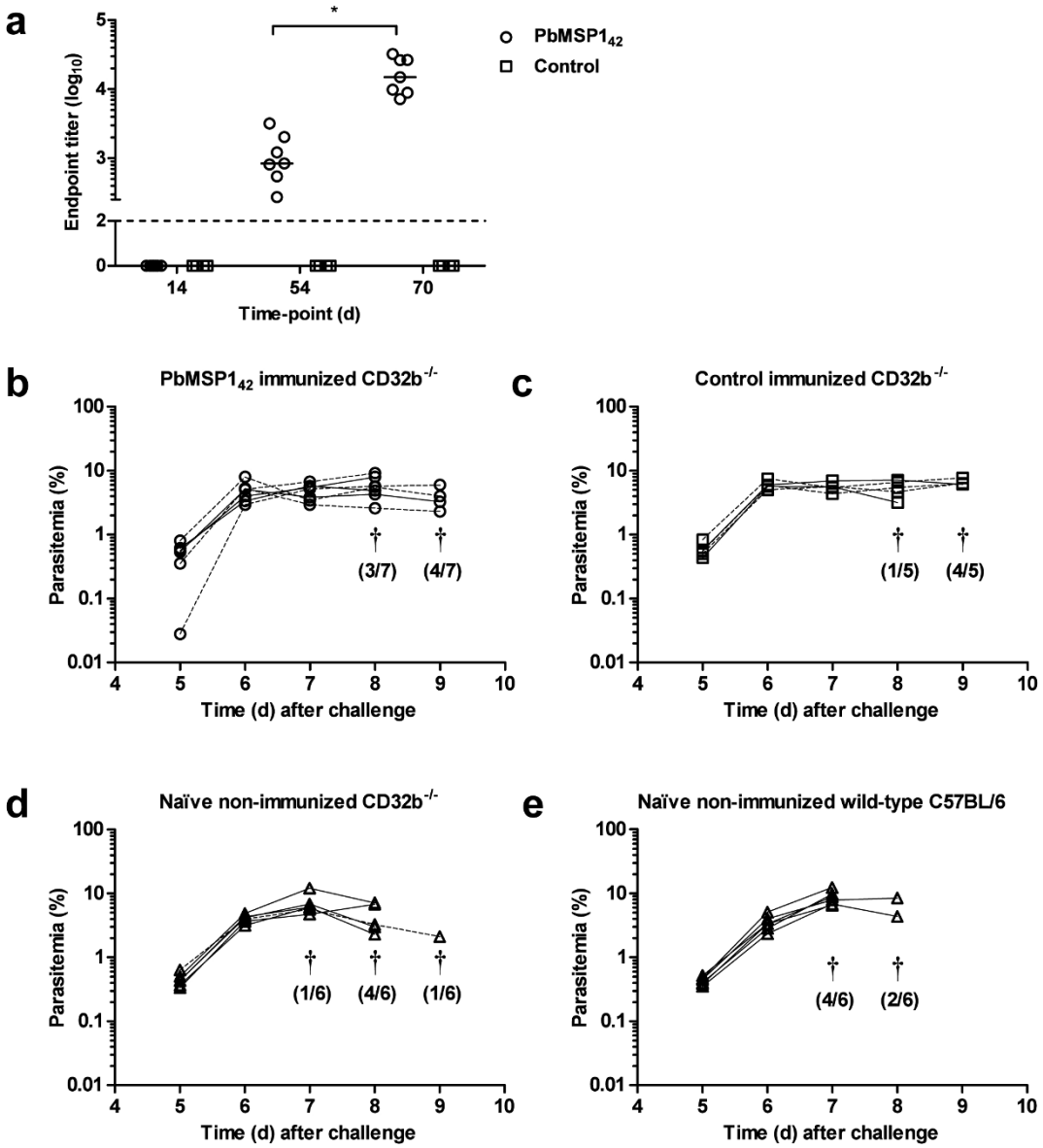

Figure 7 | Efficacy of AdHu5-MVA PbMSP1$_{42}$ in CD32b$^{-/-}$ mice. CD32b$^{-/-}$ mice were immunized i.d. with 1×10^{10} vp AdHu5 and boosted eight weeks later i.d. with 1×10^{7} pfu MVA encoding either no antigen (Control) or PbMSP1$_{42}$. (a) On days 14, 54 (pre-boost) and 70 (2 weeks post-boost) sera were collected and PbMSP1$_{19}$-specific total IgG measured by ELISA. Dots represent individual mice and bars show median endpoint titre. * $P = 0.02$ between d54 and d70 by Wilcoxon matched pairs signed rank test. The dashed line indicates the lower limit of detection. Two weeks post-boost (b) PbMSP1$_{42}$ immunized CD32b$^{-/-}$ mice and (c) control immunized mice were challenged with 5×10^{2} *P. berghei* ANKA pRBC and monitored for parasitemia. Groups of (d) non-immunized (naïve) CD32b$^{-/-}$ and (e) wild-type C57BL/6 mice were also challenged. Lines represent individual mice of 5–7 mice/group (females solid lines and males dashed lines). † indicates that mice were culled. The number culled that day from the total number of mice in the group is shown in brackets.

vaccine efficacy in wild-type mice was not due to inhibitory signalling through FcγRIIb preventing activatory IgG2a-mediated signals.

The role of sequestration in antibody-mediated protection against *P. berghei* merozoites. We also tested a final hypothesis as to whether sequestration of *P. berghei* parasites was affecting the efficacy of the anti-merozoite vaccines. We considered that sequestration of infected erythrocytes might reduce the period of exposure of merozoites to antibody. Studies have shown sequestration of *P. berghei* pRBC in bone marrow, thereby bringing parasites in close proximity to immature RBCs in particular[55]. Such attributes of the parasite could potentially favour rapid RBC invasion by newly released merozoites, leaving minimal time in which antibodies could interfere with this process. To test this, we made use of a recently described *P. berghei* parasite line that cannot sequester, due to ablation of CD36 binding as a result of deletion of the *smac* gene[56]. Studies with this parasite have indeed suggested that pRBC sequestration may confer advantages to parasite growth in addition to avoidance of splenic removal[56]. BALB/c mice were immunized with the AdHu5-MVA vaccines encoding PbMSP1$_{42}$, PbAMA1 or PbMSP9, or a co-administered mixture of all three, and subsequently challenged with Δ*smac* pRBCs. However, similar to all the other studies, no vaccine efficacy was apparent in this case. Parasitemias were comparable between the vaccinees and controls (Supplemental Figure 3), and all mice were culled on day 10 post challenge.

Discussion

Here we report on the ability of subunit vaccines targeting the MSP1, AMA1 and MSP9 antigens to protect against blood-stage *P. berghei*

parasites. Active immunization with subunit vaccines targeting these merozoite antigens has previously been reported to protect against blood-stage *P. berghei* infection in only four studies[30–33]. Following active immunization of BALB/c mice here with AdHu5-MVA vectors expressing these three *P. berghei* merozoite antigens, antibodies capable of binding to native parasite antigen were observed, indicating that all three antigen-delivery regimes were immunogenic. In addition, vector immunization against $PbMSP1_{42}$ induced $PbMSP1_{19}$-specific IgG that reached high endpoint titers in an ELISA assay. However, despite induction of these responses, no protection was afforded against *P. berghei* ANKA pRBC challenge. We observed similar results in a variety of inbred mouse strains, including C3H mice that developed the highest antibody titers. If protection is antibody-mediated in this mouse model, we cannot exclude the possibility that titers induced by viral vector immunization were too low to protect, or that the fine specificity of the antibody response was qualitatively non-protective. Also, it is possible the PbMSP1 vaccines targeted an incorrect region of the molecule; given responses against the PbMSP1 N-terminal region may be important (as reported for a recombinant *Salmonella*-based vaccine[31]). However, this is not the case in the *P. yoelii* and *P. chabaudi* models and is therefore unlikely. Delivery of the orthologous $PyMSP1_{42}$ or PcAMA1 antigens respectively by AdHu5-MVA is protective against blood-stage challenge in mice (shown here and in previous reports[18,19]). Similarly immunization with $MSP1_{19}$ protein in Freund's adjuvant (a regime capable of inducing extremely high titer responses) was shown here to afford sterilizing immunity against *P. yoelii* (in agreement with an earlier report of the same regime[20]), but no efficacy against either wild-type *P. berghei* or the chimeric Pb-PfM19 parasite.

Initial pRBC challenge experiments were performed using *P. berghei* ANKA. However in the published examples of protection against wild-type blood-stage *P. berghei* by subunit vaccination, the challenge strain was *P. berghei* NK65 rather than ANKA[30–32]. *P. berghei* NK65 infection results in a greater number of 'latent merozoites' than ANKA, meaning merozoites are in the blood for longer (and thus potentially more accessible to antibody) before invading a new RBC[57]. NK65 is also known to i) have a greater predilection to invade immature RBC (reticulocytes); ii) be more likely to multiply infect the same cell; and iii) produce more ring forms early in infection[43], leading to a less synchronous infection than the ANKA strain. Challenge experiments were therefore repeated using *P. berghei* NK65 but, despite these differences, no efficacy was seen. It cannot be excluded that the failure to protect against *P. berghei* NK65 may be due to differences in homology between the vaccine antigens and NK65 challenge strain (in the case of $PbMSP1_{42}$ and PbAMA1). However, assessment of sequence data from NK65 and ANKA *P. berghei* parasite lines found no differences in PbAMA1 and only one SNP in PbMSP1 (CJJ and BMF-F, unpublished observations), and in the case of PbMSP9 (based on the NK65 sequence) antibodies raised by AdHu5-MVA vaccination were capable of recognizing *P. berghei* ANKA schizonts in the IFA. Moreover, no protection was also observed against sporozoite challenge (bar in the case of one animal immunized with PbAMA1), despite the possibility of viral vectored immunization leading to the development of $CD8^+$ T cells capable of targeting *P. berghei* liver-stage forms. Such liver-stage protection has been reported using the same vectors in the $PyMSP1_{42}$ model[24]. Overall these data are in agreement with more recently published studies showing a lack of anti-$PbMSP1_{42}$ and -PbAMA1 vaccine efficacy in mice[36,37], and add to the growing body of evidence that *P. berghei* may possess mechanisms that can circumvent such responses.

Studies with a non-lethal/attenuated strain *P. berghei* XAT (derived from *P. berghei* NK65[58]) have shown that protection requires IFN-γ and IgG2a[59]. Similarly, FcR were shown to be essential in mediating blood-stage protection against Pb XAT following infection of FcR common γ-chain knockout mice[51]. Protection was also reported against the Pb-PfM19 chimeric parasite using a human IgG1 mAb against $PfMSP1_{19}$, but only in transgenic mice expressing the human FcγRI (CD64)[46], but not for an isotype switched human IgA mAb against the same epitope in human FcαRI (CD89) transgenic mice[60]. In agreement with this, a similar experiment with the 'non-cytophilic' mouse IgG1 isotype failed to show protection in wild-type mice (although 'cytophilic' IgG2a was not tested)[61]. We therefore tested here whether vaccine-induced protection against the merozoite antigens could be improved in mice lacking the inhibitory FcγRIIb (CD32b). This knockout would be more likely to enhance the action of mouse IgG1 (due to the greater inhibition of this isotype and low activatory-to-inhibitory (A:I) ratio[62]) but could still also enhance the action of vaccine-induced IgG2a and IgG2b – known to be induced by AdHu5-MVA vaccination[18,48,54]. Immunization of $CD32b^{-/-}$ mice might also be expected to enhance immune responses to the vaccine due to removal of the regulatory/inhibitory FcγR signalling at the time of immunization. This has been reported in studies for antibodies[63,64] as well as tumor-specific $CD8^+$ T cells[65]. Wild-type and $CD32b^{-/-}$ mice were not directly compared in this study, and in future it will be important to establish the impact of CD32b absence on vaccine immunogenicity. However, IgG responses were clearly primed and boosted against $PbMSP1_{19}$ in the knockout mice but, despite the absence of possible inhibitory FcγRIIb signalling, no vaccine efficacy was observed. No difference was also observed between naïve wild-type and knockout mice, again in contrast to the *P. chabaudi* infection model that observed reduced parasitemia in $CD32b^{-/-}$ mice[66].

In contrast to subunit vaccines against the merozoite, whole parasite immunization protocols of mice can afford significant protection against subsequent parasite challenge. Such protocols may involve drug-cure, immunization with killed parasites in adjuvant or exposure to attenuated/non-lethal infection. Interpretation of such studies is complicated by the significant variability observed with each different protocol and each unique inbred mouse and parasite strain combination[67]. Nonetheless, following such 'immunization' protocols mice are typically protected against homologous challenge, whilst outcome of heterologous challenge varies significantly. However, early studies clearly document reduced levels of efficacy afforded by exposure to replicating *P. berghei* parasites as compared to the other rodent malaria species[68]. In 1977 Playfair *et al.* also documented differences in the ease with which protection could be achieved between rodent malaria species following administration of a killed blood-stage vaccine. Whilst lysed and fixed *P. yoelii* pRBC induced homologous protection that was at least partly antibody-mediated, similar experiments with *P. berghei* pRBC led to little or no protection[69]. Although formalin-killed blood-stage parasites are able to protect against *P. berghei* this protection is incomplete and mice develop parasitemia levels of ~10%. The mechanism of this protection appears to be at least partly antibody-mediated as delay to parasitemia occurred following the transfer of immune sera to non-immunised mice[70]. Protective immunity in mice against blood-stage challenge could also be induced by self-limiting infections with growth-attenuated *P. berghei* ANKA parasites mutants lacking plasmepsin 4[71]. Following challenge, the trophozoite/schizont-infected RBCs were trapped in the spleen resulting in rapid and efficient removal of parasites from the circulation. These observations indicate that the protective blood-stage immunity induced in this case was mediated against pRBC rather than merozoite invasion. Proposed reasons for the difficulty in protecting against *P. berghei* include sequestration of the parasite, immune suppression or evasion, and antigenic variation. *P. berghei* pRBC sequester in a similar way to *P. falciparum*, through adherence to the host receptor CD36 and such sequestration appears to be beneficial for parasite growth[56]. However, anti-merozoite vaccine efficacy was not improved here when tested against the Δ*smac* parasite line, suggesting this

sequestration phenotype is unlikely to be helping *P. berghei* parasites circumvent such immune responses. Another possible contributor to the difficulty in protecting against blood-stage *P. berghei* might be the suppression of the immune system during parasite infection. Blood-stage *P. berghei* can rapidly suppress MHC class I and class II presentation of both malarial and other antigens by dendritic cells within 4 days of infection[72]. Such a phenomenon may prevent the essential development of *de novo* protective immune responses or the boosting of vaccine-induced antibodies. However, the vaccines tested here were ineffective against the first 4 days of parasite growth – a period in which pre-existing vaccine-induced responses are probably critical, and which probably precedes the development of either parasite-induced immunosuppression or substantial infection-induced adaptive immune responses.

Protection against a chimeric *P. berghei* line expressing *P. falciparum* $MSP1_{19}$ was also assessed here. Similar to other reported studies using baculoviral-based vaccines[36], or $PfMSP1_{19}$ protein in Freund's adjuvant[80], we again failed to protect against this parasite in two strains of mice following active subunit vaccine immunization. Passive transfer of IgG from $PfMSP1_{19}$-immunized rabbits or a human mAb has been demonstrated to protect against challenge with blood-stage Pb-PfM19 parasites[46,47]. However in our hands polyclonal purified IgG, from PfMSP1 immunized rabbits, did not protect against Pb-PfM19. It remains wholly possible that the antibody titers here were too low to protect, or that the antibodies were of incorrect fine specificity. Protection of mice (made semi-immune to this parasite line by drug-cure) from homologous pRBC challenge correlated with $PfMSP1_{19}$-specific inhibitory antibodies, but not with titers of total PfMSP-1_{19} IgG[34]. However, these PfMSP1 immunogens have been previously shown to be recognized by conformational inhibitory anti-$PfMSP1_{19}$ mAbs and to raise IgG in rabbits that show functional activity *in vitro*[48]. Intriguingly, in both reported results to date where passive immunization was successful[46,47], sterilizing immunity was observed. Partial efficacy or blood-stage control has not been reported. Successful outcome may therefore rely on an immediate clearance mechanism of the challenge inoculum rather than reduced rates of RBC invasion, though this requires further investigation. The Pb-PfM19 parasite used here also lacks the first four αα of $PfMSP1_{19}$ and instead expresses the first 4 αα of $PbMSP1_{19}$[34], and this may affect the ability of IgG to inhibit $MSP1_{42}$ processing. Although protection against this parasite by transfer of $PfMSP1_{42}$-specific IgG has been observed[47], the independently developed chimeric parasite developed by Cao *et al.*[35], possesses the full length $PfMSP1_{19}$ sequence and may be a more suitable parasite line for future experiments.

Overall, these data suggest a failure of vaccines against commonly studied merozoite antigens to protect against *P. berghei* blood-stage infection, in contrast to many similar studies using *P. yoelii* and *P. chabaudi*. Although qualitative differences in the immune responses induced by the *P. berghei* vaccines tested here may explain the failure to protect, this remains unlikely given the success of both viral vector and protein/adjuvant vaccine platforms targeting the same antigens from other rodent malaria species and in raising IgG that is functional against *P. falciparum in vitro*. A fraction of parasite-derived material containing unknown antigens was recently reported to be protective in this model[73], and thus vaccines targeting other blood-stage proteins may yet prove to be more effective. Despite supporting data obtained using the *P. yoelii* and *P. chabaudi* models, protein/adjuvant and viral vectored PfMSP1- and PfAMA1-based vaccines have faced significant disappointment in Phase IIa/b clinical trials to-date[6,8]. It may well be that the seemingly more stringent *P. berghei* mouse model will prove to be a better predictor of *P. falciparum* clinical vaccine efficacy. These observations have important implications for the development of chimeric parasite models used to test *P. falciparum* vaccine candidates, and the interpretation of future studies demonstrating protection in one or other mouse model[16]. Further studies remain warranted to establish whether or not *P. falciparum* does indeed share mechanisms with *P. berghei* that potentially enable this rodent malaria to circumvent immune responses induced by vaccines against merozoite antigens such as MSP1 and AMA1.

Methods

Viral vector vaccine generation. Recombinant human adenovirus serotype 5 (AdHu5) and modified vaccinia Ankara (MVA) viral vector vaccines expressing candidate antigens were designed according to previously published methods[18]. All vectors encoded the transgene of interest (listed below), with an N-terminal in-frame signal sequence from human tissue plasminogen activator (tPA). Adenoviral vaccines were grown in HEK293 cells, purified by CsCl centrifugation[74] and titered by UV spectrophotometry to give units of viral particles (vp/mL)[18], whilst MVA vaccines were grown in chicken embryo fibroblasts (CEFs), purified by centrifugation through a sucrose cushion[75] and titered by fluorescence plaque assay using the GFP marker to give plaque-forming units (pfu/mL)[18].

PbMSP9. PbMSP9 NK65 strain (GenBank AY302245) commenced at amino acid (αα) 23 (histidine) and was truncated at αα 392 (glutamic acid)[30]. In order to remove potential sites of N-linked glycosylation, the serine residues at αα sites 96, 155, 171, 301 and 338 were substituted with alanine. An additional serine residue at αα site 284 was substituted with alanine to remove an active serine protease site and avoid potential toxicity *in vitro* during virus propagation (ADD, SJD unpublished observations). The transgene was synthesized by GeneArt GmbH (Regensburg, Germany) and codon optimized for expression in mice.

PbAMA1. PbAMA1 ANKA strain (GenBank U45969) commenced at αα 21 (cysteine) and was truncated at αα 478 (glutamic acid). In order to remove potential sites of N-linked glycosylation, serine residues at αα sites 160, 233, 251, 290, 407 and 412 were substituted with alanine, and asparagine at αα site 189 was substituted with glutamine. The gene was synthesized as for PbMSP9.

$PbMSP1_{42}$. The 42 kDa C-terminus of *P. berghei* MSP1 was amplified from genomic DNA extracted from wild-type *P. berghei* ANKA strain (Pb-PbM19)[34] using the following oligonucleotide primers (see below for description of parasites and PCR method): forward primer 5′-TCC GAA AAT GCA CAA GAA AAA AAT A-3′; reverse primer 5′-TCC CAT AAA GCT GGA AGA GCT ACA GAA-3′. Primers were designed to amplify DNA from αα site 1416 (serine) and finish prior to the GPI anchor at αα site 1776 (glycine) (PlasmoDB: PBANKA_083100).

PfMSP1. Vaccines encoding the *P. falciparum* MSP1 inserts termed PfM115 and PfM128 have been described previously[48]. Briefly, the PfM115 insert encodes a 115 kDa composite *P. falciparum* MSP1 antigen. From N- to C-terminus it includes the conserved blocks of MSP1 sequence (blocks 1, 3, 5 and 12) from the 3D7 clone, followed by Wellcome strain block 16 ($MSP1_{33}$), linked by a glycine-proline linker to 3D7 clone blocks 16 and 17 ($MSP1_{42}$). PfM128 encodes a 128 kDa insert as described above for PfM115, except the Wellcome strain $MSP1_{33}$ sequence was replaced with the sequence encoding $MSP1_{42}$.

$PyMSP1_{42}$. AdHu5 and MVA vectors encoding $MSP1_{42}$ from *P. yoelii* YM have been previously described[18].

Ovalbumin and no malaria antigen controls. AdHu5 and MVA vectors encoding hen ovalbumin (OVA) have been previously described[39], as have AdHu5 lacking a transgene amd MVA encoding only GFP[38].

Protein vaccines. Generation of recombinant *P. yoelii* strain YM $MSP1_{19}$-glutathione S-transferase (GST) fusion protein has been described previously[18]. Recombinant $PyMSP1_{19}$ and $PfMSP1_{19}$ fused to IMX108 (mouse complement C4 binding protein, C4bp)[41] were kindly provided by Dr F. Hill (Imaxio, France). Recombinant $PbMSP1_{19}$ was produced as a GST fusion protein using previously published methods[18]. To generate the vector, $PbMSP1_{19}$ (αα methionine 1669 – glycine 1776) was amplified from the $PbMSP1_{42}$ sequence described above by PCR and cloned into the expression vector pGEX-2T (Amersham Biosciences, Bucks., UK).

Animals and immunization studies. All procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act Project Licence and were approved by the University of Oxford Animal Care and Ethical Review Committee (PPL 30/2414). Six to eight week old female BALB/c ($H\text{-}2^d$), C3H ($H\text{-}2^k$), C57BL/6 ($H\text{-}2^b$) and CD1 (outbred) mice were obtained from Harlan, UK, and housed in specific pathogen-free conditions. Unless otherwise stated, mice were immunized intramuscularly (i.m.) with 1×10^{10} vp AdHu5 vaccines and then boosted eight weeks later with 1×10^7 pfu MVA expressing the same antigen, as per a previously established immunization regime[18,24]. Protein vaccines were administered at doses of 20 μg at weeks 0 (subcutaneously, s.c.), 3 (s.c.), 6 (intraperitoneally, i.p.), 8 (i.p.) and 9 (i.p.). The adjuvant used for subcutaneous injection at week 0 was complete Freund's adjuvant (CFA) and at weeks 3, 6 and 8 was incomplete (IFA). At week 9 protein was administered in PBS. This regime was based on a published protocol[20] shown to induce very high-titer antibody responses. Sera were collected from tail vein bleeds as described in Results.

Fcγ receptor IIb (CD32b) deficient mice on a C57BL/6 background ($CD32b^{-/-}$)[63] were provided from the Queen's Medical Centre, Nottingham, UK and bred at the Wellcome Trust Centre for Human Genetics, Oxford, UK. Genotype was confirmed using DNA extracted from tissue samples and the Expand High Fidelity PCR Kit (Roche Diagnostics, UK) and previously described reaction conditions[76], except the primers were: 5′-CTC GTG CTT TAC GGT ATC GCC-3′ (Mutant); 5′-AAA CTC GAC CCC CCG TGG ATC-3′ (Common); and 5′-TTG ACT GTG GCC TTA AAC GTG TAG-3′ (Wild-type).

New Zealand white rabbits were used for all rabbit experiments. Vaccines were shipped from Oxford and the immunization of rabbits and collection of sera was performed by Agrobio, France. Rabbits were immunized i.m. on day 0 with 5×10^{10} vp AdHu5 or ChAd63 expressing PfM128 or no malaria insert (control) and then boosted i.m. on day 56 with 1×10^{8} pfu MVA expressing PfM128 or GFP (control). Serum was collected pre-immunization on day 0 as well as two weeks post-boost on day 70.

Rabbit IgG purification. Polyclonal rabbit IgG was purified from serum samples by Protein G affinity chromatography using a buffer system (Immunopure, Pierce) according to manufacturer's instructions. IgG was quantified using a spectrophotometer at 280 nm. The extinction coefficient for rabbit IgG is 1.44 (i.e. 1 mg/mL of rabbit IgG has an OD of 1.44 at 280 nm). The concentration of antibody was therefore calculated as follows: Concentration of rabbit IgG (mg/mL) = (OD × dilution factor)/1.44.

Immunogenicity assays. Mouse and rabbit total IgG ELISAs were performed according to previously published methods[48]. Briefly, protein was coated onto ELISA plates at a concentration of 2 μg/mL in PBS. Test sera were applied in duplicate wells and serially diluted. The endpoint titers were taken as the x-axis intercept of the dilution curve at an absorbance value 3 × standard deviations greater than the $OD_{405\ nm}$ for naïve mouse sera.

Immunofluorescence assays (IFA) were performed using methods based on those described previously[48]. Briefly, blood from *P. berghei* infected mice was cultured *in vitro* overnight to enable development of schizonts. Slides were prepared with a thin smear of this blood. Slides were fixed with 4% formaldehyde and 0.1% NP40 for 15 min at room temperature (RT). Mouse sera were diluted 1 : 100 in PBS and incubated on slides for 45 min. Slides were washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG for 30 min. Slides were washed in PBS, DAPI was applied, and the slides were viewed under a fluorescence microscope.

Parasites. *P. berghei* NK65 (MR4 Number: MRA-268) was obtained through the Malaria Research and Reference Reagent Resource Centre (MR4) and was deposited by Prof. V. Nussenzweig, New York University, USA. *P. berghei* strain ANKA clone 234, strain NK65 or *P. yoelii* strain YM parasitized red blood cell (pRBC) challenges were carried out as previously described[18,77]. Briefly, donor mice were infected by i.p. injection of cryopreserved infected blood stocks, before passage into experimental mice which were infected by intravenous (i.v.) injection with 500 (*P. berghei*) or 10,000 (*P. yoelii*) pRBCs unless otherwise stated. Parasitemia was monitored from day 3 post-challenge by Giemsa-stained thin blood smear and was calculated as a percentage of infected RBC. Mice were considered uninfected if no parasites were observed in 50 fields of view. For *P. berghei* strain ANKA 234 sporozoite challenge studies, infected *Anopheles stephensi* mosquitoes were prepared as previously described[78]. Salivary glands were collected by dissection and placed in a tissue homogenizer with RPMI 1640 (Sigma) to release the sporozoites, which were then counted using a haemocytometer. Unless otherwise stated, mice were challenged i.v. with 500 sporozoites and monitored for blood-stage infection from day 5 as for pRBC challenge. In some cases, a linear-regression model was generated to predict time until 1% parasitemia as previously described[79].

A chimeric parasite line known as Pb-PfM19 and a control parasite line, Pb-PbM19, were kindly provided by Dr B. S. Crabb and Dr T. F. de Koning-Ward. These chimeric parasite lines were generated using an allelic replacement approach, on a *P. berghei* ANKA background[34]. Pb-PfM19 expresses *P. falciparum* strain D10 $MSP1_{19}$ (MAD20/ETSR allele) in place of wild-type $PbMSP1_{19}$, and Pb-PbM19 was generated as a transfection control, replacing wild-type $PbMSP1_{19}$ with the identical wild-type sequence. The identity of the two parasite lines was confirmed before the studies by PCR. Infected blood at 5–10% parasitemia was harvested from BALB/c donor mice by cardiac exsanguination into 10 mM EDTA. Blood was made up to 5 mL with PBS, and white blood cells filtered out using a Plasmodipur filter (Euro-Diagnostica, The Netherlands). Erythrocytes were washed in PBS before extraction of parasite DNA using a QIAamp Blood Mini Kit (Qiagen). Parasite identity was confirmed by Expand High Fidelity PCR (Roche Diagnostics, UK) according to the manufacturer's instructions and using 4 mM $MgCl_2$ and an annealing temperature of 52°C. Species-specific primers were used for $MSP1_{19}$: $PfMSP1_{19}$ (5′-ATG CGT AAA AAA ACA ATG TCC AGA AAA T-3′; 5′-GTT AGA GGA ACT GCA GAA AAT ACC ATC G-3′) and $PbMSP1_{19}$ (5′-CTG CAA ATG CTG GAT GTT TTA GAT A-3′; 5′-CAT CAT AAT ATG CAT TAG GGG TTG G-3′). Amplified DNA fragments were of the expected fragment sizes ($PbMSP1_{19}$ = 228 bp, $PfMSP1_{19}$ = 271 bp) and identities for their respective parasites (data not shown).

P. berghei ANKA parasites lacking the schizont membrane-associated cytoadherence protein (SMAC) have previously been described (Δ*smac*; reference 56; mutant 1160cl7; RMgm ID: RMgm-661). Δ*smac* parasites exhibit reduced CD36-mediated sequestration and reduced growth rates in wild-type mice.

Statistics. Data were analyzed using GraphPad Prism v5.03. Continuous outcomes in two independent groups were compared using a Mann-Whitney U test, or using the Wilcoxon matched pairs signed rank test for paired data. A Kruskal-Wallis test with post-hoc Dunn's analysis was used to compare responses between more than two groups. A Friedman test was used for paired data when there were more than two time-point observations. In all cases $P \leq 0.05$ was considered significant (* $P \leq 0.05$ and ** $P \leq 0.01$).

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Author contributions

A.L.G., E.K.F., A.C.M., S.C.G., A.V.S.H., R.J.P. and S.J.D. conceived, planned and designed experiments, including design of viral vectors. C.J.J. and B.M.F. designed and provided the Δ*smac* parasites. A.G.G., E.K.F., A.R.W., A.D.D., S.C.D.C., K.B., S.B., M.D.J.D. and D.L. conducted the experiments. A.L.G., E.K.F. and S.J.D. analyzed the data. A.L.G., E.K.F. and S.J.D. wrote the paper. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: ALG, EKF, ARW, ADD, SCdC, MDJD, ACM, SCG, AVSH, RJP and SJD are named inventors on patent applications covering malaria vectored vaccines and/or immunization regimes. KB, SB, DL, CJJ and BMF have no competing financial interests.

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