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A conserved malaria parasite antigen Pb22 plays a critical role in male gametogenesis in *Plasmodium berghei*

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Abstract

Gametogenesis, the formation of gametes from gametocytes, an essential step for malaria parasite transmission, is targeted by transmission-blocking drugs and vaccines. We identified a conserved protein (PBANKA_0305900) in *Plasmodium berghei*, which encodes a protein of 22 kDa (thus named *Pb22*) and is expressed in both asexual stages and gametocytes. Its homologs are present in all *Plasmodium* species and its closely related *Hepaticystis*, but not in other apicomplexans. *Pb22* protein was localized in the cytosols of schizonts, as well as male and female gametocytes. During gamete-to-ookinete development, *Pb22* became localized on the plasma membranes of gametes and ookinetes. Compared to the wild-type (WT) parasites, *P. berghei* with *pb22* knockout (KO) showed a significant reduction in exflagellation (~89%) of male gametocytes and ookinete number (~97%) during *in vitro* ookinete culture. Mosquito feeding assays showed that ookinete and oocyst formation of the *pb22*-KO line in mosquito midguts was almost completely abolished. These defects were rescued in parasites where *pb22* was restored. Cross-fertilization experiments with parasite lines defective in either male or female gametes confirmed that the defects in the *pb22*-KO line were restricted to the male gametes, whereas female gametes in the *pb22*-KO line were fertile at the WT level. Detailed analysis of male gametogenesis showed that 30% of the male gametocytes in the *pb22*-KO line failed to assemble the axonemes, whereas ~48.9% of the male gametocytes formed flagella but failed to egress from the host erythrocyte. To explore its transmission-blocking potential, recombinant *Pb22* (r*Pb22*) was expressed and used to immunize

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ARTHOR CONTRIBUTIONS

F.L., L.C., Y.C. designed the project. F.L., F.Y., Y.W., M.S., W.Z., H.M., D.L., Y.J. performed experiments. F.L., F.Y., T.T. analyzed the results. F.L., T.T., L.C., Y.C. wrote the manuscript. L.C., Y.C. acquired funding for this project.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

mice. *In vitro* assays showed that the rPb22-antisera significantly inhibited exflagellation by ~64.8% and ookinete formation by ~93.4%. Mosquitoes after feeding on rPb22-immunized mice also showed significant decreases in infection prevalence (83.3–93.3%) and oocyst density (93.5–99.6%). Further studies of the Pb22 orthologs in human malaria parasites are warranted.

Keywords

Plasmodium berghei; exflagellation; sexual development; cross fertilization; transmission-blocking vaccine

1 INTRODUCTION

Malaria is caused by unicellular parasites of the *Plasmodium* genus that are transmitted to humans by *Anopheles* mosquitoes. In the last decade, a significant decrease in the global morbidity and mortality of malaria has been achieved through expanded funding for malaria interventions such as long-lasting insecticide-treated bed nets and artemisinin combination therapies. However, the control efforts are thwarted by the emergence of insecticide-resistant mosquitoes and drug-resistant parasites (WHO, 2019). Transmission-blocking vaccines (TBVs) are a promising strategy for effectively reducing malaria transmission and achieving the goal of malaria elimination (Nilsson *et al.*, 2015). Currently, only a few parasite-derived proteins such as P25, P28, P48/45, P230, and HAP2/GCS1 are considered lead TBV candidates. Thus, efforts to discover novel TBV antigens are urgently needed.

In the life cycle of the *Plasmodium* parasites, gametocytes formed in the human blood are the sexual precursor stage needed for transmission to the mosquitoes. Once ingested by susceptible mosquitoes, gametocytes in the blood meal, in response to environmental changes such as a drop in temperature, an increase of pH, and the presence of xanthurenic acid, transform into gametes (Carter *et al.*, 1977, Nijhout, 1979, Billker *et al.*, 2000, Kehrer *et al.*, 2016). Whereas each female gametocyte forms a single immotile macrogamete, a male gametocyte generates up to eight flagella-like microgametes in a process called “exflagellation”. The egress of fully differentiated gametes from the erythrocyte requires the lysis of two membranes surrounding the parasite: the inner parasitophorous vacuole membrane (PVM) and the outer erythrocyte membrane (Sologub *et al.*, 2011, Deligianni *et al.*, 2013). The activated gametocytes emerging from the erythrocyte follow a sequential, inside-out mode, in which rupture of PVM occurs during the first minutes after gametocyte activation, while the rupture of erythrocyte membrane occurs several minutes later (Andreadaki *et al.*, 2018). Subsequently, the micro- and macro-gametes fuse to form a diploid zygote, which transforms into a motile ookinete that penetrates the midgut epithelium to form an oocyst (Paul *et al.*, 2002, Bousema *et al.*, 2011). A better understanding of the molecular mechanisms of sexual development of the malaria parasites will help identify novel targets for blocking transmission.

TBV targets include parasite proteins that are expressed in sexual stages and mosquito midgut proteins (Wu *et al.*, 2015). So far, a handful of proteins expressed during sexual stages have been evaluated for their transmission-blocking (TB) potential. The gamete-specific surface proteins P48/45 and HAP2, required for gamete adhesion and fusion, have

been identified for the pre-fertilization phase. HAP2 is localized on the plasma membrane of microgametocytes and microgametes and has demonstrated potent TB efficacy in the rodent malaria parasite *P. berghei* and human malaria parasite *P. falciparum*. Anti-*P. berghei* HAP2 serum significantly inhibits ookinete and oocyst formation but does not affect microgametocyte exflagellation (Liu *et al.*, 2008, Blagborough *et al.*, 2009). P48/45 belongs to the 6-Cys protein family and is expressed on the surface membranes beginning in gametocyte stages and lasting through zygote stages in the mosquito midgut (van Dijk *et al.*, 2001, van Dijk *et al.*, 2010). Sera from mice immunized with the full-length recombinant Pfs48/45 protein display nearly complete inhibition of oocyst formation (Chowdhury *et al.*, 2009). Another 6-Cys protein P230, exclusively expressed on the *P. falciparum* gametes, physically interacts with Pfs48/45 (Kumar, 1987, Eksi *et al.*, 2002). Antibodies against Pfs230 can reduce oocyst development by lysing gametes in a complement-dependent manner (Read *et al.*, 1994, Healer *et al.*, 1997, Eksi *et al.*, 2006). The *P. yoelii* microgamete surface protein (PyMiGS), localized to the male osmiophilic bodies (OBs) in male gametocytes and the surface of microgametes, though dispensable for male gametocyte egress from red blood cells (RBCs), plays an important role in the exflagellation process. Antibodies against PyMiGS severely reduced the formation of oocysts (Tachibana *et al.*, 2018). After fertilization, P25 and P28 are both expressed on the surfaces of zygotes and ookinetes, and antibodies against the *P. falciparum* protein Pfs25 elicit long-lasting TB activity (Tomas *et al.*, 2001). The limited number of TBV candidates in the vaccine development pipeline demands further efforts in antigen discovery.

The advancement in “omics” technology opens an unprecedented opportunity for vaccine discovery. Previous proteomic analysis characterized the proteomes of the *P. berghei* parasites in mosquito stages. Based on *in silico* prediction, 506 of 624 proteins have been described for both cellular localization and function in the microgametes, among which 103 are transmembrane proteins and 129 extracellular or membrane-associated (Hall *et al.*, 2005, Wass *et al.*, 2012). Since membrane or surface localization in the sexual stages is a critical parameter for TBV prediction, it is logical to explore these proteins as novel TBV targets. Among these latter proteins, the *P. berghei* gene *PBANKA_0305900* (designated hitherto as *Pb22* based on the molecular weight of the encoded protein) is expressed in asexual stages and gametocytes (plasmodb.org), and is dispensable for the asexual intraerythrocytic development in a large-scale gene knockout (KO) study in *P. berghei* (Fonager *et al.*, 2012). Here, we pursued the functional analysis of this gene during parasite development and assessed its TB potential using both *in vitro* and *in vivo* assays.

2 RESULTS

2.1 Pb22 is a conserved in *Plasmodium* and *Hepaticystis*

Proteomic analyses of male and female gametocytes in *P. berghei* (Khan *et al.*, 2005) and *P. falciparum* (Lasonder *et al.*, 2016, Miao *et al.*, 2017) identified many proteins with putative signal peptides suggestive of secreted proteins. Here, we characterized one such gene in *P. berghei*, *PBANKA_0305900* (*Pb22*), which is predicted to encode a sexual-stage protein of 218 amino acids (aa) with a molecular weight of 22 kDa. *Pb22* has a putative signal peptide (aa 1–18), suggesting that it might be secreted (Figure S1A). *Pb22* did not contain any

domains of known functions, while the SMART program (<http://smart.embl-heidelberg.de/>) detected two low-complexity regions spanning aa 63–75 and aa 166–185, which contain compositionally biased amino acids (Wootton, 1994). P22 is highly conserved among *Plasmodium* species (Figure S1B). BLAST search also identified a homolog of this protein in *Hepaticocystis*, which is considered to be descended from *Plasmodium* with the loss of blood schizogony (Aunin *et al.*, 2020). Transcriptomic analysis of asexual stages and separated male and female gametocytes showed that *pb22* is expressed in both asexual stages and gametocytes, while in gametocytes *pb22* expression level is about five times higher in female than male gametocytes (Yeoh *et al.*, 2017). Consistent with *pb22* expression in asexual stages, the Pb22 protein was identified in proteomic analysis of proteins in membrane fractions of schizont-infected RBCs (Fonager *et al.*, 2012). Its ortholog in *P. falciparum* PF3D7_0208800 was also predominantly expressed in gametocytes, but mass spectrometry analysis only identified this protein in female gametocytes (Lasonder *et al.*, 2016, Miao *et al.*, 2017).

We first attempted to confirm these earlier results about Pb22 expression. To generate antibodies against Pb22, we expressed the recombinant Pb22 (rPb22) protein in *Escherichia coli* Rosetta-gami B (DE3) and purified it by using Ni-NTA affinity chromatography. Analysis on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel showed that the purified rPb22 protein was relatively pure and with an estimated molecular weight of 21.9 kDa, which is in accord with its predicted size (Figure 1A). Pb22 is predicted to contain seven antigenic peptides based on the B cell epitope prediction program (<http://imed.med.ucm.es>) (Figure S1C). Mice immunized with rPb22 developed a strong immune response with rPb22-specific antibody titer reaching 1:256000 after the final booster (Figure S2). As a control, we also expressed and purified a recombinant glutathione S-transferase (GST) protein (Figure 1A) and immunized mice with GST (Figure S2A, B).

To confirm the specificity of anti-rPb22 sera, we purified schizonts, gametocytes, and ookinetes of the wild-type (WT) parasites (Figure S3). Western blotting of lysates from these purified parasites showed that the anti-rPb22 antisera recognized a band of approximately 22 kDa (Figure 1B). No signal was detected in uninfected erythrocyte lysates. Pb22 protein expression levels appeared similar in these three stages.

For the cross-validation purpose, we generated a Pb22-HA-tagged parasite line (Figure S4A). Integration-specific PCR showed the correct tagging of Pb22 in the transgenic line (Figure S4B). Western blot using lysates from purified schizonts, gametocytes, and ookinetes (Figure S3) of the Pb22-HA line probed with anti-HA monoclonal antibody (mAb) again detected the ~22 kDa band in each stage analyzed (Figure S4C). No bands were detected by the anti-HA mAb with lysates from WT gametocytes and uninfected RBCs.

2.2 Pb22 is expressed in both asexual and sexual stages and associated with plasma membranes of gametes and ookinetes

To further characterize Pb22 protein expression, an indirect immunofluorescence assay (IFA) was performed on schizonts, female and male gametocytes, female and male gametes, and ookinetes. The control sera against GST as well as secondary antibodies alone did not

produce any detectable staining of ookinetes (Figure 2). In the erythrocytic stages, Pb22 was restricted to the parasites in schizonts and gametocytes, and fluorescence was observed in these stages only after membrane permeabilization (Figure 2). The detection of Pb22 in membrane components of schizonts-infected RBCs in a proteomic study suggests an association of the Pb22 with membrane structure in schizonts (Fonager *et al.*, 2012). Despite that the *pb22* mRNA was much more abundant in female than in male gametocytes (Yeoh *et al.*, 2017), IFA revealed abundant expression of the Pb22 protein in both male and female gametocytes. To differentiate male and female parasites, we used a female-specific marker P47 and male-specific marker α -tubulin II to stain gametocytes and gametes (Figure 2). It is noteworthy that anti- α -tubulin antibodies stained both male and female gametocytes, but the fluorescent signal was much stronger in male gametocytes (Figure S5). We also used antibodies against a nuclear marker, the SET protein, which strongly accumulates in male gametocytes, to differentiate the sex of gametocytes (Pace *et al.*, 2006). In the exflagellating male gametes, fluorescence was observed on the residual body as well as the flagella. Consistent with Pb22 as a potentially secreted protein, strong fluorescence was observed on gametes and ookinetes even without membrane permeabilization (Figure 2), indicating that Pb22 was on the plasma membranes of these stages. Transcriptomic and proteomic analyses also detected the orthologs of Pb22 (PVX_003895 in *P. vivax* and PF3D7_0208800 in *P. falciparum*) in salivary gland sporozoites (Swearingen *et al.*, 2016, Zanghi *et al.*, 2018, Vivax Sporozoite, 2019).

To verify these observations on Pb22 expression and localization, we performed IFA using transgenic parasites with HA-tagged Pb22. A similar fluorescent pattern was observed with the Pb22-HA parasites probed with the anti-HA monoclonal antibody (Figure S6). As negative controls, no fluorescence was detected in HA-tagged ookinetes probed with only the Alexa Fluor 488- and 555-labeled secondary antibodies or in WT ookinetes probed with the anti-HA monoclonal antibody (Figure S6). We also used the female-specific marker G377 of the OBs and cytoplasmic marker Hsp70 to further define the localization of Pb22. IFA with these markers confirmed Pb22 localization in the cytoplasm of gametocytes (Figure S7). These data collectively showed that Pb22 was expressed in schizonts, gametocytes, gametes, and ookinetes, and was associated with the plasma membrane of gametes and ookinetes.

2.3 *Pb22* deletion results in a significant reduction in infectivity to mosquitoes

Pb22 was shown to be dispensable for asexual erythrocytic development during a large-scale functional screen (Fonager *et al.*, 2012). To study its functions during *P. berghei* parasite development, we generated a *Pb22* KO line (Figure 3A). To verify that defects in the *pb22*-KO line were indeed due to *pb22* deletion, a *pb22*-restored (RE) line was then generated using the ‘gene insertion/marker out’ (GIMO) strategy after selection with 5-fluorocytosine (Lin *et al.*, 2011) (Figure 3A). Diagnostic PCR was used to verify independent *pb22* deletion and restoration clones (Figure 3A, B). Western blot and IFA analysis using gametocytes purified from different parasite lines further confirmed *pb22* deletion in the *pb22*-KO line and *pb22* restoration in the *pb22*-RE line (Figure S8A, B).

Although *Pb22* is expressed in schizonts, *pb22* deletion did not result in significant changes in asexual growth of the parasites (Figure S9A), a finding that agrees with the earlier *pb22*-deletion study (Fonager *et al.*, 2012). In addition, *pb22* deletion did not affect the formation of either male or female gametocytes, as gametocytemia and female/male ratio were all comparable among *pb22*-KO, *pb22*-RE, and the WT parasites ($P > 0.05$; Figure S9B, C). To determine whether *pb22* KO affects subsequent sexual development, we performed both *in vitro* ookinete culture and mosquito feeding experiments using WT, *pb22*-KO, and *pb22*-RE parasites. *In vitro* analysis showed that *pb22*-KO male gametocytes showed a ~89% reduction in the exflagellation centers formed as compared to the WT control ($P < 0.0001$; Figure 4A). To differentiate the female gametes from gametocytes, we performed labeling of the RBC membrane with the TER-119 antibody, and labeling of female gametocytes and gametes with the anti-P47a antiserum. We found that in the WT, *pb22*-KO, and *pb22*-RE parasite lines, macrogamete numbers were similar between the *pb22*-KO and WT ($P > 0.05$; Figure 4B), and there was no defect in the formation and egress of female gametes in the *pb22*-KO line (Figure 5A, B). *In vitro* ookinete culture assay showed that the ookinete number in *pb22*-KO line was reduced by ~97% compared to WT control ($P < 0.0001$; Figure 4C). To determine whether *pb22* deletion impacted subsequent development in mosquitoes, the *pb22*-KO and *pb22*-RE clones and WT parasites were used to infect mice, and mosquito feeding was conducted. After blood feeding, ookinete formation in the blood boluses of mosquito midguts was assessed at 24 h, while oocyst number was determined at 12 days. Similar to the *in vitro* assay, ookinete formation was almost completely blocked in the mosquitoes feeding on the *pb22*-KO parasite-infected mice with only one midgut in 30 midguts dissected containing 5 ookinetes, as compared to 1187–2280 ookinetes/midgut in mosquitoes feeding on WT *P. berghei*-infected mice (Figure 4D, Table 1). Furthermore, *pb22* deletion completely blocked the formation of oocysts in the midguts, and none of the mosquitoes feeding on mice infected with the *pb22*-KO parasite developed oocysts ($P < 0.0001$; Figure 4E, Table 1). In comparison, all defects in exflagellation of male gametocytes, ookinete and oocyst formation were effectively restored in the *pb22*-RE line (Figure 4, Table 1), indicating that these defects were indeed due to *pb22* deletion. These results indicate that *Pb22* is essential for male gametogenesis, fertilization and possibly subsequent development of the parasites.

2.4 The *pb22*-KO line is defective in male gametogenesis

To further investigate the exflagellation defect in *pb22*-KO parasites, we compared WT and *pb22*-KO parasites by IFA to differentiate defects in flagella formation and egress. At 15 min after induction with xanthurenic acid, male parasites were stained with antibodies against α -tubulin II, nucleus by Hoechst 33258, and RBC membrane by TER-119. DNA replication appeared normal, as DNA contents of microgametocytes in the *pb22*-KO line and WT were similar (Figure S9D). Also, there were about the same proportions of the microgametocytes showing no signs of activation (Figure 5C, D). The major defects in the *pb22*-KO line appeared to be at the egress step since 78.9% of the microgametocytes in the *pb22*-KO line were in the stage of aborted exflagellation with the RBC membrane retained. For microgametocytes with aborted exflagellation, 30% failed to assemble the axonemes and 48.9% were egress-defective (flagella formed but failed to egress from the RBC). Time-lapse recording for 30 min confirmed that most of the activated microgametocytes in the *pb22*-KO

line with flagella ('bag of worms' phenotype in Movie S1) or without flagella (Movie S2) failed to egress from the RBC. Eventually, only 15.5% of the microgametocytes in *pb22*-KO line egressed from the RBC, among which 10% formed exflagellation centers and the remaining male gametes did not interact with the RBCs (arrested exflagellation), as compared to >91.1% male gametes in the WT *P. berghei* having egressed and 88.9% forming exflagellation centers ($P < 0.0001$, Figure 5C, D).

2.5 The *pb22*-KO female gametocytes are competent to form ookinetes

To confirm that *pb22*-KO line was defective only in male fertility, we performed *in vitro* cross-fertilization experiments between gametes of different mutants (van Dijk *et al.*, 2010). For this purpose, two clones of *p47* (male fertile, female infertile) and *p48/45* (male infertile, female fertile) each were used in cross-fertilization. Both the *p47* and *p48/45* lines showed self-fertilization rates that were reduced by more than 99% compared to that in WT (Figure 6). *In vitro* ookinete cultures demonstrated that both the *p47* (C1 and C2) and *p48/45* (C1 and C2) lines contained clusters of unfertilized female gametes, and the *pb22*-KO line showed a similar defect in *in vitro* self-fertilization. Consistent with earlier findings (van Dijk *et al.*, 2010), the cross-fertilization rates between *p47* and *p48/45* lines were not significantly different from that of the WT (Figure 6), confirming that only one sex in each of these deletion lines was defective. In cross-fertilization experiments, the *pb22*-KO line and female-defective *p47* (C1 and C2) lines were able to cross-fertilize at the rates comparable to that of the WT, whereas the *pb22*-KO line and the male-defective *p48/45* lines were unable to fertilize to produce ookinetes (Figure 6), confirming that the *pb22*-KO line was defective only in male fertility.

2.6 Pb22 possesses obvious transmission-blocking potential

Expression of Pb22 on the surfaces of sexual stages prompted us to evaluate whether this protein could be a TBV candidate. To assess the TB potential of Pb22, mice were immunized with the rPb22 protein or the recombinant GST protein as a control and then infected with the WT *P. berghei*. Parasitemia ($P > 0.05$; Figure S10A), gametocytemia ($P > 0.05$; Figure S10B) and the gametocyte sex ratio ($P > 0.05$; Figure S10C) were not significantly different among the rPb22 immunization, the GST control, and adjuvant control groups. In addition, *in vitro* assay showed that rPb22 immunization did not affect macrogamete formation as the macrogamete numbers in the three groups were also similar ($P > 0.05$; Figure 7A). However, compared with the GST immunization and adjuvant control groups, the number of exflagellation centers in parasitized blood 3 days post-infection was reduced by ~65% in the rPb22 immunization group ($P = 0.0026$; Figure 7B), which led to a ~93% reduction of ookinetes formed in *in vitro* culture ($P < 0.001$; Figure 7C). When immunized mice were used for mosquito feeding, consistent and significant reduction in ookinete formation, oocyst density and infection prevalence was observed in the rPb22-immunization group as compared with the two control groups. Specifically, ookinete numbers in the midguts 24 h after blood feeding showed 83.3–93.3% reduction in the rPb22-immunization group as compared to the GST-immunization group ($P < 0.001$; Figure 7D, Table 2). The mean oocyst number/midgut in mosquitoes that fed on the GST-immunization group was 102.9–110.4, which was reduced to 0.4–6.7 in those that fed on rPb22-immunized mice, corresponding to 93.5–99.6% reduction in oocyst density ($P < 0.0001$, Figure 7E,

Table 2). Furthermore, compared to an average infection prevalence of 93.1–100% in mosquitoes feeding on the GST control mice, mosquitoes feeding on rPb22-immunized mice had an infection prevalence of 6.7–10%, corresponding to 83.3–93.3% reduction in infection prevalence ($P < 0.0001$; Table 2). It is noteworthy that there were no significant differences in these parameters examined between the GST-immunization group and the adjuvant control group (Figure 7 and Table 2).

We performed *in vitro* assays to further illustrate that the TB activity observed with the rPb22 immunization was due to specific antibodies against Pb22. Among the immune sera collected from the three groups of mice, only the rPb22 immune sera detected the ~22 kDa protein in gametocyte lysates of the WT parasite in Western blot (data not shown) and the rPb22 antisera reacted with the surface of male gametes and ookinetes (Figure S11A). Using the *in vitro* assay, we showed that the effect of rPb22 immune sera on exflagellation and ookinete formation was concentration-dependent. Regardless of the dilution, the GST control sera showed no differences in the formation of exflagellation centers or ookinetes (Figure S11B, C). At the 1:5 dilution, the rPb22 immune sera conferred a 56.3% decrease in exflagellation centers (Figure S9B) and an 84.8% decrease in ookinete number (Figure S11C) as compared with the GST control sera. However, the inhibitory effects of the rPb22-immune sera were attenuated at the 1:10 dilution (Figure S11B, C).

3 DISCUSSION

In this study, we characterized a protein, Pb22, which is expressed in both asexual and sexual stages of *P. berghei* but is functionally essential only for male gametogenesis. *Pb22* KO did not affect asexual growth or gametocytogenesis, but it led to defective male gametogenesis at multiple steps. As a result, egress, fertilization, ookinete formation and mosquito infectivity were all severely affected. The localization of this protein on the plasma membrane of both male and female gametes and subsequently on ookinetes prompted us to investigate its TB potential. Antibodies against rPb22 demonstrated strong TB activity in both *in vitro* ookinete culture and mosquito feeding assays.

Gametocytes formed in the vertebrate hosts are obligative for the transmission of malaria parasites to the mosquitoes. Within 15–20 min of blood ingestion, gametogenesis occurs in the mosquito midgut to produce male and female gametes. Whereas a female gametocyte only forms one spherical macrogamete, a male gametocyte undergoes three rounds of DNA replication to produce eight flagella-like gametes, each consisting of an axoneme and a nucleus encased by a flagellar membrane (Kuehn *et al.*, 2010). Egress from the erythrocyte membrane is an important step of gametogenesis and requires multiple factors for the sequential lysis of PVM and the erythrocyte membrane (Sologub *et al.*, 2011, Wirth *et al.*, 2012, Deligianni *et al.*, 2013, Andreadaki *et al.*, 2018). Several factors affect the egress of both male and female gametocytes. MDV1/PEG3 (male development-1/protein of early gametocyte 3) is important for the lysis of PVM; *MDV1/PEG3* deletion blocks the egress of both male and female gametes (Ponzi *et al.*, 2009). Similarly, male and female gametocytes lacking the GEST (gamete egress and sporozoite traversal) protein also are not able to lyse the PVM or egress from the RBC (Talman *et al.*, 2011). MTRAP (merozoite-specific thrombospondin-related anonymous protein) is essential for the disruption of the gamete-

containing PVM, and MTRAP-deficient male and female gametocytes are wrapped in intact PVM and RBC membrane (Bargieri *et al.*, 2016, Kehrer *et al.*, 2016). In this study, We found that despite Pb22 expression in asexual stages and female gametocytes, *pb22* deletion did not affect asexual growth (Fonager *et al.*, 2012), gametocytogenesis or the formation of female gametes. The male-specific defects have been confirmed by IFA to show egress of the macrogametes from the RBCs and by cross-fertilization experiments with male-deficient *p48/45* and female-deficient *p47* parasite lines.

Male gametogenesis is a dramatic event and occurs very rapidly within the mosquito midgut. Six minutes after the activation, three rounds of DNA replication are complete (Fang *et al.*, 2017). DNA replication is physically linked to the simultaneous assembly of eight axonemes, followed by cytokinesis to form individual male gametes. Gamete escape from erythrocyte is mediated by the secretion of the content of OBs, which contain membranolytic factors, into the parasitophorous vacuole (Olivieri *et al.*, 2015). Several protein kinases and phosphatases have been identified as key regulators of male gametogenesis. Calcium-dependent protein kinase (CDPK) 4 is involved in the initiation of DNA replication, assembly of the mitotic spindle, and finally motility of the male flagellum (Fang *et al.*, 2017). Whereas CDPK1 deletion in *P. berghei* only delays the exflagellation process (Sebastian *et al.*, 2012), PfCDPK1 KO parasites show no flagellar movement or exflagellation (Bansal *et al.*, 2018). The mitogen-activated protein kinase 2 (MAP-2) affects the final stages of male gamete formation in both *P. berghei* and *P. falciparum* (Tewari *et al.*, 2005, Hitz *et al.*, 2020). Recent studies have revealed the participation of some structural and motor proteins in axoneme assembly (Marques *et al.*, 2015, Depoix *et al.*, 2020). Later in male gametogenesis, the egress process requires the release of lytic factors (e.g., the plasmodial perforin-like protein 2 [PPLP2], a ferlin-like protein FLP, the serine protease SUB1, and the gamete egress protein GEP) from secretory vesicles or OBs to lyse the PVM and the RBC membrane (Wirth *et al.*, 2014, Obrova *et al.*, 2019, Pace *et al.*, 2019, Andreadaki *et al.*, 2020). For example, upon *PbPPLP2* deletion, male gametes can disrupt the PVM normally but fail to rupture the RBC membrane, thus forming a super-flagellum, where multiple axonemes are sheathed together by the RBC membrane (Deligianni *et al.*, 2013, Wirth *et al.*, 2014). A phospholipase (PfPATPL1) has been found to be important for the re-localization of the PPLP2-containing vesicles, thus affecting gamete egress (Singh *et al.*, 2019). Interestingly, the male OB-associated protein PyMiGS in *P. yoelii* does not affect egress or formation of flagella, but it affects exflagellation (Tachibana *et al.*, 2018). In this study, the male defects in gametogenesis upon *Pb22* deletion seemed to involve both axoneme assembly and egress. While 30% of the microgametocytes have successfully finished DNA replication but failed to form axonemes, ~49% have formed flagella but the microgametes could not egress from the RBC membrane. Its cytoplasmic localization in gametocytes shows a punctate pattern, suggesting it could be present in vesicular structures, OBs, or granules. Consistent with its cytosolic localization, *Pb22* does not seem to affect DNA replication as male gametocyte DNA contents after activation were similar between WT and *Pb22* KO parasites. Given the involvement of OBs in male gamete egress, those displaying the “bag of worms” phenotype may be defective in the formation of OBs or secretion of the OB content. Like PyMiGS, *Pb22* is localized in the cytoplasm of gametocytes but then appeared on the plasma membrane of gametes, suggesting it might be

released from the vesicular structure during gametogenesis. Future fine structure analysis and identification of proteins interacting with Pb22 will help to elucidate its subcellular localization and functions.

After fertilization in the midgut, zygote develops into a motile ookinete, which traverses the midgut wall forming an oocyst to establish infection in the mosquito. In addition to the defect in male gamete formation, the *pb22*-KO parasites may have additional defects in fertilization, ookinete development, and midgut invasion, since mosquitoes feeding on the *pb22*-KO-infected mice developed no oocysts. Despite ~10% *pb22*-KO male gametocytes could form exflagellation centers, very few ookinetes were formed with the *pb22*-KO x *p48/45* cross. Such a defect indicates that the surface-localized Pb22 may play a direct or indirect role in male-female gamete interaction and fertilization. Furthermore, although residual ookinetes (<3% of the WT level) were formed during *in vitro* culture of *pb22*-KO-infected blood, no oocysts were formed during the mosquito feeding assay, suggesting that the ookinetes lacking surface Pb22 protein may have additional defects in midgut traversal and subsequent development.

During gamete formation to ookinete development, Pb22 protein is localized on the plasma membrane of parasites, since IFA without membrane permeabilization still detected Pb22 on the surface of these stages. Pb22 has a predicted signal peptide, but lacks identifiable transmembrane domains, suggesting that surface localization of Pb22 may rely on the interactions with other proteins on the surface of these stages. It has been shown that two male fertility factors P48/45 and P230 form a complex on the surface of the male gametes (Kumar, 1987, Eksi *et al.*, 2006, Cao *et al.*, 2018). It would be interesting to determine how Pb22 is localized on the surface of the parasites, and whether Pb22 deletion affects the surface localization of these male fertility factors.

An important criterion to select TBV candidates is that the target protein is localized on the sexual stages of the parasite (Nunes *et al.*, 2014). TBV development has focused on the pre-fertilization proteins P230 and P48/45, as well as post-fertilization proteins P28/25 (Baton *et al.*, 2005, MacDonald *et al.*, 2016, Lennartz *et al.*, 2018). A TBV candidate expressed during both pre- and post-fertilization, like Pb22, would offer the advantages of blocking parasite development at multiple time points. Although *pb22* mRNA expression was only detected in female gametocytes (Yeoh *et al.*, 2017), Pb22 protein was detected in both male and female gametocytes (Khan *et al.*, 2005). Our study further confirmed Pb22 protein expression in asexual stages and in both male and female gametocytes. Its surface localization on male and female gametes, and also during ookinete development led us to investigate its TB potential. In this study, we provided evidence that antibodies against Pb22 could inhibit exflagellation. We reason that the permeabilization of the PVM and RBC membrane during the gamete egress process will likely allow the antibodies to gain access to Pb22 on male gametes and block their release. The *in vivo* analysis of TB activity showed that mosquitoes that fed on mice immunized with the rPb22 had up to 93.3% reduction in the prevalence of infected mosquitoes and 99.6% reduction in oocyst density. This excellent TB activity of Pb22 in the rodent parasite and conservation of this gene in *Plasmodium* suggest that it might be a promising TBV candidate and is worth of evaluation in the human malaria parasites.

In conclusion, we have characterized a conserved *Plasmodium* protein that plays an important role in male gametogenesis and potentially subsequent sexual development. Despite the expression of Pb22 in both male and female gametocytes, the defect of *pb22* deletion appears to be restricted to male gametes. Being a surface protein during gamete to ookinete development, Pb22 demonstrated excellent TB activity which supports further exploration of P22 as a TBV candidate antigen in human malaria parasites.

4 EXPERIMENTAL PROCEDURES

4.1 Animals, parasites, and mosquitoes

General maintenance of parasites and mosquitoes was described previously (Zheng *et al.*, 2016). Briefly, *P. berghei* ANKA 2.34 parasites were maintained in 6–8 week-old female Balb/c mice by serial mechanical passages (up to a maximum of eight passages). *Anopheles stephensi* mosquitoes (Hor strain) were bred in an insectary under 25°C and 50–80% humidity. Mice and rabbits were kept at the central animal facilities of China Medical University under the care of trained veterinary staff. All animal experiments were approved by the university animal ethics committee.

4.2 Generation of transgenic parasites

Several transgenic parasites were generated using the double-crossover homologous recombination strategy described previously (Janse *et al.*, 2006). To tag the endogenous Pb22 with an HA tag, the 5' fragment was amplified with the primers HA-5UTR-F and HA-5UTR-R, while the 3' fragment was amplified with primers HA-3UTR-F and HA-3UTR-R (Table S1). The fragments were cloned into vector PL0035 (containing the human *dhfr* cassette, *yfcu* cassette and *ha* tag). Plasmids (10 µg) were linearized and electroporated into 1×10^7 purified *P. berghei* schizonts. After transfection, parasites were mixed with 50 µl complete culture medium and intravenously injected into mice. Transgenic parasites were selected 24 h later with 70 µg/ml pyrimethamine (Sigma Aldrich, St. Louis, MO, USA) added in drinking water for mice. Parasite genomes were extracted from infected blood to examine the transgenic parasite by integration-specific PCR. HA-tagged parasites were cloned using a limiting dilution method (Guttery *et al.*, 2014).

KO lines for *pb22* were generated similarly. Primers for the 5' and 3' recombination fragments of these genes are listed in Table S1. Purified and digested PCR fragments were cloned into the PL0034 vector. The *p48/45* and *p47* lines were generated earlier and used to study sex-specific defects (Zhu *et al.*, 2019). To restore the *pb22* gene in the *pb22*-KO line by GIMO (Lin *et al.*, 2011), the primers KO-5UTR-F and KO-3UTR-R were used to amplify a reconstructed fragment. Purified PCR fragments were digested with *HindIII* and *NotI*, then transfected into the *pb22*-KO schizonts as described above. Parasites with the *pb22* gene restored (*pb22*-RE) were selected by intraperitoneal injection of 0.4 mg/kg/day of 5-fluorocytosine (Sigma) for 4 days, beginning at 24 h after transfection. For the final proof of successful gene KO and restoration, diagnostic PCR and Western blots were performed.

4.3 Expression of rPb22 and other recombinant proteins

The sequence corresponding to Ser₁₉-Ser₂₁₈ (entire protein minus signal peptide) of Pb22 was amplified by PCR using primers pET32a-Pb22-F and R (Table S1). In addition to the *Bam*HI site, the forward primer contains a sequence (GACGACGACGACAAG) for peptide Asp-Asp-Asp-Asp-Lys, which is the enterokinase cleavage sequence. The PCR product was cloned into the *Bam*HI/*Not*I sites of the expression vector pET32a, producing a fusion protein under the control of the T7 *lac* promoter. The plasmids were transformed into *E. coli* Rosetta-gami B (DE3) cells and the rPb22 protein expression was induced with 1 mM isopropyl-D-1-thiogalactoside at 19°C for 4 h. Recombinant proteins were purified by affinity chromatography on nickel-nitrilotriacetic acid agarose (Ni-NTA, Millipore, Germany) under denaturing conditions. Purified rPb22 was dialyzed using phosphate-buffered saline (PBS, pH 7.4) at 4°C overnight. Eluted fusion protein rPb22 was digested by recombinant enterokinase at 25°C for 16 h in a Tris buffer (0.2 mM Tris-HCl, 100 mM NaCl, pH 8.0). The digested proteins were further purified on a Ni-NTA column, and the released rPb22 protein was analyzed by 10% SDS-PAGE. A recombinant GST protein was similarly expressed in *E. coli* using the empty vector pGEX-4T-1. It was used as an immunization control since this GST has limited sequence identity (28%) to the *P. berghei* GST sequence.

We also expressed recombinant P47 and SET for antibody production. The DNA sequences encoding amino acids I₂₃-L₁₅₄ for P47 (PBANKA_1359700) and M₁-D₂₆₇ for SET (PBANKA_0819900) were amplified from *P. berghei* cDNA using primers listed in Table S1. The PCR products were cloned into pET32a and transformed into DE3. The recombinant proteins were purified from *E. coli* as described for rPb22. Purified proteins were quantified using a BCA Protein Assay Kit (Beyotime, Haimen, China).

4.4 Production of antisera

Polyclonal antisera against rPb22 were raised in female BALB/c mice (n = 5) by subcutaneously injecting 50 µg purified rPb22 emulsified in complete Freund's adjuvant (Sigma). Boosting on day 15 and 30 was performed via subcutaneous injection with 30 µg rPb22 in incomplete Freund's adjuvant. The same immunization procedure was performed for the GST immunization control and the adjuvants only control. Sera were collected and pooled 10 days after the last immunization.

To generate polyclonal antisera against P47 and SET, New Zealand white rabbits were immunized subcutaneously with 250 µg of purified recombinant protein with Freund's complete adjuvant, followed by two boosters with 250 µg of purified recombinant protein in Freund's incomplete adjuvant at 3-week intervals. Antisera were collected 14 days after the last immunization. Rabbit anti- α -tubulin II antibodies were custom-made against the peptide CDGEGEDEGYE by Genscript (Nanjing, China).

4.5 Enzyme-linked immunosorbent assay (ELISA)

To estimate the antibody titers of antisera from rPb22 immunized group, ELISA was performed as previously described (Miura *et al.*, 2008). The microtiter plate was coated with purified proteins (5 µg/ml) in 0.05 M sodium carbonate buffer (pH 9.6) at 4°C overnight.

Then the plate was washed with PBS-T (0.05% Tween-20 in PBS) three times and blocked with 1% bovine serum albumin (BSA, Sigma) at 37°C for 1 h. To each well of the plate, 100 µl of mouse sera, serially diluted with 1% BSA in PBS from 1:1000 to 1:512000, were added and incubated at 37°C for 2 h. After three washes with PBS-T, 100 µl HRP-conjugated goat anti-mouse secondary antibodies (1:5000, ThermoFisher, 62–6520) were added and incubated at 37°C for 2 h. After additional six washes, 100 µl of substrate solution (tetramethyl benzidine, Amresco, USA) were added and incubated in the dark for 5 min. The reaction was stopped by adding 50 µl of 1 mM H₂SO₄ and the absorbance at 490 nm was read immediately. The value for the final dilution of the antisera was defined as that above the cut-off value of the control antisera + 3 × standard deviation (SD).

4.6 Western blot

For Western blot analysis, total proteins were prepared from schizonts, gametocytes and ookinetes as previously described (Kou *et al.*, 2016). Briefly, BALB/c mice were intraperitoneally injected with 1×10⁶ WT *P. berghei*, *pb22-KO*, *pb22-RE* and *Pb22-HA* infected RBCs (iRBCs). Heparinized blood on day 5 post-infection was cultured in a complete schizont culture buffer (RPMI 1640, 50 mg/l penicillin, 50 mg/l streptomycin, 100 mg/l neomycin, 25% [vol/vol] heat-inactivated fetal calf serum, 6 U/ml heparin) at 37°C for 16 h and schizonts were fractionated on a 55% (v/v) Nycodenz (Axis-Shield, Oslo, Norway) gradient. Gametocyte purification was performed based on a previously described protocol (Beetsma *et al.*, 1998). BALB/c mice were pre-treated with 25 mg/ml phenylhydrazine in saline solution to induce hyper-reticulocytosis. Three days later mice were injected intraperitoneally with 1×10⁶ WT iRBCs. Four days post-infection, mice were treated with sulfadiazine (Sigma) at 20 mg/l in drinking water for 48 h to kill other parasite stages. Gametocytes were purified on a 48% (v/v) Nycodenz gradient at 4°C. For ookinetes, purified gametocytes were cultured in a complete ookinete culture medium (RPMI 1640, 50 mg/l penicillin, 50 mg/l streptomycin, 100 mg/l neomycin, 25% [v/v] heat-inactivated fetal calf serum, 6 U/ml heparin, 100 µM xanthurenic acid, pH 8.0) in plastic Petri dishes at 19°C for 24 h and ookinetes were isolated with 62% Nycodenz.

Proteins were extracted with 2% SDS containing protease inhibitors. Equal amounts of parasite lysates (10 µg) were separated by 10% SDS-PAGE under reducing conditions. Proteins were transferred to PVDF membranes (BioRad, Hercules, CA, USA), blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20) at room temperature for 2 h, and probed with mouse anti-rPb22 antisera (1:200) or mouse anti-HA monoclonal antibody (1:1000; Invitrogen, Carlsbad, CA, USA). After three washes with TBST, the membrane was incubated with HRP-conjugated anti-mouse secondary antibody. An ECL Western Blot Kit (Pierce, Rockford, IL, USA) was used to visualize protein via chemiluminescence. Protein loading was estimated using the anti-rHsp70 sera (Hsp70, PBANKA_0711900) produced in the laboratory.

4.7 IFA

Pb22 localization was determined by IFA using a modified protocol (Tonkin *et al.*, 2004). WT and *Pb22-HA* parasites were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde (Sigma) in PBS for 30 min at room temperature. After washing with PBS,

cells were treated with or without 0.1% Triton X-100 (Sigma) in PBS for 10 min and then blocked with 3% BSA/PBS for 60 min. Following another PBS wash, mouse anti-rPb22 and anti-HA mAb (1:500; Invitrogen, 326700) were added, and samples were incubated for 1 h at 37°C. After washing with PBS, the parasites without membrane permeabilization were treated with 0.1% Triton X-100 and then blocked with 3% BSA/PBS for 60 min. Subsequently, parasites were co-incubated with rabbit antisera against PbMSP1 (1:500), P47 (1:500), α -tubulin II (1:500), SET (1:500) and PSOP25 (1:500) as stage-specific markers for schizonts, female gametocytes/gametes, male gametocytes/gametes, nucleus, and ookinetes, respectively (Pace *et al.*, 2006, Molina-Cruz *et al.*, 2017, Zheng *et al.*, 2017, Liu *et al.*, 2018, Liu *et al.*, 2019). After three washes with PBS, Alexa Fluor-488-conjugated goat anti-mouse IgG antibodies (1:500; Invitrogen, A32723) and Alexa Fluor-555-conjugated goat anti-rabbit IgG antibodies (1:500; Abcam, ab50078) were added and incubated for 1 h. Except for the commercial antibodies, antibodies against PbMSP1, P47, Pbg377, SET, Pbs21, and PSOP25 were made in our laboratory. The nucleus was counterstained with Hoechst 33258 (1:1000; Invitrogen). Negative controls for IFA included WT ookinetes incubated with the anti-HA antibody, GST immune sera, and secondary antibodies only, as well as Pb22-HA ookinetes incubated with only the secondary antibodies. Parasites were visualized on a Nikon C2 fluorescence confocal laser scanning microscope (Japan).

4.8 Phenotype analysis of *pb22-KO* and *pb22-RE* parasites

To confirm the absence and presence of Pb22 expression in *pb22-KO* and *pb22-RE* parasites, respectively, gametocytes of *pb22-KO* and *pb22-RE* lines were purified and used for Western blot analysis and IFA using the anti-rPb22 sera and antisera for additional markers. To study the function of *Pb22*, three groups of mice (3/group) were infected with 1×10^6 iRBCs of WT, *pb22-KO* and *pb22-RE* lines, respectively. Parasitemia was monitored every other day from day 3 post infection. Another three groups of mice (3/group) were used for gametocyte induction as described in 4.6. Five days after infection, gametocytes per 10^4 RBCs and gametocyte sex ratios from counting 100 gametocytes were estimated in Giemsa-stained thin blood films (Marques *et al.*, 2015). Mature male and female gametocytes were differentiated based on staining with male- and female-specific markers as described above. Exflagellation centers (male gamete interactions with RBCs), macrogamete and ookinete numbers were monitored using *in vitro* culture (Eksi *et al.*, 2006). Briefly, $\sim 10 \mu\text{l}$ infected blood containing equal numbers of mature gametocytes were mixed with ookinete culture medium in a total volume of $50 \mu\text{l}$ at 25°C to induce gamete formation. Fifteen minutes later, $1 \mu\text{l}$ was placed on a glass slide (Matsunami Glass IND., LTD, Japan) and exflagellation centers (10 view fields per mouse) were enumerated under a phase-contrast microscope at 400 \times magnification for 10 min. An exflagellation center is defined as an exflagellating male gametocyte with more than four tightly associated RBCs (Templeton *et al.*, 1998). The time-lapse recording of exflagellation centers was captured with a camera mounted on an Olympus microscope. To count macrogametes and ookinetes, $\sim 10 \mu\text{l}$ infected blood containing equal numbers of mature gametocytes were mixed with the ookinete medium in a total volume of $500 \mu\text{l}$ at 25°C for 15 min. The total number of macrogametes in $0.5 \mu\text{l}$ (equivalent to $0.01 \mu\text{l}$ of infected blood) was counted after labeling with the anti-Pbs21 mAb (1:500) and Alexa-488-conjugated anti-mouse IgG antibodies (1:500) without permeabilization (van Dijk *et al.*, 2001). The culture was continued at 19°C for 24 h to allow

ookinete formation. Similarly, the number of ookinetes in 0.5 μ l of culture was counted after labeling with the anti-Pbs21 mAb (1:500).

4.9 Mosquito feeding

For direct mosquito-feeding assays, mice were infected with parasites as described above. When asexual parasitemia was approximately 5–7% after 3 days of infection, ~50 4-day-old, pre-starved *An. stephensi* mosquitoes were allowed to feed on each infected mouse for 1 h. At 24 h after the feeding, approximately 10 engorged mosquitoes per group were ground to enumerate ookinetes in each mosquito using Giemsa-stained films. One mosquito was ground in 50 μ l PBS, and 1 μ l of the mixture was used to enumerate ookinetes. At 12 days after blood feeding, midguts were dissected from ~30 mosquitoes of each group and stained with 0.5 % mercurochrome for 10 min. Oocysts were counted and expressed as the number of oocysts per positive midgut. Finally, the proportion of infected mosquitoes among all mosquitoes was determined.

4.10 Gametocyte egress assay

WT- and *pb22*-KO-infected blood samples with gametocytes were mixed with the ookinete culture medium and incubated at 25°C for 15 min. Cells were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde for 30 min and proceeded for IFA with permeabilization as described above. Samples were labeled with either rabbit anti-P47 antiserum (1:500) or rabbit anti- α -tubulin II antiserum (1:500) as previously described, followed by incubation with Alexa Fluor 555-conjugated goat anti-rabbit IgG antibodies (1:500 dilution). RBC membranes were labeled with rat anti-TER119 antibody conjugated to Alexa Fluor 488 (1:200 dilution, Invitrogen, 4322597). Cell nuclei were counterstained with Hoechst 33258 (1:1000). At least 30 egressed or un-egressed parasites were differentiated under a fluorescence microscope (Bansal *et al.*, 2017, Tachibana *et al.*, 2018). To quantify the nuclear DNA content of male gametocytes, gametocytes were mixed with ookinete culture medium at 25°C, fixed, and permeabilized as described above at 6 min when the male gametocytes should have completed three rounds of genome replication (Fang *et al.*, 2017). The cells were incubated with 3% BSA/PBS for 1 h, washed with PBS, and then incubated with rabbit anti- α -tubulin II serum for 1 h. After washing with 1 ml PBS, they were incubated with Alexa Fluor 555-conjugated goat anti-rabbit IgG antibodies for 1 h, and counterstained with Hoechst 33258 for 10 min. Images of the male gametocytes (Alexa Fluor 555 positive) were captured under a Nikon C2 confocal laser scanning microscope using identical settings. The fluorescent signal intensity of 30 parasites from each group was measured using the Nikon software (Wang *et al.*, 2020).

4.11 Cross-fertilization study

To study whether the *pb22*-KO parasites was defective in either male or female fertility, *in vitro* cross-fertilization assay was performed using either the *p47* line (female defective, male fertile) or the *p48/45* line (PBANKA_1359600, male defective, female fertile) (van Dijk *et al.*, 2010). Equal numbers of mature gametocytes of each clone (counted by Giemsa staining) were mixed and incubated for 24 h. Ookinetes and unfertilized gametes from a total of 100 parasites were counted separately. Fertilization rate is the percentage of female gametes that develop within 24 h into ookinetes *in vitro*.

4.12 TB activity

To evaluate the TB activity of anti-rPb22 sera in *in vivo* assay, 6 mice/group immunized with the rPb22 protein, GST or adjuvants only, respectively. Ten days after the third booster, 3 mice were infected with 1×10^6 parasites. Parasitemia, gametocytemia, and gametocyte sex ratios were counted on day 3 post infection. For *in vitro* analysis, exflagellation centers, macrogametes and ookinetes were counted as described above. The other three mice were fed to starved female *An. stephensi*, and 24 h later, ookinete numbers in mosquitoes were calculated using Giemsa-stained films. The numbers of oocysts and infected mosquitoes were determined at 12 days after feeding.

In vitro assay was further performed using different dilutions of the immune sera. Both the anti-rPb22 sera and the GST sera were diluted at 1:5 and 1:10 with the ookinete culture medium and mixed with 10 μ l of infected mouse blood in a total volume of 50 μ l. The exflagellation of male gametocytes was quantified as described above. For ookinete culture, serum samples were diluted at 1:5 and 1:10 with the ookinete culture medium and mixed with 10 μ l of infected mouse blood in a total volume of 500 μ l. The number of ookinetes in 0.5 μ l culture was counted at 24 h.

4.13 Statistical analyses

Student's *t*-test and ANOVA were used to compare antibody titers, parasitemia, gametocytemia, gametocyte sex ratio, exflagellation and ookinete numbers among groups. Chi-square test was used to compare the proportions. Mann-Whitney *U* test was used to analyze oocyst density (oocysts number/midgut), while Fisher's exact test was used to analyze infection prevalence. All data are presented as mean \pm SD. Statistical analyses were carried out using the SPSS software, version 21.0 (IBM, North Castle, NY, USA). $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Take Away

- We characterized Pb22 that is expressed in schizonts-ookinetes in *P. berghei*
- *Pb22* KO showed defects in male gametogenesis and ookinete development
- Most *Pb22* KO male gametocytes failed in axoneme assembly or egress
- Cross-fertilization confirmed defects restricted to male gametes only
- Recombinant *Pb22* showed obvious transmission-blocking potential

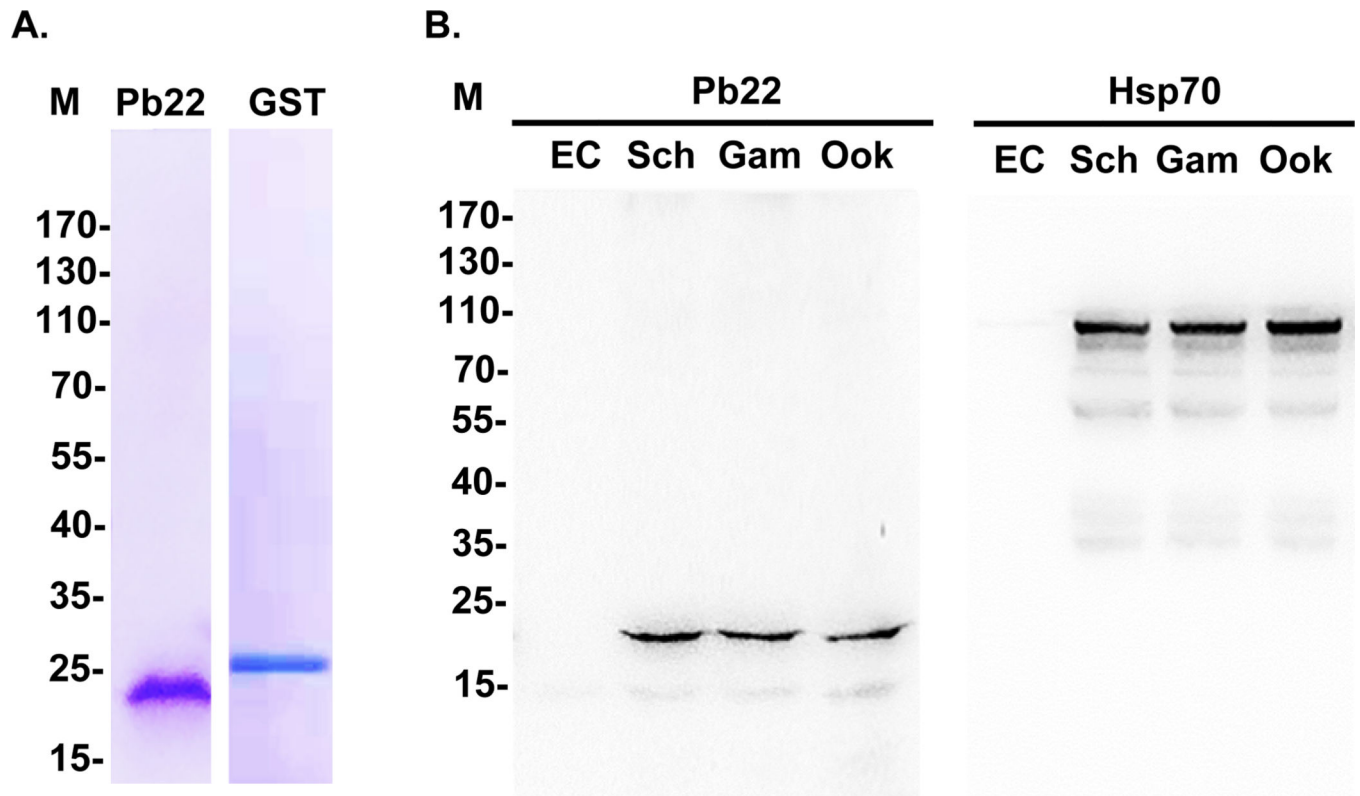


FIGURE 1. Purification of the rPb22 protein and detection of Pb22 expression during development.

A. Purified recombinant Pb22 and GST protein were separated on 10% SDS-PAGE gels and stained with Coomassie brilliant blue. **B.** Western blot analysis of protein extracts from purified schizonts (Sch), gametocytes (Gam) and ookinetes (Ook) of WT *P. berghei* parasites and uninfected erythrocytes (EC). The blots were probed with the anti-rPb22 antisera (Pb22, left panel), while protein loading was estimated by the anti-rHsp70 sera (Hsp70, right panel). M, molecular markers in kDa.

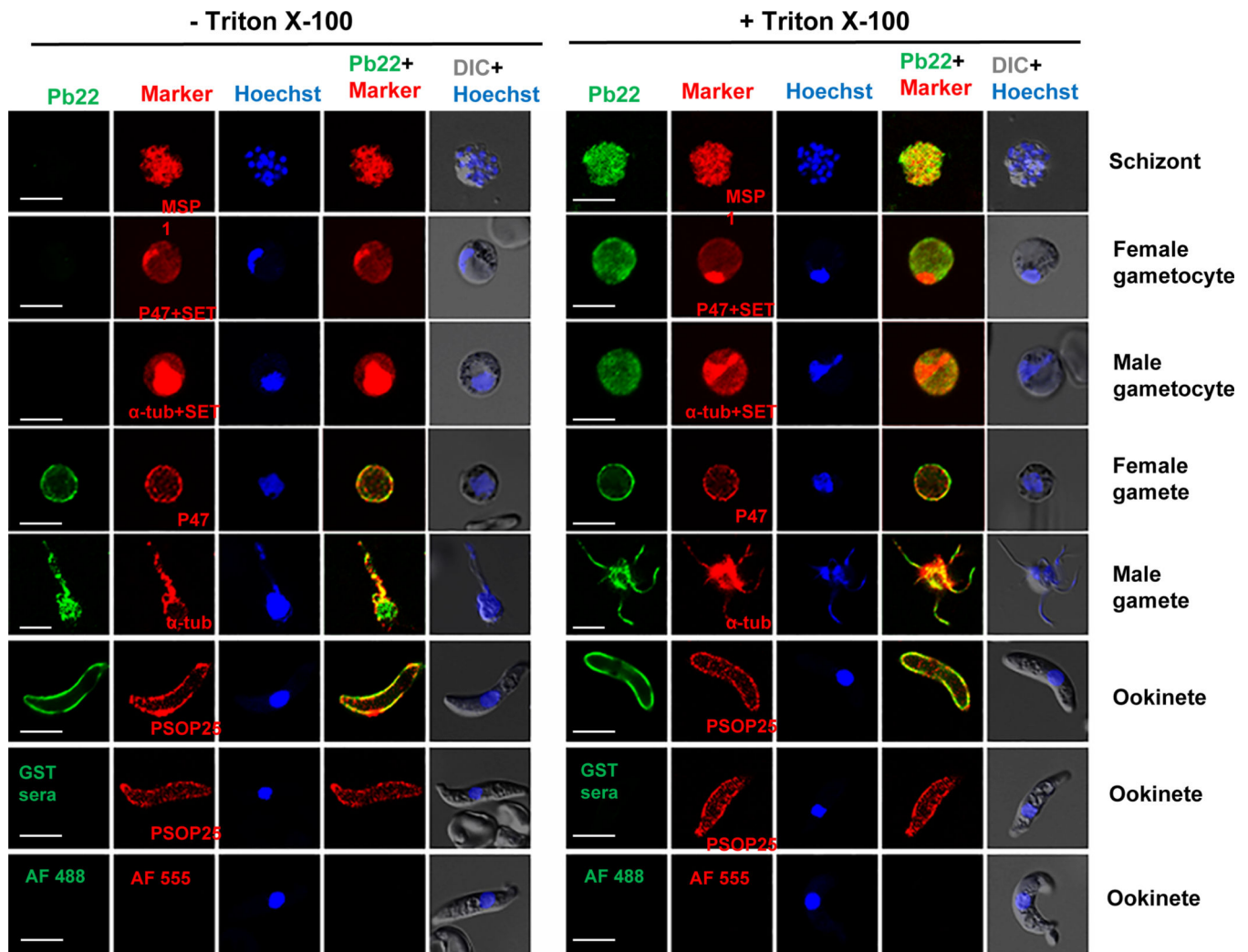


FIGURE 2. Localization of Pb22 protein by IFA.

Representative photomicrographs of fluorescent staining in WT parasites at different developmental stages. The parasites were incubated with anti-rPb22 (1:500) as the primary antibodies (green) without (left panel, -Triton X-100) or with (right panel, +Triton X-100) membrane permeabilization. The parasites were also labeled with antibodies against the markers for different stages (red) after membrane permeabilization. Markers include PbMSP1 for schizonts, P47 for female gametocytes and gametes, α -tubulin II (α -tub) for male gametocytes and gametes, SET for nuclei of gametocytes, and PSOP25 for ookinetes. Alexa Fluor 488 (AF488)-conjugated goat anti-mouse IgG antibodies and Alexa Fluor 555 (AF555)-conjugated goat-anti-rabbit IgG antibodies were used as the secondary antibodies. WT ookinetes labeled with anti-GST sera and with the secondary antibodies only were used as negative controls. Nucleus was stained with Hoechst-33258 (blue). Images were obtained under the same conditions at a magnification of 1000 \times . DIC, differential interference contrast microscopy. Scale bar = 5 μ m.

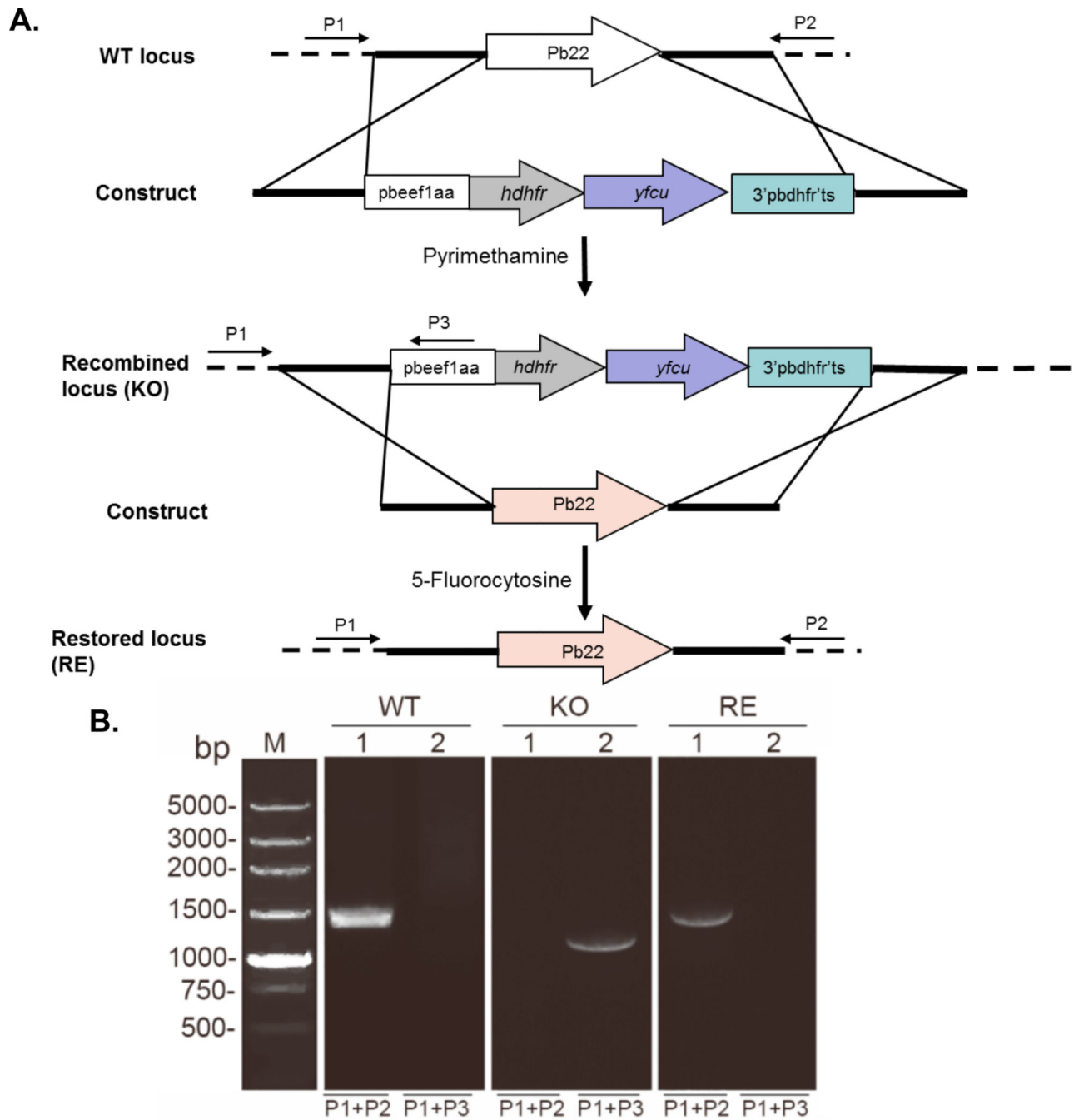


FIGURE 3. Generation of *pb22* knockout and restoration parasites.

A. Schematic representation of the WT *pb22* locus, the transfection construct, the recombined locus and reconstructed locus. The construct contains a *dhfr* expression cassette, directed by the *pbeef1aa* promoter, for selection of the *pb22* knockout (KO) parasites with pyrimethamine and *yfcu* expression cassette for selection of the *pb22*-restored (RE) parasites with 5-fluorocytosine. Primers used to detect *pb22* deletion and restoration are marked. **B.** Diagnostic PCR of WT, KO and RE parasites. Lane 1, primers 1 + 2 (1449 bp); Lane 2: primer 1 + 3 (1180 bp).

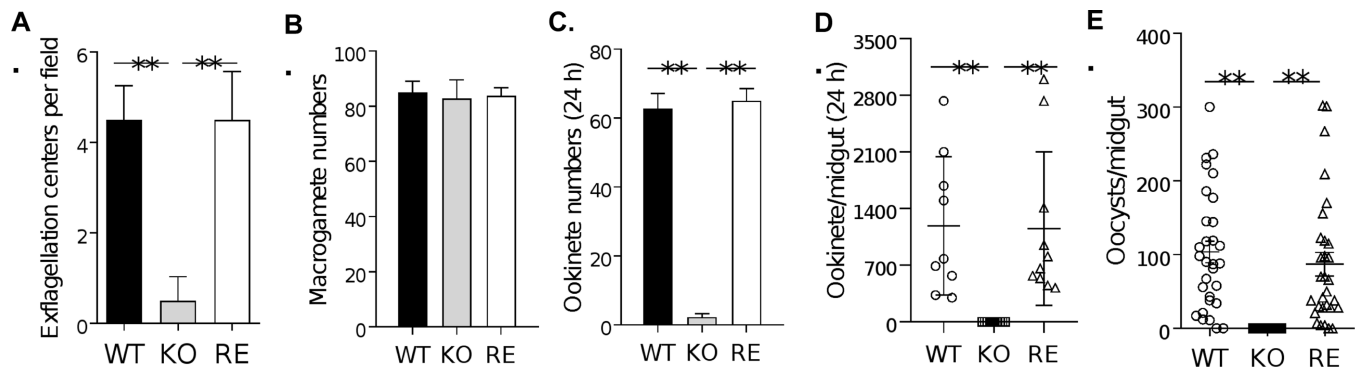


FIGURE 4. Phenotypic analyses of *pb22*-KO and *pb22*-RE lines.

BALB/c mice were infected by intraperitoneal (i.p.) injection with 1×10^6 WT, *pb22*-KO (KO), or *pb22*-RE (RE) parasites. **A.** Exflagellation centers per field at 400 \times magnification. **B.** Macrogamete numbers in 0.01 μ l of infected blood. **C.** Ookinete numbers in an *in vitro* culture assay (the same number of mature gametocytes among WT, KO and RE groups). **D.** Ookinete numbers in midguts 24 h post blood meal. Horizontal bars indicate the mean number (n = 10). **E.** Oocyst numbers per midgut in mosquitoes 12 days after feeding on WT, KO and RE parasites infected mice. Horizontal bars indicate the mean number (n = 30). **D** and **E** are representative graphs of experiment 1 in Table 1. Error bars indicate SD. **, $P < 0.01$. All experiments included three biological replicates.

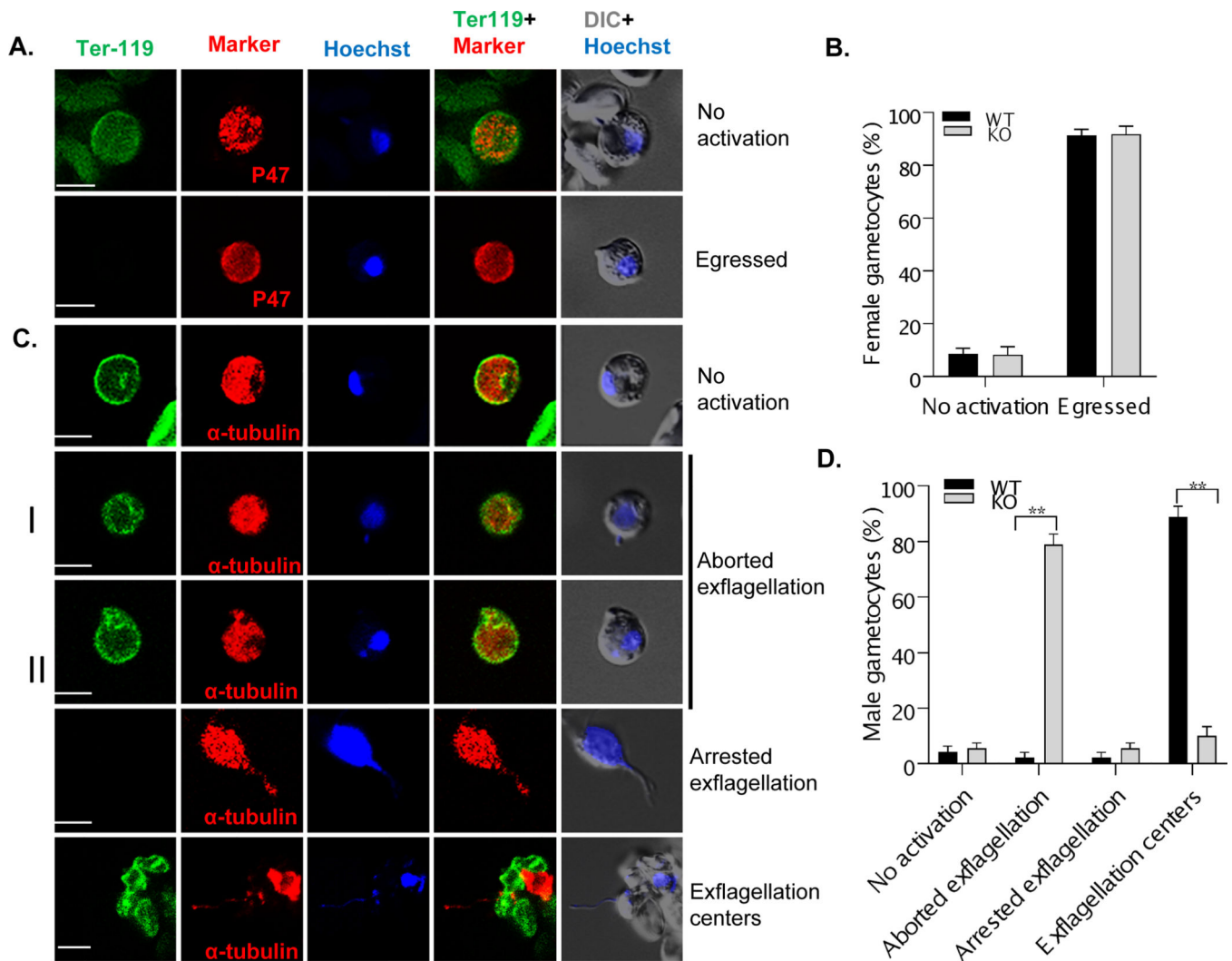


FIGURE 5. Male gametogenesis defects of the *pb22*-KO line determined by IFA.

Blood with mature gametocytes in WT and *pb22*-KO parasites induced for gametogenesis for 15 min were stained with antibodies against P47 (red) for female gamete and α -tubulin II (red) for male gamete, TER-119 (green) for mouse RBC membrane, and Hoechst-33258 (blue) for the nucleus. **A.** Female gametocytes and gametes were stained with P47 and Ter-119. The presence of the RBC membrane indicates ‘No activation’ of the gametocytes, whereas the lack of the RBC membrane indicates ‘egressed’ macrogametes. **B.** The proportions of female gametocytes showing no signs of activation and egressed macrogametes in the blood of mice infected with the WT and *pb22*-KO parasites. **C.** Male gametocytes and gametes were stained with α -tubulin II and Ter-119. Gametocytes were grouped as ‘No activation’ (no DNA replication and RBC membrane present), ‘Aborted exflagellation’ (I – DNA replication occurred, no flagella formed; and II [“bag of worms” phenotype] – flagella formed but RBC membrane present), ‘Arrested exflagellation’ (flagella formed and egressed, but did not interact with RBCs), and ‘Exflagellation centers’. **D.** The proportions of male gametocytes and gametes in C were compared between the WT

(n=30) and *pb22*-KO (n=30) parasites. Data are presented as mean \pm SD. **, $P < 0.01$. Data is a representative graph of three independent experiments.

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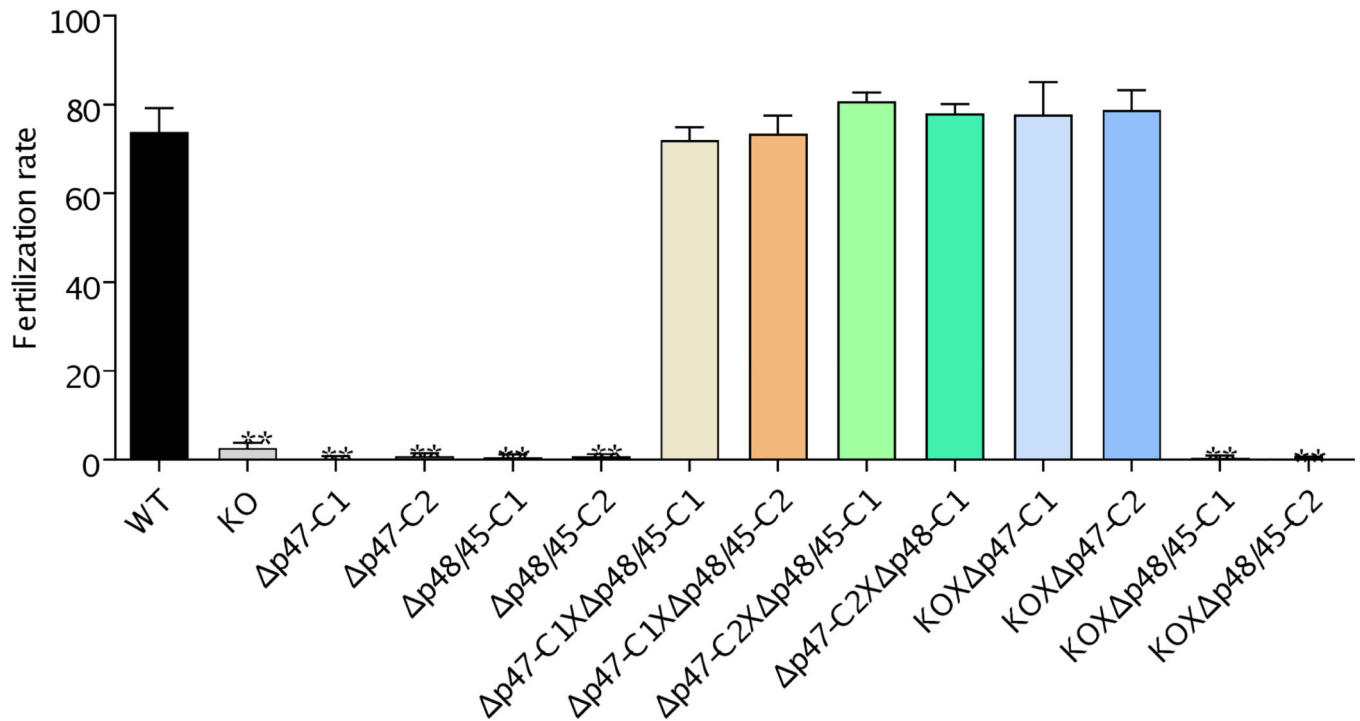


FIGURE 6. Cross-fertilization of KO line with *p47* and *p48/45*.

The fertilization rate was calculated as the percentage of female gametes that develop into mature oocytes. Self-fertilization and cross-fertilization rates of WT, *pb22*-KO, *p47* and *p48/45*. The *pb22*-KO female gametes were fertile and fertilized with *p47* at the level similar to the WT, whereas the *pb22*-KO male gametes were unable to fertilize with male-defective *p48/45* (n=100). Data indicate mean \pm SD. ** indicates $P < 0.01$ between the marked group with the WT group. Data are representative of three independent experiments.

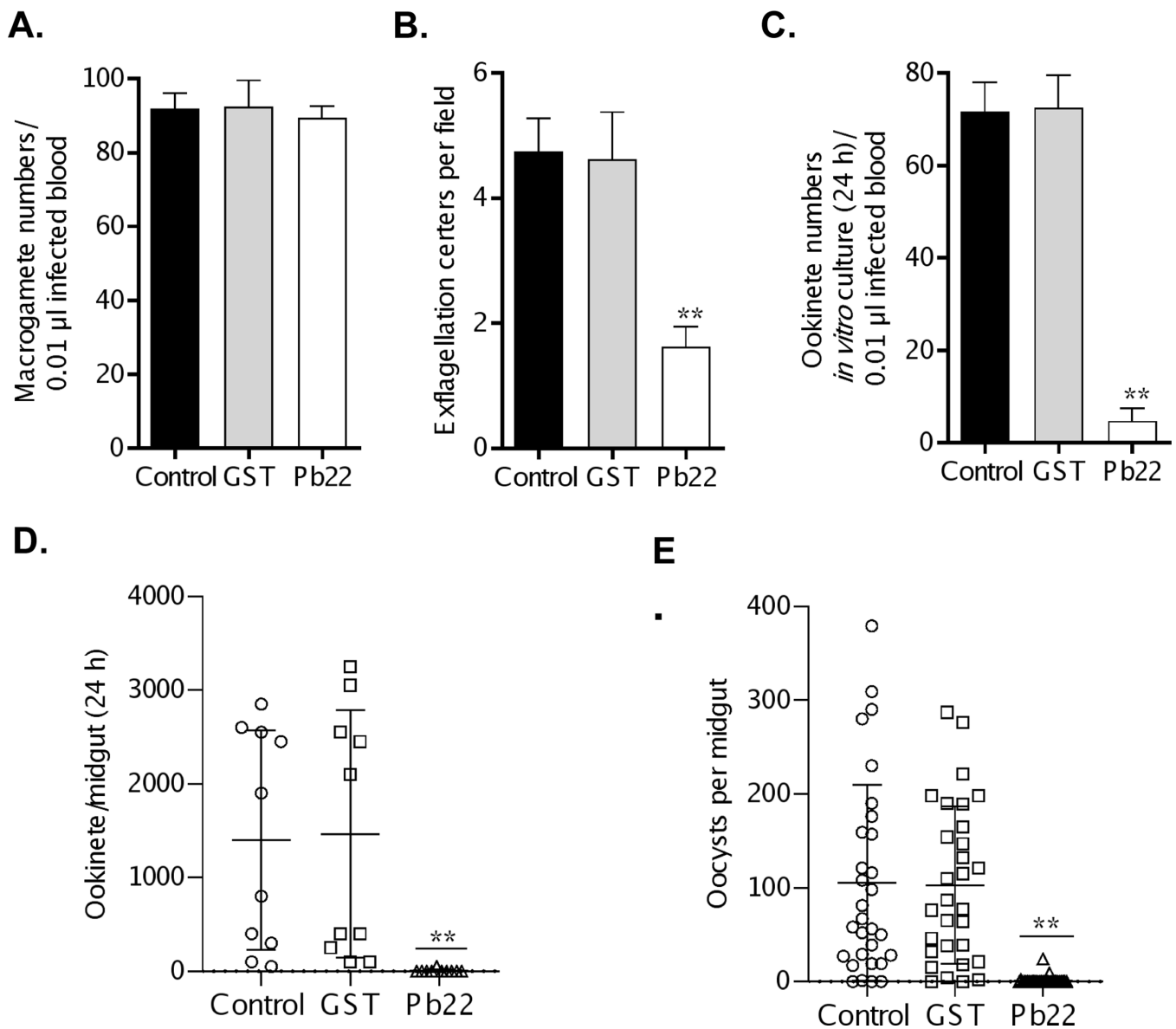


FIGURE 7. *In vivo* transmission-blocking activity of rPb22 protein.

Six mice immunized with rPb22 and GST as well as the adjuvant control groups were infected with *P. berghei* parasites 10 days after the third immunization. **A-C.** Tail blood was collected at 3 days post-infection, and *in vitro* ookinete culture assay was used to obtain the macrogamete numbers in 0.01 μ l of infected blood (**A**), exflagellation centers per field (**B**), and the ookinete numbers in 0.5 μ l of culture (roughly equivalent to 0.01 μ l of infected blood) (**C**). **D.** Representative graph of ookinete numbers in 10 midguts 24 h post blood meal. **E.** Representative graph of oocyst numbers per midgut 12 days post blood meal in all immunization groups. The **D** and **E** are representative graphs of experiment 1 in Table 2. Data are presented as mean \pm SD. ** ($P < 0.01$) represents significance between GST-immunized and rPb22-immunized groups. The results are from three different biological replicates.

Table 1

Ookinete numbers, oocyst density and prevalence in mosquitoes fed on WT, *pb22-KO* and *pb22-restored (pb22-RE)* parasites.

Exp.	Parasite	Ookinete formation in midgut at 24 h			Oocyst formation at 12 days		
		Ookinete-positive midguts (no. dissected)	No. ookinetes/positive midgut (range)	Infection prevalence (no. dissected) ^a	Reduction in prevalence ^b	Oocyst density [Mean (range)] ^c	
1	WT	9 (9)	1187 (300–2730)	93.3% (30)		103.7 (0–300)	
	<i>pb22-KO</i>	0 (10)	0	0 (30)	93.3%	0	
	<i>pb22-RE</i>	10 (10)	1154 (420–3000)	93.3% (30)		86.9 (0–302)	
2	WT	10 (10)	2230 (500–4550)	86.7% (30)		100.7 (0–319)	
	<i>pb22-KO</i>	0 (10)	0	0 (29)	86.7%	0	
	<i>pb22-RE</i>	10 (10)	2065 (750–5300)	96.6% (29)		97.5 (0–303)	
3	WT	10 (10)	2280 (1000–3700)	93.1% (29)		110.6 (0–309)	
	<i>pb22-KO</i>	1 (10)	5 (0–5)	0 (28)	93.1%	0	
	<i>pb22-RE</i>	9 (9)	1985 (600–5050)	86.7% (30)		96.8 (0–302)	

^aThe infection prevalence was calculated by the number of mosquitoes with oocysts / number of mosquitoes dissected in each group × 100%.

^bThe percent reduction in oocyst prevalence was calculated as % mean prevalence wt – % mean prevalence *pb22-KO*.

^cMean number and range of oocysts per mosquito midgut.
Exp., Experiment.

Table 2
Evaluation of transmission-blocking effect of rPb22 immunization in mosquito feeding assays

Experiment	Group	No. midguts dissected	No. ookinetes/midgut	Mean (range)	Infection prevalence (no. dissected) ^b	Reduction in prevalence ^c	Oocyst density Mean (range) ^d	Reduction in oocyst density ^e
1	Control	9	1400	(50–2850)	90% (30)		105.3	(0–379)
	GST	10	1465	(100–3250)	93.3% (30)		102.9	(0–287)
	rPb22	10	5	(0–50)	10% (30)	83.3%	1.2	(0–24)
2	Control	10	1025	(0–4400)	89.7% (29)		103	(0–298)
	GST	10	1055	(0–4700)	100% (28)		110.4	(5–330)
	rPb22	10	30	(0–250)	6.7% (30)	93.3%	6.7	(0–198)
3	Control	10	2300	(350–4000)	96.4% (28)		114.1	(0–309)
	GST	10	2465	(0–4500)	93.1% (29)		106.9	(0–298)
	rPb22	9	45	(0–400)	7.1% (28)	86%	0.4	(0–9)

^aMean number and range of ookinetes per mosquito midgut inside the blood bolus dissected at 24 h after blood feeding.

^bThe prevalence of infection was calculated by the number of mosquitoes with oocysts / total mosquitoes dissected in each group \times 100%.

^cThe percent reduction of prevalence was calculated as % mean prevalence GST – % mean prevalence rPb22.

^dMean number and range of oocysts per mosquito midgut.

^eThe percent reduction in oocyst density was calculated as (mean oocyst density control – mean oocyst density Pb22) / mean oocyst density control \times 100%.