IAI Journals.ASM.org

Immunization with the MAEBL M2 Domain Protects against Lethal *Plasmodium yoelii* Infection

Juliana A. Leite,[a] Daniel Y. Bargieri,[b] Bruna O. Carvalho,[a,c] Letusa Albrecht,[a,d] Stefanie C. P. Lopes,[a,e] Ana Carolina A. V. Kayano,[a] Alessandro S. Farias,[a] Wan Ni Chia,[f] Carla Claser,[f] Benoit Malleret,[f] Bruce Russell,[f*] Catarina Castiñeiras,[a] Leonilda M. B. Santos,[a] Marcelo Brocchi,[a] Gerhard Wunderlich,[b] Irene S. Soares,[g] Mauricio M. Rodrigues,[h†] Laurent Rénia,[f] Fabio T. M. Costa[a]

Department of Genetics, Evolution and Bioagents, University of Campinas—UNICAMP, Campinas, SP, Brazil[a]; Department of Parasitology, University of São Paulo—USP, São Paulo, SP, Brazil[b]; Instituto Oswaldo Cruz, Fundação Oswaldo Cruz—FIOCRUZ, Rio de Janeiro, RJ, Brazil[c]; Instituto Carlos Chagas, Fundação Oswaldo Cruz—FIOCRUZ, Curitiba, PR, Brazil[d]; Instituto Leônidas e Maria Deane, Fundação Oswaldo Cruz—FIOCRUZ, Manaus, AM, Brazil[e]; Singapore Immunology Network, Agency for Science, Technology and Research (A*STAR), Singapore[f]; Department of Clinical and Toxicological Analysis, Pharmaceutical Sciences, University of São Paulo—USP, São Paulo, SP, Brazil[g]; Center for Cellular and Molecular Therapy—CTCMol, Federal University of São Paulo, São Paulo, SP, Brazil[h]

Malaria remains a world-threatening disease largely because of the lack of a long-lasting and fully effective vaccine. MAEBL is a type 1 transmembrane molecule with a chimeric cysteine-rich ectodomain homologous to regions of the Duffy binding-like erythrocyte binding protein and apical membrane antigen 1 (AMA1) antigens. Although MAEBL does not appear to be essential for the survival of blood-stage forms, ectodomains M1 and M2, homologous to AMA1, seem to be involved in parasite attachment to erythrocytes, especially M2. MAEBL is necessary for sporozoite infection of mosquito salivary glands and is expressed in liver stages. Here, the *Plasmodium yoelii* MAEBL-M2 domain was expressed in a prokaryotic vector. C57BL/6J mice were immunized with doses of *P. yoelii* recombinant protein rPyM2-MAEBL. High levels of antibodies, with balanced IgG1 and IgG2c subclasses, were achieved. rPyM2-MAEBL antisera were capable of recognizing the native antigen. Anti-MAEBL antibodies recognized different MAEBL fragments expressed in CHO cells, showing stronger IgM and IgG responses to the M2 domain and repeat region, respectively. After a challenge with *P. yoelii* YM (lethal strain)-infected erythrocytes (IE), up to 90% of the immunized animals survived and a reduction of parasitemia was observed. Moreover, splenocytes harvested from immunized animals proliferated in a dose-dependent manner in the presence of rPyM2-MAEBL. Protection was highly dependent on $CD4^+$, but not $CD8^+$, T cells toward Th1. rPyM2-MAEBL antisera were also able to significantly inhibit parasite development, as observed in *ex vivo P. yoelii* erythrocyte invasion assays. Collectively, these findings support the use of MAEBL as a vaccine candidate and open perspectives to understand the mechanisms involved in protection.

Malaria remains one of the most devastating infectious diseases in intertropical countries, affecting mainly children under the age of 5 years and pregnant women. Approximately 600,000 deaths occur every year (1). People repeatedly exposed to malarial infections in areas where malaria is endemic develop immunity to clinical disease and subsequently to parasitemia (2–5). Antibodies have been shown to be responsible for naturally acquired immunity, since passive transfer of immune IgG from adults can protect against *Plasmodium falciparum* blood-stage infection (2, 6–8), suggesting that a malaria vaccine based on asexual antigens is feasible. Unfortunately, none of the vaccines currently tested achieved a convincing rate of protected individuals (9–11) and the observed protection was often short-lived or highly strain specific (12–16).

The stakes for blood-stage vaccines are even higher when malaria eradication is the goal because the vaccines must not only reduce disease but also reduce the parasitic burden to a degree that reduces transmission (17). Despite considerable efforts, none of the blood-stage vaccine candidates have exhibited satisfactory clinical and sterile protection in field tests (18, 19).

Many of the current vaccine candidates were encountered on the basis of the finding that partly immune individuals possess high titers of antibodies against the antigens tested. Recently, the finding that antibodies against PfRH5 are highly effective in blocking merozoite reinvasion but are rarely detected in significant quantities in semi-immune carriers was reported (20). This suggests that other merozoite-exposed antigens to which no significant response is developed in natural infections may also be effective as vaccines.

MAEBL is a 200-kDa type 1 membrane protein that belongs to the erythrocyte binding protein (*ebl*) family (21–24). It has a carboxy-terminal cysteine-rich region homologous to region IV of the Duffy binding-like (DBL) erythrocyte binding protein. Additionally, the MAEBL amino-terminal cysteine-rich domains (M1 and M2 ligand domains) exhibit partial similarity to apical mem-

Received 2 March 2015 **Returned for modification** 30 March 2015
Accepted 3 July 2015

Accepted manuscript posted online 13 July 2015

Citation Leite JA, Bargieri DY, Carvalho BO, Albrecht L, Lopes SCP, Kayano ACAV, Farias AS, Chia WN, Claser C, Malleret B, Russell B, Castiñeiras C, Santos LMB, Brocchi M, Wunderlich G, Soares IS, Rodrigues MM, Rénia L, Costa FTM. 2015. Immunization with the MAEBL M2 domain protects against lethal *Plasmodium yoelii* infection. Infect Immun 83:3781–3792. doi:10.1128/IAI.00262-15.

Editor: J. H. Adams

Address correspondence to Fabio T. M. Costa, fabiotmc72@gmail.com.

* Present address: Bruce Russell, Department of Microbiology, National University of Singapore—NUS, Singapore.

† Deceased.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00262-15.

doi:10.1128/IAI.00262-15

brane antigen 1 (AMA1) (23, 25). The M1 and M2 domains of *P. yoelii* have been shown to be functionally equivalent to the DBL ligand domain, as they bind to mouse erythrocytes (25).

MAEBL is essential for the development of the parasite during sporozoite infection of mosquito salivary glands (26, 27) and is also expressed in the salivary gland sporozoite and during the late liver stage (28). Weak expression of MAEBL can also be detected in blood-stage merozoite forms, although *maebl* deletion has no impact on blood-stage parasite development (26). Coincidently, only few antibodies are found in naturally infected individuals from areas with low transmission rates (29). The gene for MAEBL is highly conserved between evolutionarily distinct *Plasmodium* species (25). Among the clones of *P. falciparum* and field isolates, there is little amino acid sequence variation in the M1 and M2 domains (21). Because the gene for MAEBL is well conserved and expressed at different parasite stages, MAEBL is considered an interesting potential vaccine candidate (30).

The current knowledge of the mechanisms of and interactions during *Plasmodium* invasion of erythrocytes is still limited, which impairs the development of ways to block this essential step in *Plasmodium* biology. As blocking of erythrocyte invasion strategies is part of the rationale for several vaccines based on merozoite antigens, approaches designed to elucidate the invasion phenomenon might facilitate the validation and identification of potential antigens that could be used as vaccine targets.

In this study, we investigated the immunogenicity of the MAEBL M2 domain of *P. yoelii*. We amplified, cloned, and expressed the MAEBL M2 domain as a prokaryotic fusion protein (rPyM2-MAEBL). Mice received four doses of the recombinant antigen emulsified in Freund's adjuvant. Immunization induced robust protection against a lethal challenge with asexual forms of *P. yoelii* YM. Protection was dependent on $CD4^+$, but not $CD8^+$, T cells toward Th1. By adapting an *ex vivo* invasion assay, we could show that sera from immunized mice inhibited invasion of parasite blood-stage forms. These results demonstrate that MAEBL can be used as an antigen in antimalarial vaccine formulations.

MATERIALS AND METHODS

Parasites and animals. Six- to 7-week-old C57BL/6J mice were purchased from the University of Campinas Animal Center (CEMIB-UNICAMP). Animals were kept in a mouse pathogen-free facility. All experiments and procedures were approved by the Ethical Committee for Animal Research of the University of Campinas (protocol no. 1437-1). Blood forms of *P. yoelii* were obtained after the intraperitoneal (i.p.) injection of thawed, glycerin-frozen stocks.

Generation of the recombinant *P. yoelii* MAEBL M2 domain. DNA fragments encoding the MAEBL M2 domain were obtained by PCR amplification with Platinum *Taq* high-fidelity DNA polymerase (Invitrogen) with *P. yoelii* genomic DNA from infected mice as a template. For amplification of the sequence corresponding to the M2 domain of the MAEBL antigen, we used synthetic oligonucleotides based on the sequence of the *P. yoelii yoelii* gene encoding amino acids 589 to 992 of MAEBL (GenBank accession number AF031886). The wing oligonucleotides used for this were pET28aM2F (5′-CGC GGA TCC CTT AAC AAA TAT ATG AAA TCT AAT GTT GAA CTT-3′) and antisense pIgSPM2R (5′-CTC GAA TTC CTA CGA TTC ATC GGT ATT TCT TGT AG-3′), which contain BamHI and EcoRI sites and were purchased from Invitrogen. The resulting PCR product was purified and digested with both restriction enzymes and inserted into the pET28a vector (Novagen). The resulting plasmid was designated pET28aM2. To verify that the reading frame was correct, inserts were sequenced with vector-specific oligonucleotides covering the entire cloned sequence.

The recombinant protein (rPyM2-MAEBL) was expressed and purified as previously described (31), with slight modifications. *E. coli* BL21(DE3) (Novagen) was transformed with the pET28aM2 plasmid and cultivated at 37°C in Luria-Bertani broth in the presence of 30 μg/ml kanamycin (Sigma). Protein expression was induced at an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 nm with 0.1 mM isopropyl-β-D-thiogalactopyranoside (Invitrogen) for 4 h. After centrifugation, the bacterial pellet was resuspended and sonicated in phosphate-buffered saline (PBS) supplemented with 1 mg/ml lysozyme (Sigma) and 100 mM phenylmethylsulfonyl fluoride (Sigma). The sonicated material was then centrifuged, and the supernatant was separated from the pellet. Under these conditions, rPyM2-MAEBL was found in the insoluble fraction. For solubilization and purification, the pellet was resuspended in a mixture of 100 mM Tris-HCl, 8 M urea, and 100 mM dithiothreitol at pH 8.0. Complete solubilization was achieved by passage of the solution through a 21-gauge needle. The solution was then centrifuged, and the supernatant was applied to a column with N^{2+}-nitrilotriacetic acid-agarose resin (Qiagen). After extensive washing, the recombinant protein was eluted with a buffer at pH 5.0 and the eluted protein was dialyzed against PBS with 2 M urea. Protein expression and concentration were determined by SDS-PAGE and immunoblotting analysis.

Generation of a pDisplay library containing fragments of the MAEBL immunogen. Primers were designed to be in frame, complementary, and specific to different regions of MAEBL without self-annealing sequences with the software Amplify3x. Three different regions (MAEBL-3, MAEBL-4, and MAEBL-5) encompassing the M2, repeat, and C-terminal cysteine regions, respectively, were designed. Six smaller overlapping regions of the MAEBL-3 region (which contained different segments of the M2 region, the interdomain between M2 and the repeats, or the repeats) were designed and then subcloned into the pDisplay vector (Invitrogen) together with MAEBL-4 and MAEBL-5 constructs. For the primer sequences used to clone these fragments of MAEBL, see Table S1 in the supplemental material.

Genes of interest were amplified with Phusion Hot Start II polymerase (Finnzymes) according to the manufacturer's instructions by using the Bio-Rad Peltier thermal cycler. The PCR products were ligated into the XmaI- and SacII (NEB)-linearized pDisplay vector according to the manufacturer's recommendations (In-Fusion Cloning; Clontech) before heat shock transformation into XL10-Gold bacteria (Agilent). Bacterial clones were screened for positive transformants by colony PCR with T7 and BgH primers. They were then sent for sequencing to ensure that the codons were in frame. The plasmids were purified with the Nucleobond AX Endotoxin-Free Midiprep kit (Macherey-Nagel) before transfection. CHO (Chinese hamster ovary) cells were used for the expression of recombinant MAEBL proteins. Cells were grown in F12 complete medium containing 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco) in a 37°C incubator with 5% CO_2. Sterile EDTA diluted to a final concentration of 2 mM in PBS was used to detach transfected cells/stable cell lines. EndoFectin (GeneCopoeia) was used to transfect the recombinant pDisplay plasmid into CHO cells. CHO cells were seeded into 6-cm cell culture dishes 1 day before transfection at a density that would give approximately 70 to 80% confluence on the day of transfection. To transfect the cells, 9 μl of EndoFectin was added dropwise with vortexing to 3 μg of plasmid diluted in F12 complete medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin for 6-cm dishes. The DNA-EndoFectin complex was incubated at room temperature for 25 min before it was added dropwise to the cells. The plates or dishes were swirled gently to distribute the transfection mixture before being returned to the 37°C incubator overnight. The transfection medium was replaced with fresh F12 complete medium the following day.

Immunoblotting analysis. Recombinant protein samples were subjected to SDS-PAGE under denaturing conditions (2-mercaptoethanol).

Proteins were then transferred onto a nitrocellulose membrane (GE Healthcare). Immunoblotting was conducted with an anti-His_6 antibody (Life Technologies) at a 1:1,500 dilution. Antibody binding was detected by incubation with peroxidase-coupled goat anti-mouse IgG (Sigma) at a 1:2,000 dilution. Bands were detected with a solution containing 50 mM Tris-HCl (pH 7.5), 0.6 mg/ml 3,3′-diaminobenzidine, and 1 μl/ml 0.03% H_2O_2 (Sigma) solution.

Immunization regimen. Groups of 6 to 10 C57BL/6J male mice (6 to 7 weeks old) were injected subcutaneously four times at 3-week intervals with 5 μg of rPyM2-MAEBL 1:1 (vol/vol) emulsified in complete Freund's adjuvant (CFA; Sigma) for the first dose or incomplete Freund's adjuvant (IFA) for the subsequent doses. As a control group, animals were injected with 1:1 (vol/vol) adjuvant (CFA and IFA) in PBS. Sera were collected from immunized mice immediately before each dose and 3 weeks after the last dose (see Fig. 2A).

ELISA. Anti-rPyM2-MAEBL antibodies titers were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (32). Plates were coated with recombinant protein at 200 ng/well. Mouse sera were tested at serial dilutions starting at 1:100. A 1:2,000 dilution of peroxidase-coupled goat anti-mouse IgG (Sigma) conjugated with Alexa Fluor 568 was used as the secondary antibody. Specific anti-MAEBL titers were determined as the highest dilution yielding an OD_{492} of >0.1. Detection of IgG subclasses was performed as described above, except that the secondary antibody used was specific for mouse IgG1, IgG2b, and IgG2c (Southern Technologies).

Epitope mapping. Transfected cells (150,000 per well) were transiently transfected and seeded into 96-well plates (Nunc). They were stained with serum diluted in fluorescence-activated cell sorter (FACS) buffer (PBS plus 10% FBS) by incubation for 1 h with agitation at 4°C. The different MAEBL constructs are flanked by a myc epitope at the N terminus and a hemagglutinin (HA) epitope at the C terminus when expressed on the CHO cell surface, and both epitopes were used to assess protein expression on CHO cells with rabbit anti-myc IgG (Upstate) or rabbit anti-HA IgG (Sigma) diluted 1:100. Untransfected parental CHO cells were stained with the same sera and used as a negative control. For determination of serum recognition, the different test sera from mice immunized with MAEBL protein or the control vehicle DMSO were diluted 1:100 and incubated with the cells for 1 h with agitation at 4°C and then washed once with FACS buffer. The secondary antibody, either goat anti-rabbit IgG-Alexa Fluor 488 (Molecular Probes, Invitrogen) diluted 1:500 for checking protein expression or goat anti-mouse IgM- or IgG-Alexa Fluor 488 (Molecular Probes) diluted 1:500 and mixed with propidium iodide diluted to 0.001 mg/ml for the serum screening assay, was incubated for 30 min with agitation at 4°C. Labeled samples were analyzed with the BD Accuri high-throughput flow cytometer (Becton Dickinson). FlowJo (Tree Star Inc.) was used for all flow cytometry analysis. Serum response was determined as the percentage of cells recognized by serum divided by the percentage of cells expressing the construct of interest, as illustrated in the following formula: Serum response = [(% of cells recognized by immune serum − % of cells recognized by naive serum)/(% of protein-of-interest-expressing cells)] × 100%.

The 20% cutoff for positivity was determined as the mean plus 6 standard deviation of the quadruplicate serum recognition values obtained for sera from naive animals. This high threshold was used to reduce the possibility of false-positive results.

Cellular proliferation and cytokine quantification. Ten million splenic cells collected from animals in different immunization groups ($n = 3$) were cultured in a 96-well plate in a final volume of 200 μl. rPyM2-MAEBL protein was added to the culture at a final concentration of 1 or 10 μg/ml. As a control, some wells received concanavalin A (ConA; 50 μg/ml). Lymphocyte proliferation was assessed by the addition of *methyl*-[^{3}H]thymidine ([^{3}H]TdR; GE Healthcare) at 0.5 mCi/well to the splenocytes after 48 h of culture. Splenocytes were cultured for an additional 18 h, and plates were frozen. For later analysis, plates were thawed and the culture was collected on filter paper with a cell harvester. Tritium incorporation was measured as radioactivity with a 1450 MicroBeta TriLux scintillation and luminescence counter (PerkinElmer). The results are expressed as the average of triplicate cultures.

In parallel, culture supernatants were collected after 120 h and the amounts of secreted gamma interferon (IFN-γ), tumor necrosis factor (TNF), interleukin-2 (IL-2), IL-4, and IL-5 were simultaneously detected with the BD Cytometric Bead Array (CBA) Mouse Th1/Th2 Cytokine kit. Cytokine concentrations in each sample were determined with standard curves of known concentrations of all five recombinant proteins. Samples were read in a FACSCalibur flow cytometer (Becton Dickinson). The detection limit of the assay was 0.5 pg/ml.

IF assays. Slides were prepared by collecting blood from BALB/c mice infected with *P. yoelii* YM on day 5 postinfection (p.i.). Selection of mature stages of the parasite was performed with LS-MACS columns (Miltenyi Biotec), and thin blood smears were made, air dried, and stored until immunofluorescence (IF) assay preparation and microscopy analysis. IF assays were performed after the blood smears were fixed with ice-cold acetone for 20 min and air dried. Well diameters were established with a Dako-Pen (Dako), and blocking was performed by 30 min of incubation at 37°C in PBS containing 3% BSA (USB). Pooled sera from the different immunization groups and *P. yoelii* hyperimmune sera were diluted 1:100 in PBS supplemented with 3% BSA, applied to the slides, and incubated for 1 h at 37°C. Slides were washed three times in PBS, incubated with Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen) for 1 h at 37°C in the dark, washed three times in PBS, and then incubated with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Invitrogen) diluted in ultrapure (Millipore) water for 10 min at room temperature. After another round of washing, FluorSave (Calbiochem) was added and the slides were sealed with coverslips. Parasites were visualized with a Nikon TS100 epifluorescence microscope. *P. yoelii* hyperimmune sera were obtained from BALB/c mice infected with *P. yoelii* 17XNL clone 1.1 (nonlethal strain) once a month for a total of 7 months. In parallel, to confirm specificity, we also conducted IF assays with *Leishmania amazonensis* promastigote forms (kindly donated by Selma Giorgio, UNICAMP) and anti-rPyM2-MAEBL antisera.

Mouse challenge and parasitemia assessment. The levels of protection against blood-stage forms were determined by immunized mouse survival of a lethal challenge via i.p. injection of 10^6 *P. yoelii* YM IE. Parasitemia was monitored (see Fig. 2A) between 3 and 10 days p.i. by counting at least 1,000 erythrocytes from thin blood smears stained with the Panótico Rápido kit (Laborclin).

$CD4^+$ and $CD8^+$ T lymphocyte depletion. Groups of 15 C57BL/6J mice were immunized (see Fig. 6A). Three weeks after the fourth dose, five animals each were injected with 0.5 mg of anti-CD4 (clone GK1.5) or anti-CD8 (clone YTS169.4) monoclonal antibody (BioXCell) for depletion of the corresponding lymphocyte population. The *in vivo* depletion efficacy of this antibody dose was determined in a preliminary experiment, as assessed by flow cytometry of $CD8^+$ and $CD4^+$ T cells with specific monoclonal antibodies recognizing different epitopes in the CD4 and CD8 molecules. As a control, five immunized animals were each injected with 0.5 mg of purified unrelated mouse IgG (Sigma). On the following day, animals were challenged with 10^6 *P. yoelii* YM IE (lethal strain). The survival of animals was monitored and parasitemia was evaluated as described previously.

***Ex vivo* invasion assays of *P. yoelii* YM merozoites.** To obtain high levels of reticulocytes to be used as target cells in the invasion assay, reticulocytemia was induced in groups of four to six naive mice by repeated daily retro-orbital bleeding for 6 days. Leukocyte removal from the reticulocyte-enriched blood was achieved after two cycles of CF11 filtration (33).

Infected erythrocytes were enriched and matured to schizonts. Enrichment was achieved by bleeding *P. yoelii* YM-infected mice ($n = 3$) on day 5 p.i. (>60% parasitemia) and isolating mature forms (15 to 18 h of development) by magnetic concentration as previously described (34). A blood smear was made after the procedure to determine the concentration

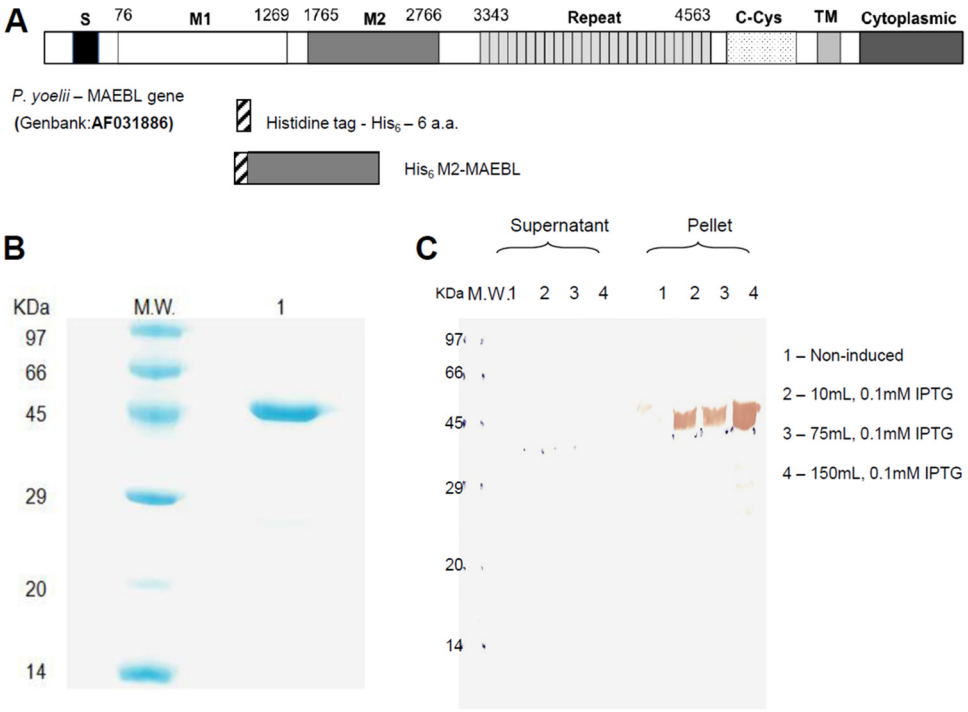


FIG 1 (A) Schematic representation and characterization of the gene for *P. yoelii* MAEBL. Abbreviations: S, signal peptide; C-Cys, cysteine-rich domain; TM, transmembrane region; a.a., amino acids. (B) SDS-PAGE analysis of rPyM2-MAEBL. Lane M.W., molecular size markers (1). Detection of a 45-kDa band corresponding to rPyM2-MAEBL. The lane was loaded with approximately 1 μg of protein and run through a 12% polyacrylamide gel. The gel was further stained with Coomassie blue under denaturing conditions (2-mercaptoethanol). (C) Immunoblot analysis with anti-His antibodies confirms the expression of rPyM2-MAEBL. After visualization on SDS-PAGE, rPyM2-MAEBL was transferred to a nitrocellulose membrane and immunoblot analysis was performed with anti-His antibodies at a 1:1,500 dilution and goat anti-mouse IgG peroxidase antibodies.

of parasites and verify the complete elimination of leukocytes. Next, to synchronize parasites to at least 80% at the schizont stage, the pellet corresponding to the mature forms of *P. yoelii* was resuspended in McCoy's 5A medium (Sigma) supplemented with glucose at 2.4 g/liter, gentamicin (Sigma) at 40 mg/ml, and 20% human AB serum (UNICAMP blood bank), inactivated, and allowed to adsorb to murine red blood cells, which were cultivated for 3 to 6 h in 5% CO_2 at 37°C for maturation.

Finally, invasion assays adapted from an assay for *P. vivax* (35) were performed by mixing *P. yoelii* concentrated schizonts and target cells (enriched reticulocyte-rich blood) at a 1:10 ratio, corresponding to approximately 10% of the initial parasitemia. The mixture was diluted to a 4% hematocrit in 100 μl of McCoy's 5A medium supplemented with 2.4 g/liter glucose and 40 mg/ml gentamicin and then cultured for approximately 20 h in a candle jar (36).

Immunized sera used in the invasion assays were heat inactivated by incubation at 56°C for 40 min. Pooled sera from animals immunized with four doses of rPyM2-MAEBL diluted 1:100 were added to the culture. As controls, the pooled sera of animals injected four times with CFA and IFA at the same dilution were used, and E64 (Sigma) at 100 μM was used as a positive inhibition control because it ensures no schizont rupture. As a negative inhibition control, the assay was performed without the addition of any drug or serum. Freshly infected erythrocytes were determined by light microscopy, and parasitemia of young forms (rings and young trophozoites) was determined as previously described. For details of the standardized methodology used for the *P. yoelii* invasion assays, see Fig. 7.

Statistical analysis. Survival curves of immunized animals after a lethal challenge were evaluated by the log-rank test. Comparison of the means of three or more groups was performed by one-way analysis of variance (ANOVA), and the statistical significance of differences between two groups was assessed by the Bonferroni multiple-comparison test when data assumed a Gaussian distribution, as assessed by the D'Agostino and Pearson normality test. For samples with no Gaussian distribution, group comparison was performed with the Kruskal-Wallis test, followed by Dunn's test. For assessment of the statistical significance of differences between two groups, the Mann-Whitney U test was used. All tests were performed with Prism GraphPad software version 5.0a. The results were considered significant at $P < 0.05$.

RESULTS

Cloning and expression of the MAEBL M2 domain. The region of the MAEBL M2 domain expressed as a recombinant bacterial protein (rPyM2-MAEBL) is depicted in Fig. 1A. rPyM2-MAEBL was expressed as an insoluble polypeptide and was therefore purified from the pellet after solubilization in buffer with 8 M urea after several passages through a needle. SDS-PAGE analysis of the purified product revealed a single band of approximately 45 kDa (Fig. 1B). The recombinant protein generated (rPyM2-MAEBL) was recognized by anti-histidine tag antibodies in the bacterial pellet (Fig. 1C).

Immunogenicity of rPyM2-MAEBL in mice. Two groups of mice ($n = 8$ to 10) were immunized in accordance with the protocol described in Fig. 2A. Sera from immunized animals were collected 3 weeks after each immunization, and antibody titers against rPyM2-MAEBL were measured by ELISA. As shown in Fig. 2B, the antibody titers obtained with one single dose of rPyM2-MAEBL ($\log_{10}$ 4.7 ± 0.51) increased significantly after the second dose in the rPyM2-MAEBL group ($\log_{10}$ 6.0 ± 0.15) and remained high until the fourth dose ($\log_{10}$ 6.2 ± 0.17). As expected, no specific antibodies were detected in the serum of ani-

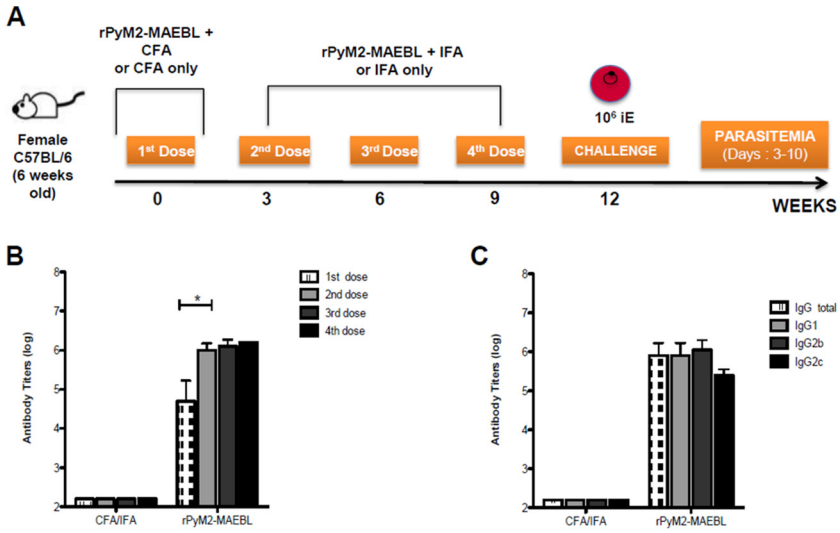


FIG 2 Immunogenicity of rPyM2-MAEBL. (A) Female C57BL/6J mice were immunized four times with 5 μg of rPyM2-MAEBL emulsified in CFA and IFA (1:1, vol/vol) or only with the adjuvants (control group). (B) Induction of anti-MAEBL M2 total IgG responses in mice after the administration of priming and booster doses. Pools of sera from immunized mice after each dose were quantified by ELISA, revealing high titers of antibodies (*, $P < 0.05$ [Kruskal-Wallis test]). (C) IgG subclass determination in mice immunized with rPyM2-MAEBL. The results are expressed as the mean log of the antibody titer ± the standard deviation of triplicates.

mals injected with adjuvant alone. These data demonstrate that rPyM2-MAEBL is highly immunogenic in C57BL/6J mice.

Next, we determined the IgG subclass profile and the IgG1/IgG2c ratio. As shown in Fig. 2C, mice immunized with the recombinant protein produced high titers of IgG1 ($\log_{10}$ 5.9 ± 0.32) and IgG2c ($\log_{10}$ 5.4 ± 0.15), corresponding to an IgG1/IgG2c ratio of 3.16. When tested by flow cytometry against various MAEBL fragments expressed at the CHO cell surface (Fig. 3A), anti-MAEBL IgM and IgG antibodies recognized different fragments in the M2 region and the repeat region of MAEBL. It is interesting that the IgM response was strongest against the M2 region (MAEBL-3 fragments) and the IgG response was strongest against the repeat region (MAEBL-4 fragments) (Fig. 3B). As expected, no responses were observed against the MAEBL-5 fragments, which encode the cysteine and transmembrane domains of MAEBL and were not included in the MAEBL vaccine used in this study.

We next performed IF assays to determine whether the antibodies present in the sera of immunized mice recognized MAEBL in its native form. As shown in Fig. 4, sera from immunized, but not control, mice recognized *P. yoelii* schizont forms. *P. yoelii*-hyperimmune sera recognized schizonts of *P. yoelii*, whereas pooled sera from immunized mice did not recognize the promastigote forms of the parasite *L. amazonensis* used as control for specificity (data not shown).

Immunization with rPyM2-MAEBL protects against a challenge with blood-stage *P. yoelii* YM. In order to explore the protective effect of anti-MAEBL antibodies, immunized animals were challenged with 10^6 blood-stage *P. yoelii* YM (lethal strain) parasites 15 days after the last booster dose. As shown in Fig. 5A, 75% of the animals that received only the adjuvant succumbed to the infection after 6 to 8 days. In contrast, 90% the group immunized with rPyM2-MAEBL were significantly protected ($P = 0.0034$). Moreover, the group of animals that received the recombinant protein exhibited reduced parasite growth (Fig. 5B), demonstrating lower parasitemia than the CFA and IFA (Fig. 5C) group observed on day 5 p.i., the peak of parasitemia. Replicates of immunization experiments were performed (see Fig. S1 in the supplemental material).

$CD4^+$ cells are essential for protection. Protection against the blood-stage forms of the parasite is mediated predominantly by antibodies and $CD4^+$ T cells after vaccination with various antigenic formulations (37, 38). Thus, we investigated the involvement of $CD4^+$ and $CD8^+$ T lymphocytes in the protection of animals immunized with rPyM2-MAEBL before a challenge with *P. yoelii*. To this end, 15 C57BL/6J mice were immunized with the rPyM2-MAEBL protein in accordance with the protocol depicted in Fig. 6A. Three weeks after the fourth dose, five animals immunized with rPyM2-MAEBL were treated with depleting anti-CD4 or anti-CD8 monoclonal antibodies. The next day, the mice were infected with 10^6 *P. yoelii* IE. Control animals ($n = 5$) were injected with purified rat IgG at the same concentration. As shown in Fig. 6B, 100% of the $CD4^+$-depleted animals died between days 5 and 6 p.i. In contrast, animals depleted of $CD8^+$ cells or treated with the IgG control exhibited the same levels of protection as undepleted mice (Fig. 5).

To further characterize the cell-mediated immune response induced by vaccination with rPyM2-MAEBL or with CFA and IFA, splenocytes collected from immunized mice were cultured in the presence of the rPyM2-MAEBL protein (1 or 10 μg/ml) for 18 h. A significant and specific level of cell proliferation was observed in rPyM2-MAEBL splenocyte cultures stimulated with 10 μg/ml of the recombinant protein (Fig. 6C). Splenocytes from animals injected with Freund's adjuvant exhibited lower proliferation levels when stimulated with the rPyM2-MAEBL protein. As expected, cell stimulation with the mitogen ConA led to strong, nonspecific proliferation in both groups. Moreover, spleen cells harvested from mice immunized with rPyM2-MAEBL secreted higher levels of IFN-γ after 120 h than animals injected only with Freund's adjuvant. Additionally, the

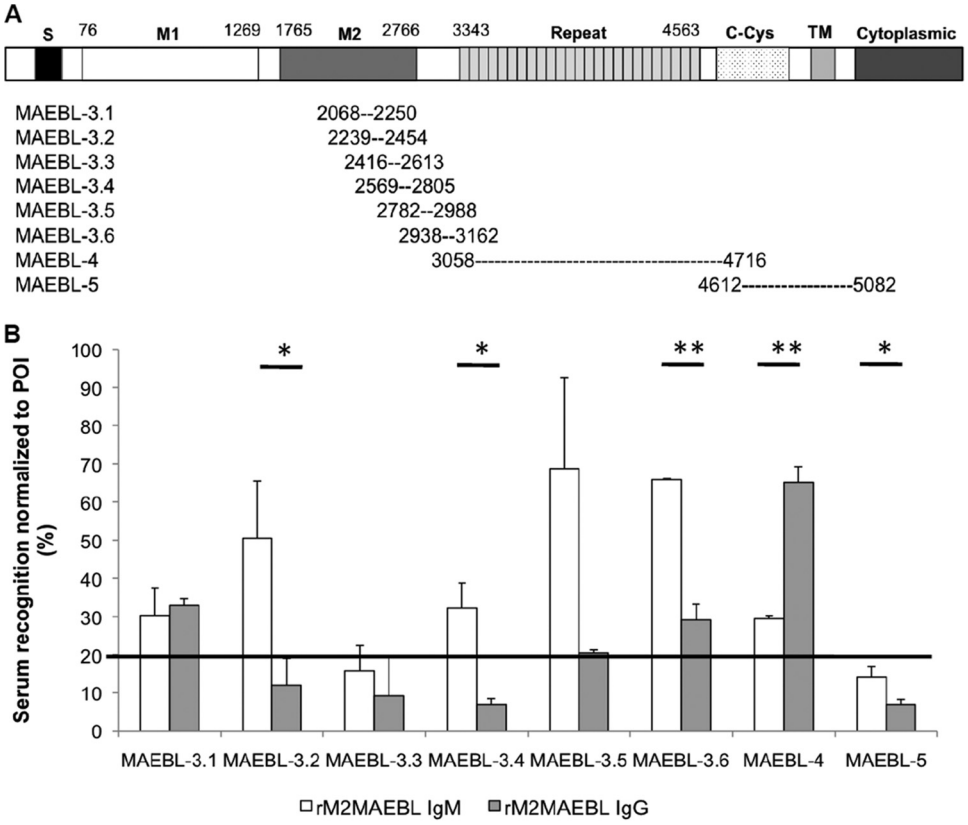


FIG 3 rPyM2-MAEBL-specific IgM and IgG antibodies generated in vaccinated mice recognized multiple epitopes in the MAEBL protein. (A) Localization of various fragments of the MAEBL protein. These fragments were cloned into the pDisplay vector and expressed at the CHO cell surface after transfection. (B) Sera from vaccinated mice were tested on transfected cells by flow cytometry. Serum response was calculated as defined in Materials and Methods. Responses above the cutoff line of 20% were considered positive. *, $P < 0.05$; **, $P < 0.005$.

amount of IFN-γ increased when we raised the concentration of rM2-MAEBL used for stimulation with a maximum of 152.10 pg/ml when stimulated with 10 μg/ml (Fig. 6D). The same dose-dependent pattern was observed for the TNF dosage. Accordingly, after stimulation with 10 μg/ml protein, TNF was detected at 638.38 pg/ml (Fig. 6E). Although the absolute levels of IL-2 were low, with a maximum of 5.36 pg/ml, they were higher than those of animals injected only with Freund's

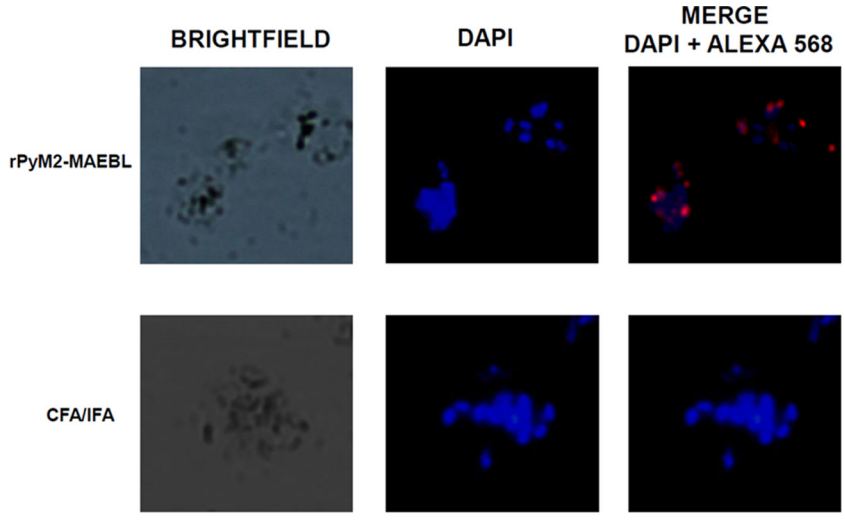
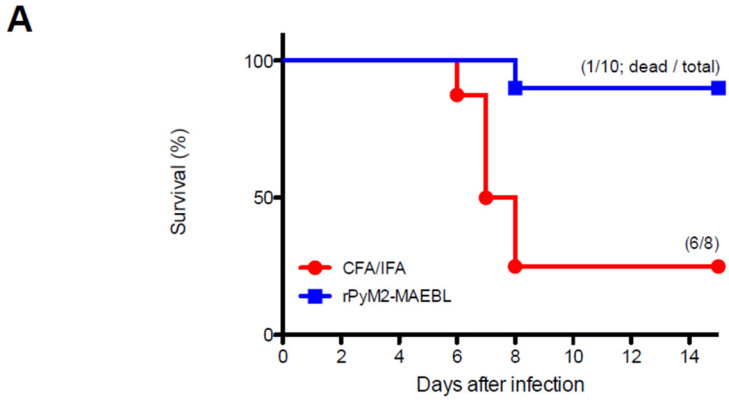
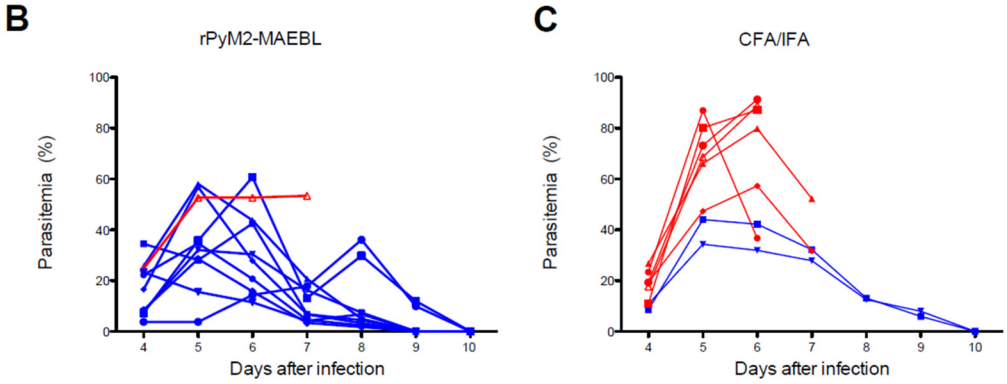


FIG 4 rPyM2-MAEBL-specific antibodies generated in vaccinated mice recognize the native protein expressed by *P. yoelii*. Fixed IFA slides of mature *P. yoelii* schizonts were incubated with pooled sera from mice immunized as indicated in a 1:100 dilution. Bound IgG was stained with Alexa Fluor 568, and the parasite nuclei were stained with DAPI.





D

Means of parasitemia levels per group.

Immunization groups	Days after infection						
	4	5	6	7	8	9	10
CFA/IFA	17,105 ± 6,46	62,58 ± 18,65	64,41 ± 25,18	36,00 ± 10,99	12,88 ± 0,41	7,00 ± 1,41	0
rPyM2-MAEBL	17,48 ± 10,14	34,61 ± 17,53	32,02 ± 15,84	14,70 ± 14,97	9,72 ± 12,52	11,00 ± 1,41	0
Statistics (Mann-Whitney U test)	ns p=0,9682	** p=0,0085	* p= 0,0155	* p=0,0240	N too small	N too small	N too small

FIG 5 (A) Recombinant MAEBL M2 vaccine induces solid protection against a lethal challenge with *P. yoelii* YM. Mice from all of the immunized groups were challenged with 1×10^6 *P. yoelii* YM IE 3 weeks after the fourth immunization. The percent survival and number of dead animals/total number of animals are shown. rPyM2-MAEBL immunization protected 90% of the infected animals, in contrast to CFA and IFA administration ($P = 0.0034$ [log-rank test]). (B, C) Parasitemia levels (percent) of immunized mice were evaluated after infection with *P. yoelii* YM 3 weeks after the fourth dose in the control (C) and rPyM2-MAEBL (B) groups. Immunization with rPyM2-MAEBL exhibited superior infection control and reduced parasite growth after infection with 10^6 *P. yoelii* IE. The red lines represent animals that succumbed to the infection. (D) Summary of the mean parasitemia levels per group ± the standard deviation and *P* values. For assessment of the statistical significance of differences between two groups, the Mann-Whitney U test was used. *, $P < 0.05$; **, $P < 0.01$; ns, no significant difference.

adjuvant (Fig. 6F). The levels of secreted IL-4 and IL-5 were below the detection limit of the assay (data not shown).

Anti-MAEBL M2 antibodies inhibit *P. yoelii* merozoite invasion. Recently, Russell et al. designed a reliable assay to study *P. vivax* invasion (35). Thus, to evaluate the inhibitory potential of anti-PyM2-MAEBL antibodies against *P. yoelii* invasion of red blood cells, we adapted this *ex vivo* invasion assay, which was successfully used for *P. vivax* (35) (Fig. 7). To obtain blood containing a high level of reticulocytes to be used in the invasion assays important for *P. yoelii* YM parasites (39), groups of four to six naive mice were bled daily to induce anemia and thus increase the proportion of reticulocytes. Reticulocyte-rich blood was obtained after 6 days of bleeding, reaching 46% ± 10% reticulocytes (Fig. 8A). Next, this blood was mixed with heat-inactivated pooled

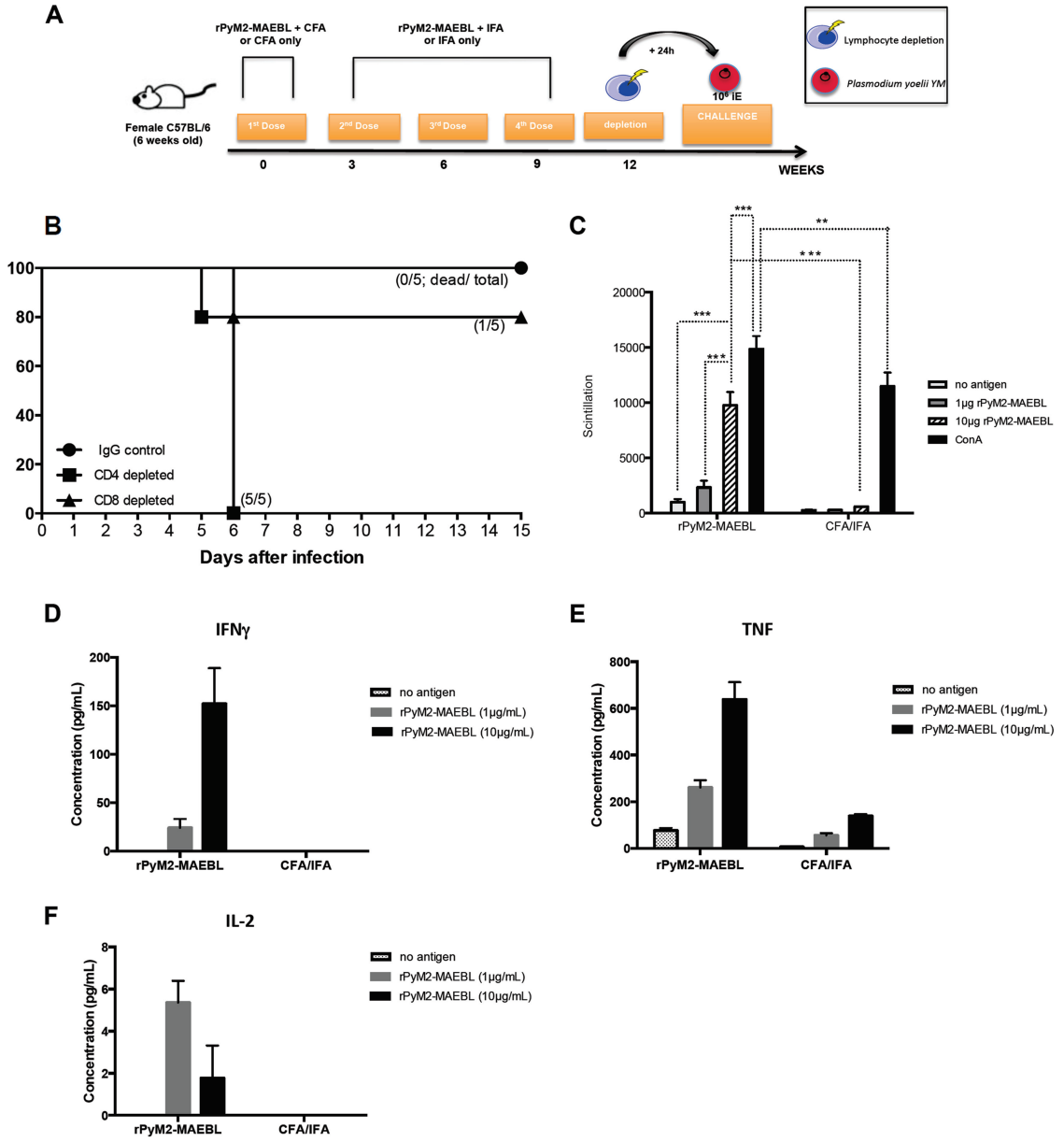


FIG 6 CD4$^+$, but not CD8$^+$, T cells are essential for protection against a lethal challenge. (A) Five mice were immunized with rPyM2-MAEBL, and 3 weeks after the fourth and final immunizing dose, they were injected with an anti-CD4 or anti-CD8 monoclonal antibody and on the following day were infected with 10^6 *P. yoelii* IE. The control animals were injected with control IgG at the same concentration as the others. (B) Survival of immunized mice after CD4$^+$ or CD8$^+$ lymphocyte depletion after a challenge with *P. yoelii* YM. The percent survival and number of dead animals/total number of animals are shown. Statistical analysis was performed by the log-rank test: anti-CD4 versus IgG control, $P = 0.0039$; anti-CD4 versus anti-CD8, $P = 0.0145$. (C) Proliferation of cultured spleen cells from vaccinated mice. Splenocytes were collected from immunized mice and cultured in the presence of the rPyM2-MAEBL protein (1 or 10 μg/ml) for 120 h. As a control, some wells received ConA (50 μg/ml). T and B lymphocyte proliferation was assessed by the addition of [^{3}H]TdR at 0.5 mCi/well. Tritium incorporation was measured as radioactivity with the 1450 MicroBeta TriLux scintillation and luminescence counter (PerkinElmer). The results are expressed as the average of triplicate cultures. Data are representative of three animals per group. Comparison of the means of the groups was done by one-way ANOVA, followed by Bonferroni's multiple-comparison test. **, $P < 0.01$; ***, $P < 0.001$. (D, E, F) The amounts of secreted cytokines in cell culture supernatants were estimated with the BD CBA Th1/Th2 Mouse Cytokine kit. Concentrations of IFN-γ (D), TNF (E), and IL-2 (F) in each sample were determined by using standard curves with known concentrations of all of the recombinants. The detection limit of the assay was 0.5 pg/ml. The results are expressed as the average of triplicate cultures. Data are representative of three animals per group. Statistical analysis was performed with the Kruskal-Wallis test (no significant difference).

sera from immunized animals and a schizont preparation. As a negative control for inhibition, the assay was performed in the presence of nonimmune sera (CFA and IFA) or with medium alone. As a positive control for inhibition and to validate our assay, we added E64 to the culture mixture. E64 is a protease inhibitor that has been shown to prevent schizont rupture and thus prevent merozoite release and invasion. As expected, in E64-treated parasites, 94.91% ± 3.24% inhibition was observed (Fig. 8B). When sera from animals immunized with rPyM2-MAEBL at a 1:100 dilution were used, up to 40% inhibition of merozoite invasion rel-

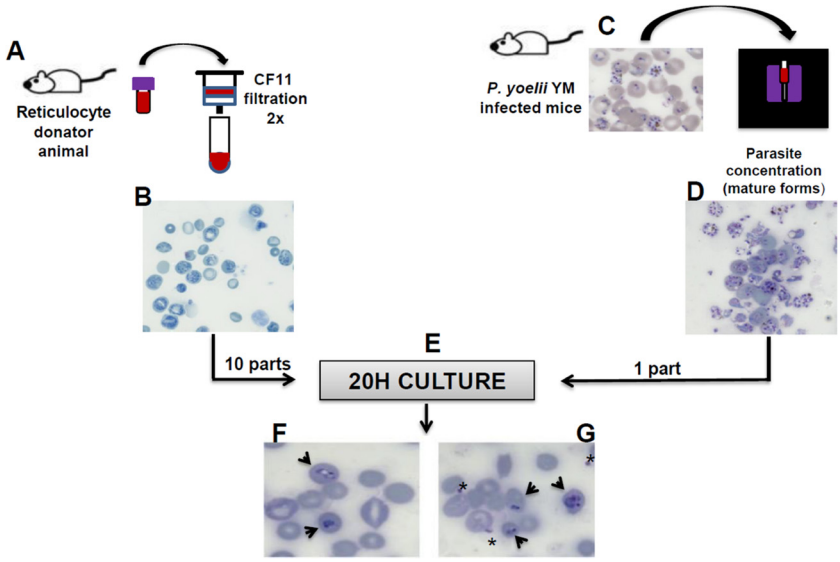


FIG 7 Overview of the *P. yoelii ex vivo* invasion assay methodology used in this study. Thin smears stained with new methylene blue (B) or Giemsa (C, D, F, G), illustrating the key methodological steps of the *ex vivo P. yoelii* invasion assay. (A) Blood from reticulocyte donator animals contained a mixture of normocytes, reticulocytes (35 to 50%), and leukocytes. (B) Reticulocyte-rich blood was collected and submitted to two rounds of filtration on a CF-11 cellulose column for leukocyte depletion. (C) Thin smears demonstrating different stages of parasite maturation typically observed on asynchronous infections with *P. yoelii*. (D) Infected animals were bled on days 5 and 6 p.i. and submitted to an enrichment protocol through midi-MACS LS columns. Enriched parasites were matured for 3 to 6 h. Concentrated reticulocyte target cells (B) and mature *P. yoelii* schizonts (D) were mixed at a ratio of 1:10 for the invasion assay (E). After culturing for 20 h, the invasion assay mixture reveals remnants of ruptured schizonts (asterisks) and newly invaded red blood cells (arrows) (F, G).

ative to the control was observed. This inhibition was significant, as no inhibition was observed when sera from mice injected with CFA and IFA alone were used (Fig. 8B). We also tested sera without complement heat inactivation and did not observe any difference in the invasion pattern (data not shown). This shows that rPyM2-MAEBL-immunized mice produce antibodies that specifically interfere with the invasion processes.

DISCUSSION

Here, we evaluated the immunogenic properties of a recombinant protein based on the M2 domain of the *P. yoelii* MAEBL antigen. We demonstrated that immunization with rPyM2-MAEBL emulsified in Freund's adjuvant generated high levels of specific antibodies capable of recognizing different epitopes in the recombinant protein or its native form in parasite schizonts. These antibodies were able to significantly inhibit *P. yoelii* merozoite invasion *ex vivo*.

In vivo experiments revealed that immunization with four doses of rPyM2-MAEBL was able to reduce the parasite load and prevented death in 90% of the animals challenged with the lethal *P. yoelii* YM strain. Although the immunization data presented in this study correspond to a single experiment, we performed five sets of immunization experiments. In three of them, we challenged mice (for the results, see Fig. S1 in the supplemental material). As shown in Fig. S1A, three doses of rPyM2-MAEBL did not induce a significant protective immune response ($P = 0.5431$ [log-rank test]), in contrast to that seen in animals immunized four times (see Fig. S1B and C in the supplemental material; $P = 0.0031$ and $P = 0.0034$ [log-rank test], respectively). Consequently, we adopted the four-dose strategy in order to maximize the immune response and to ensure that all of the animals had similarly high-titer and mature antibodies. Previous results had shown a similar effect, and it was noted that more booster doses were more effective in generating high antibody levels that can be maintained for long periods (40). Importantly, the proportions of protection levels remained similar between the groups in two experiments, demonstrating the reproducibility of the results (see Fig. S1 in the supplemental material).

The finding that four immunizations were more effective in terms of protection suggested a role for T cells. First, we observed that immunization with rPyM2-MAEBL induced a T-cell response that could be reproduced *in vitro*. Next, depletion experiments demonstrated that $CD4^+$ but not $CD8^+$ cells were also involved in protection. Our data point to a role for $CD4^+$ T cells, but it should be noted that other cell types (i.e., NK T cells) can be positive for the CD4 marker and thus maybe depleted by the GK1.5 antibody that was used here. These cells have been shown to have a role in innate immunity during the early phase of infection with rodent malaria parasites (41). Defining the role of the CD4 cells involved in the protection conferred by rPyM2-MAEBL is important and deserves further studies.

Because depletions were performed before a challenge, when antibodies against rPyM2-MAEBL are already present in the sera of immunized mice, our data indicate that both mechanisms may be complementary for full protection. An analysis of the antibody isotype profile revealed a balanced immune response, as IgG1 and IgG2c were strongly induced and no significant differences among the IgG subclasses were detected. Thus, this experiment could not answer if immunization with rPyM2-MAEBL preferentially induces a Th1- or Th2-type response. To explore this matter further, five Th1/Th2 cytokine levels were measured simultaneously with the BD CBA Th1/Th2 Mouse Cytokine kit. As a result, we detected larger amounts of Th1 cytokines (IFN-γ and IL-2). IL-4 and IL-5 were not detected.

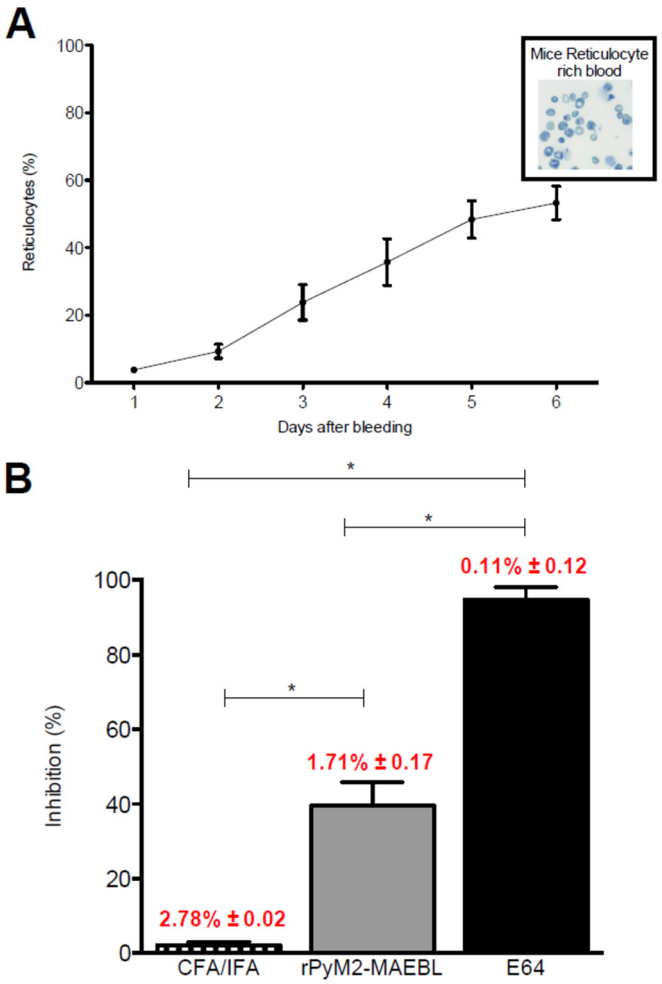


FIG 8 (A) Production of reticulocyte-rich blood to be used as target cells in *ex vivo* cultures of *P. yoelii*. Groups of naive mice were bled for 6 days to induce anemia and thus increase the population of reticulocytes. The percentage of reticulocytes in the blood of animals was assessed daily in smears stained with Accustain reticulocyte stain (Sigma). The day before the onset of bleeding, naive animals exhibited 1 to 3% reticulocytes; after 6 days of bleeding, 35 to 50% reticulocytes was observed. The results are expressed as the mean ± the standard deviation. (B) Sera from mice immunized with rPyM2-MAEBL inhibit invasion of red blood cells by *P. yoelii* YM. Parasites were cultured *in vitro* in the presence of serum (pool of the fourth dose) from mice immunized with rPyM2-MAEBL diluted 1:100. After 20 h of incubation, parasitemia was assessed on thin smears by counting at least 1,000 erythrocytes and is represented by the red values. The results are expressed as the mean ± the standard deviation of three cultures. *, $P < 0.05$ (Kruskal-Wallis test).

Little is known about the invasion pathways of *P. yoelii*, mainly because of the absence of a robust *ex vivo* invasion assay. To date, the elucidation of the *P. yoelii* invasion process has been limited to complicated and time-consuming *in vivo* assays (42). Here we present a simple *ex vivo* assay to study the process of *P. yoelii* invasion that could be helpful in assessing the steps involved in parasite adhesion to and invasion of red blood cells, thereby creating/adapting an important methodology to validate antibodies able to inhibit parasite invasion, a crucial strategy in vaccine design. The invasion assay developed allowed us to test the ability of rPyM2-MAEBL antibodies to inhibit the invasion of red blood cells by *P. yoelii* YM. Sera from animals immunized with rPyM2-MAEBL were able to inhibit invasion by blood-stage forms of *P. yoelii* YM up to 40%. The fact that complete inhibition of invasion by rPyM2-MAEBL antisera was not obtained can be explained by the possibility that either only a subset of the antibodies induced was inhibitory or those merozoites use multiple pathways to invade red blood cells. The latter possibility is supported by data from previous experiments in which MAEBL knockout *P. berghei* ANKA parasites were still able to invade red blood cells *in vivo* (43). Also, *in vitro* invasion assays never show complete inhibition (44, 45) but still point to an inhibitory potential of the developed antibody. From our experiments, it is not clear if MAEBL functions in the primary attachment of merozoites to the red blood cell membrane or in later steps, when the tight junction has been formed.

Although MAEBL was first described in blood-stage merozoites (23), its expression was also detected in midgut and salivary gland sporozoites (26, 46, 47). In addition, antibodies capable of recognizing the *P. yoelii* MAEBL antigen also inhibit parasite development in primary hepatocyte cultures (28). It is also possible that MAEBL contains T-cell epitopes, which may be targetable on infected liver cells. Therefore, MAEBL appears to be strongly associated with protection and may present a promising candidate for a multistage vaccine comprising several components. The data presented here set the foundation for the exploration of M2 domain-based vaccines with other adjuvants and immunization regimens alone or in combination with other malaria antigens for the development of an efficient malaria vaccine.

ACKNOWLEDGMENTS

We dedicate this article to our friend and brilliant scientist, Professor Mauricio Martins Rodrigues (in memoriam), who dedicated his life to understand the immunoprotective mechanism against protozoan parasites in order to develop vaccines to protect those who are/have been neglected.

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), the Instituto Nacional de Ciência e Tecnologia de Vacinas (INCTV), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). J.A.L., B.O.C., L.A., S.C.P.L., A.C.A.V.K., D.Y.B., and A.S.F. were sponsored by FAPESP fellowships. C.C. was supported by a CNPq fellowship. F.T.M.C., I.S.S., M.M.R., L.M.B.S., M.B., and G.W. are CNPq research fellows. We thank Ana Leda F. Longhini for the help with the CBA kit analysis. This study, B.M., C.C., and B.R. received funding from SIgN under the Agency for Science, Technology and Research (A*STAR, Singapore). W.N.C. is supported by a postgraduate scholarship from the Yong Loo Lin School of Medicine, National University of Singapore. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. **World Health Organization.** 2014. World malaria report 2014. World Health Organization, Geneva, Switzerland. http://www.who.int/malaria/publications/world_malaria_report_2014/wmr-2014-no-profiles.pdf.
2. **Doolan DL, Dobano C, Baird JK.** 2009. Acquired immunity to malaria. Clin Microbiol Rev **22:**13–36. http://dx.doi.org/10.1128/CMR.00025-08.
3. **Langhorne J, Ndungu FM, Sponaas AM, Marsh K.** 2008. Immunity to malaria: more questions than answers. Nat Immunol **9:**725–732. http://dx.doi.org/10.1038/ni.f.205.
4. **Marsh K, Kinyanjui S.** 2006. Immune effector mechanisms in malaria. Parasite Immunol **28:**51–60. http://dx.doi.org/10.1111/j.1365-3024.2006.00808.x.
5. **Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, Garver LS, Miller LH, Barillas-Mury C, Pierce SK.** 2014. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious dis-

ease. Annu Rev Immunol **32:**157–187. http://dx.doi.org/10.1146/annurev-immunol-032713-120220.
6. **McGregor IA.** 1964. The passive transfer of human malarial immunity. Am J Trop Med Hyg **13:**237–239.
7. **Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, Foucault C, Chongsuphajaisiddhi T, Druilhe P.** 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am J Trop Med Hyg **45:**297–308.
8. **Cohen S, McGregor IA, Carrington S.** 1961. Gamma-globulin and acquired immunity to human malaria. Nature **192:**733–737. http://dx.doi.org/10.1038/192733a0.
9. **Heppner DG.** 2013. The malaria vaccine—status quo 2013. Travel Med Infect Dis **11:**2–7. http://dx.doi.org/10.1016/j.tmaid.2013.01.006.
10. **Vaughan AM, Kappe SH.** 2012. Malaria vaccine development: persistent challenges. Curr Opin Immunol **24:**324–331. http://dx.doi.org/10.1016/j.coi.2012.03.009.
11. **Arama C, Troye-Blomberg M.** 2014. The path of malaria vaccine development: challenges and perspectives. J Intern Med **275:**456–466. http://dx.doi.org/10.1111/joim.12223.
12. **Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Ouattara A, Kone AK, Guindo AB, Traore K, Traore I, Kouriba B, Diallo DA, Diarra I, Daou M, Dolo A, Tolo Y, Sissoko MS, Niangaly A, Sissoko M, Takala-Harrison S, Lyke KE, Wu Y, Blackwelder WC, Godeaux O, Vekemans J, Dubois MC, Ballou WR, Cohen J, Thompson D, Dube T, Soisson L, Diggs CL, House B, Lanar DE, Dutta S, Heppner DG, Jr, Plowe CV.** 2011. A field trial to assess a blood-stage malaria vaccine. N Engl J Med **365:**1004–1013. http://dx.doi.org/10.1056/NEJMoa1008115.
13. **Sagara I, Ellis RD, Dicko A, Niambele MB, Kamate B, Guindo O, Sissoko MS, Fay MP, Guindo MA, Kante O, Saye R, Miura K, Long C, Mullen GE, Pierce M, Martin LB, Rausch K, Dolo A, Diallo DA, Miller LH, Doumbo OK.** 2009. A randomized and controlled phase 1 study of the safety and immunogenicity of the AMA1-C1/Alhydrogel + CPG 7909 vaccine for Plasmodium falciparum malaria in semi-immune Malian adults. Vaccine **27:**7292–7298. http://dx.doi.org/10.1016/j.vaccine.2009.10.087.
14. **Sheehy SH, Douglas AD, Draper SJ.** 2013. Challenges of assessing the clinical efficacy of asexual blood-stage Plasmodium falciparum malaria vaccines. Hum Vaccin Immunother **9:**1831–1840. http://dx.doi.org/10.4161/hv.25383.
15. **Sheehy SH, Duncan CJ, Elias SC, Choudhary P, Biswas S, Halstead FD, Collins KA, Edwards NJ, Douglas AD, Anagnostou NA, Ewer KJ, Havelock T, Mahungu T, Bliss CM, Miura K, Poulton ID, Lillie PJ, Antrobus RD, Berrie E, Moyle S, Gantlett K, Colloca S, Cortese R, Long CA, Sinden RE, Gilbert SC, Lawrie AM, Doherty T, Faust SN, Nicosia A, Hill AV, Draper SJ.** 2012. ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans. Mol Ther **20:**2355–2368. http://dx.doi.org/10.1038/mt.2012.223.
16. **Sirima SB, Cousens S, Druilhe P.** 2011. Protection against malaria by MSP3 candidate vaccine. N Engl J Med **365:**1062–1064. http://dx.doi.org/10.1056/NEJMc1100670.
17. **Kappe SH, Vaughan AM, Boddey JA, Cowman AF.** 2010. That was then but this is now: malaria research in the time of an eradication agenda. Science **328:**862–866. http://dx.doi.org/10.1126/science.1184785.
18. **Ellis RD, Sagara I, Doumbo O, Wu Y.** 2010. Blood stage vaccines for Plasmodium falciparum: current status and the way forward. Hum Vaccin **6:**627–634. http://dx.doi.org/10.4161/hv.6.8.11446.
19. **Chia WN, Goh YS, Rénia L.** 2014. Novel approaches to identify protective malaria vaccine candidates. Front Microbiol **5:**586.
20. **Douglas AD, Williams AR, Illingworth JJ, Kamuyu G, Biswas S, Goodman AL, Wyllie DH, Crosnier C, Miura K, Wright GJ, Long CA, Osier FH, Marsh K, Turner AV, Hill AV, Draper SJ.** 2011. The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody. Nat Commun **2:**601. http://dx.doi.org/10.1038/ncomms1615.
21. **Blair PL, Kappe SH, Maciel JE, Balu B, Adams JH.** 2002. *Plasmodium falciparum* MAEBL is a unique member of the *ebl* family. Mol Biochem Parasitol **122:**35–44. http://dx.doi.org/10.1016/S0166-6851(02)00067-1.
22. **Kappe SH, Curley GP, Noe AR, Dalton JP, Adams JH.** 1997. Erythrocyte binding protein homologues of rodent malaria parasites. Mol Biochem Parasitol **89:**137–148. http://dx.doi.org/10.1016/S0166-6851(97)00113-8.
23. **Kappe SH, Noe AR, Fraser TS, Blair PL, Adams JH.** 1998. A family of chimeric erythrocyte binding proteins of malaria parasites. Proc Natl Acad Sci U S A **95:**1230–1235. http://dx.doi.org/10.1073/pnas.95.3.1230.
24. **Noe AR, Adams JH.** 1998. Plasmodium yoelii YM MAEBL protein is coexpressed and colocalizes with rhoptry proteins. Mol Biochem Parasitol **96:**27–35. http://dx.doi.org/10.1016/S0166-6851(98)00084-X.
25. **Michon P, Stevens JR, Kaneko O, Adams JH.** 2002. Evolutionary relationships of conserved cysteine-rich motifs in adhesive molecules of malaria parasites. Mol Biol Evol **19:**1128–1142. http://dx.doi.org/10.1093/oxfordjournals.molbev.a004171.
26. **Kariu T, Yuda M, Yano K, Chinzei Y.** 2002. MAEBL is essential for malarial sporozoite infection of the mosquito salivary gland. J Exp Med **195:**1317–1323. http://dx.doi.org/10.1084/jem.20011876.
27. **Saenz FE, Balu B, Smith J, Mendonca SR, Adams JH.** 2008. The transmembrane isoform of Plasmodium falciparum MAEBL is essential for the invasion of Anopheles salivary glands. PLoS One **3:**e2287. http://dx.doi.org/10.1371/journal.pone.0002287.
28. **Preiser P, Renia L, Singh N, Balu B, Jarra W, Voza T, Kaneko O, Blair P, Torii M, Landau I, Adams JH.** 2004. Antibodies against MAEBL ligand domains M1 and M2 inhibit sporozoite development in vitro. Infect Immun **72:**3604–3608. http://dx.doi.org/10.1128/IAI.72.6.3604-3608.2004.
29. **Medeiros MM, Fotoran WL, dalla Martha RC, Katsuragawa TH, Pereira da Silva LH, Wunderlich G.** 2013. Natural antibody response to Plasmodium falciparum merozoite antigens MSP5, MSP9 and EBA175 is associated to clinical protection in the Brazilian Amazon. BMC Infect Dis **13:**608. http://dx.doi.org/10.1186/1471-2334-13-608.
30. **Singh N, Preiser P, Renia L, Balu B, Barnwell J, Blair P, Jarra W, Voza T, Landau I, Adams JH.** 2004. Conservation and developmental control of alternative splicing in *maebl* among malaria parasites. J Mol Biol **343:**589–599. http://dx.doi.org/10.1016/j.jmb.2004.08.047.
31. **Bargieri DY, Leite JA, Lopes SC, Sbrogio-Almeida ME, Braga CJ, Ferreira LC, Soares IS, Costa FT, Rodrigues MM.** 2010. Immunogenic properties of a recombinant fusion protein containing the C-terminal 19 kDa of Plasmodium falciparum merozoite surface protein-1 and the innate immunity agonist FliC flagellin of Salmonella typhimurium. Vaccine **28:**2818–2826. http://dx.doi.org/10.1016/j.vaccine.2010.02.004.
32. **Barbedo MB, Ricci R, Jimenez MC, Cunha MG, Yazdani SS, Chitnis CE, Rodrigues MM, Soares IS.** 2007. Comparative recognition by human IgG antibodies of recombinant proteins representing three asexual erythrocytic stage vaccine candidates of Plasmodium vivax. Mem Inst Oswaldo Cruz **102:**335–339. http://dx.doi.org/10.1590/S0074-02762007005000040.
33. **Sriprawat K, Kaewpongsri S, Suwanarusk R, Leimanis ML, Lek-Uthai U, Phyo AP, Snounou G, Russell B, Renia L, Nosten F.** 2009. Effective and cheap removal of leukocytes and platelets from Plasmodium vivax infected blood. Malar J **8:**115. http://dx.doi.org/10.1186/1475-2875-8-115.
34. **Trang DT, Huy NT, Kariu T, Tajima K, Kamei K.** 2004. One-step concentration of malarial parasite-infected red blood cells and removal of contaminating white blood cells. Malar J **3:**7. http://dx.doi.org/10.1186/1475-2875-3-7.
35. **Russell B, Suwanarusk R, Borlon C, Costa FT, Chu CS, Rijken MJ, Sriprawat K, Warter L, Koh EG, Malleret B, Colin Y, Bertrand O, Adams JH, D'Alessandro U, Snounou G, Nosten F, Renia L.** 2011. A reliable ex vivo invasion assay of human reticulocytes by Plasmodium vivax. Blood **118:**e74–81. http://dx.doi.org/10.1182/blood-2011-04-348748.
36. **Trager W, Jensen JB.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675. http://dx.doi.org/10.1126/science.781840.
37. **Amante FH, Crewther PE, Anders RF, Good MF.** 1997. A cryptic T cell epitope on the apical membrane antigen 1 of Plasmodium chabaudi adami can prime for an anamnestic antibody response: implications for malaria vaccine design. J Immunol **159:**5535–5544.
38. **Hirunpetcharat C, Tian JH, Kaslow DC, van Rooijen N, Kumar S, Berzofsky JA, Miller LH, Good MF.** 1997. Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1[19]) of Plasmodium yoelii expressed in Saccharomyces cerevisiae: correlation of protection with antigen-specific antibody titer, but not with effector $CD4^+$ T cells. J Immunol **159:**3400–3411.
39. **Fahey JR, Spitalny GL.** 1984. Virulent and nonvirulent forms of *Plasmodium yoelii* are not restricted to growth within a single erythrocyte type. Infect Immun **44:**151–156.
40. **Jeamwattanalert P, Mahakunkijcharoen Y, Kittigul L, Mahannop P, Pichyangkul S, Hirunpetcharat C.** 2007. Long-lasting protective immune response to the 19-kilodalton carboxy-terminal fragment of *Plasmodium*

yoelii merozoite surface protein 1 in mice. Clin Vaccine Immunol **14:**342–347. http://dx.doi.org/10.1128/CVI.00397-06.

41. **Stevenson MM, Riley EM.** 2004. Innate immunity to malaria. Nat Rev Immunol **4:**169–180. http://dx.doi.org/10.1038/nri1311.
42. **Swardson-Olver CJ, Dawson TC, Burnett RC, Peiper SC, Maeda N, Avery AC.** 2002. Plasmodium yoelii uses the murine Duffy antigen receptor for chemokines as a receptor for normocyte invasion and an alternative receptor for reticulocyte invasion. Blood **99:**2677–2684. http://dx.doi.org/10.1182/blood.V99.8.2677.
43. **Fu J, Saenz FE, Reed MB, Balu B, Singh N, Blair PL, Cowman AF, Adams JH.** 2005. Targeted disruption of *maebl* in *Plasmodium falciparum*. Mol Biochem Parasitol **141:**113–117. http://dx.doi.org/10.1016/j.molbiopara.2004.12.017.
44. **Kocken CH, Narum DL, Massougbodji A, Ayivi B, Dubbeld MA, van der Wel A, Conway DJ, Sanni A, Thomas AW.** 2000. Molecular characterisation of Plasmodium reichenowi apical membrane antigen-1 (AMA-1), comparison with P. falciparum AMA-1, and antibody-mediated inhibition of red cell invasion. Mol Biochem Parasitol **109:**147–156. http://dx.doi.org/10.1016/S0166-6851(00)00250-4.
45. **Woehlbier U, Epp C, Hackett F, Blackman MJ, Bujard H.** 2010. Antibodies against multiple merozoite surface antigens of the human malaria parasite Plasmodium falciparum inhibit parasite maturation and red blood cell invasion. Malar J **9:**77. http://dx.doi.org/10.1186/1475-2875-9-77.
46. **Kappe SH, Gardner MJ, Brown SM, Ross J, Matuschewski K, Ribeiro JM, Adams JH, Quackenbush J, Cho J, Carucci DJ, Hoffman SL, Nussenzweig V.** 2001. Exploring the transcriptome of the malaria sporozoite stage. Proc Natl Acad Sci U S A **98:**9895–9900. http://dx.doi.org/10.1073/pnas.171185198.
47. **Ghai M, Dutta S, Hall T, Freilich D, Ockenhouse CF.** 2002. Identification, expression, and functional characterization of MAEBL, a sporozoite and asexual blood stage chimeric erythrocyte-binding protein of Plasmodium falciparum. Mol Biochem Parasitol **123:**35–45. http://dx.doi.org/10.1016/S0166-6851(02)00123-8.