Synergistic malaria vaccine combinations identified by systematic antigen screening

Leyla Y. Bustamante,1,2, Gareth T. Powell,1,3, Yen-Chun Lin, Michael D. Macklin, Nadia Cross, Alison Kemp, Paula Cawkili, Theo Sanderson, Cecile Crosnier, Nicole Muller-Sienert, Ogobara K. Doumbou, Boubacar Traore, Peter D. Crompton, Pietro Cicuta, Tuan M. Tran, Gavin J. Wright, and Julian C. Rayner

Malaria Programme, Wellcome Trust Sanger Institute, Cambridge CB10 1SA, United Kingdom; 3Cell Surface Signalling Laboratory, Wellcome Trust Sanger Institute, Cambridge CB10 1SA, United Kingdom; Cavendish Laboratory, University of Cambridge, Cambridge CB3 0HE, United Kingdom; Division of Infectious Diseases, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202; 4Malaria Research and Training Centre, Department of Epidemiology of Parasitic Diseases, International Centre of Excellence in Research, University of Sciences, Techniques and Technologies of Bamako, Bamako, Mali; and Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852

Edited by Rino Rappuoli, GlaxoSmithKline Vaccines, Siena, Italy, and approved September 25, 2017 (received for review February 24, 2017)

A highly effective vaccine would be a valuable weapon in the drive toward malaria elimination. No such vaccine currently exists, and only a handful of the hundreds of potential candidates in the parasite genome have been evaluated. In this study, we systematically evaluated 29 antigens likely to be involved in erythrocyte invasion, an essential developmental stage during which the malaria parasite is vulnerable to antibody-mediated inhibition. Testing antigens alone and in combination identified several strain-transcending targets that had synergistic combinatorial effects in vitro, while studies in an endemic population revealed that combinations of the same antigens were associated with protection from febrile malaria. Video microscopy established that the most effective combinations targeted multiple discrete stages of invasion, suggesting a mechanistic explanation for synergy. Overall, this study both identifies specific antigen combinations for high-priority clinical testing and establishes a generalizable approach that is more likely to produce effective vaccines.

malaria | vaccine | Plasmodium falciparum | antigen combinations | erythrocyte invasion

Malaria caused by Plasmodium falciparum parasites remains one of the most significant global public health challenges, with more than 200 million cases and 438,000 deaths in 2015 (1). There has recently been significant progress in reducing malaria mortality (2), but the emergence and spread of parasites resistant to current frontline antimalarial artemisinin (3) threaten current control methods and emphasize the need for novel control and intervention tools, such as an effective vaccine. Malaria vaccine development has been challenging, with only one vaccine, RTS,S (Mosquirix), reaching phase III trials, where it had limited, albeit consistent, efficacy (4). While the WHO has recommended that RTS,S be advanced to large-scale pilots in Africa, the well-established partial efficacy coupled with concerns about strain-specific responses (5) makes identifying additional components to include in a second-generation P. falciparum vaccine an urgent priority.

Two significant challenges confront P. falciparum antigen identification—the complexity of the parasite life cycle, which presents a large number of potential targets, and the depth of genomic diversity across global parasite populations (6), which makes the development of strain-transcending protection difficult. Given these twin challenges, an effective second-generation vaccine will almost certainly need to target multiple components simultaneously (7). Despite this fact, malaria vaccine development has so far primarily focused on a very limited number of targets, leaving the vast majority of potential candidates encoded by the >5,000-gene P. falciparum genome unexplored (8). The search for vaccines targeting erythrocyte invasion is a microcosm of this broader challenge. Erythrocyte invasion, the process by which P. falciparum merozoites recognize, form protein–protein interactions with, and then actively invade human erythrocytes, is essential for parasite survival and is the only window during blood stage development when the parasite is extracellular and therefore exposed to antibody-mediated inhibition. It is also a very complex process, potentially involving more than 400 genes, including more than 100 that may encode for surface-exposed proteins (9). Until now, however, invasion-blocking vaccines have focused on only a handful of targets, which not coincidentally were also among the first P. falciparum genes ever sequenced (8).

A reverse vaccinology approach will be needed to identify new targets from this long candidate list, incorporating systematic screens of a larger number of antigens and using data from multiple sources to identify potentially synergistic combinations. We have previously used a mammalian expression system to express a library of entire ectodomains, up to 200 kDa in length, from merozoite-expressed Plasmodium proteins that are thought to be involved in erythrocyte recognition and entry (10). Expressing full-length

Significance

Malaria still kills hundreds of thousands of children each year. Malaria vaccine development is complicated by high levels of parasite genetic diversity, which makes single target vaccines vulnerable to the development of variant-specific immunity. To overcome this hurdle, we systematically screened a panel of 29 blood-stage antigens from the most deadly human malaria parasite, Plasmodium falciparum. We identified several targets that were able to inhibit erythrocyte invasion in two genetically diverse strains. Testing these targets in combination identified several pairs that blocked invasion more effectively in combination than in isolation. Video microscopy and studies of natural immune responses to malaria in patients suggest that targeting multiple steps in invasion is more likely to produce a synergistic vaccine response.


Conflict of interest statement: Three of the authors (J.C.R., G.J.W., and L.Y.B.) are named on patent applications relating to some of the vaccine candidates studied in this work. This article is a PNAS Direct Submission.

This is an open access article distributed under the PNAS license.

1. L.Y.B. and G.T.P. contributed equally to this work.
2. Present address: Department of Cell and Developmental Biology, University College London, London WC1E 6BT, United Kingdom.
3. Present address: Division of Infectious Diseases, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202.
4. To whom correspondence should be addressed. Email: julian.rayner@sanger.ac.uk.

This article contains supportting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702944114/-/DCSupplemental.
protein ectodomains in the context of a eukaryotic secretory pathway allows disulphide bonds to form and maximizes the chance that the recombinant antigens will fold correctly to mimic the function and antigenicity of native P. falciparum proteins, all of which pass through the P. falciparum secretory pathway. This library has been used to identify new protein–protein interactions (11), perform detailed biochemical analysis of known interactions (12), and underpin large-scale immunoenepidemiological studies to identify targets of protective immunity (13). In this study, we tested whether this ectodomain library could be used to identify new erythrocyte invasion-blocking vaccine combinations by raising antibodies against multiple P. falciparum proteins, systematically testing their ability to inhibit invasion, and incorporating immunoenepidemiological and mechanistic data to identify synergistic combinations.

Results

Systematic Screening Identifies Strain-Transcendent Vaccine Candidates.

The extracellular domain of each target protein, based on the sequence from the ref. 3D7 P. falciparum genome, was produced in a soluble recombinant form by transient transfection of HEK239E cells (14). We down-selected 29 targets for further investigation, based both on the diversity of their known or inferred subcellular location and on pragmatic considerations such as protein expression level (Fig. S1 and Table S1). We purified 0.4–1.0 mg of each protein using nickel affinity chromatography and used them to raise polyclonal rabbit antibodies. Total IgG antibodies were purified using protein G columns and tested by ELISA to confirm binding activity against the immunizing antigens (Fig. S2) before being used in growth inhibition activity (GIA) assays.

To establish whether each antibody alone could inhibit parasite growth, late trophozoite stage P. falciparum parasites were cultured in the presence of IgG at the maximum concentration that could be purified from the polyclonal antiserum. Parasites were incubated with each IgG for 24 h to allow sufficient time for erythrocyte invasion to occur, before parasitemia was measured using flow cytometry (15). Antigen polymorphism has been a major cause of failure for previous P. falciparum candidate antigens. To incorporate testing for strain-transcending inhibition at the earliest stage of candidate down-selection, antibodies against all 29 targets were tested against both 3D7 and Dd2 parasites, which have genome sequences broadly representative of West African and Asian P. falciparum parasites, respectively, and differ at >15,000 nucleotide positions across the 23-Mb genome (16). IgG purified from polyclonal antibodies raised against PIRh5, a leading strain-transcending blood-stage vaccine candidate (17, 18), was used as a comparator in this and subsequent experiments. Antibodies raised against the 3D7 variants of PMSP1, PISERA9, PIMSRP5, PIEBA181, PCyRPA, and PIRAMA all had a strong inhibitory effect on the growth of both 3D7 and Dd2 parasites, inhibiting 3D7 growth to a similar extent as anti-PIRh5 antibodies (Fig. 1). PIEBA181, PIMSRP5, and PISERA9 are members of gene families in which at least one other gene is known or suspected to play a role in erythrocyte invasion [PIMSP7-like (19), EBL (20), PISERA (21)], so the inhibitory effects of each of these antibodies could in part be explained by cross-reactivity against multiple members of each protein family. We therefore tested the ability of the purified IgG for each of these proteins to recognize other members of each family, but only in the case of PIMSRP5-specific IgG was there any evidence for cross-reactivity (Fig. S3), suggesting the IgG responses are largely target-specific. PCyRPA is a member of a protein complex that includes PIRh5 (22, 23), while little is known about the function of PIRAMA, although antibodies against it have previously been associated with protection against malaria (24). PMSP1 is an extensively studied vaccine target with known allele-specific effects (25) and so was excluded from further study.

To quantitatively compare the inhibitory potential of IgG specific for the remaining five targets, GIA assays were performed using increasing concentrations of purified IgG to generate IC50 values. All exhibited a clear dose-dependent inhibitory effect on the growth of both 3D7 and Dd2 parasites (Fig. S4), with IC50 values ranging from 0.25 mg/mL to 1.5 mg/mL total IgG (Table S2). Antibodies raised against PISERA9, PIMSRP5, and PIRh5 did not show any evidence of strain specificity, with almost no difference in IC50 values between the two strains. Antibodies against PIEBA181, PCyRPA, and PIRAMA all showed some reduction in efficacy against Dd2 relative to 3D7 parasites, with an accompanying 1.7-fold (PCyRPA and PIRAMA) and 2.7-fold (PIEBA181) increase in IC50 values. However, the difference in IC50 values for PCyRPA and PIRAMA against 3D7 and Dd2 parasites was relatively minor and of a similar magnitude to shifts in IC50 values between different strains for anti-PIRh5 antibodies (18), and moreover, PCyRPA has previously been reported to have broadly strain-transcendent effects (22, 26). Strain specificity is therefore only an immediate concern in the case of PIEBA181.

Candidates Identified in Vitro Are Associated with Protection from Clinical Malaria in Vivo.

Repeated exposure to P. falciparum malaria generates immune responses to a large number of antigens (13, 27), which can result in clinical immunity. To investigate whether antibodies to these vaccine targets contribute to clinical immunity, we tested for the presence of naturally acquired IgG in uninfected Malian individuals enrolled in a prospective cohort study in which we previously described an association between PfRh5-specific IgG and protection from febrile malaria (28). At the uninfected baseline before the 6-mo malaria season, antigen-specific IgG levels and seroprevalence increased with age for all antigens except for PCyRPA, which demonstrated poor natural immunogenicity, similar to its binding partner PIRh5 (Fig. 2 A and B). We next evaluated whether these baseline IgG levels predicted protection from febrile malaria during the ensuing malaria season. Associations between risk of febrile malaria, as measured by time-to-first febrile malaria episode after incident blood-stage infection, and IgG levels for each antigen, alone and in combination, were examined using a Cox regression model that included age, sickle cell trait, gender, and anemia as covariates. Although IgG specific for PCyRPA, PIEBA181, PIMSRP5, PIRAMA, or PISERA9 did not predict protection individually, the combined presence of IgG specific for PIEBA181, PIMSRP5, or PIRAMA with PIRh5-specific IgG was associated with reduced malaria risk relative to
PIRh5-specific IgG alone, albeit with overlapping confidence intervals (CIs) (Table S3). A significantly reduced hazard ratio for the anti-PIEBA181 IgG + anti-PIRH5 IgG combination relative to anti-PIEBA181 IgG alone suggests that antibodies generated against these two antigens may provide malaria-protective synergy (Table S3). Notably, two combinations that did not contain anti-PIRh5 IgG (anti-PIEBA181 IgG + anti-PMSRP5 IgG + anti-PIRAMA IgG and anti-PMSRP5 IgG + anti-PIRAMA IgG) also predicted protection from malaria (Table S3). The protective effect of anti-PIEBA181 IgG + anti-PMSRP5 IgG + anti-PIRAMA IgG, but not PMSRP5 IgG + anti-PIRAMA IgG, remained significant even after controlling for reactivity against other P. falciparum antigens, including PIRh5 (Fig. 2C). To determine whether positive IgG responses affected parasite growth, we compared in vivo parasite multiplication rates (PMRs) between negative and positive responders for each antigen combination among individuals for whom parasite density data were available. Although IgG responses to PMSRP5 + PIRh5 and PIRAMA associated with lower PMRs in univariate analyses, these associations were not significant after adjusting for multiple testing or in logistic regression models that included age, sickle cell trait, anemia, and gender as covariates (Table S4).

Combining Targets at Multiple Steps of Invasion Can Increase Synergy. These data suggest that IgG responses to these antigens contribute to naturally acquired immunity to malaria and also suggest that combining these antigens in a vaccine could improve protective efficacy. We therefore assessed whether combining purified total IgG from different targets could act synergistically to inhibit parasite growth in vitro. The amount of anti-PIRAMA IgG was limiting, so we were only able to test this antibody in combination with anti-PIRh5 and anti-PICyRPA IgG. Antibody interactions were evaluated over a range of concentrations by a fixed-ratio method (29), and IC50 values were used to calculate the 50% fractional inhibitory concentration (FIC50). FIC50 values at different concentration ratios were used to construct isobolograms for each antibody combination (Fig. 3). Several combinations of antibodies showed deviations from the diagonal line that would indicate a purely additive interaction under Loewe additivity. Combinations of anti-PICyRPA/PISERA9, anti-PICyRPA/PIRAMA, anti-PIRh5/PIRAMA, and anti-PIRh5/PMSRP5 all showed deviations below the diagonal, indicating a trend toward synergy (Fig. 3 C and D). However, the combinations of anti-PIEBA181/PICyRPA and anti-PIEBA181/PISERA9 showed curves above the diagonal, indicating a trend toward antagonism (Fig. 3 A and E). Several combinations of antibodies with anti-PIRh5 had different effects at high and low concentrations of anti-PIRh5 (FIC50 > 1) (Fig. 3 D, I, and J), emphasizing that the ratio in which two antibodies are combined can determine whether a given combination has a greater effect than that achieved by each antibody alone. To test the statistical significance of these interactions, we calculated an interaction index for each combination and modeled its associated 95% CI using Monte Carlo simulation of the measurement errors in the IC50 values (Fig. S5). This analysis showed no combination had CIs that did not include one at all concentrations tested, meaning that none were unfailingly either synergistic or antagonistic. However, the
Combinatorial screening reveals antibody pairs with synergistic effects. Isobolograms showing the combined effect of fixed-ratio mixtures (5:0, 4:1, 3:2, 2:3, 1:4, and 0:5) of purified polyclonal antibodies on erythrocyte invasion by *P. falciparum* 3D7. In each case x and y axes represent the FIC values associated with the two antibodies in each combination: EBA181/CyRPA (A), MSPRS5/CyRPA (B), SERA9/CyRPA (C), Rh5/CyRPA (D), SERA9/EBA181 (E), CyRPA/RAMA (F), Rh5/RAMA (G), Rh5/MSRPS5 (H), SERA9/Rh5 (I), and EBA181/Rh5 (J). Dashed lines represent the expected result under Loewe additivity (sum of FICs equal to 1). Points below this line are suggestive of synergistic inhibition, while points above it suggest antagonistic inhibition (38).

Combinations of anti-PfRh5/PfRAMA and anti-PfCyRPA/PfRAMA were those with the most consistently synergistic interactions, while as noted above, several combinations including anti-PfRh5 combinations were significantly synergistic at low concentrations of anti-PfRh5 but not high concentrations (Fig. S5). Combinations that included PfMSRP5 had particularly wide CIs, decreasing the statistical weight that can be put to them.

One potential explanation for the combinatorial effects observed is that simultaneously blocking targets that act at different steps during invasion may not have the same effect as blocking targets that act at the same step of invasion. While the specific function of PfRh5 during invasion has been studied in detail (30), the role of the other antigens is much less well-defined. We therefore carried out video microscopy studies, incubating purified late schizonts from the 3D7 *P. falciparum* strain with purified IgG at the maximal concentration for each target. Schizont egress and subsequent merozoite–erythrocyte interactions were recorded using a recently described imaging platform (31), and multiple invasion associated parameters were quantified from these videos. All antibodies decreased the number of merozoite–erythrocyte contacts made after each egress, but anti-PfMSRP5 had the most significant effect at this very early stage of invasion (Fig. 4A and Movies S1 and S2), consistent with its presence on the merozoite surface. Anti-PfEBA181 was the only IgG that had a marked effect on prolonging the duration of merozoite–erythrocyte interactions (Fig. 4B and Movie S3), suggesting that it acts after the initial contact has occurred but before active invasion has begun, as has been suggested for other members of the same family of invasion ligands (30). By contrast, anti-PfRh5, PfCyRPA, and PISERA9 had no effect on the duration of contacts but did have a significant effect on the severity of erythrocyte deformations (Fig. 4C and Movies S4–S6), with the majority of merozoites inducing little to no deformation (Fig. 4D). Action at this late stage of invasion, interpreted as representing tight junction formation, has been previously reported for PfRh5 (30). Finally, anti-PfRAMA IgG had no effect on the number or severity of deformation events (Fig. 4D and E and Movie S7), indicating that it inhibits invasion much later in the process after the tight junction has been established. Taking the data together, these targets seem to function at discrete temporal steps during invasion, as summarized in Fig. 4: (i) PfMSRP5, (ii) PfEBA181, (iii) PfRh5/PfCyRPA/PISERA9, and (iv) PfRAMA. It is notable that combinations of antibodies that target both steps 3 and 4 (PfRh5/PfRAMA and PfCyRPA/PfRAMA) had particularly wide CIs, decreasing the statistical weight that can be put to them.

**Fig. 3.** Combinatorial screening reveals antibody pairs with synergistic effects. Isobolograms showing the combined effect of fixed-ratio mixtures (5:0, 4:1, 3:2, 2:3, 1:4, and 0:5) of purified polyclonal antibodies on erythrocyte invasion by *P. falciparum* 3D7. In each case x and y axes represent the FIC values associated with the two antibodies in each combination: EBA181/CyRPA (A), MSPRS5/CyRPA (B), SERA9/CyRPA (C), Rh5/CyRPA (D), SERA9/EBA181 (E), CyRPA/RAMA (F), Rh5/RAMA (G), Rh5/MSRPS5 (H), SERA9/Rh5 (I), and EBA181/Rh5 (J). Dashed lines represent the expected result under Loewe additivity (sum of FICs equal to 1). Points below this line are suggestive of synergistic inhibition, while points above it suggest antagonistic inhibition (38).

**Fig. 4.** Video microscopy of erythrocyte invasion reveals discrete roles for candidate antigens. More than 40 egress events were recorded in the presence of IgG against each target, and a range of invasion-related phenotypes was quantitated. (A) Number of merozoite contacts that occur after each egress event. (B) Time taken (s) between merozoite contact and erythrocyte deformation, sometimes referred to as preinvasion, quantitated for merozoites that went on to productively invade erythrocytes (invaders) and those that did not (noniniders). Total number of events analyzed in A and B: control, 63; anti-PfMSRP5, 65; anti-PfEBA181, 49; anti-PfSERA9, 53; anti-PfRh5, 50; anti-PfRAMA, 55; anti-PfCyRPA, 52. (C) Examples of deformation scores from 0 (no deformation) to 3 (strong deformation). (D) Range of deformation scores for every merozoite–erythrocyte interaction, quantitated for both invaders and noniniders. (E) Number of deformation events that take place after each egress event. *P < 0.06, **P < 0.001, ***P < 0.0005. (F) Schematic summarizing the data in this study for each antigen: the phase of invasion they function in, as determined by video microscopy (numbered black boxes one to four); the synergistic (dark blue arrows), antagonistic (black bars), and mixed synergistic/antagonistic interactions (purple diamonds) between antibodies targeting these antigens based on isobologram analyses; the immunoprotective combinations of antigens revealed in the Malian cohort study (connected circles); and the physical location of the proteins on merozoites (beige boxes).
PIRAMA) were the most consistently synergistic in GIA assays. Similarly, the combinations of antigen-specific IgG responses that were associated with protection from febrile malaria in immunoepidemiological studies (Fig. 2) all targeted antigens operating at multiple distinct steps of invasion, rather than multiple antigens operating at the same step. Focusing combinatorial strategies on nonoverlapping steps during invasion may therefore maximize the chance of synergistic effects.

**Discussion**

Malaria vaccine candidate identification has so far largely consisted of detailed preclinical studies targeting a single antigen at a time, with two significant detrimental consequences. Firstly, few antigens can be studied, leaving the majority of potential targets in the *P. falciparum* genome unexplored. Secondly, such single targets are particularly susceptible to failure due to allele-specific responses, as if variants in that antigen evolve that are not recognized by the dominant vaccine-induced response and there is nothing to prevent vaccine escape. Next-generation malaria vaccines will need to target multiple antigens, preferably in combinations that induce synergistic responses. Such a design would mimic what is now becoming clear about natural immunity to malaria, where the breadth of response to multiple antigens is a much stronger predictor of protection than the response to any single antigen (13). This study performed a systematic screen of 29 *P. falciparum* antigens alone and together and complemented in vitro inhibition and mechanistic studies with an investigation of immune responses in the field to prioritize candidates and combinations. Multiple targets were identified that induced antibodies that inhibited *P. falciparum* growth, and several combinations of these antibodies were synergistic in vitro, mirroring immunoepidemiological data that combinations of antibodies against the same targets were associated with protection in vivo at a field site in Mali. This study also highlights the complexity of antibody interactions. For example, in in vitro invasion studies, anti-PIRH5 and anti-PIEBA181 IgG interact antagonistically at high levels of anti-Rh5 IgG but synergistically at low levels of anti-Rh5 IgG. Intriguingly, the latter scenario is consistent with seroepidemiological data that have shown that anti-Rh5 IgG associates with malaria protection despite having relatively low reactivity (28).

Although screening 29 antigens in this manner is a significant step forward, it represents perhaps only one-third of the antigens that are exposed on the parasite surface during erythrocyte invasion. The number of antigens that could be screened was in part limited by the HEK293E expression system. This system has several advantages, most significantly that by being eukaryotic it is more likely to produce antigens that are functional and will therefore best mimic their native counterparts. However, significant amounts of antigen (up to 1 mg) are required for immunization studies such as these, and in general only 70–80% of antigens can be expressed at these levels in the HEK293E system (10, 32). The HEK293E expression system may also not suit all antigens, so there is a risk of false negatives, as there is in any systematic screen. This may explain the absence of inhibitory antibodies generated against MSP2, for example, which has previously been extensively investigated as a blood-stage vaccine candidate (33). Alternative eukaryotic expression systems such as previously been extensively investigated as a blood-stage vaccine candidate (33). Alternative eukaryotic expression systems such as the insect cell system used recently for successful expression of PIRh5 (34), or the wheat germ cell-free system used for its binding partner PIRIPR (35), may be required in addition to perform truly comprehensive blood-stage antigen screens. Testing for effective strain transcendence is also a critical consideration. Now that the true extent of global genomic variation has been revealed by large-scale genome sequencing studies (6), it is apparent that there are several geographic regions that have distinct genomic repertoires but for which there are no commonly available in vitro adapted isolates for testing. Identification, expansion, and distribution of *P. falciparum* isolates from these areas, specifically East Africa, India/Bangladesh, and Papua New Guinea, will be an essential step to enable more comprehensive assessment of strain transcendence. In addition, efforts to standardize growth inhibition assays need to focus on miniaturization, to allow testing of a larger number of isolates when antibody volume is limiting, as it was here.

Despite these limitations, several antigens and combinations were identified in this study. As well as identifying targets, this work also suggests a logical rationale to guide the selection of potentially synergistic combinations—targeting multiple independent steps in the same pathway, in this case erythrocyte invasion. The mechanism by which a given combination results in synergy is not known, but it has been previously suggested that kinetics could play a role, where binding to some merozoite surface antigens might slow invasion down sufficiently to allow other antibodies to bind (36). This model certainly fits with some mechanisms uncovered by video microscopy, such as anti-EBAI81, which appears to increase the duration of merozoite–erythrocyte contacts and shows some evidence for synergy with anti-Rh5. However, further work is clearly required to truly understand the mechanisms of inhibition, and the strategy of targeting multiple steps is not the only viable approach, as recent studies of the AMA1–RON2 interaction that forms a complex late during erythrocyte invasion show that in this case inhibiting both members of the same complex is more effective than targeting either alone (37).

Targeting multiple antigens will require parasites to simultaneously evolve variants in multiple antigens to avoid vaccine-induced immune responses, which should slow the emergence of resistance at the population level. Targeting antigens that operate at distinct steps in the same pathway offers an additional level of redundancy at the single parasite level by requiring individual merozoites to avoid inhibition of multiple temporally distinct steps. This same theoretical approach may be applied to other malaria vaccine targets, such as the recognition and invasion of hepatocytes by *Plasmodium* sporozoites for pre-erythrocytic stage vaccines or gamete development within the *Anopheles* midgut for transmission-blocking vaccines. A deeper understanding of all of these biological processes coupled with more systematic reverse vaccinology approaches will help further drive the development of the next generation of more complex, and more effective, malaria vaccine combinations.

**Methods**

**Recombinant Merozoite Protein Production.** Recombinant extracellular domains of merozoite proteins were produced by transient transfection of HEK293 cells, as previously described (11). Culture supernatants were collected after 6 d and tested for expression of recombinant proteins by ELISA, using a mouse monoclonal antibody that binds the CD4 tag (OX68) to detect expressed protein.

**Protein Purification and Quality Assessment.** Recombinant merozoite proteins were purified from pooled transfection supernatants using HisTrap HP columns (GE Healthcare). Proteins were eluted using an elution buffer containing 400 mM imidazole and then dialyzed against PBS (dialyzer; Novagen). The concentration of protein samples was determined by absorbance at 280 nm, using in silico predicted extinction coefficients (DS Gene version 1.5; Accelrys), and quality assessed by ELISA and reducing SDS/PAGE.

**Antibody Purification and Quality Assessment.** Rabbit polyclonal antibodies were raised against purified recombinant proteins by Cambridge Research Biochemicals after ethical assessment. Sera was tested for activity against the appropriate antigen by ELISA and then purified using a HitTrap Protein G HP column (GE Healthcare). Purified antibodies were dialyzed against PBS (or RPMI 1640 for invasion assays), tested for activity against the appropriate antigen by ELISA, and quality assessed by reducing SDS/PAGE.

**Parasite Culture and GIA Assays.** *P. falciparum* 3D7 and Dd2 parasites were cultured in GIA assays. Parasites were infected in 96-well plates, with a culture volume of 100 μL per well at a hematocrit of 2%. Synchronized parasites were incubated with antibodies for 24 h at 37 °C before being stained with 1:5,000 SYBR Green I (Invitrogen) to detect parasite DNA (12). Invasion efficiency was calculated by comparing invasion in

Bustamante et al.  PNAS Early Edition | 5 of 6
the presence of a given antibody concentration to invasion in the absence of antibodies. All experiments were carried out in triplicate. Use of erythrocytes from noninfected donors of P. falciparum culture was approved by the NHS Cambridge Biophysics Group Ethical Committee, and all donors supplied written informed consent.

Human Cohort Study. The details of the Malian prospective cohort study have been described (28). The Ethics Committee of the Faculty of Medicine, Pharmacy and Dentistry at the University of Sciences, Technique and Technology of Bamako, and the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health approved the Malian cohort study, which is registered on https://clinicaltrials.gov/ (no. NCT01322581).

Written, informed consent was obtained from adult participants and from the parents or guardians of participating children. For this study, malaria episodes were defined as an asexual parasite density by peripheral blood smear of ≥2,500 parasites per μL, an axillary temperature of ≥37.5 °C within 24 h, and no other cause of fever discernible by physical examination.

Immunoenpidemiology. Plasma IgG levels against target antigens were determined by ELISA as previously described (26) and outlined in detail in Methods. In brief, plasma samples from each individual were tested in duplicate, alongside the same positive controls (hyperimmune plasma) and negative controls (unexposed donor plasma). ODs, adjusted for background, were converted to arbitrary units (AU) by dividing the test OD by the mean OD of negative controls plus 3 SDs, and AU > 1 was defined as a positive IgG response. A base Cox proportional hazards model was used to determine whether positive IgG responses to any of the 63 possible reactivity combinations was associated with a reduction in risk of clinical malaria, using time from first P. falciparum blood-stage inoculum (estimated as the midpoint between the first microscopy-positive visit and the previous Plasmodium falciparum PCR-positive visit) to first febrile malaria episode as the dependent variable and controlling for potential confounding variables (Table S3). In vivo PMRs were estimated using qPCR-determined parasite density at the first PCR-positive visit and the number of days between the first P. falciparum blood-stage inoculum and the first smear-positive visit. Analyses were performed in R version 3.3.0 (www.r-project.org) or Prism version 5.02 (GraphPad Software).

Isologram Analyses. Dose–response assays were first carried out to obtain the IC50 of the individual antibodies. Interactions were then assessed over a range of concentrations by a fixed-ratio method based on the IC50 values (29). FIC≤50 were calculated on the basis of the IC50 obtained per assay for each antibody (the FIC≤50 is equal to the IC50 of antibody A in combination with antibody B/Ic50 of antibody A alone) and used to plot isolograms. An interaction index was calculated by summing the FIC≤50 derived from each of the two antibodies in any combination and CIs calculated using Monte Carlo simulation based on the error terms in the fitted IC50 curves.

Video Microscopy. Highly synchronous P. falciparum 3D7 late-stage schizonts were purified using a magnetic column (Miltenyi Biotec) and placed in a Secure- Seal hybridization chamber (Sigma-Aldrich) mounted on a glass slide. All live-cell interventions were performed to Artemisia truncatulosa extract or Artemisia annua (1:250)–positive visit to Artemisia truncatulosa extract or Artemisia annua (1:250)–positive visit) to first febrile malaria episode as the dependent variable and controlling for potential confounding variables (Table S3). In vivo PMRs were estimated using qPCR-determined parasite density at the first PCR-positive visit and the number of days between the first P. falciparum blood-stage inoculum and the first smear-positive visit. Analyses were performed in R version 3.3.0 (www.r-project.org) or Prism version 5.02 (GraphPad Software).

Isologram Analyses. Dose–response assays were first carried out to obtain the IC50 of the individual antibodies. Interactions were then assessed over a range of concentrations by a fixed-ratio method based on the IC50 values (29). FIC≤50 were calculated on the basis of the IC50 obtained per assay for each antibody (the FIC≤50 is equal to the IC50 of antibody A in combination with antibody B/Ic50 of antibody A alone) and used to plot isolograms. An interaction index was calculated by summing the FIC≤50 derived from each of the two antibodies in any combination and CIs calculated using Monte Carlo simulation based on the error terms in the fitted IC50 curves.

Video Microscopy. Highly synchronous P. falciparum 3D7 late-stage schizonts were purified using a magnetic column (Miltenyi Biotec) and placed in a Secure-S Seal hybridization chamber (Sigma-Aldrich) mounted on a glass slide. All live-cell interventions were performed to Artemisia truncatulosa extract or Artemisia annua (1:250)–positive visit to Artemisia truncatulosa extract or Artemisia annua (1:250)–positive visit) to first febrile malaria episode as the dependent variable and controlling for potential confounding variables (Table S3). In vivo PMRs were estimated using qPCR-determined parasite density at the first PCR-positive visit and the number of days between the first P. falciparum blood-stage inoculum and the first smear-positive visit. Analyses were performed in R version 3.3.0 (www.r-project.org) or Prism version 5.02 (GraphPad Software).

Isologram Analyses. Dose–response assays were first carried out to obtain the IC50 of the individual antibodies. Interactions were then assessed over a range of concentrations by a fixed-ratio method based on the IC50 values (29). FIC≤50 were calculated on the basis of the IC50 obtained per assay for each antibody (the FIC≤50 is equal to the IC50 of antibody A in combination with antibody B/Ic50 of antibody A alone) and used to plot isolograms. An interaction index was calculated by summing the FIC≤50 derived from each of the two antibodies in any combination and CIs calculated using Monte Carlo simulation based on the error terms in the fitted IC50 curves.

Video Microscopy. Highly synchronous P. falciparum 3D7 late-stage schizonts were purified using a magnetic column (Miltenyi Biotec) and placed in a Secure-S Seal hybridization chamber (Sigma-Aldrich) mounted on a glass slide. All live-cell interventions were performed to Artemisia truncatulosa extract or Artemisia annua (1:250)–positive visit to Artemisia truncatulosa extract or Artemisia annua (1:250)–positive visit) to first febrile malaria episode as the dependent variable and controlling for potential confounding variables (Table S3). In vivo PMRs were estimated using qPCR-determined parasite density at the first PCR-positive visit and the number of days between the first P. falciparum blood-stage inoculum and the first smear-positive visit. Analyses were performed in R version 3.3.0 (www.r-project.org) or Prism version 5.02 (GraphPad Software).