Naturally acquired immunity against immature *Plasmodium falciparum* gametocytes

Kathleen W. Dantzler[1,2], Siyuan Ma[3*], Priscilla Ngotho[2*], Will J.R. Stone[4,5], Dingyin Tao[6,7], Sanna Rijpma[4], Mariana De Niz[2], Sandra K. Nilsson Bark[1], Matthijs M. Jore[4], Tonke K. Raaijmakers[4], Angela M. Early[8], Ceereena Ubaida-Mohien[9], Leandro Lemgruber[2], Joseph J. Campo[10], Andy A. Teng[10], Timothy Q. Le[10], Cassidy L. Walker[10], Patricia Hermand[11], Philippe Deterre[11], D. Huw Davies[12], Phil Felgner[12], Isabelle Morlais[13], Dyann F. Wirth[1], Daniel E. Neafsey[8], Rhoel R. Dinglasan[6,14], Miriam Laufer[15], Curtis Huttenhower[3], Karl Seydel[16,17], Terrie Taylor[16,17], Teun Bousema[4]*, Matthias Marti[1,2]*

[1]Department of Immunology and Infectious Disease, Harvard T.H. Chan School of Public Health, Boston, MA; [2]Wellcome Centre for Molecular Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom; [3]Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA; [4]Radboud Institute for Health Sciences, Radboud University Medical Center, The Netherlands; [5]Immunology and Infection Department, London School of Hygiene and Tropical Medicine, London, United Kingdom; [6]W. Harry Feinstone Department of Molecular Microbiology and Immunology and the Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD; [7]National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD; [8]Broad Institute of Harvard and MIT, Cambridge, MA; [9]National Institute on Aging, National Institutes of Health, Baltimore, MD [10]Antigen Discovery, Inc., Irvine, CA; [10]Sorbonne Universités, UPMC Univ Paris 06, Inserm, Centre d'Immunologie et des Maladies Infectieuses (Cimi-Paris), UMR 1135, ERL CNRS 8255 F-75013 Paris, France; [12]Division of Infectious Diseases, Department of Medicine, University of California, Irvine, CA; [13]UMR MIVEGEC UM1-CNRS 5290-IRD 224, Institut de Recherche pour le Développement, Montpellier Cedex, France; [14]Emerging Pathogens Institute, Department of Infectious Diseases & Immunology, University of Florida College of Veterinary Medicine, Gainesville, FL; [15]Division of Malaria Research, Institute for Global Health, University of Maryland School of Medicine, Baltimore, MD; [16]Department of Osteopathic Medical Specialties, College

of Osteopathic Medicine, Michigan State University, E. Lansing, MI; [17]Blantyre Malaria Project, University of Malawi College of Medicine, Blantyre, Malawi

*=Contributed equally to this work

Overline: Malaria

One sentence Summary: Naturally acquired immune responses target infected red blood cell surface antigens of immature malaria transmission stages.

Abstract

The recent decline in global malaria burden has stimulated efforts towards *Plasmodium falciparum* elimination. Understanding the biology of malaria transmission stages may provide opportunities to reduce or prevent onward transmission to mosquitoes. Immature *P. falciparum* transmission stages, termed stage I-IV gametocytes, sequester in human bone marrow before release into the circulation as mature stage V gametocytes. This process likely involves interactions between host receptors and potentially immunogenic adhesins on the infected red blood cell (iRBC) surface. Here we developed a flow cytometry assay to examine immune recognition of live gametocytes of different developmental stages by naturally exposed Malawians. We identified strong antibody recognition of the earliest immature gametocyte-iRBCs (giRBCs) but not mature, stage V giRBCs. Candidate surface antigens (n=30), most of them shared between asexual- and gametocyte-iRBCs and others enriched in giRBCs, were identified by mass spectrometry and mouse immunizations, as well as correlations between responses by proteome microarray and flow cytometry. Naturally acquired responses to a subset of candidate antigens were associated with reduced asexual and gametocyte density, and plasma samples from malaria-infected individuals were able to induce immune clearance of giRBC *in vitro*. Infected RBC surface expression of 6 select candidate antigens was validated using specific antibodies in fluorescent microscopy and flow cytometry experiments, and genetic analysis revealed a subset with minimal variation across strains. Our data demonstrate that humoral immune responses to immature giRBCs and shared iRBC antigens are naturally acquired following malaria exposure. These humoral immune responses may have consequences for malaria transmission potential by clearing developing gametocytes, which could be leveraged for malaria intervention.

Introduction

Plasmodium falciparum malaria morbidity and mortality has decreased substantially in the last decade (*1*). These recent gains are threatened by the spread of artemisinin-resistant parasites (*2*) and insecticide-resistant mosquitoes (*3*). The recent achievements in malaria control and necessity to contain artemisinin resistance have stimulated malaria elimination initiatives that require a thorough understanding of the biology and epidemiology of malaria transmission and alternative transmission-reducing interventions (*4*).

P. falciparum transmission to mosquitoes is initiated when a small subset of asexually replicating blood stage parasites produce sexual progeny, or gametocytes. Gametocytes develop in human red blood cells (RBC) along 5 morphological transitions (Stage I-V); stage I-IV development takes place predominantly in the extravascular niche of the bone marrow and spleen (*5-7*). Mature Stage V gametocytes are released into the peripheral blood circulation where they may be ingested by a blood-feeding mosquito upon which they egress from RBCs as activated gametes and fuse and form motile zygotes. Further sporogonic development renders the mosquito infectious to humans. Several sexual stage proteins have been identified that have no function in gametocyte development but are essential for gamete fertilization (e.g. Pfs48/45 and Pfs230) or post-fertilization development in the mosquito (e.g. Pfs25, Pfs28) (*8*).

There is currently incomplete evidence for immune responses that affect gametocyte formation, maturation or circulation time (*9*). Several field studies suggested mature gametocyte clearance after repeated malaria exposure (*10-13*) and antibody responses against uncharacterized targets on mature gametocyte-infected red blood cells (giRBCs) have been associated with lower gametocyte densities (*12, 14*). Another field study identified antibodies that bound the surface of stage II-V giRBCs and distorted early gametocyte morphology and maturation (*15*). Depending on which stage(s) they target, anti-gametocyte immune responses could be involved in blocking extravascular adhesion of immature giRBCs and/or clearance of circulating mature giRBCs, in a manner similar to antibodies against the asexual antigen PfEMP1. PfEMP1 is an immunodominant antigen on the surface of RBCs infected with asexual

parasites (aiRBC); anti-PfEMP1 antibodies have an established role in immune clearance by inhibiting vascular adhesion and by opsonizing aiRBCs for phagocytic clearance (*16, 17*). aiRBC surface antigens other than PfEMP1 exist (*18*), and are associated with phagocytosis and cytotoxicity (*19*). The ligands involved in giRBC adherence may be different from those involved in endothelial binding of aiRBCs; giRBCs are localized to an extravascular compartment (*5, 7*), show limited binding to human endothelial cell lines and harbor minimal PfEMP1 on their surface (*20*). Whilst no specific giRBC ligand has been identified, one tenth of the early gametocyte proteome consists of putatively exported antigens called *P. falciparum* gametocyte-exported proteins (PfGEXPs) (*21*).

Hypothesizing that developing gametocytes could be targets of antibody responses in the human host**,** we performed a systematic characterization of gametocyte stage-specific immune recognition and clearance. We demonstrate naturally acquired human immune responses targeting immature (stage I-III) but not more mature stage V giRBCs. Experiments using whole cells and surface-intact and surface-depleted membrane fractions of diverse parasite strains provide evidence for giRBC surface antigens, most of them shared with aiRBCs. We further demonstrate that natural immunity to shared iRBCs correlates with reduced asexual and gametocyte burden and that a subset of the target antigens shows minimal sequence diversity.

Results

Human immune responses recognize secreted gametocyte proteins

We first probed a *Plasmodium falciparum* peptide array enriched for proteins expressed in the gametocyte and gamete stages *(22)* with human plasma samples from 579 asymptomatically infected individuals from Cameroon, Burkina Faso, and the Gambia (*22*)(**table S1**) to examine natural immunity. Proteins were clustered based on their stage-specific abundance in blood and mosquito stages in proteomics studies (*21, 23, 24*) and by cellular localization; localization was divided into those proteins that are parasite internal (internal/unknown localization) or secreted onto the merozoite or gamete surface or into the host cell in intra-erythrocytic stages (secreted)(**fig. 1A-B**, see also **table S2**). Five stage-specific clusters (gametocyte

specific or shared with asexual stages) were enriched in secreted antigens (**Figure 1A),** and secreted antigens showed higher antibody responses compared to internal antigens for shared, gametocyte-specific (p-value = 8.86 x 10^{-288}) and gametocyte/mosquito stage proteins (p-value = 4.31 x 10^{-119})**(fig. 1C)**. Responses to shared secreted proteins increased with age while responses to secreted gametocyte or mosquito stage proteins or to parasite internal proteins did not. Correlations were highly significant for a total of 121 individual peptides (adjusted p-value < 0.05, **table S3**). Although responses to numerous protein fragments showed progressive increases with age **(fig. S1A),** responses to other antigens, including PTP6 (*25*) and GEXP08, reached a plateau in the 12-30 year old group **(fig. S1B).** These results indicate that humoral responses to secreted parasite antigens (shared and gametocyte-specific) are correlated with cumulative exposure to malaria.

Immune responses target the immature but not the mature giRBC surface

Detection of immune responses against secreted gametocyte proteins prompted us to directly examine immune recognition of giRBC surface antigens among an independent population. In a cross-sectional study, we collected plasma samples from 244 individuals with suspected malaria from southern Malawi (see Materials and Methods and **table 1**). A subset of rapid diagnostic test (RDT)+ samples and an RDT-control (representative of the entire Malawian study population in terms of age and sex distribution) was incubated with *P. falciparum* NF54 stage II/III giRBC, stage V giRBCs, or activated gametes. Surface reactivity was measured by comparing the percentage of IgG-positive cells between incubations with Malawian and naïve control sera (see **fig. S2 and S3**). To differentiate non-activated gametocytes (i.e., intact giRBCs) from activated ones (i.e., free gametes), stage V incubations were co-stained with antibodies recognizing the gametocyte/gamete surface antigen Pfs48/45 (which becomes accessible upon RBC rupture and giRBC activation) and the RBC surface antigen Glycophorin C. Highest surface reactivity was found for gametes (mean 25.80% recognized cells), with substantial reactivity also observed for stage II/III (mean 6.22%) but not for stage V giRBCs (**fig. 2A**). The relatively low percentage of giRBCs recognized suggests low abundance, accessibility and/or immunogenicity of

putative antigen targets. Of the Malawian plasma samples tested, 75.00% (n/N=18/24) and 95.83% (n/N=23/24) recognized stage II/III giRBCs or gametes respectively, whereas no samples were positive for stage V recognition (**fig. 2B**).

We further investigated antibody specificity to immature giRBC surface antigens as compared to aiRBCs using a transgenic version of the Ghanaian *P. falciparum* parasite Pf2004 (*26, 27*), Pf2004_164/TdTom. This parasite expresses the TdTomato reporter under the control of the *PF10_0164* promoter (*28*) that allows detection by fluorescence microscopy and flow cytometry of gametocytes of all stages except the first 30 hours of development (**fig. 2C**). Among 244 Malawian plasma samples, the strongest responses to aiRBCs correlated with the strongest responses to giRBCs, whereas 14 samples were uniquely positive for giRBCs (**fig. 2D-E**). No differences between RDT+ and RDT- individuals in antibody responses for any antigen class was observed **(fig. S4A)**. When we repeated our surface recognition experiments with the 3D7 reference strain (a clone of NF54 used in **fig. 2A**, potentially expressing different surface proteins than Pf2004), we observed lower surface antigen expression and lower non-specific IgG labeling from naïve serum compared to Pf2004 (**fig. S4B-C**). These strain disparities are consistent with previous work observing differential reactivity of Kenyan plasma samples to parasite strains of different genetic origins (*18*). Surface protein removal with trypsin/chymotrypsin revealed that both specific and non-specific binding of IgG involved antigens on the surface of aiRBCs and giRBCs (**fig. S4D-E**). Further experiments using the same patient sera and naïve controls revealed no IgM binding above background and therefore excluded IgM binding as an explanation for the observed non-specific surface recognition (**fig. S5**). These data provide strong evidence for IgG-targeted antigens that are shared between asexual and gametocyte stages.

The prevalence (number of samples with substantial aiRBC and/or giRBC recognition) and magnitude (median fluorescence intensity) of iRBC reactivity was significantly higher for adults compared to children (**fig. 2F**). The increased aiRBC reactivity with age (top panel) corroborates the well-characterized pattern of increasing breadth of antibody response to asexual parasites with cumulative exposure (*29-31*). The slower age-dependent increase for giRBC responses (bottom panel) may reflect the

lower abundance of immature gametocytes and suggests that giRBC responses differ from those against gametocyte/gamete antigens Pfs48/45 and Pfs230 that appear short-lived *(22, 32, 33)*. We then probed a subset of the Malawian plasma samples (representing a range of reactivity by flow cytometry) on the peptide array to identify recognized targets. Recognition of the giRBC surface by flow cytometry was correlated with mean array responses for shared asexual-gametocyte and gametocyte-specific secreted antigens **(fig. 2G)** but not internal proteins. Individuals recognizing giRBCs by flow cytometry had significantly higher reactivity (p-value <0.05) to a subset of 22 protein fragments (including 4 shared and 13 gametocyte-specific) compared to individuals with minimal reactivity to giRBCs (**table S4** and **fig. S1C)**. Altogether these data demonstrate that plasma samples recognizing both aiRBCs and giRBCs show the highest magnitude in reactivity, and this signal is driven by antibody responses against secreted antigens across all age groups.

Antigens on the giRBC surface are predominantly shared with aiRBC

TdTomato fluorescence increases with later stage gametocytes (**Figure 3A**) and microscopy and flow experiments indicated that "weak TdTomato+" corresponded to stage I/II gametocytes and "strong TdTomato+" to stage II/III gametocytes. Three lines of evidence suggest that giRBC surface reactivity is specific for stage I/II gametocytes: i) a higher percentage of stage I/II, weak TdTomato signal consistently corresponded to a higher percentage of cells staining positive for the surface (**Figure 3B**); ii) the intensity of IgG staining correlated with the percentage of weak TdTomato positive cells (**Figure 3C**); iii) microscopy confirmed significantly higher percentages of surface labeling of aiRBCs and stage I/II giRBCs compared to stage II/III giRBCs (**Figure 3D**). These results demonstrate that giRBC reactivity is highest in early stage gametocytes (stage I/II) and decreases during gametocyte development.

To identify the target giRBC surface antigens, we probed aiRBC and stage I-III giRBC membranes +/- treatment with trypsin/chymotrypsin (hereafter referred to as +trypsin and –trypsin samples) with Malawian plasma samples by Western blot. By comparing differential bands between surface-intact (-trypsin) and surface-depleted (+trypsin) samples, we identified both shared (aiRBC-giRBC) and giRBC-

specific trypsin-sensitive protein bands (**Figure 4A**), demonstrating the presence of immunogenic antigens on the giRBC surface. Next, we performed mass spectrometry-based proteomics of stage I-III giRBC membrane samples and assessed reactivity of sera from mice immunized with the same giRBC membrane samples. These results were combined with the proteins recognized by individuals with giRBC reactivity by flow cytometry in experiments described above to form an initial list of potential giRBC surface antigens.

In the first approach, we performed whole lane in-gel digestion with 3 biological replicates of +trypsin *vs*. –trypsin giRBC membranes and identified differentially enriched protein bands between the two conditions by mass spectrometry (**fig 4B; table S5**). Overall, 72.20% of proteins identified in – trypsin samples were shared between all 3 replicates and 92.21% of proteins were identified unequivocally in at least 2 of the 3 replicates. Out of all 235 proteins that were >1.25x enriched in the – trypsin sample (**table S5)**, a subset of 30 (12.77%) secreted proteins were considered putative surface antigen candidates. Secreted proteins were defined by the presence of at least one transmembrane domain (TM, including the N-terminal signal sequence) and either known localization to membrane/surface or host cell or unknown localization. Within this set of 30 candidates, 28 (93.33%; 11.91% of total candidate list) showed evidence for export into the host cell based on predicted PEXEL motif (21 proteins) or PEXEL/HT negative exported protein (PNEP) annotation (7 proteins) and 23 were expressed in both asexual and gametocyte stages. Importantly, this candidate list includes several previously identified secreted antigens such as multiple Plasmodium helical interspersed subtelomeric (PHIST) family proteins (*21, 34, 35*), PIESP2 (*35-37*), and GEXP02 (*21, 38*).

In a complementary antigen-discovery approach, we immunized mice with the same surface-intact (-trypsin) or surface-depleted (+trypsin) giRBC membranes used for proteomics and probed sera on our gametocyte-enriched protein array. Several bands on Western blot were present only in experiments using sera from mice immunized with surface-intact giRBC membranes, and were reduced in intensity when surface-depleted membranes were probed with these sera compared to surface-intact membranes (**fig. 4C**). Sera from all mice showed similar responses to parasite-internal peptides on the array, but sera from

mice immunized with –trypsin preparations showed significantly higher responses to secreted proteins compared to mice immunized with +trypsin preparations (p-value=0.04315)(**fig. 4D**). Due to lower background using mouse sera compared to human sera, many normalized mean response values were negative; however, the significant differential responses were consistent with observed reduced band intensity after trypsin treatment by Western blot **(fig. 4C)** and with the same array probed with human plasma samples described earlier. Consistent with our previous results using the peptide array, 16 individual protein fragments elicited significantly higher differential responses with sera from mice immunized with surface-intact membranes **(fig. 4E, table S6**). Notably, GEXP07 and GEXP10, two proteins on the iRBC surface that can bind to the chemokine CX3CL1 (*37*) were recognized both by sera from mice immunized with intact and surface-depleted membranes **(fig. 4F),** suggesting that their ectodomain is trypsin insensitive.

In total, we identified an overlapping set of 68 initial candidate giRBC surface antigens: 22 proteins with significantly correlated array *vs.* flow cytometry responses (**table S4**), 30 proteins from mass spectrometry-based proteomics (**table S5)**, and 16 proteins eliciting significantly higher responses from sera from mice immunized with surface-intact (compared to surface-depleted) giRBC membranes (**table S6**). This list was then filtered based on detection by gametocyte surface proteomics and presence of at least one TM; subsequently any proteins with confirmed localization within the parasite or parasitophorous vacuole or Maurer's clefts were removed. The remaining 30 proteins were therefore deemed potential giRBC surface antigens (**table S7**): 26 were identified by surface proteomics, 3 by the parallel mouse immune profiling experiment and 1 hit was identified only by correlating protein array responses and surface reactivity of patient plasma samples. Of the 30 candidate antigens, 26 (86.7%) showed evidence of export into the host cell based on the presence of a PEXEL (23 proteins) or PNEP (3 proteins) motif, and the majority of the identified proteins (23: 76.7%) were expressed both in asexual and gametocyte stages (i.e., shared expression profile). Importantly, there is independent evidence for localization at the iRBC periphery and/or surface for 12 out of these 30 candidates from previous studies (**Supplementary table S7**), further supporting our data.

242

243 ***Validation of giRBC antigen surface localization***

244 From the 30 proteins, we selected 9 for experimental validation of surface expression using antibodies
245 against peptides (PF3D7_0402000, PF3D7_0702500, PF3D7_0936800, PTP5, PTP6, GEXP02 and
246 GEXP10; GEXP07 and GEXP10 (*37*)), or recombinant protein (PF3D7_0532400 (*39*)) **(table S8)** in
247 Western blots (**fig. 5A**), flow cytometry (**fig. 5B**), and live immunofluorescence assays **(fig. 5B-C)**. In
248 addition, we performed IFAs using fixed, permeabilized cells to determine the cellular distribution of the
249 candidate proteins (**fig. 5D**). We obtained a band of the expected size by Western blot, and candidate
250 antigens showed variable degrees of trypsin sensitivity **(fig. 5A and fig. S6)**. All antibodies except PTP6,
251 which did not detect giRBCs, were then tested by flow cytometry **(fig. 5B)** and immunofluorescence
252 microscopy **(fig. 5B-C)** using live Pf2004/164TdTomato parasites. By flow cytometry, all antibodies,
253 except those against GEXP10 and GEXP07, showed significantly reduced recognition of surface-depleted
254 asexual stages and early gametocytes although cell binding was low for some antibodies **(fig. 5B, right**
255 **panel)**. The overall percentage of cells labeled, as well as the magnitude of decreased labeling after
256 trypsin treatment, were higher by live IFA **fig. 5B, left panel; fig. 5C)** compared to flow cytometry.
257 Again, GEXP10 and GEXP07 appeared insensitive to trypsin treatment in these assays. Apart from
258 trypsin sensitivity we quantified the proportion of surface-labeled aiRBCs and giRBCs, the fluorescence
259 intensity of surface labeling, and the average percentage of surface coverage among labeled cells by live
260 microscopy **(fig. 5C, left panel)**. Whereas GEXP10 and GEXP07 showed high levels for all 3
261 measurements, other antibodies had high values for one or two parameters **(fig. 5C)**. Automatic
262 independent clustering by all 3 measurements simultaneously confirmed 6 candidates - PTP5, GEXP02,
263 PF3D7_0936800, GEXP07, GEXP10 and PF3D7_0702500. In contrast, the two candidates with major
264 expression in asexual stages and minimal expression in gametocytes based on our proteomic clustering
265 (PF3D7_0402000, PF3D7_0532400) showed the lowest levels of giRBC surface staining by live
266 microscopy. Finally, immunofluorescence microscopy using fixed and permeabilized cells confirmed
267 significant labeling at the iRBC periphery, and in addition, co-labeling with the Maurer's Cleft marker

SBP1, for 3 of these candidates across asexual and immature gametocyte stages **(fig. 5D)**. Antibodies against all three candidates showed markedly weaker labeling in gametocytes compared to asexual parasites. Altogether, analysis of a subset of candidates using peptide antibodies validated our analysis pipeline and confirmed six proteins as giRBC surface antigens.

A subset of secreted parasite antigens shows minimal sequence diversity and elicits responses that are correlated with reduced gametocyte burden

To determine the extent of sequence polymorphisms amongst the antigens analyzed in this study, we measured signatures of selection in the encoding genes from clinical isolates collected from two patient populations in Senegal and Malawi **(table S9)**. Analysis of nonsynonymous pairwise nucleotide diversity (π_{NS}) demonstrated significantly elevated levels of genetic diversity in genes encoding secreted compared to internal antigens across all stages (**fig. 6A and S7A;** Mann-Whitney U test, $p = 8x10^{-13}$ (Senegal), $p = 3.7x10^{-11}$ (Malawi)). Genes with Tajima's *D* values above the genome-wide 95th percentile (*D*>-0.343), indicating balancing selection, were also enriched in secreted relative to internal antigens (**fig. S7B**; Fisher's Exact Test, p=0.0153 (Senegal), p=0.00660 (Malawi)). These data support the hypothesis that acquired immunity drives genetic diversity in genes encoding secreted *P. falciparum* blood stage antigens (both shared and gametocyte-specific). Indeed, we measured a positive correlation between immune responses against secreted antigens and the levels of π_{NS} of the encoding genes (Pearson's correlation; r=0.221, p=0.000141 (Senegal); r=0.199, p=0.000623 (Malawi)). Levels of π_{NS} were significantly increased at mean responses greater than 0.5 across secreted antigens, suggesting a threshold effect inducing positive selection through antibody-mediated immunity (**fig. 6A;** Mann-Whitney U test, p=0.0265 (Senegal), p=0.0441 (Malawi)). We also quantified genetic differentiation between the two geographically separated parasite populations in Malawi and Senegal using the fixation index (F_{ST}). This analysis demonstrated that genes encoding secreted antigens show significantly higher F_{ST} indices (Mann-Whitney U test, $p=2.3x10^{-5}$), and that the majority of genes had high corresponding indices (F_{ST} > 0.1)(**fig. 6B;** Mann-Whitney U test, $p=2.3x10^{-5}$). Amongst our 30 candidate antigens, 9 showed minimal

levels of nucleotide diversity across parasite populations in Malawi and Senegal (**fig. S7C,** and **table S9**). Seven antigens, including the validated surface antigens GEXP07 and PTP5, show both minimal levels of nucleotide diversity across parasite populations and low levels of population divergence between populations (**fig. S7C,** and **table S9**). Altogether, genetic analysis demonstrates that genes encoding secreted antigens show significantly higher signatures of selection compared to internal antigens whilst a subset of eight antigens show minimal levels of genetic diversity and may thus elicit strain-transcending immunity (**table 2**).

It is currently unknown whether antibodies recognizing shared or gametocyte-specific surface antigens may inhibit giRBC binding/sequestration and/or increase phagocytosis efficiency by opsonization - as implicated in responses to PfEMP1 (*18, 40, 41*) and merozoite antigens (*42, 43*). To directly test this hypothesis, we opsonized iRBCs with the same Malawian plasma samples used for iRBC surface labeling and determined the level of iRBC phagocytosis by THP-1 cells (*18*). Significant levels of iRBC phagocytosis were detected (**fig. 6C**), and the magnitude of surface reactivity was significantly correlated with induction of phagocytosis both for aiRBCs and giRBCs (**fig. 6D**). Altogether these data demonstrate existence of functional antibodies targeting both aiRBCs and giRBCs, and provide evidence for antibody-mediated clearance of giRBCs. In support of these functional assays, the intensity of recognition of shared secreted antigens by plasma samples from individuals in Cameroon, Burkina Faso, or the Gambia was overall negatively associated with the gametocyte fraction in these individuals (quantified by coefficients of regressing antigen response on logit-transformed gametocyte fraction). In contrast, normalized recognition of asexual antigens was overall negatively associated with asexual stage and gametocyte load (also quantified by regression coefficients, antigen response on log-transformed asexual/gametocyte load), whereas normalized recognition of gametocyte-specific antigens did not show any negative association **(fig. 6E)**. Furthermore, the proportion of total parasites that were gametocytes was negatively associated with breadth of response to the 76 fragments representing the 31 candidate surface antigens on the peptide array (coefficient, -0.002 (95% CI -0.004/-0.0004), p=0.019). Importantly, responses to a total of 12 candidate surface antigens, including three of our final candidates (Table 2)

showed significant (p<0.05) negative correlation between immune response and both peripheral gametocyte and asexual stage load **(fig. 6F and S8,** and **table S10)**. These data support the phagocytosis data and suggest that iRBC immunity may be able to simultaneously reduce total parasite burden and gametocyte burden.

Discussion

In this study, we systematically addressed immune recognition of antigens on the surface of giRBCs and provide evidence for the identity of these proteins. Our combination of a flow cytometry assay using distinct gametocyte stages, immune profiling by protein microarray, 3 parallel methods of antigen discovery, and a functional assay to quantify antibody-mediated iRBC phagocytosis, provides evidence for naturally acquired antibodies recognizing shared asexual/gametocyte and gametocyte-specific antigens on the surface of immature giRBCs.

Two previous studies reported immune recognition of mature giRBCs (*12, 14*) but did not specifically control for gametocyte activation. We regularly observed glycophorin-negative gametocyte populations where the giRBC membrane was lost due to activation or permeabilization. It is conceivable that earlier studies have similarly experienced a loss in RBC integrity and may thus have detected antibodies against gamete proteins, that are common in endemic populations (*22*), instead of mature giRBC responses. Less stringent methods of giRBC purification also could have hindered detection of responses targeting the most immature stages. When we carefully prevented activation by using a compound that prevents gamete egress (*44*), and confirmed the intact RBC membrane by counterstains (the gamete surface antigen Pfs48/45 and the RBC surface antigen Glycophorin C), we did not detect significant recognition of stage V giRBC. In addition, we observed strong reactivity to stage I/II gametocytes but negligible reactivity to stage V gametocytes in our highly synchronous TdTomato transgenic parasite line (*45*). Our data demonstrate that plasma from naturally exposed individuals strongly recognizes early stage I/II giRBCs and aiRBCs; the majority of immunogenic giRBC antigens in our study are also expressed in asexual stage parasites. These observations have potential implications for

346 our understanding of parasite biology. Asexual and early gametocyte stages of *P. falciparum*, *P. vivax,*
347 and *P. berghei* are abundantly present in the bone marrow parenchyma (*5, 7, 46, 47*), suggesting
348 environmental characteristics supporting both gametocyte development and a genuine asexual replication
349 cycle. An independent study recently confirmed that both bone marrow and spleen represent major
350 reservoirs for parasite development in rodent malaria (*48*). We hypothesize that shared antigens present
351 on aiRBC and giRBC surfaces are involved in cellular interactions in the bone marrow parenchyma and
352 critical for the maturation of both asexual and gametocyte stages. In such a model, the aiRBC surface
353 serves the dual purpose of vascular adherence and extravascular binding, while the giRBC surface is
354 optimized for extravascular binding only. Indeed, recent work demonstrated trypsin-sensitive binding of
355 aiRBCs and immature but not mature giRBCs to human bone marrow mesenchymal stromal cells (*49*).
356 Interestingly, two antigens we identified on the giRBC and aiRBC surface, GEXP07 and GEXP10, were
357 recently described as aiRBC surface proteins that bind the chemokine CX3CL1 (*37*). As expression of
358 this chemokine on bone marrow stromal cells is involved in homing and retention of monocytes (*50*), it is
359 tempting to speculate that GEXP07 and GEXP10 are involved in such interactions between iRBCs and
360 other cell types. It remains to be determined why human IgG levels recognizing giRBC antigens are
361 generally lower compared to aiRBCs and why recognition is restricted to young gametocyte stages,
362 despite their continued presence in the extravascular niche until maturity. Although we only examined
363 stage I/II, III and V gametocytes, and not the intermediate stage IV, our data suggest reduced antigen
364 expression on the giRBC surface over the course of gametocyte development, the mechanism of which
365 could include a combination of membrane remodeling, protease activity, or release via extracellular
366 vesicles. As the molecular mechanisms of the bone marrow sequestration process become further
367 elucidated, the ability and function of natural antibodies to access this compartment in meaningful
368 concentrations and effectively target parasites in this niche is likely to also be revealed.
369 Our data reveal a positive correlation between antibody-mediated immunity and genetic diversity in
370 secreted parasite antigens. Nevertheless, we identified a small set of immunogenic candidate antigens
371 with minimal genetic diversity within and between populations, suggesting that they may induce strain-

transcendent immunity. Our plasma samples were from cross-sectional surveys in asymptomatic populations. Whilst this makes it unlikely that inflammation or acute disease have influenced the results, our sampling approach means we were lacking details on gametocyte commitment and maturation, and were thus unable to test causality between antibody responses and parasite and gametocyte dynamics. We observed that the proportion of the total parasite biomass that is gametocyte (indicating what fraction of parasites successfully develops into circulating mature gametocytes (*51*)) was reduced in infections of individuals who responded to peptides mapping to shared asexual/gametocyte antigens. The negative associations between responses to asexual secreted antigens and asexual parasite load suggest a specific role for these proteins in reducing asexual parasite burden, in addition to the established contribution of anti-PfEMP1 antibodies (*18, 19*). Importantly a total of 12 candidate antigens, including 3 of our 8 top candidates with low sequence diversity, showed negative correlations between antibody titer and both asexual and gametocyte load, suggesting an association with reduced parasite growth and gametocyte maturation or clearance. This possible phenotype of the detected antibody responses is supported by our finding that plasma samples with increased aiRBC and giRBC surface recognition demonstrate increased phagocytosis of aiRBC and giRBC by THP-1 cells. This phenotype and the identification of a small set of target immunogenic antigens present on the giRBC surface with low sequence diversity, provides a rationale for a novel transmission blocking vaccine strategy that may interfere with gametocyte maturation. Such a vaccine approach would reduce the number of gametocytes in the circulation and hence transmission potential.

Altogether, we provide compelling evidence for natural immune responses targeting young gametocytes and their antibody-mediated immune clearance. We identify a small set of 8 candidate antigens that are i) expressed in gametocytes (7 of them are also expressed in asexual stages), ii) elicit natural antibody responses and iii) display low sequence diversity.

Materials and Methods

Study design

For the Malawi study, samples were collected over 4 weeks in July/August 2013. Two weeks were spent in Chikhwawa, as this region had higher malaria transmission during this time of year and one week each in Ndirande and Thyolo. All individuals receiving an RDT at the clinic were referred to our study and samples were taken from all of those individuals who consented to the study. The end of data collection was not determined by any factor other than the end of the defined sample collection period. Samples from two individuals who withdrew their consent after participation were discarded; all other samples were shipped to the US for further experiments. We aimed to detect natural antibody responses among the study participants that recognize giRBCs and then to determine the targets of these antibody responses. To examine antibody binding to the giRBC surface, we used a surface reactivity flow cytometry assay, immunofluorescence microscopy and a protein array enriched for proteins expressed during gametocyte stages. In these experiments, samples were identified only by number and patient age and corresponding clinical data was unblinded only after experiments finished. Three technical replicates were used for all samples and two biological replicates were performed for a subset of samples. In cases where the result from one technical replicate was of a different magnitude than the other 2 replicates, this value was removed. To determine the identity of antigens targeted by the identified antibodies, we used mass spectrometry and immunization of mice with giRBC membranes, each using 3 biological replicates for preparation of giRBC membranes. Surface expression of candidate antigens was validated by Western blot, flow cytometry, and immunofluorescence microscopy. Functional activity was assessed using a THP-1 cell phagocytosis assay. Sequence diversity was assessed using standard methods (nonsynonymous pairwise nucleotide diversity, balancing selection measured by Tajima's D, genetic differentiation measured by the fixation index).

Statistical analysis

The appropriate statistical test for each experiment was determined based on the type of data being compared. FDR corrections were performed for all analyses involving multiple comparisons and *p*-values <0.05 were considered significant. Simple univariate linear regressions were performed for examining the

correlation between levels of IgG responses against individual fragments on the protein array and covariates including (ordinally categorized) age, burden, and iRBC recognition by flow cytometry. *P*-values across fragments were corrected with Bonferroni method. Pairwise, two-sided student t-tests were used to test for difference in mean IgG response against proteins across stages. Linear regressions were used to test for associations between IgG response against fragments and parasite load, gametocyte load and gametocyte fraction, with adjustment for age by including age groups as covariates. The regression t-statistics (estimated coefficients / standard error) of internal and secreted protein fragments are compared by two-sided Mann-Whitney U test. The association of gametocyte fraction and breadth of response (number of proteins seropositive) was conducted on gametocyte positive individuals for whom asexual and gametocyte stages had been quantified. Analysis on breadth and fraction on continuous scales was performed with linear regression, adjusting for gametocyte density. Analysis with breadth as a binary variable was performed with logistic regression, adjusting for gametocyte density. Throughout the manuscript significant *p*-values are reported either as is or with the corresponding alpha-level (all < 0.05).

Supplementary materials

Table S2. Annotation of proteins on the array.

Table S3. Correlations between mean responses and age.\

Table S4. Correlations between mean responses and giRBC surface reactivity by flow cytometry.

Table S5. Proteomics hits identified by LC-MS/MS.

Table S6. IgG responses from mice immunized with gametocyte ghosts.

Table S7. Candidate gametocyte antigens identified by three complementary methods (expanded from table 2).

Table S8. Amino acid sequences for peptide antibodies generated in this study.

Table S9. Genetic diversity data for all genes analyzed in this study.

Table S10. Correlations between mean responses by array with parasite load.

Data file S1. Primary data

References and Notes

1. S. Bhatt, D. J. Weiss, E. Cameron, D. Bisanzio, B. Mappin, U. Dalrymple, K. E. Battle, C. L. Moyes, A. Henry, P. A. Eckhoff, E. A. Wenger, O. Briet, M. A. Penny, T. A. Smith, A. Bennett, J. Yukich, T. P. Eisele, J. T. Griffin, C. A. Fergus, M. Lynch, F. Lindgren, J. M. Cohen, C. L. Murray, D. L. Smith, S. I. Hay, R. E. Cibulskis, P. W. Gething, The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature* **526**, 207-211 (2015).
2. E. A. Ashley, M. Dhorda, R. M. Fairhurst, C. Amaratunga, P. Lim, S. Suon, S. Sreng, J. M. Anderson, S. Mao, B. Sam, C. Sopha, C. M. Chuor, C. Nguon, S. Sovannaroth, S. Pukrittayakamee, P. Jittamala, K. Chotivanich, K. Chutasmit, C. Suchatsoonthorn, R. Runcharoen, T. T. Hien, N. T. Thuy-Nhien, N. V. Thanh, N. H. Phu, Y. Htut, K. T. Han, K. H. Aye, O. A. Mokuolu, R. R. Olaosebikan, O. O. Folaranmi, M. Mayxay, M. Khanthavong, B. Hongvanthong, P. N. Newton, M. A. Onyamboko, C. I. Fanello, A. K. Tshefu, N. Mishra, N. Valecha, A. P. Phyo, F. Nosten, P. Yi, R. Tripura, S. Borrmann, M. Bashraheil, J. Peshu, M. A. Faiz, A. Ghose, M. A. Hossain, R. Samad, M. R. Rahman, M. M. Hasan, A. Islam, O. Miotto, R.

Amato, B. MacInnis, J. Stalker, D. P. Kwiatkowski, Z. Bozdech, A. Jeeyapant, P. Y. Cheah, T. Sakulthaew, J. Chalk, B. Intharabut, K. Silamut, S. J. Lee, B. Vihokhern, C. Kunasol, M. Imwong, J. Tarning, W. J. Taylor, S. Yeung, C. J. Woodrow, J. A. Flegg, D. Das, J. Smith, M. Venkatesan, C. V. Plowe, K. Stepniewska, P. J. Guerin, A. M. Dondorp, N. P. Day, N. J. White, C. Tracking Resistance to Artemisinin, Spread of artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med* **371**, 411-423 (2014).

3. J. Hemingway, H. Ranson, A. Magill, J. Kolaczinski, C. Fornadel, J. Gimnig, M. Coetzee, F. Simard, D. K. Roch, C. K. Hinzoumbe, J. Pickett, D. Schellenberg, P. Gething, M. Hoppe, N. Hamon, Averting a malaria disaster: will insecticide resistance derail malaria control? *Lancet* **387**, 1785-1788 (2016).

4. P. L. Alonso, G. Brown, M. Arevalo-Herrera, F. Binka, C. Chitnis, F. Collins, O. K. Doumbo, B. Greenwood, B. F. Hall, M. M. Levine, K. Mendis, R. D. Newman, C. V. Plowe, M. H. Rodriguez, R. Sinden, L. Slutsker, M. Tanner, A research agenda to underpin malaria eradication. *PLoS medicine* **8**, e1000406 (2011).

5. R. Aguilar, A. Magallon-Tejada, A. H. Achtman, C. Moraleda, R. Joice, P. Cistero, C. S. Li Wai Suen, A. Nhabomba, E. Macete, I. Mueller, M. Marti, P. L. Alonso, C. Menendez, L. Schofield, A. Mayor, Molecular evidence for the localization of Plasmodium falciparum immature gametocytes in bone marrow. *Blood* **123**, 959-966 (2014).

6. E. Farfour, F. Charlotte, C. Settegrana, M. Miyara, P. Buffet, The extravascular compartment of the bone marrow: a niche for Plasmodium falciparum gametocyte maturation? *Malar J* **11**, 285 (2012).

7. R. Joice, S. K. Nilsson, J. Montgomery, S. Dankwa, E. Egan, B. Morahan, K. B. Seydel, L. Bertuccini, P. Alano, K. C. Williamson, M. T. Duraisingh, T. E. Taylor, D. A. Milner, M. Marti, Plasmodium falciparum transmission stages accumulate in the human bone marrow. *Science translational medicine* **6**, 244re245 (2014).

8. R. W. Sauerwein, T. Bousema, Transmission blocking malaria vaccines: Assays and candidates in clinical development. *Vaccine* **33**, 7476-7482 (2015).
9. C. J. Sutherland, Surface antigens of Plasmodium falciparum gametocytes--a new class of transmission-blocking vaccine targets? *Mol Biochem Parasitol* **166**, 93-98 (2009).
10. J. T. Bousema, L. C. Gouagna, C. J. Drakeley, A. M. Meutstege, B. A. Okech, I. N. Akim, J. C. Beier, J. I. Githure, R. W. Sauerwein, Plasmodium falciparum gametocyte carriage in asymptomatic children in western Kenya. *Malaria journal* **3**, 18 (2004).
11. S. Dunyo, P. Milligan, T. Edwards, C. Sutherland, G. Targett, M. Pinder, Gametocytaemia after drug treatment of asymptomatic Plasmodium falciparum *PloS Clinical Trials* **1**, e20 (2006).
12. M. Saeed, W. Roeffen, N. Alexander, C. J. Drakeley, G. A. Targett, C. J. Sutherland, Plasmodium falciparum antigens on the surface of the gametocyte-infected erythrocyte. *PLoS One* **3**, e2280 (2008).
13. J. K. Baird, T. R. Jones, Purnomo, S. Masbar, S. Ratiwayanto, B. Leksana, Evidence for specific suppression of gametocytemia by Plasmodium falciparum in residents of hyperendemic Irian Jaya. *The American journal of tropical medicine and hygiene* **44**, 183-190 (1991).
14. B. Dinko, E. King, G. A. Targett, C. J. Sutherland, Antibody responses to surface antigens of Plasmodium falciparum gametocyte-infected erythrocytes and their relation to gametocytaemia. *Parasite Immunol* **38**, 352-364 (2016).
15. N. Tonwong, J. Sattabongkot, T. Tsuboi, H. Iriko, S. Takeo, J. Sirichaisinthop, R. Udomsangpetch, Natural infection of Plasmodium falciparum induces inhibitory antibodies against gametocyte development in human hosts. *Japanese journal of infectious diseases* **65**, 152-156 (2012).
16. J. A. Chan, F. J. Fowkes, J. G. Beeson, Surface antigens of Plasmodium falciparum-infected erythrocytes as immune targets and malaria vaccine candidates. *Cell Mol Life Sci* **71**, 3633-3657 (2014).

17. A. Bengtsson, L. Joergensen, T. S. Rask, R. W. Olsen, M. A. Andersen, L. Turner, T. G. Theander, L. Hviid, M. K. Higgins, A. Craig, A. Brown, A. T. Jensen, A novel domain cassette identifies Plasmodium falciparum PfEMP1 proteins binding ICAM-1 and is a target of cross-reactive, adhesion-inhibitory antibodies. *Journal of immunology* **190**, 240-249 (2013).

18. J. A. Chan, K. B. Howell, L. Reiling, R. Ataide, C. L. Mackintosh, F. J. Fowkes, M. Petter, J. M. Chesson, C. Langer, G. M. Warimwe, M. F. Duffy, S. J. Rogerson, P. C. Bull, A. F. Cowman, K. Marsh, J. G. Beeson, Targets of antibodies against Plasmodium falciparum-infected erythrocytes in malaria immunity. *The Journal of clinical investigation* **122**, 3227-3238 (2012).

19. G. Arora, G. T. Hart, J. Manzella-Lapeira, J. Y. Doritchamou, D. L. Narum, L. M. Thomas, J. Brzostowski, S. Rajagopalan, O. K. Doumbo, B. Traore, L. H. Miller, S. K. Pierce, P. E. Duffy, P. D. Crompton, S. A. Desai, E. O. Long, NK cells inhibit Plasmodium falciparum growth in red blood cells via antibody-dependent cellular cytotoxicity. *eLife* **7**, (2018).

20. M. Tiburcio, F. Silvestrini, L. Bertuccini, A. F. Sander, L. Turner, T. Lavstsen, P. Alano, Early gametocytes of the malaria parasite Plasmodium falciparum specifically remodel the adhesive properties of infected erythrocyte surface. *Cellular microbiology*, (2012).

21. F. Silvestrini, E. Lasonder, A. Olivieri, G. Camarda, B. van Schaijk, M. Sanchez, S. Younis Younis, R. Sauerwein, P. Alano, Protein export marks the early phase of gametocytogenesis of the human malaria parasite Plasmodium falciparum. *Molecular & cellular proteomics : MCP* **9**, 1437-1448 (2010).

22. W. J. R. Stone, J. J. Campo, A. L. Ouedraogo, L. Meerstein-Kessel, I. Morlais, D. Da, A. Cohuet, S. Nsango, C. J. Sutherland, M. van de Vegte-Bolmer, R. Siebelink-Stoter, G. J. van Gemert, W. Graumans, K. Lanke, A. D. Shandling, J. V. Pablo, A. A. Teng, S. Jones, R. M. de Jong, A. Fabra-Garcia, J. Bradley, W. Roeffen, E. Lasonder, G. Gremo, E. Schwarzer, C. J. Janse, S. K. Singh, M. Theisen, P. Felgner, M. Marti, C. Drakeley, R. Sauerwein, T. Bousema, M. M. Jore, Unravelling the immune signature of Plasmodium falciparum transmission-reducing immunity. *Nat Commun* **9**, 558 (2018).

23. E. Lasonder, C. J. Janse, G. J. van Gemert, G. R. Mair, A. M. Vermunt, B. G. Douradinha, V. van Noort, M. A. Huynen, A. J. Luty, H. Kroeze, S. M. Khan, R. W. Sauerwein, A. P. Waters, M. Mann, H. G. Stunnenberg, Proteomic profiling of Plasmodium sporozoite maturation identifies new proteins essential for parasite development and infectivity. *PLoS pathogens* **4**, e1000195 (2008).

24. S. C. Oehring, B. J. Woodcroft, S. Moes, J. Wetzel, O. Dietz, A. Pulfer, C. Dekiwadia, P. Maeser, C. Flueck, K. Witmer, N. M. Brancucci, I. Niederwieser, P. Jenoe, S. A. Ralph, T. S. Voss, Organellar proteomics reveals hundreds of novel nuclear proteins in the malaria parasite Plasmodium falciparum. *Genome biology* **13**, R108 (2012).

25. A. G. Maier, M. Rug, M. T. O'Neill, M. Brown, S. Chakravorty, T. Szestak, J. Chesson, Y. Wu, K. Hughes, R. L. Coppel, C. Newbold, J. G. Beeson, A. Craig, B. S. Crabb, A. F. Cowman, Exported proteins required for virulence and rigidity of Plasmodium falciparum-infected human erythrocytes. *Cell* **134**, 48-61 (2008).

26. S. R. Elliott, P. D. Payne, M. F. Duffy, T. J. Byrne, W. H. Tham, S. J. Rogerson, G. V. Brown, D. P. Eisen, Antibody recognition of heterologous variant surface antigens after a single Plasmodium falciparum infection in previously naive adults. *The American journal of tropical medicine and hygiene* **76**, 860-864 (2007).

27. M. Hommel, S. R. Elliott, V. Soma, G. Kelly, F. J. Fowkes, J. M. Chesson, M. F. Duffy, J. Bockhorst, M. Avril, I. Mueller, A. Raiko, D. I. Stanisic, S. J. Rogerson, J. D. Smith, J. G. Beeson, Evaluation of the antigenic diversity of placenta-binding Plasmodium falciparum variants and the antibody repertoire among pregnant women. *Infection and immunity* **78**, 1963-1978 (2010).

28. M. Aingaran, R. Zhang, S. K. Law, Z. Peng, A. Undisz, E. Meyer, M. Diez-Silva, T. A. Burke, T. Spielmann, C. T. Lim, S. Suresh, M. Dao, M. Marti, Host cell deformability is linked to transmission in the human malaria parasite Plasmodium falciparum. *Cellular microbiology* **14**, 983-993 (2012).

29. K. Marsh, S. Kinyanjui, Immune effector mechanisms in malaria. *Parasite immunology* **28**, 51-60 (2006).
30. L. Schofield, I. Mueller, Clinical immunity to malaria. *Current molecular medicine* **6**, 205-221 (2006).
31. V. Ryg-Cornejo, A. Ly, D. S. Hansen, Immunological processes underlying the slow acquisition of humoral immunity to malaria. *Parasitology* **143**, 199-207 (2016).
32. T. Bousema, W. Roeffen, H. Meijerink, H. Mwerinde, S. Mwakalinga, G. J. van Gemert, M. van de Vegte-Bolmer, F. Mosha, G. Targett, E. M. Riley, R. Sauerwein, C. Drakeley, The dynamics of naturally acquired immune responses to Plasmodium falciparum sexual stage antigens Pfs230 & Pfs48/45 in a low endemic area in Tanzania. *PLoS One* **5**, e14114 (2010).
33. C. J. Drakeley, J. T. Bousema, N. I. Akim, K. Teelen, W. Roeffen, A. H. Lensen, M. Bolmer, W. Eling, R. W. Sauerwein, Transmission-reducing immunity is inversely related to age in Plasmodium falciparum gametocyte carriers. *Parasite immunology* **28**, 185-190 (2006).
34. T. J. Sargeant, M. Marti, E. Caler, J. M. Carlton, K. Simpson, T. P. Speed, A. F. Cowman, Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. *Genome biology* **7**, R12 (2006).
35. S. K. Nilsson Bark, R. Ahmad, K. Dantzler, A. K. Lukens, M. De Niz, M. J. Szucs, X. Jin, J. Cotton, D. Hoffmann, E. Bric-Furlong, R. Oomen, M. Parrington, D. Milner, D. E. Neafsey, S. A. Carr, D. F. Wirth, M. Marti, Quantitative Proteomic Profiling Reveals Novel Plasmodium falciparum Surface Antigens and Possible Vaccine Candidates. *Molecular & cellular proteomics : MCP* **17**, 43-60 (2018).
36. L. Vincensini, S. Richert, T. Blisnick, A. Van Dorsselaer, E. Leize-Wagner, T. Rabilloud, C. Braun Breton, Proteomic analysis identifies novel proteins of the Maurer's clefts, a secretory compartment delivering Plasmodium falciparum proteins to the surface of its host cell. *Molecular & cellular proteomics : MCP* **4**, 582-593 (2005).

37. P. Hermand, L. Ciceron, C. Pionneau, C. Vaquero, C. Combadiere, P. Deterre, Plasmodium falciparum proteins involved in cytoadherence of infected erythrocytes to chemokine CX3CL1. *Scientific reports* **6**, 33786 (2016).

38. S. J. Tarr, R. W. Moon, I. Hardege, A. R. Osborne, A conserved domain targets exported PHISTb family proteins to the periphery of Plasmodium infected erythrocytes. *Molecular and biochemical parasitology* **196**, 29-40 (2014).

39. A. Oberli, L. M. Slater, E. Cutts, F. Brand, E. Mundwiler-Pachlatko, S. Rusch, M. F. Masik, M. C. Erat, H. P. Beck, I. Vakonakis, A Plasmodium falciparum PHIST protein binds the virulence factor PfEMP1 and comigrates to knobs on the host cell surface. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **28**, 4420-4433 (2014).

40. J. G. Beeson, E. J. Mann, S. R. Elliott, V. M. Lema, E. Tadesse, M. E. Molyneux, G. V. Brown, S. J. Rogerson, Antibodies to variant surface antigens of Plasmodium falciparum-infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. *J Infect Dis* **189**, 540-551 (2004).

41. A. Ghumra, P. Khunrae, R. Ataide, A. Raza, S. J. Rogerson, M. K. Higgins, J. A. Rowe, Immunisation with recombinant PfEMP1 domains elicits functional rosette-inhibiting and phagocytosis-inducing antibodies to Plasmodium falciparum. *PloS one* **6**, e16414 (2011).

42. D. L. Hill, E. M. Eriksson, C. S. Li Wai Suen, C. Y. Chiu, V. Ryg-Cornejo, L. J. Robinson, P. M. Siba, I. Mueller, D. S. Hansen, L. Schofield, Opsonising antibodies to P. falciparum merozoites associated with immunity to clinical malaria. *PloS one* **8**, e74627 (2013).

43. F. H. Osier, G. Feng, M. J. Boyle, C. Langer, J. Zhou, J. S. Richards, F. J. McCallum, L. Reiling, A. Jaworowski, R. F. Anders, K. Marsh, J. G. Beeson, Opsonic phagocytosis of Plasmodium falciparum merozoites: mechanism in human immunity and a correlate of protection against malaria. *BMC Med* **12**, 108 (2014).

628 44. R. G. Donald, T. Zhong, H. Wiersma, B. Nare, D. Yao, A. Lee, J. Allocco, P. A. Liberator,
629 Anticoccidial kinase inhibitors: identification of protein kinase targets secondary to cGMP-
630 dependent protein kinase. *Mol Biochem Parasitol* **149**, 86-98 (2006).
631 45. N. M. Brancucci, I. Goldowitz, K. Buchholz, K. Werling, M. Marti, An assay to probe
632 Plasmodium falciparum growth, transmission stage formation and early gametocyte development.
633 *Nature protocols* **10**, 1131-1142 (2015).
634 46. N. Obaldia, 3rd, E. Meibalan, J. M. Sa, S. Ma, M. A. Clark, P. Mejia, R. R. Moraes Barros, W.
635 Otero, M. U. Ferreira, J. R. Mitchell, D. A. Milner, C. Huttenhower, D. F. Wirth, M. T.
636 Duraisingh, T. E. Wellems, M. Marti, Bone Marrow Is a Major Parasite Reservoir in Plasmodium
637 vivax Infection. *MBio* **9**, (2018).
638 47. M. De Niz, E. Meibalan, P. Mejia, S. Ma, N. M. B. Brancucci, C. Agop-Nersesian, R. Mandt, P.
639 Ngotho, K. R. Hughes, A. P. Waters, C. Huttenhower, J. R. Mitchell, R. Martinelli, F.
640 Frischknecht, K. B. Seydel, T. Taylor, D. Milner, V. T. Heussler, M. Marti, Plasmodium
641 gametocytes display homing and vascular transmigration in the host bone marrow. *Sci Adv* **4**,
642 eaat3775 (2018).
643 48. R. S. Lee, A. P. Waters, J. M. Brewer, A cryptic cycle in haematopoietic niches promotes
644 initiation of malaria transmission and evasion of chemotherapy. *Nat Commun* **9**, 1689 (2018).
645 49. V. Messina, M. Valtieri, M. Rubio, M. Falchi, F. Mancini, A. Mayor, P. Alano, F. Silvestrini,
646 Gametocytes of the Malaria Parasite Plasmodium falciparum Interact With and Stimulate Bone
647 Marrow Mesenchymal Cells to Secrete Angiogenetic Factors. *Frontiers in cellular and infection*
648 *microbiology* **8**, 50 (2018).
649 50. P. Hamon, P. L. Loyher, C. Baudesson de Chanville, F. Licata, C. Combadiere, A. Boissonnas,
650 CX3CR1-dependent endothelial margination modulates Ly6Chigh monocyte systemic
651 deployment upon inflammation in mice. *Blood* **129**, 1296-1307 (2017).

51. C. Drakeley, C. Sutherland, J. T. Bousema, R. W. Sauerwein, G. A. Targett, The epidemiology of Plasmodium falciparum gametocytes: weapons of mass dispersion. *Trends in parasitology* **22**, 424-430 (2006).

52. K. G. Pelle, K. Oh, K. Buchholz, V. Narasimhan, R. Joice, D. A. Milner, N. M. Brancucci, S. Ma, T. S. Voss, K. Ketman, K. B. Seydel, T. E. Taylor, N. S. Barteneva, C. Huttenhower, M. Marti, Transcriptional profiling defines dynamics of parasite tissue sequestration during malaria infection. *Genome medicine* **7**, 19 (2015).

53. L. Florens, X. Liu, Y. Wang, S. Yang, O. Schwartz, M. Peglar, D. J. Carucci, J. R. Yates, 3rd, Y. Wub, Proteomics approach reveals novel proteins on the surface of malaria-infected erythrocytes. *Mol Biochem Parasitol* **135**, 1-11 (2004).

54. C. Harris, I. Morlais, T. S. Churcher, P. Awono-Ambene, L. C. Gouagna, R. K. Dabire, D. Fontenille, A. Cohuet, Plasmodium falciparum produce lower infection intensities in local versus foreign Anopheles gambiae populations. *PloS one* **7**, e30849 (2012).

55. I. Morlais, S. E. Nsango, W. Toussile, L. Abate, Z. Annan, M. T. Tchioffo, A. Cohuet, P. H. Awono-Ambene, D. Fontenille, F. Rousset, A. Berry, Plasmodium falciparum mating patterns and mosquito infectivity of natural isolates of gametocytes. *PloS one* **10**, e0123777 (2015).

56. C. J. Drakeley, I. Secka, S. Correa, B. M. Greenwood, G. A. Targett, Host haematological factors influencing the transmission of Plasmodium falciparum gametocytes to Anopheles gambiae s.s. mosquitoes. *Trop Med Int Health* **4**, 131-138 (1999).

57. C. J. Drakeley, N. I. Akim, R. W. Sauerwein, B. M. Greenwood, G. A. Targett, Estimates of the infectious reservoir of Plasmodium falciparum malaria in The Gambia and in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**, 472-476 (2000).

58. G. Targett, C. Drakeley, M. Jawara, L. von Seidlein, R. Coleman, J. Deen, M. Pinder, T. Doherty, C. Sutherland, G. Walraven, P. Milligan, Artesunate reduces but does not prevent posttreatment transmission of Plasmodium falciparum to Anopheles gambiae. *The Journal of infectious diseases* **183**, 1254-1259 (2001).

59. S. Dunyo, R. Ord, R. Hallett, M. Jawara, G. Walraven, E. Mesa, R. Coleman, M. Sowe, N. Alexander, G. A. Targett, M. Pinder, C. J. Sutherland, Randomised trial of chloroquine/sulphadoxine-pyrimethamine in Gambian children with malaria: impact against multidrug-resistant P. falciparum. *PLoS Clin Trials* **1**, e14 (2006).

60. R. L. Hallett, S. Dunyo, R. Ord, M. Jawara, M. Pinder, A. Randall, A. Alloueche, G. Walraven, G. A. Targett, N. Alexander, C. J. Sutherland, Chloroquine/sulphadoxine-pyrimethamine for gambian children with malaria: transmission to mosquitoes of multidrug-resistant Plasmodium falciparum. *PLoS Clin Trials* **1**, e15 (2006).

61. C. J. Sutherland, R. Ord, S. Dunyo, M. Jawara, C. J. Drakeley, N. Alexander, R. Coleman, M. Pinder, G. Walraven, G. A. Targett, Reduction of malaria transmission to Anopheles mosquitoes with a six-dose regimen of co-artemether. *PLoS medicine* **2**, e92 (2005).

62. A. L. Ouedraogo, B. P. Goncalves, A. Gneme, E. A. Wenger, M. W. Guelbeogo, A. Ouedraogo, J. Gerardin, C. A. Bever, H. Lyons, X. Pitroipa, J. P. Verhave, P. A. Eckhoff, C. Drakeley, R. Sauerwein, A. J. Luty, B. Kouyate, T. Bousema, Dynamics of the Human Infectious Reservoir for Malaria Determined by Mosquito Feeding Assays and Ultrasensitive Malaria Diagnosis in Burkina Faso. *The Journal of infectious diseases* **213**, 90-99 (2016).

63. D. F. Da, T. S. Churcher, R. S. Yerbanga, B. Yameogo, I. Sangare, J. B. Ouedraogo, R. E. Sinden, A. M. Blagborough, A. Cohuet, Experimental study of the relationship between Plasmodium gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. *Exp Parasitol* **149**, 74-83 (2015).

64. D. P. Mathanga, E. D. Walker, M. L. Wilson, D. Ali, T. E. Taylor, M. K. Laufer, Malaria control in Malawi: current status and directions for the future. *Acta Trop* **121**, 212-217 (2012).

65. D. H. Davies, X. Liang, J. E. Hernandez, A. Randall, S. Hirst, Y. Mu, K. M. Romero, T. T. Nguyen, M. Kalantari-Dehaghi, S. Crotty, P. Baldi, L. P. Villarreal, P. L. Felgner, Profiling the humoral immune response to infection by using proteome microarrays: High-throughput vaccine and diagnostic antigen discovery. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 547-552 (2005).

66. B. M. Bolstad, R. A. Irizarry, M. Astrand, T. P. Speed, A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185-193 (2003).

67. Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing. . *Journal of the Royal Statistical Society* **Series B 57**, 289-300 (1995).

68. W. Trager, J. B. Jensen, Human malaria parasites in continuous culture. *Science* **193**, 673-675 (1976).

69. K. Buchholz, T. A. Burke, K. C. Williamson, R. C. Wiegand, D. F. Wirth, M. Marti, A high-throughput screen targeting malaria transmission stages opens new avenues for drug development. *J Infect Dis* **203**, 1445-1453 (2011).

70. Q. L. Fivelman, L. McRobert, S. Sharp, C. J. Taylor, M. Saeed, C. A. Swales, C. J. Sutherland, D. A. Baker, Improved synchronous production of Plasmodium falciparum gametocytes in vitro. *Mol Biochem Parasitol* **154**, 119-123 (2007).

71. D. Tao, C. Ubaida-Mohien, D. K. Mathias, J. G. King, R. Pastrana-Mena, A. Tripathi, I. Goldowitz, D. R. Graham, E. Moss, M. Marti, R. R. Dinglasan, Sex-partitioning of the Plasmodium falciparum stage V gametocyte proteome provides insight into falciparum-specific cell biology. *Molecular & cellular proteomics : MCP* **13**, 2705-2724 (2014).

72. R. C. Team, R: A language and environment for statistical computing. *R Foundation for Statistical Computing, Vienna, Austria*, http://www.R-project.org (2014).

73. A. Teo, W. Hasang, P. Boeuf, S. Rogerson, A Robust Phagocytosis Assay to Evaluate the Opsonic Activity of Antibodies against Plasmodium falciparum-Infected Erythrocytes. *Methods Mol Biol* **1325**, 145-152 (2015).

74. B. S. Weir, C. C. Cockerham, Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358-1370 (1984).

Acknowledgements: Antibodies to full length PF3D7_0532400 were kindly provided by Dr. Hans-Peter Beck (Swiss Tropical and Public Health Institute, Basel), and to N-terminal SBP-1 by Dr. Tobias Spielmann (Bernhard-Nocht-Institut für Tropenmedizin, Hamburg). Rebecca Tweedell (Johns Hopkins) helped with protein sample in-gel digestion for mass spectrometry. We would like to acknowledge the following people for their help collecting plasma samples in Blantyre: Nelson Chimbiya, Chiledso Mlangali, Alexious Mwafulirwa, Dr. Atupele Kapito-Tembo, Paul Pensulo, Andy Bauleni, Alex Saidi, Esther Gondwe, and the entire Facility-based surveillance team (Malawi ICEMR). We also thank all of the individuals who participated in the study, as well as their families.

Funding: This work was supported by the U.S. NIH (M.M.: R01A1077558, P.F.: AI095916 and U19 AI089686, T.T.: U19AI089683, D.T.: Intramural Research Program of the NIH, National Center for Advancing Translational Sciences, C.U.: Intramural Research Program of the NIH, National institute on Aging), a career development award from the Burroughs Wellcome Fund (M.M.), European Research Council award BoneMalar ERC-2015-CoG 682360 to M.M., Sanofi Innovation Award and a Wellcome Trust Center award (104111). D.T., C.U. and R.R.D. were supported by the Bloomberg Family Foundation through the Johns Hopkins Malaria Research Institute. S.R., W.J.R.S and T.B. are supported by the Netherlands Organization for Scientific Research through a VIDI fellowship grant to T.B. (number 016.158.306) and a fellowship from the European Research Council (ERC-2014-StG 639776) to T.B**.** K.W.D. was supported by a Herchel Smith Graduate Fellowship. M.D.N. was supported by a postdoctoral fellowship from the Swiss National Science Foundation (P2BEP3_165396) and S.N.B. by a postdoctoral fellowship from the American Heart Association. P.H. and P.D. were supported by “Fondation pour la Recherche Médicale “ (Equipes FRM 2016) and by “Agence Nationale de la Recherche" (grant CE-15-0019-01, CMOS).

Author contributions:

K.W.D., S.R., T.B. and M.M. designed the experiments in this study. K.W.D. performed flow cytometry experiments, Western blots, preparation of giRBC membranes, and immunofluorescence microscopy with help from S.R., M.M.J. and T.R. (flow cytometry with stage V gametocytes and gametes), S.K.N.B. (preparation of aiRBC membranes and design of mouse immunization studies), and M.D.N. (live immunofluorescence microscopy). S.M. and C.H. designed and executed statistical analysis of protein array data. D.T., C.U-M. and R.D. conducted mass spectrometry-based proteomics. W.J.R.S., T.B. and J.J.C. developed the protein array down-selected from a larger array originally developed by H.D. and P.F. J.J.C. and A.A.T. designed protein microarray experiments, A.A.T. produced gametocyte protein arrays, and T.Q.L. and C.L.W. generated protein array data. P.N. performed phagocytosis assays and protein clustering analysis. L.L. performed high content image analysis of iRBCs. A.M.E. and D.N. performed population diversity analysis. T.T., K.S. and M.L. helped set up the data collection in Malawi while I.M. set up the cohorts in Burkina Faso, Cameroon, and the Gambia. P.H. and P.D. provided GEXP10 and GEXP07 antibodies and helped with data interpretation. D.F.W. was involved in study design and helped with data interpretation. K.W.D., T.B. and M.M. wrote the manuscript with input from all co-authors.

Competing interests:

P.F. is inventor on patent application # US20180016299A1 submitted by University of California that covers Protein Microarray Construction.

Data and availability:

All data associated with this study are present in the paper or Supplementary Materials. Sample meta data and protein microarray data are available at https://datadryad.org/resource/doi:10.5061/dryad.8bp05. Whole genome sequence data for the Senegal and Malawi samples are publicly available on the MalariaGen Pf3k website (https://www.malariagen.net/projects/pf3k). Scripts and example input files used for calculating population genetics statistics are available at https://github.com/amearly/Dantzler_et_al_Diversity_Calcs.

781

782

Figures legends

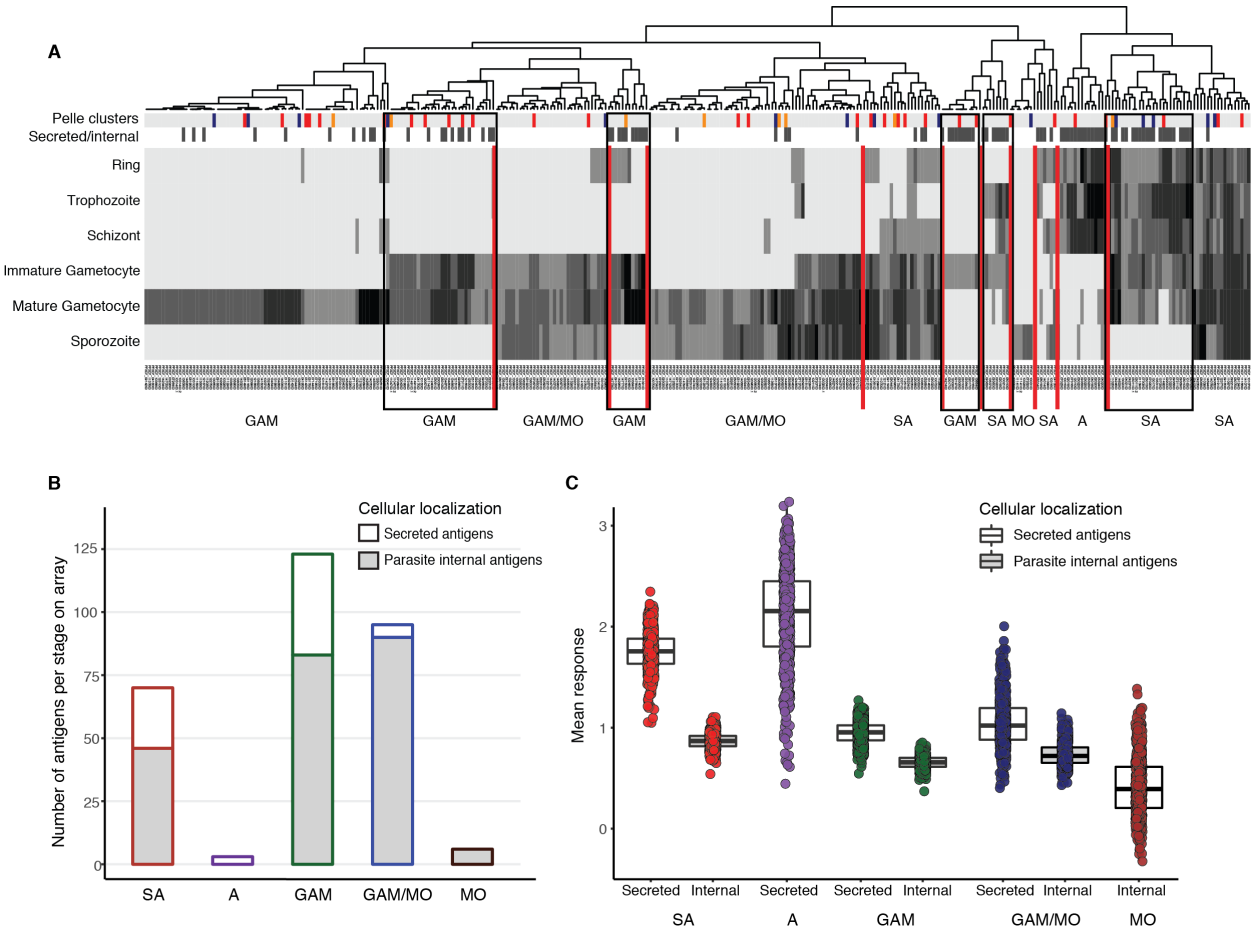


Figure 1: Human plasma samples recognize secreted asexual (aiRBC) and gametocyte (giRBC) surface antigens.

A. Heat map of 344 *P. falciparum* antigens from 3D7 genome (PlasmoDB Release 31) clustering proteins on the array by timing of protein expression (log read counts of number of peptides sequences). Additional annotations are indicated by color bars at the top of the heat map: first row indicates cluster stage annotation from (*52*) (orange: gametocyte rings, red: immature gametocytes, blue: mature gametocytes, grey: others), and second row indicates cellular localization (black: secreted, white: internal/unknown). Vertical red lines separate stage-specific clusters. Black boxes highlight 5 clusters of shared or gametocyte-specific secreted antigens. **B.** Distribution of 528 *P. falciparum* protein fragments on the peptide array (developed in (*22*)) by stage and location. The proteins were selected based on

795 expression during gametocyte stages and predicted export (details in **table S2**). **C.** Mean responses across
796 3 malaria-exposed populations are quantified by peptide array (after normalization to controls and
797 quantile normalization), stage of protein expression, and whether they are secreted or not (see **table S1**).
798 GAM=Gametocyte, GAM/MO=Gametocyte/Mosquito stages, MO=Mosquito stages, A=Asexual stages,
799 SA=Shared antigens.
800

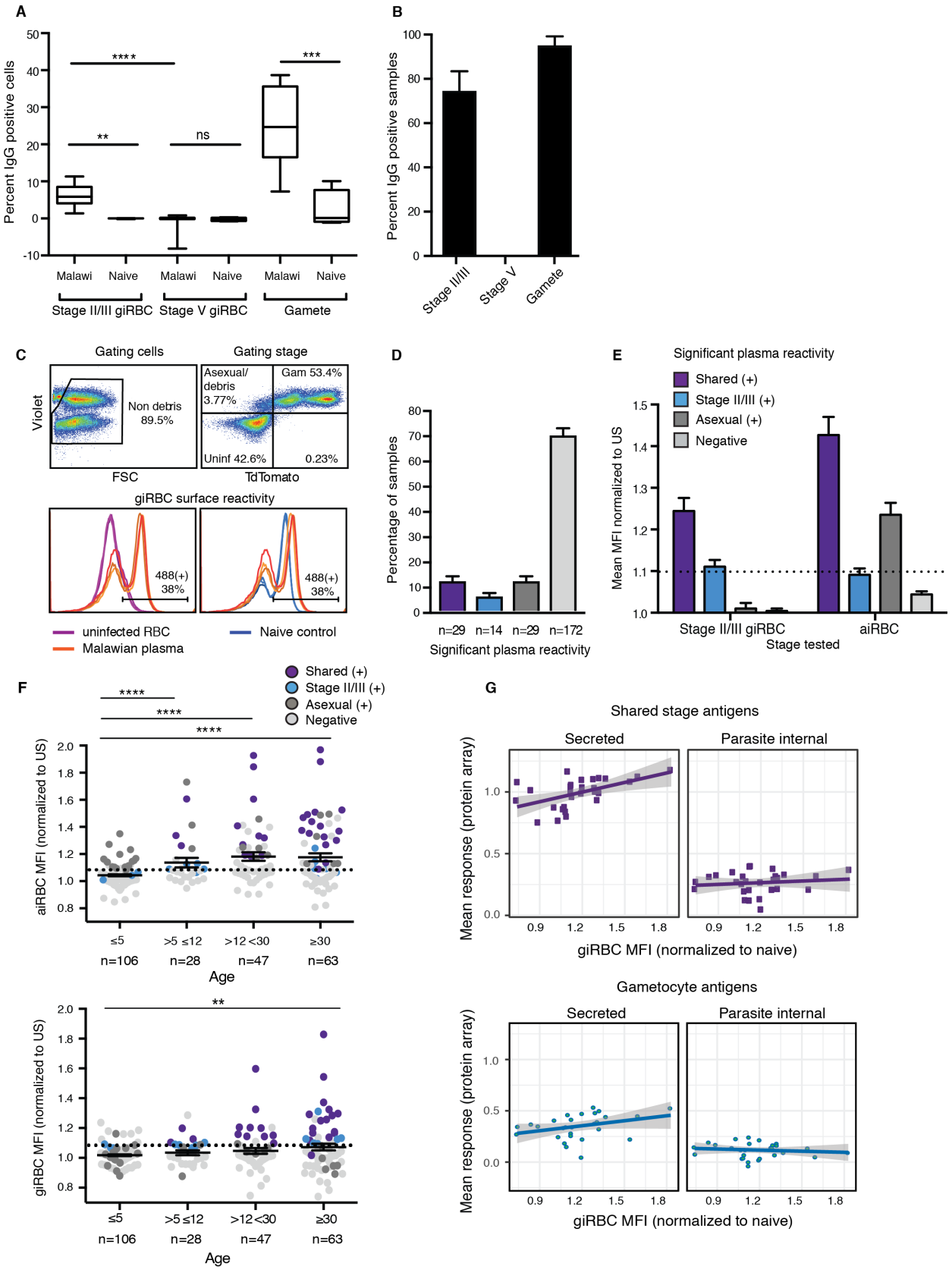
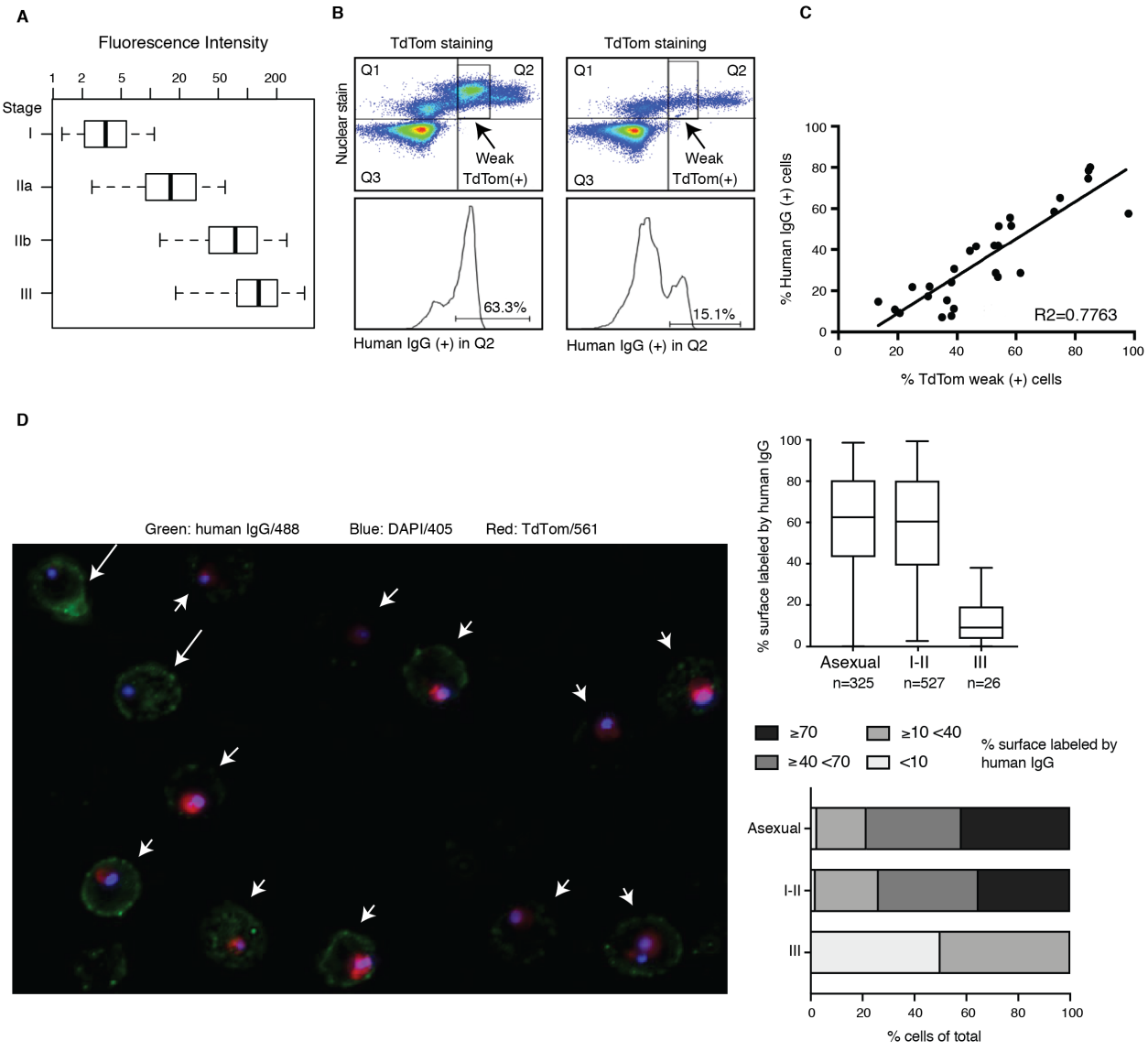

A
Percent IgG positive cells
50
40
30
20
10
0
-10
****
**
ns
***
Malawi
Naive
Malawi
Naive
Malawi
Naive
Stage II/III giRBC
Stage V giRBC
Gamete
B
Percent IgG positive samples
100
80
60
40
20
0
Stage II/III
Stage V
Gamete
C
Gating cells
Gating stage
Violet
Non debris
89.5%
FSC
Asexual/
debris
3.77%
Gam 53.4%
Uninf 42.6%
0.23%
TdTomato
giRBC surface reactivity
488(+)
38%
488(+)
38%
uninfected RBC
Malawian plasma
Naive control
D
Percentage of samples
80
70
60
50
40
30
20
10
0
n=29
n=14
n=29
n=172
Significant plasma reactivity
E
Significant plasma reactivity
Shared (+)
Stage II/III (+)
Asexual (+)
Negative
Mean MFI normalized to US
1.5
1.4
1.3
1.2
1.1
1.0
Stage II/III giRBC
aiRBC
Stage tested
F
Shared (+)
Stage II/III (+)
Asexual (+)
Negative
****
****
****
aiRBC MFI (normalized to US)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
≤5
>5 ≤12
>12 <30
≥30
n=106
n=28
n=47
n=63
Age
**
giRBC MFI (normalized to US)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
≤5
>5 ≤12
>12 <30
≥30
n=106
n=28
n=47
n=63
Age
G
Shared stage antigens
Secreted
Parasite internal
Mean response (protein array)
1.0
0.5
0.0
0.9
1.2
1.5
1.8
giRBC MFI (normalized to naive)
Gametocyte antigens
Secreted
Parasite internal
Mean response (protein array)
1.0
0.5
0.0
0.9
1.2
1.5
1.8
giRBC MFI (normalized to naive)

Figure 2: Immune responses target the immature but not the mature giRBC surface.

A-B. Results from a pilot flow cytometry study testing reactivity of 24 Malawian plasma samples (22 from Chikhwawa, a high transmission region, and 2 from Ndirande, a low transmission region) and 5 naïve controls against stage II/III and stage V gametocytes and gametes. Positive surface reactivity (> 3 standard deviations above mean of naive controls) is shown both as percentage of significantly positive samples of all those tested (**A**) and percentage of positive cells among those incubated with an individual plasma sample (**B**). **C**. Schematic for gating strategy of giRBC surface detection in 244 Malawian plasma samples by flow cytometry. IgG positivity is determined using the Pf2004_164/TdTom line that allows selection of the parasite population (positive for DNA dye) and TdTomato (positive for gametocytes). Top panel: Cells are first gated for live cells and single cells by forward and side scatter (left). After debris is gated out, quadrant gates separate gametocytes (Violet+/TdTomato+), asexual/lysed cells/debris (Violet+/TdTomato-), and uninfected cells (right). Bottom panel: AlexaFluor488 surface fluorescence (human IgG-secondary antibody conjugates) is compared between uninfected cells and gametocytes (left), and between infected cells incubated with naïve controls and Malawian plasma samples (right). Technical replicates are shown as individual lines. **D-E.** Positive recognition of aiRBCs and stage II/III giRBCs (determined by t-tests comparing Malawi samples to naïve US controls using the Holm-Sidak method with alpha=0.05) by 244 Malawian plasma samples is shown as prevalence **(D)** and as significant fold change in AlexaFluor488 median fluorescence compared to naïve controls **(E)**. The threshold for specific positive reactivity was set to 1.1 based on the highest level of non-specific reactivity (i.e. reactivity to aiRBCs of human plasma significantly positive for stage II/III giRBC but negative for aiRBC). **F.** Correlation of human plasma recognizing aiRBCs (top panel) and giRBCs (bottom panel) by flow cytometry with age. **G**. Correlation of antigen responses by peptide array *vs.* surface recognition by flow cytometry. For the same set of Malawi plasma samples, normalized peptide array signal intensities were averaged across all shared stage antigens (top panel) or gametocyte-specific antigens (bottom panel). These mean responses were correlated with giRBC recognition by flow cytometry as measured by median fluorescence (AlexaFluor488) fold change compared to naïve controls. Overall, mean responses of shared

828 secreted antigens are significantly correlated with giRBC recognition (p=0.004), whereas the other
829 correlations are non-significant. Significance values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p <$
830 0.0001.
831



832

833 **Figure 3: Human IgG selectively recognizes the early (stage I/II) giRBC surface.**

834 **A.** Stage-specific expression of the TdTomato reporter in transgenic Pf2004/164TdTom parasites by flow

835 cytometry. Reporter expression is shown by fluorescence intensity in a time course across stage I-III

836 gametocytes. **B.** The Violet+TdTomato+ gametocyte population detected by flow cytometry can be

837 separated into weak TdTomato+ (stage I/II gametocytes) and strong TdTomato+ (stage II/III) subgroups.

838 C. Correlation between TdTom signal and human IgG based on flow cytometry data (Pearson's

839 correlation, $p<0.0001$). **D.** Fluorescence microscopy analysis using the same antibody and reporter

840 combination as above. Left panel: Surface labeling is present on both asexual parasites (arrows) and early

gametocytes (arrowheads). Right panels: High content image quantification of fluorescence microscopy data, based on proportion of the cell surface that is labeled (top panel) and stratified by intensity (bottom panel).

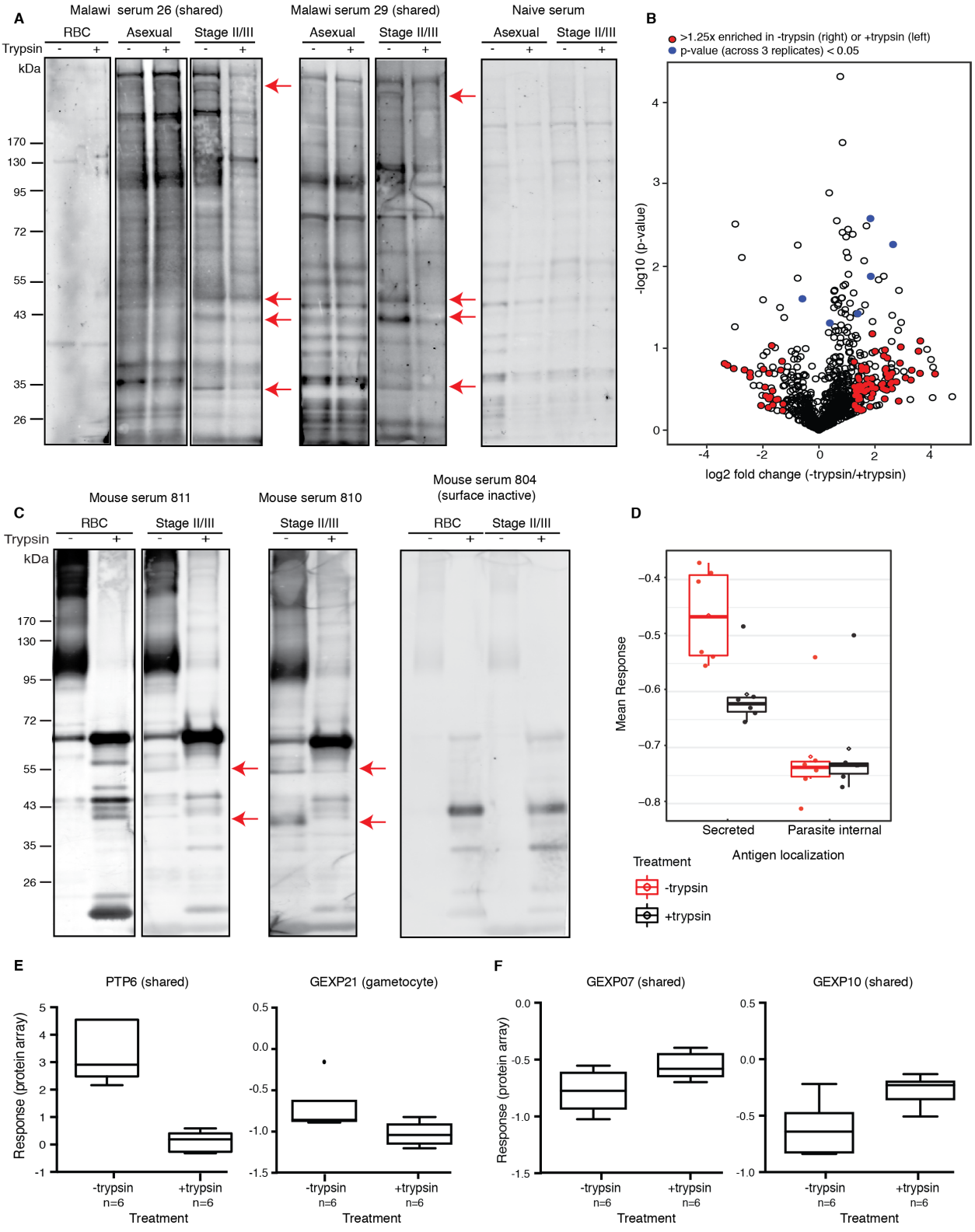


Figure 4: Immunogenic gametocyte antigens identified by 3 complementary approaches. A. Surface-depleted *vs.* surface-intact uninfected and infected RBC membranes were probed with Malawian plasma

samples and naïve US sera by Western blot. Each lane represents protein extract from 2.5×10^6 uRBC or iRBC. Differential band patterns between the trypsin(+) and trypsin(-) samples are marked with red arrows. **B.** Volcano plot showing human and *Plasmodium* proteins identified by comparing surface-intact *vs.* surface-depleted giRBC membranes. X-axis represents log2 fold change of -trypsin/+trypsin and Y-axis shows the T-test *p*-value ($p<0.05$ corresponds to *p*-value 0.0004 after Benjamin-Hochberg correction) of -trypsin/+ trypsin biological replicates (n=3). *Plasmodium* proteins with a log2 fold change >1.25 are marked in red and significant *Plasmodium* proteins across 3 replicates are marked in blue. **C.** Surface-depleted (+trypsin/chymotrypsin) *vs.* surface intact (-trypsin/chymotrypsin) uRBC and giRBC membranes were probed with sera from mice (6 per group) immunized with surface-intact or surface-depleted giRBCs by Western blot. Each lane represents protein extract from 2.5×10^6 uRBC or iRBC. Differential band patterns between the trypsin(+) and trypsin(-) giRBC samples are shown in red. **D.** The array described in **Figure 1** was probed with sera from mice immunized with either surface-depleted or surface-intact giRBC membranes. Responses were normalized to controls and then quantile normalized. **E.** PTP6 and GEXP21 differential responses between sera from mice immunized with surface-intact (-trypsin) giRBC membranes and surface-depleted (+trypsin) giRBC membranes. **F.** GEXP07 and GEXP10 differential responses from sera from mice immunized with intact and surface-depleted membranes. See **table S7** for complete data set.

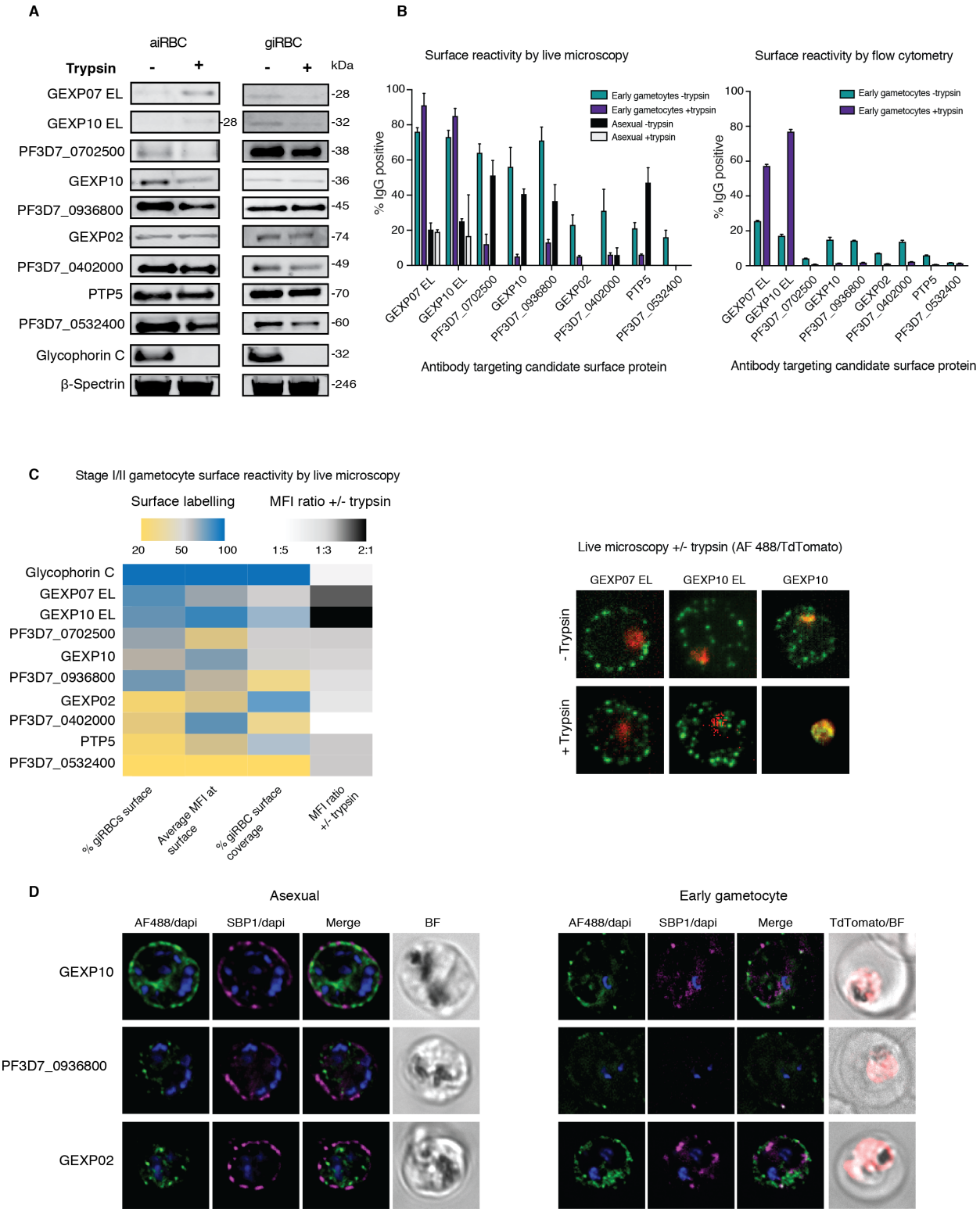


Figure 5: Six candidate antigens expressed during gametocyte stages are validated on the giRBC surface.

Previously published GEXP07 and GEXP10 antibodies (*37*) target the putative extracellular loops of these proteins and will be referred to as "GEXP07 EL" and "GEXP10 EL" to distinguish from our newly produced peptide antibodies targeting the same proteins. **A.** MACS purified aiRBC or giRBC membranes (+/- pre-treatment with trypsin/chymotrypsin: hereafter referred to as +/- trypsin) are probed with polyclonal antibodies targeting candidate antigens by Western blot (see full blots in **Supplemental Figure S6**). Antibodies against Glycophorin C (trypsin-sensitive, surface expressed) and β-spectrin (trypsin resistant, internally localized) are included as controls. Each lane represents protein extract from 2.5×10^6 iRBC. **B.** Reactivity of candidate antibodies to surface of MACS-purified Pf2004/164TdTomato iRBCs (+/- trypsin/chymotrypsin) was detected by live microscopy (left panel) and flow cytometry (right panel), using the same sample preparations in parallel. For live microscopy, the percentage of surface labeled aiRBCs or stage I giRBCs (weak TdTomato+) are shown for all antibodies tested. No asexual samples were tested for GEXP02 and PF3D7_0402000. For flow cytometry, cells were gated for live cells, single cells, and then uRBCs and giRBCs were gated based on Vybrant Violet and TdTomato fluorescence and surface reactivity measured using AlexaFluor488-conjugated secondary antibody . The TdTomato positive population was further split into "weak TdTomato+" (corresponding to earlier gametocytes) and "strong TdTomato+" (corresponding to later gametocytes) populations. **C.** Antibodies were clustered (automatic independent clustering) based on the imaging parameters shown in the heatmap: percentage of labeled giRBCs, average MFI at the giRBC cell surface, % giRBC surface covered, and ratio of MFI at the surface of –trypsin samples compared to +trypsin. Glycophorin C is included as a control. Live representative images of early giRBCs +/-trypsin treatment are shown for GEXP07 and GEXP10. **D.** Immunofluorescence analysis of the localization of GEXP02, GEXP10, and PF3D7_0936800 (detected with anti-peptide antibodies) in fixed, permeabilized aiRBCs and giRBCs (days 2 and 4 of the induction, corresponding to stages I-IIA and IIA-IIB, respectively). Candidate protein is shown in green, SBP1 in magenta, TdTomato in red, and nuclear staining in blue.

895

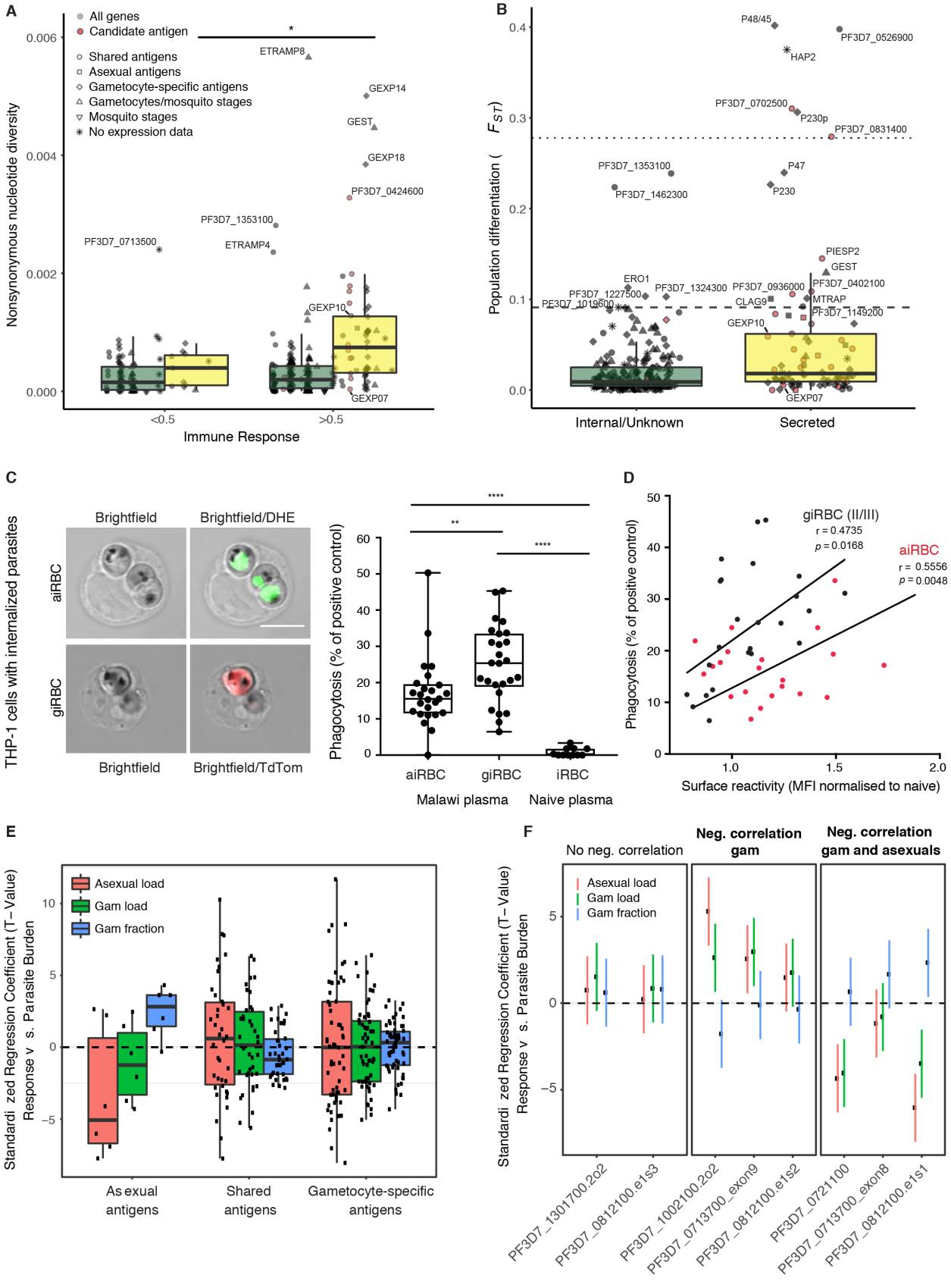

A
All genes
Candidate antigen
Shared antigens
Asexual antigens
Gametocyte-specific antigens
Gametocytes/mosquito stages
Mosquito stages
No expression data
Nonsynonymous nucleotide diversity
Immune Response
<0.5
>0.5
ETRAMP8
GEXP14
GEST
GEXP18
PF3D7_0424600
PF3D7_1353100
PF3D7_0713500
ETRAMP4
GEXP10
GEXP07
B
Population differentiation (F_{ST})
Internal/Unknown
Secreted
P48/45
PF3D7_0526900
HAP2
PF3D7_0702500
P230p
PF3D7_0831400
PF3D7_1353100
P47
PF3D7_1462300
P230
PIESP2
GEST
ERO1
PF3D7_1324300
PF3D7_0936000
PF3D7_0402100
PF3D7_1227500
PF3D7_1019600
CLAG9
MTRAP
PF3D7_1149200
GEXP10
GEXP07
C
THP-1 cells with internalized parasites
Brightfield
Brightfield/DHE
aiRBC
giRBC
Brightfield
Brightfield/TdTom
Phagocytosis (% of positive control)
aiRBC
giRBC
iRBC
Malawi plasma
Naive plasma
D
Phagocytosis (% of positive control)
giRBC (II/III)
r = 0.4735
p = 0.0168
aiRBC
r = 0.5556
p = 0.0048
Surface reactivity (MFI normalised to naive)
E
Standardized Regression Coefficient (T−Value)
Response vs. Parasite Burden
Asexual load
Gam load
Gam fraction
Asexual antigens
Shared antigens
Gametocyte-specific antigens
F
No neg. correlation
Neg. correlation gam
Neg. correlation gam and asexuals
Standardized Regression Coefficient (T−Value)
Response vs. Parasite Burden
Asexual load
Gam load
Gam fraction
PF3D7_1301700.2o2
PF3D7_0812100.e1s3
PF3D7_1002100.2o2
PF3D7_0713700_exon9
PF3D7_0812100.e1s2
PF3D7_0721100
PF3D7_0713700_exon8
PF3D7_0812100.e1s1

Figure 6: Candidate gametocyte surface antigens elicit responses correlated with reduced gametocyte burden and a subset show minimal genetic diversity.

A. Nonsynonymous nucleotide diversity for all antigens present on the protein array, stratified by stage, localization and level of immune response (Mann-Whitney U Test, $p < 0.05$). Genome data are from a set of parasite samples in Senegal. **B**. Population differentiation between Senegal and Malawi parasite samples for secreted and internal proteins (F_{ST} at nonsynonymous sites; Mann-Whitney U Test, $p = 2.0 \times 10^{-5}$). The dotted and dashed lines mark the 99th and 95th percentile of genome-wide nonsynonymous F_{ST} values. **C.** Left panel: internalized aiRBCs and giRBCs upon phagocytosis by THP-1 cells. aiRBCs are stained with the nuclear dye dihydroethidium (DHE) and giRBCs show TdTomato (TdTom) reporter fluorescence. Right panel: phagocytosis index of Malawi plasma samples relative to positive control (rabbit anti-human RBC) and naive US serum. **D.** iRBC phagocytosis vs. surface recognition. **E.** Associations were estimated between gametocyte fraction (gametocytes/total parasites), gametocyte and asexual parasite load, and secreted antigen fragment responses by peptide array (after normalization to IVTT controls and quantile normalization as in **Figure 1**) across 3 malaria-exposed populations. Median standardized regression coefficient -0.86, Wilcoxon test p=0.087. **F.** Regression coefficients (and 95% confidence intervals) between individual protein fragments of the prioritized candidate antigens and parasite parameters (either gametocyte fraction, gametocyte load or asexual parasite load). Fragments are stratified by their correlation with parasite parameters. Significance values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Tables:

Age (years)		Gender		Location		RDT status	
≤5	106 (43.44%)	Male	102 (41.8%)	Chikhwawa	171 (70.1%)	+	169 (69.3%)
>5,≤12	28 (11.48%)	Female	142 (58.2%)	Ndirande	35 (14.3%)	-	75 (30.7%)
>12,<30	47 (19.26%)			Thyolo	38 (15.6%)		
≥30	63 (25.82%)						
Total							**244**

Table 1. Characteristics of Malawian study population. Chikhwawa has year-round malaria transmission whereas Ndirande and Thyolo have more seasonal transmission peaking during the rainy season each year. RDT=rapid diagnostic test.

925

Accession ID	Export motif	Protein description	Stage annotation	Previously described localizations	Detection method	Host phenotypes	Conservation	Validated surface expression
PF3D7_0601900	PNEP	Conserved Plasmodium protein, unknown function	Shared	Maurer's clefts	Proteomics		Pf	
PF3D7_0713700	**NO**	**Conserved Plasmodium protein, unknown function**	**Gam/Mo**	**Unknown**	**Proteomics**	**Neg correlation with asex./gam. load**	**Pf, Pv, Pb**	
PF3D7_0721100	**PEXEL**	**Conserved Plasmodium protein, unknown function**	**Shared**	**RBC surface**	**Proteomics Array/flow correlation**	**pos. correlation with age/neg. correlation with asex./gam. load**	**Pf, Pv, Pb**	
PF3D7_0812100	**NO**	**Conserved Plasmodium protein, unknown function**	**Shared**	**Unknown**	**Proteomics**	**pos. correlation with age/neg correlation with asex./gam. load**	**Pf, Pv, Pb**	
PF3D7_0831400	PEXEL	Plasmodium exported protein, unknown function (Hyp12)	Shared	Unknown	Proteomics		Pf	
PF3D7_1002100	PEXEL	EMP1-trafficking protein (PTP5)	Shared	Maurer's clefts	Proteomics		Pf	X
PF3D7_1038000	NO	Antigen UB05	Shared	Unknown	Proteomics		Pf, Pv, Pb	
PF3D7_1301700	PEXEL	Plasmodium exported protein (hyp8), unknown function (GEXP07)	Shared	RBC surface	Proteomics/ Mouse sera array (+trypsin enriched)		Pf	X

926

927 **Table 2: Prioritized candidate gametocyte antigens.** 8 candidate gametocyte antigens were identified by:

928 1) predicted or known host secretion, 2) proteomics of trypsin-treated and –untreated giRBC membranes, 3)

929 correlations between plasma reactivity by protein array and flow cytometry, 4) array reactivity of serum from

930 mice immunized with trypsin-treated and –untreated giRBC membranes, 5) exposure-dependent increase of

931 IgG in malaria-positive individuals, 6), negative correlation with asexual and gametocyte load, and 7) low
932 genetic diversity and divergence. 3 candidates (marked in bold) fulfill all criteria (1-6). Previously described
933 stage annotation and localization was retrieved from plasmodb.org (*21, 23, 24, 53*). Further details on
934 candidates are provided in **table S7** and for 3 top candidates in **fig. S8**. Pf=*P. falciparum*; Pv=*P. vivax*,
935 Pb=*P. berghei*.

936

937
938