High-density Peptide Arrays Help to Identify Linear Immunogenic B-cell Epitopes in Individuals Naturally Exposed to Malaria Infection

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In Brief
Within this study, a novel high-throughput peptide array technology enabled the correlation between the clinical phenotype and the antibody response against linear epitopes of the *P. falciparum* proteome. Peptides from twelve known vaccine candidates and a bioinformatical selection were screened. Strong reactivities to epitopes derived from known vaccine candidates as well as new immunogenic proteins/epitopes were identified, which may serve as vaccine targets and new biomarkers.

Graphical Abstract

**Highlights**

- Profiling antibody responses of patients with naturally acquired malaria immunity.
- High-density peptide arrays featuring linear epitopes.
- Epitope mapping of known and potential novel vaccine candidates.
- Novel immunogenic epitopes discovered, and known antibody target motifs confirmed.
High-density Peptide Arrays Help to Identify Linear Immunogenic B-cell Epitopes in Individuals Naturally Exposed to Malaria Infection*

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High-density peptide arrays are an excellent means to profile anti-plasmodial antibody responses. Different protein intrinsic epitopes can be distinguished, and additional insights are gained, when compared with assays involving the full-length protein. Distinct reactivities to specific epitopes within one protein may explain differences in published results, regarding immunity or susceptibility to malaria. We pursued three approaches to find specific epitopes within important plasmodial proteins, (1) twelve leading vaccine candidates were mapped as overlapping 15-mer peptides, (2) a bioinformatical approach served to predict immunogenic malaria epitopes which were subsequently validated in the assay, and (3) randomly selected peptides from the malaria proteome were screened as a control. Several peptide array replicas were prepared, employing particle-based laser printing, and were used to screen 27 serum samples from a malaria-endemic area in Burkina Faso, West Africa. The immunological status of the individuals was classified as “protected” or “unprotected” based on clinical symptoms, parasite density, and age. The vaccine candidate screening approach resulted in significant hits in all twelve proteins and allowed us (1) to verify many known immunogenic structures, (2) to map B-cell epitopes across the entire sequence of each antigen and (3) to uncover novel immunogenic epitopes. Predicting immunogenic regions in the proteome of the human malaria parasite Plasmodium falciparum, via the bioinformatics approach and subsequent array screening, confirmed known immunogenic sequences, such as in the leading malaria vaccine candidate CSP and discovered immunogenic epitopes derived from hypothetical or unknown proteins. Molecular & Cellular Proteomics 18: 642–656, 2019. DOI: 10.1074/mcp.RA118.000992.

Malaria caused by the parasite Plasmodium falciparum (Pf) is one of the major causes of death in young children and pregnant women in Sub-Saharan Africa (1). Malaria control primarily relies on vector control and access to diagnosis and treatment (2). Currently available subunit vaccine candidates have shown only moderate protection rates (3, 4) whereas approaches based on whole sporozoites were successful in early clinical testing (5–10), yet still facing challenges regarding wide-scale human use (11). In endemic areas, humans eventually develop immunity to malaria (NAI, naturally acquired immunity), which is established gradually over time and maintained by repeated exposure to the parasite (12, 13). NAI results in protection against clinical disease, although sterile immunity to infection is typically never acquired (14). To date, the human immune response and the specific mechanisms mediating both protection from disease in NAI and sterilizing immunity upon whole sporozoite vaccinations are still poorly understood. Identification of the antigens that are responsible for these protective immune responses remains a challenge but will ultimately create new opportunities for vaccine development.

Humoral responses triggered upon a Plasmodium infection act at the pre-erythrocytic stage, by decreasing parasite in-
vasion of hepatocytes and, moreover, are the central immune effector mechanisms at the pathogenic asexual blood stage (15–17). It was already shown in the early sixties that IgG antibodies from adults living in malaria-endemic areas can mediate remission of acute clinical malaria in recipients (18). Many have investigated humoral immune responses and protection against a limited selection of single antigens, but only few considered multiantigen responses (17). Some recent studies used protein microarrays, covering the proteome of \emph{P. falciparum} in a range from 5 to 91% to profile antibody responses, triggered by natural and/or experimental exposure to \emph{Plasmodium} (5, 19–24), revealing distinct antibody patterns for serum donor or patient groups and numerous highly reactive antigens. However, statistical significance of association with protection often varied between different studies. For example, for the vaccine candidates LSA-3, MSP-1, and MSP-2, no statistically significant association with protection from uncomplicated malaria in Malian children was detected (20), whereas the same antigens were correlated with protection from symptomatic malaria in Kenyan children (24). Beside protein microarrays, another high-throughput screening approach to profile antibody responses used a \emph{PF} blood-stage cDNA expression library in conjunction with sera of children, which were either susceptible or protected from severe malaria (25). The authors could show that antibodies against the previously uncharacterized protein \emph{P/SEA-1} can protect from severe malaria.

Our high-density peptide arrays are created with the particle-based laser printing technology (26), where the printer directs solid particles, which incorporate amino acid monomers, to defined areas of a functionalized glass substrate. Thereby, peptides are synthesized directly \textit{in situ} on a glass surface. Clinically well-characterized serum samples from individuals living in the Nouna Health District, Burkina Faso, West Africa, were investigated. Based on clinical symptoms, parasite density, and age, the immunologic status of the individuals was classified as “protected” or “unprotected.”

In this methodological proof-of-principle study, we successfully validated strong reactivity to previously known epitopes in vaccine candidates as well as in other not yet described immunogenic proteins.

\footnote{The abbreviations used are: ACS-1a, acyl-CoA synthetase 1a; ACS-1b, acyl-CoA synthetase 1b; AMA-1, apical membrane antigen 1; CSP, circumsporozoite protein; ETRAMP-(5/13), early transcribed membrane protein (5/13); EXP-1, circumsporozoite protein related antigen; GLURP, glutamate-rich protein; HDSS, health and demographic surveillance system; LSA-1, liver stage antigen 1; MSP-(1, 2, 3, 7, 8), merozoite surface protein (1, 2, 3, 7, 8); NAI, naturally acquired immunity; Pf, \textit{Plasmodium falciparum}; RBB, rockland blocking buffer; SEA-1, schizont egress antigen 1; SSP3, sporozoite surface protein 3; STAR, sporozoite threonine and asparagine-rich protein; TRAP, thrombospondin-related adhesive protein.}

### EXPERIMENTAL PROCEDURES

#### Study Population—Blood samples were collected during a cross-sectional survey in the rainy season of 2009 in the Nouna Health District, North-Western Burkina Faso. The study site is in a holoendemic, highly seasonal malaria transmission area (27). The survey was part of a study assessing genotypic drug resistance over time and was already described elsewhere (28, 29). Briefly, every six months the inhabitants of Bourasso village (~15 km south of the district capital Nouna) were invited to participate using a random household list generated from the data of the Health and Demographic Surveillance System (HDSS). The Nouna’s HDSS is part of the INDEPTH network (30). Written informed consent was obtained from every participant. Study subjects received a physical examination by a trained medical professional and were screened for malaria on the spot using microscopy. Filter papers from finger prick blood were prepared for the genetic analysis and 5 ml venous blood was taken from smear-positive individuals in a serum tube (no anti-coagulants, preservatives, or protease inhibitors), stored in a coolbox after collection (approx. 5 °C). After coagulation on the same day, the serum was separated with a pipette and immediately frozen at ~80 °C. Shipping was done on dry ice and samples were stored at ~80 °C. We selected 27 patient samples, based on their parasite density, age, and clinical phenotype. Asymptomatic adult patients with negative or low-grade parasitemia were assumed to have acquired NAI, whereas symptomatic young patients or patients with high parasite density were classified as susceptible. To exclude and compare unspecific reactions, 6 naïve European sera were used as controls (Table I), taken from healthy European donors (anonymized samples, male or female, 21–42 years old, no previous malaria infection).

#### Ethics Statement—Written informed consent was obtained from every participant, or a parent or guardian of any child participant provided informed consent on their behalf. Ethical approval was obtained from the Institutional Review Boards of the Nouna Health Research Centre and the Medical Faculty of Heidelberg University Hospital.

#### Bioinformatical Peptide Preselection—The proteome of the 3D7 reference strain of \emph{P. falciparum} includes 5446 known proteins (31). We selected the regions of the proteome, which are (1) easily accessible to the immune system (i.e. external surface protein loops) and (2) not available in any human proteins.

The peptide selection procedure contained several steps. We first used TopPred (32) to predict and select proteins with transmembrane helices and obtained 2,081 protein candidates. Then, we used SignalP (33) and RPSP (34) to predict and select only proteins with signal peptides indicating export of the proteins to the cell surface. This approach reduced the set of proteins to 780 candidates. Next, we selected only external loops from these proteins and performed a virtual fragmentation into 15-mer peptides with an overlap of 14 amino acids, which is the length that results in optimum yields in the peptide synthesis (see next section). Then, this set of 286,886 unique peptides was trimmed into all possible 5-mers and 6-mers (shortest assumed B-cell epitope length) and the resulting fragments were scored by comparison with the human proteome, generating a ranked list. Less similarity to the human proteome yielded a higher rank. An ungapped local-global alignment score with a blosum62 matrix (35) was used as similarity measure. Finally, from these peptides, we selected the top 1912 peptides (because of array size limitations) with the lowest similarity to the human proteome for peptide array production (see Supporting Information SI_pf_vs_human.xlsx). These 1912 peptides were derived from 23 different proteins, listed in supplemental Table S1.

#### Peptide Arrays—The peptide arrays were produced by the company PEPperPRINT GmbH, Heidelberg, Germany. Array fabrication is based on the peptide laser printing technology (26), rendering 800
spots per cm² with a spot size of 200 µm × 400 µm and a spot-to-spot distance of 250 or 500 µm respectively. In short, the production cycle of the peptide arrays is as follows: The laser printer directs solid particles, which incorporate amino acid monomers, to defined areas of a functionalized glass substrate. In a subsequent heating step, the amino acid monomers can diffuse and couple to the functionalized surface. After a few chemical washing steps, this cycle is repeated, adding amino acid monomers layer-by-layer to the growing amino acid chains on the surface. Being an in situ synthesis technique, this method allows for a high content flexibility. Thus, the peptide content can be easily modified for each array. The surface coating of the peptide arrays is protein repelling so that only specific binding to peptide spots on the array is favored. For this study, three different types of arrays were generated:

(1) Bioinformatics approach: The arrays feature the bioinformatical pre-selection of 1912 different peptides, derived from the P. falciparum proteome, and framed by alternating control spots of the hemagglutinin or HA (YPYDVPDYA) and Flag (DYKDDDDK) peptides.

(2) Vaccine candidate approach: 12 leading vaccine candidate proteins from the literature were selected: MSP-1, MSP-2, MSP-3, GLURP, STARP, LSA-1, CSP, EXP-1, AMA-1, TRAP, Pf16, and SEA-1. Each protein sequence was cut into overlapping 15-mer peptide sequences with an overlap of 7 amino acids, removing all duplicate sequences. The proteins were mapped as 1116 overlapping 15-mer peptides with an overlap of 7 amino acids. At the top left and the bottom right of the arrays, alternating control spots of the hemagglutinin or HA (YPYDVPDYA) and Flag (DYKDDDDK) peptides were placed.

(3) Random malaria proteome approach: To validate our peptide preselections from (1) and (2), we generated a list of 1912 peptides, randomly selected from the proteome of P. falciparum. The arrays are framed by alternating control spots of the hemagglutinin or HA (YPYDVPDYA) and Flag (DYKDDDDK) peptides.

The peptide arrays were incubated as described in (36) for 10 min in phosphate buffer saline with 0.05% Tween 20 (PBS-T), followed by incubation in Rockland Blocking Buffer (RBB) for Fluorescent Western blotting (Rockland Immunochemicals, Limerick) for 30 min. After a short rinsing step with PBS-T, the arrays were incubated with 1 µl of serum, diluted 1:1000 in 900 µl PBS-T and 100 µl of RBB, overnight in the cold room (−16 h) at 4 °C. The arrays were washed three times with PBS-T (−10 s, −30 s, 1 min) and then incubated with the secondary antibody F(ab)2 goat-anti-human conjugated with the fluorescent dye DyLight 680 (Thermo Scientific), diluted 1:5000 in PBS-T with 1:10 RBB for 30 min. Subsequently, the arrays were washed twice with PBS-T (−10 s, −30 s) and, then, rinsed with Milli-Q water.

As peptide controls, we stained the HA peptide spots with monoclonal mouse-Anti-HA 12CA5 IgG antibodies (provided by Dr. G. Moldenhauer, DFKZ) conjugated with DyLight 680 (Lightning-Link, Innova Biosciences, UK). The antibodies were diluted 1:1000 in PBS-T and 1:10 RBB. Staining was performed for 30 min at room temperature in the dark followed by washing as described above.

Image Acquisition, Data Transformation, and Analysis—Fluorescence images were acquired as described in (36) with an Odyssey Infrared Imager (LI-COR). The scanner is equipped with two lasers (wavelengths 685 nm and 785 nm) and the filters are optimized for the emission wavelengths 700 nm and 800 nm. The scanner sensitivity was set to 7.0 for the 700 nm channel, the focal plane was set to ±0.7 mm.

The raw fluorescence images were acquired with the Odyssey V3.0 software. The raw 16-bit images were analyzed with PepSlide Analyzer (SICASYS Software GmbH, Heidelberg, Germany). Raw spot intensities are detected and measured in an arbitrary fluorescence intensity unit (AU) and range from 0 to 65535 AU. A spot intensity was determined as the median brightness of the measured pixels in the image corresponding to the peptide spot. The background of each array was measured in an area of the array, without serum staining. This was subtracted from the raw spot intensity. Each spot on the peptide array is generated in duplicate. The raw peptide spot fluorescence intensity is the average of the corresponding two spot duplicates.

For variance stabilization and normalization purposes, we performed the arcsin hyperbolicus (asinh) transformation (as in (37)) to account for the inherent variance-mean dependence in low and high signal intensities. Subsequently, the transformed fluorescence intensity data of the arrays was processed with the software Cluster Version 3.0 (38, 39). The clustering was performed using the city-block cluster algorithm with complete linkage. Data were neither further normalized nor centered. We visualized the data with the software TreeView (40) in a log2 scale, centered at 6.0 AU (red >> 6 AU; green < 6 AU; black = 6 AU) with contrast value of 1.0 AU.

In addition, we analyzed the three groups, as described above (malaria positive, malaria negative, control group; see study population) with a two-sided heteroscedastic Welch’s t test. This rendered the p values for the prediction of immunogenic peptides.

RESULTS

Discovering Immunogenic Plasmodial Epitopes Using Peptide Arrays—We applied a three-branched study design and generated 33 high-density peptide arrays for each approach. For each of the three approaches, we screened the IgG reactivity of the same 33 individual serum samples, 27 from Burkina Faso and 6 European controls (Table I). Because the peptide array technology has to date not been applied for the screening of antibody responses to multiple targets in malaria research, we initially designed arrays covering plasmodial proteins known vaccine candidates (41, 42) with described antibody responses acquired during natural exposure. This approach allows us not only to provide a proof-of-principle that peptide arrays represent a powerful technology to profile antibody responses elicited by Plasmodium parasites, but also to discover novel epitopes. To our knowledge this is the first study performing a systematic epitope screening on these antigen candidates.

Hence, the vaccine array contained 1116 peptides, derived from the following proteins of P. falciparum (3D7 strain): MSP-1, MSP-2, MSP-3, GLURP, STARP, LSA-1, CSP, EXP-1, AMA-1, TRAP, Pf16, and SEA-1. Moreover, with the aim to identify novel immunogenic peptides, we performed a preselection process based on bioinformatics to predict immunodominant epitopes in the proteome of P. falciparum, which resulted in a list of 1912 selected 15-mer peptides from 23 different Pf proteins (supplemental Table S1). To validate our peptide preselection from the bioinformatics approach we also generated a list of 1912 peptides, randomly selected from the proteome of P. falciparum (see Supporting Information).

For the antibody profiling, we grouped the sera into three different categories: Malaria parasite positive (parasite density > 2240/µl, presumably low level of NAI, n = 10; “unprotected”), Malaria parasite negative (except one patient with 200 Parasites/µl, age ≥ 25 years, presumably high level of NAI, n = 17; “protected”), and European controls (n = 6)
Epitope Profiling of Malaria Antibodies Using Peptide Arrays

Table I
List of screened sera. Six European healthy controls and 27 sera from African patients

| Patient-ID | Age (years) | Sex | Microscopy parasites/µl | PCR |
|------------|-------------|-----|-------------------------|-----|
| C1         | 21–42       | N.A.| N.A.                    | N.A.|
| C2         | 21–42       | N.A.| N.A.                    | N.A.|
| C3         | 21–42       | N.A.| N.A.                    | N.A.|
| C4         | 21–42       | N.A.| N.A.                    | N.A.|
| C5         | 21–42       | N.A.| N.A.                    | N.A.|
| C6         | 21–42       | N.A.| N.A.                    | N.A.|
| 18         | 6           | M   | 26000                   | +   |
| 22         | 16          | M   | 5600                    | +   |
| 31         | 12          | F   | 11200                   | +   |
| 35         | 50          | F   | 0                       | −   |
| 37         | 10          | M   | 23200                   | +   |
| 75         | 47          | F   | 0                       | +   |
| 86         | 50          | F   | 0                       | +   |
| 102        | 25          | M   | 200                     | +   |
| 121        | 48          | F   | 0                       | +   |
| 133        | 45          | M   | 0                       | +   |
| 151        | 50          | F   | 0                       | +   |
| 167        | 40          | F   | 2240                    | +   |
| 199        | 53          | F   | 0                       | −   |
| 207        | 8 months   | F   | 8000                    | +   |
| 216        | 13          | M   | 13600                   | +   |
| 223        | 32          | M   | 3040                    | +   |
| 226        | 56          | F   | 0                       | −   |
| 234        | 50          | F   | 0                       | o   |
| 235        | 18          | F   | 4240                    | +   |
| 241        | 54          | F   | 0                       | +   |
| 258        | 49          | F   | 0                       | o   |
| 261        | 7           | M   | 7080                    | +   |
| 272        | 54          | M   | 0                       | −   |
| 276        | 48          | F   | 0                       | −   |
| 280        | 50          | F   | 0                       | −   |
| 292        | 53          | F   | 0                       | −   |
| 294        | 49          | F   | 0                       | −   |

The staining results generally show clear differences in the overall number of spots and spot intensity. In supplemental Fig. S1, representative stainings show examples for all three approaches of three different phenotypes (control, un-protected, protected). In older asymptomatic individuals with low parasite density, the intensity of the staining pattern is generally higher (i.e. more peptide spots are stained and with stronger intensity) compared with young individuals with higher parasite density (supplemental Fig. S1). For the quantitative analysis, the staining intensity data were normalized and transformed. In supplemental Fig. S2, the transformed fluorescence staining intensity overview of all three approaches is shown, sorted by mean peptide reactivity over all patients (supplemental Fig. S2).

With the data set described above, we next followed two independent analysis strategies: a cluster analysis and a Welch's t test.

Automatic Classification of Patients with Cluster Analysis—First, we applied a city block cluster analyses for the 33 different serum samples, based on the acquired staining data for each of the three approaches (supplemental Fig. S3). A valid clustering result is assumed to distinguish between malaria positive, malaria negative, and control samples. However, such results can only be expected, when the peptide selection is optimized for important markers for the detection of malaria. Thus, if most of the peptides are irrelevant for the detection of NAI versus no immunity, the result should not correlate with disease status. Interestingly, the best result for clustering is achieved by the bioinformatics approach (supplemental Fig. S3), which supports the validity of the bioinformatical pre-selection.

Identification of Highly Immunogenic Epitope Structures—In the next step, we performed a Welch's t test for all data (Fig. 1). For each of the approaches, we used the three patient groups as described above. In general, we observed significant signals of recognized peptides in almost all proteins of the bioinformatics and the vaccine approach. As expected, the random approach rendered a much lower number of significant hits (Fig. 1).

Proof-of-Principle by Dissecting Humoral Responses to Known Vaccine Candidates on Epitope Level—By applying the vaccine array approach, we were aiming to provide a technological proof-of-principle and at the same time identify novel, not yet described epitope structures in known malarial antigens. By comparing humoral immune responses detected in sera of malaria susceptible versus disease-protected individuals, we were able to uncover highly immunogenic epitopes that were significantly recognized in sera obtained from disease-protected individuals (hits with p value < 0.05, see Supporting Information). In good agreement with previous reports, we discovered already mapped B cell epitopes within some of the selected antigens as well as immunogenic peptides located within protein domains, which are known to be recognized by antibodies acquired during natural exposure supporting that peptide arrays are suitable for malarial antibody profiling studies (Fig. 2). For example, we identified epitopes in CSP located in the central repeat (NANP) region as well as in the N- and C-terminal domain of the protein, all of which are well known to be recognized by antibodies from malaria-exposed individuals (43–56). As expected, epitopes originating from the central repeat region of CSP were found under the most significantly recognized sequences (Fig. 3 and supplemental Fig. S4, S5). In addition, epitopes within the N- and C-terminal domain detected in our study are overlapping with previously identified immunogenic peptide sequences recognized by sera from naturally infected individuals (44, 45, 48–50, 53). Moreover, we discovered peptide hits across the entire sequence of MSP-1 (57–59) and in particular within MSP-1-p83 (60–62), MSP-1-p38 (63), MSP1-p42 (64) and MSP-1-p19 (51, 55, 64–67) (Fig. 2). For GLURP, the R0, R1 as well as R2 region (55, 66, 68–71) and for LSA-1, the repeat region and C-terminal domain (51, 52, 54, 72, 73) were shown to be targets of antibody responses, which is consistent with
our present data (Fig. 2). Humoral responses to STARP are directed against the central repetitive domain and to the Rp10 region (54, 76) which was also shown from our data to be highly immunogenic. Moreover, antibodies recognizing the C-terminal domain of STARP are generated during natural infection (77). By employing the peptide array technology and hence performing an epitope mapping, we discovered significant peptide hits within the C-terminal domain and the Rp45 region. For SEA-1, recently described by Raj and co-workers (25), naturally acquired antibodies were shown to recognize recombinant SEA-1 (AA 810–1083). A subsequent study presented a B cell epitope mapping of this domain of SEA-1 AA 810–1083 using sera of monkeys, vaccinated with recombinant SEA-1 AA 810–1083 and identified five immunoreactive peptides, which were also recognized by sera from naturally exposed individuals (78). In good correlation with published data, we obtained significant epitope hits within the domain studied by Raj and colleagues. Our screening also showed a strong reactivity to one of the epitopes (#5 in (78)), associated with a decreased parasitemia identified by Nixon et al. and, moreover, to novel epitopes spread across the entire protein (Fig. 2). Studies on humoral responses against MSP-3 acquired during natural Plasmodium exposure demonstrated the presence of antibodies to the polymorphic and the conserved C-terminal domain of the antigen (66, 70, 71, 79–85). In our study, most significant epitopes that have been detected for MSP-3 are covering the conserved C-terminal part, most of them being already described by Singh and co-workers (79). Antibodies to AMA-1 generated in malaria-exposed populations were shown to target the entire ectodomain as well as parts thereof (51, 55, 86–90). Besides a study performed on Plasmodium vivax AMA-1 identifying an immunogenic B cell epitope within domain II (91), to our knowledge only the work from Biswas and colleagues used a synthetic peptide derived from P. falciparum AMA-1 (located in domain II) in conjunction with examining antibody responses (92). Here, by applying peptide arrays, we identified novel epitopes within domain II as being immunogenic along with few epitopes located at the C terminus.

Our vaccine peptide array approach resulted in a small number of significant antigenic peptides for TRAP, MSP-2, EXP-1 and PfS16. For TRAP, our analysis revealed immuno-
| Antigen | Published humoral responses acquired during natural exposure | Peptide array results | Literature |
|---------|-----------------------------------------------------------|----------------------|------------|
| P/CSF  | R | NAP-region | TSB (p < 0.05) | R | NAP-region | TSB (p < 0.05) | [1] Zavala et al., 1985 |
|        |               | (1-10)           | 322, 379     |               | (1-10)           | 322, 379     | [2] Del Giudice et al., 1988 |
|        |               |                  |              |               |                  |              | [3] Modiano et al., 1989 |
|        |               |                  |              |               |                  |              | [4] Shi et al., 1993 |
|        |               |                  |              |               |                  |              | [5] John et al., 2005 |
|        |               |                  |              |               |                  |              | [6] John et al., 2008 |
|        |               |                  |              |               |                  |              | [7] Ambrosino et al., 2010 |
|        |               |                  |              |               |                  |              | [8] Brown et al., 1992 |
|        |               |                  |              |               |                  |              | [9] Stüber et al., 1990 |
|        |               |                  |              |               |                  |              | [10] Bonfigli et al., 2009 |
|        |               |                  |              |               |                  |              | [11] Lopez et al., 1996 |
|        |               |                  |              |               |                  |              | [12] Guevara Patiño et al., 1997 |
|        |               |                  |              |               |                  |              | [13] Conway et al., 2000 |
|        |               |                  |              |               |                  |              | [14] Cavanagh et al., 2004 |
|        |               |                  |              |               |                  |              | [15] Nikodem et al., 2000 |
|        |               |                  |              |               |                  |              | [16] Dent et al., 2009 |
|        |               |                  |              |               |                  |              | [17] Egan et al., 1995 |
|        |               |                  |              |               |                  |              | [18] Perraut et al., 2005 |
|        |               |                  |              |               |                  |              | [19] Dau et al., 2015 |
|        |               |                  |              |               |                  |              | [20] Theisen et al., 1995 |
|        |               |                  |              |               |                  |              | [21] Dodo et al., 2000 |
|        |               |                  |              |               |                  |              | [22] Seo et al., 2004 |
|        |               |                  |              |               |                  |              | [23] Mamo et al., 2013 |
|        |               |                  |              |               |                  |              | [24] Theisen et al., 2000 |
|        |               |                  |              |               |                  |              | [25] Meraidi et al., 2004 |
|        |               |                  |              |               |                  |              | [26] Fido et al., 2014 |
|        |               |                  |              |               |                  |              | [27] Lee et al., 2008 |
|        |               |                  |              |               |                  |              | [28] Fido et al., 1997 |
|        |               |                  |              |               |                  |              | [29] Suwanchanron et al., 2013 |
|        |               |                  |              |               |                  |              | [30] Raj et al., 2014 |
|        |               |                  |              |               |                  |              | [31] Singh et al., 2004 |
|        |               |                  |              |               |                  |              | [32] Roussillon et al., 2007 |
|        |               |                  |              |               |                  |              | [33] Osier et al., 2007 |
|        |               |                  |              |               |                  |              | [34] Polley et al., 2007 |
|        |               |                  |              |               |                  |              | [35] Baumann et al., 2012 |
|        |               |                  |              |               |                  |              | [36] Polley et al., 2004 |
|        |               |                  |              |               |                  |              | [37] Cortes et al., 2003 |
|        |               |                  |              |               |                  |              | [38] Osier et al., 2008 |
|        |               |                  |              |               |                  |              | [39] Osier et al., 2010 |
|        |               |                  |              |               |                  |              | [40] Kusi et al., 2012 |
|        |               |                  |              |               |                  |              | [41] Biswas et al., 2008 |
|        |               |                  |              |               |                  |              | [42] Scarselli et al., 1993 |
|        |               |                  |              |               |                  |              | [43] Charoenwit et al., 1997 |
|        |               |                  |              |               |                  |              | [44] John et al., 2003 |
|        |               |                  |              |               |                  |              | [45] Taylor et al., 1995 |
|        |               |                  |              |               |                  |              | [46] Khosravi et al., 2011 |
|        |               |                  |              |               |                  |              | [47] Polley et al., 2006 |
|        |               |                  |              |               |                  |              | [48] Metzger et al., 2013 |
|        |               |                  |              |               |                  |              | [49] Balam et al., 2014 |
|        |               |                  |              |               |                  |              | [50] Meraidi et al., 2002 |
|        |               |                  |              |               |                  |              | [51] Graves et al., 1991 |
|        |               |                  |              |               |                  |              | [52] Baker et al., 1996 |
|        |               |                  |              |               |                  |              | [53] Skinner et al., 2015 |
|        |               |                  |              |               |                  |              | [54] Nixon et al., 2017 |

Fig. 2. Comparison of vaccine candidate peptide array results with selected literature references. Immunogenic linear peptide epitopes selected from literature (blue) and identified in the peptide array approach (orange: p-value < 0.05, red: p-value < 0.01) with 12 vaccine candidates. We excluded peptides where the p-value of “– vs C” was > 0.05.
genic epitopes only within the more C-terminal part of the protein (Fig. 2). In the literature, TRAP-specific antibodies present in the sera of malaria-exposed individuals have been detected using the recombinant protein (51, 52, 83, 93). Charoenvit et al. conducted a B cell epitope mapping on TRAP by using monoclonal antibodies and discovered two epitopes, which were also recognized by serum samples from malaria-endemic areas (94). These epitopes were also used in further studies (54, 95), but were different from the epitopes identified in the present work. Several reports demonstrated that MSP-2 is a target for antibodies generated during natural exposure (88, 96–101). The study conducted by Taylor et al. described the dimorphic and polymorphic region and, to a lesser extent the conserved region as being detected by humoral responses (96). Recently, both the dimorphic and C-terminal domains were subjected to an epitope mapping which resulted in the description of antigenic peptides (102) with two of them overlapping with our data set (Fig. 2). For EXP-1, most antibodies acquired during natural exposure recognize the C-terminal domain (70, 103) and only a few are targeting the N-terminal part (103). Our data resulted only in one epitope significantly recognized by antibodies in disease-protected individuals mapping to the C terminus of EXP-1. Finally, previous studies detected naturally occurring antibodies to Pf s16 (104–106). The use of monoclonal antibodies identified immunodominant epitopes (104), which were mapped to the N-terminal region and the utmost C-terminal part (AA 141–149) of Pf s16 in the following study by Baker and col-

### Fig. 3. Top p-value peptides comparing malaria positive and malaria negative samples from each approach.

The vaccine candidate approach rendered 8 peptides with a p-value < 10^{-3}, the bioinformatics approach 15 peptides with p-value < 10^{-3}. The random malaria proteome approach rendered 8 peptides with a p-value < 10^{-2}, one order in magnitude greater. We excluded peptides where the p-value of “- vs C” was > 0.05 and one peptide in the bioinformatics approach, which gave a higher signal in malaria + in comparison to malaria − samples (see supplemental Fig. S5 for box plots).

| Vaccine approach | Bioinformatics approach | Random approach |
|------------------|-------------------------|-----------------|
| Peptide | Malaria - | Malaria + | Control | p-value | Peptide | Malaria - | Malaria + | Control | p-value | Peptide | Malaria - | Malaria + | Control | p-value |
| CSP: FRANPAPPAWAFK | 3.08E-05 | 1.04E-04 | 1.34E-04 | 1.79E-04 | 3.98E-04 | 7.19E-04 | 7.88E-04 | 8.60E-04 |
| SEA1: RKNPPNPKY | 2.00E-05 | 4.06E-05 | 6.01E-05 | 7.63E-05 | 1.20E-04 | 2.23E-04 | 2.50E-04 | 3.50E-04 |
| GLOHP: RNKRPADPAPA | 4.77E-04 | 6.07E-04 | 6.80E-04 | 8.81E-04 | 6.90E-04 | 8.60E-04 | 8.81E-04 | 9.95E-03 |
| MSP1: VVEKDEPTIVIS | | | | | | | | |
| LSA1: VISEKDEPTIVIS | | | | | | | | |
| M16: RSF4405Y | | | | | | | | |

![Graph showing peptide reactivity](image-url)
By using non-overlapping peptides and serum samples from a different geographical region, Baker et al. demonstrated reactivities to nearly all the tested sequences but identified an immunodominant peptide located in the C-terminal half of *Pf*\(_{s16}\) (AA115–129). The most significant epitope hit from our analysis corresponds to the AA 140–154, which is in good agreement with previous results (104). Overall, this data provide evidence that peptide arrays represent a powerful tool to profile antiplasmodial antibody responses.

### Screening for Novel Immunogenic Peptides by Applying A Bioinformatical Preselection

With the aim of discovering novel immunogenic peptides in the proteome of *P. falciparum*, we next present a screening approach, applying a bioinformatical pre-selection process, which resulted in potential immunogenic regions (Fig. 4, highlighted in blue) derived from 23 proteins (Fig. 4, supplemental Table S1). Subsequent validation with peptide arrays identified highly significant reactivities in the sera of disease-protected individuals (Fig. 4, peptides with a *p*-value < 0.05 are highlighted in orange). Comparing the results in the vaccine and the bioinformatics approach for the known candidate antigens, we have a good correlation of the immunogenic areas (*e.g.* *Pf*CSP, *Pf*LSA-1, *Pf*STARP). Again, as expected, epitopes originating from the central repeat region of CSP were found under the most significantly recognized sequences, as well as, epitopes within the N- and C-terminal domain. In *Pf*LSA-1, the repeat region and the N-

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**Fig. 4. Bioinformatical pre-selection and peptide array screening results.** Peptides, which were selected by the algorithm (highlighted in blue), were synthesized and screened. Significant immunogenic linear peptide epitopes (orange, *p*-value ≤ 0.05) were identified. We excluded peptides where the *p*-value of “− vs C” was > 0.05. See supplemental Table S1 for comparison of literature reports on these proteins.
and C-terminal domains were shown to be targets of antibody responses, and in PfSTARP, the Rp10 region is highly immunogenic, which is consistent with the vaccine approach data. However, some discrepancies in our two targeted approaches can be also found, mainly because of nonexact amino acid sequence identities of the peptides in the two approaches, covering the same protein region, but differing slightly in their positions. For a p value < 0.001 in the bioinformatics approach, we obtained 15 peptides from 11 different proteins (Fig. 3): PfTRAP, PfSTARP, Merozoite surface protein MSA180, S-antigen, PfLSA-1, Peptidase M16, PfAMA-1, PfTRAP, PfCSP, GPI ethanolamine phosphate transferase 3, and the CSC1-like protein. Lowering the p value cutoff to 0.01, a total of 88 peptides from 19 of the 23 proteins show significant hits, including PfACS-1b, PfSSP3, PfMSP-7, PFE0-1, the conserved proteins PFC0330w and MAL13P1.107, PfS16, and PfETRAMP-5. For p values < 0.05, only PfMSP-8 showed no significant hits at all. To validate that the 88 peptides (p value < 0.01) were not derived from a single immunogenic sequence, we performed a MEME analysis (107), shown in supplemental Table S2. This analysis shows that the antibodies indeed mainly bind to different epitopes with different sequences.

From our bioinformatical preselection, 16 proteins have been also found to be immunogenic by previous studies using protein arrays (19, 20, 22, 24) (supplemental Table S1), corroborating that peptide arrays are suitable to study antibody responses in malaria research. In addition to the protein array data, our data map the immunogenic regions within the respective antigens on epitope level (Fig. 4). Moreover, our study identified PF08_0088 (PfSSP3), PFC0330w, and PFL0685w as novel candidates, harboring immunogenic epitopes, which were significantly detected by antibodies from disease-protected individuals.

Statistical Analysis and Age Stratification—We carried out a multivariable regression analysis with the log10 of parasite density as the dependent variable. Age was grouped into 3 categories because of the limited sample size and included as a general (dummy) variable. We can show that most of the antigens/epitopes still reach statistical significance, when age is controlled and adjusted for (see Supporting Information and supplemental Table S3). Sample size did not allow for constructing age groups with smaller ranges. However, this also serves to confirm the validity of our approach in the sense that in the presence of age as a strong confounder, we can still document a statistically significant effect of the identified antigens/epitopes.

Screening a Library of Randomly Selected Pf Peptides—In the random approach, we found 8 peptides, each from a different protein, with a significant p value of < 0.01: PF13_0024, PF14_0175, PFE0840c, PFL1620w (also detected in (20)), MAL7P1.37, PF14_0502, PFDF0665c, MAL13P1.295. Although p values are lower than those from the significant peptides from the other two approaches, these peptides might also be important hits. However, these hits could potentially also be based on cross-reactions as the peptides were selected randomly from the proteome. Therefore, we currently refrain from considering these epitopes in more detail, but we propose that they should be validated in future studies.

DISCUSSION

We are still facing major gaps in understanding protective immune responses in Plasmodium and the identification of antigen specificities in T cell and antibody-mediated defense mechanisms is one of its key aspects. In humoral responses, recent technological advances enabled antibody-profiling studies in high-throughput formats (5, 19–25, 111). The most frequently used approaches represent protein microarrays, relying on the recombinant expression of the antigens in soluble form. In this study, we applied high-density peptide microarrays to uncover antibody specificities. Therefore, we designed a three-branched approach, encompassing the mapping of 12 leading vaccine candidates (41, 42), as well as a screening of preselected, presumably immunogenic epitopes, derived from the proteome of P. falciparum, and a control approach, using randomly selected P. falciparum epitopes.

Peptide microarrays represent a powerful tool to analyze linear antibody epitopes in multiple applications, including autoimmunity (112, 113) and infectious diseases (114–119). Based on the standardized and straightforward chemical synthesis of peptides, the technology offers the identification of immunoreactive antigens and epitopes, applicable e.g. in the discovery of diagnostic biomarkers or rational vaccine design.

Accompanied using linear peptides, discontinuous epitopes are currently difficult to assess with this technique, but it is principally possible (120). In the case of protein arrays, which feature recombinantly expressed proteins (5, 19–24), the correct conformation of Pf proteins is uncertain and, thus, also possibly lacking conformational or discontinuous epitopes or missing linear epitopes because of protein misfolding. Moreover, some larger antigens, such as MSP-1 (121), need to be divided into several parts for efficient heterologous expression. This can also have an impact on discontinuous epitopes.

The vaccine array approach allowed us not only to verify that peptide arrays can effectively identify antigens and respective domains known to be targets of antibodies from naturally exposed individuals (Fig. 3), but also to simultaneously perform an epitope mapping across the entire sequence of each of the well-known vaccine candidates. The latter facilitated the discovery of novel, highly immunoreactive epitopes as for example in MSP-1, GLURP, LSA-1, and SEA-1, which might be of particular interest for further evaluation. Most studies deciphering the humoral response to MSP-1 rely on recombinant expressed domains, whereas we used, for the first time, overlapping peptides covering the entire sequence of the antigen. Strikingly, we identified two highly significantly recognized (and not yet described) epitopes in MSP-1 (Figs. 2 and 3), which can be mapped to p38 and p30. Aside from this, we found more epitopes within p38 and p33, resulting in a strong reactivity (p value < 0.01) in
disease-protected individuals (Fig. 2). Furthermore, in line with a study by Theisen and colleagues, identifying B cell epitopes within the R0 region of GLURP (74), our screening resulted in both immunogenic linear peptides reported within their study and novel epitopes in GLURP-R0. Indeed, one of the most frequently recognized epitopes published by Theisen et al. was also found under the most significant hits derived from our data (ENVETEEIDDVPS; Fig. 3). It has been also shown that antibodies present in the sera of naturally infected individuals recognize a peptide derived from the R2 region of GLURP (54). The peptide used by Ambrosino et al. covers the described antigenic sequence HEIVEVEEI, which is present in the highly repetitive, conserved repeat units of GLURP-R2 (68, 75) and discussed to be responsible for the immunodominant nature of this region. Accordingly, several epitopes identified by our screening overlap with the repetitive sequence motif HEIVEVEEI. Interestingly, as depicted in Fig. 3, our analysis yielded a second GLURP epitope under the most significant hits (QEPVPVTLNENENVT), located within the R2 region, which was not yet described. With respect to LSA-1, beside significantly recognized epitopes originating from the repeat region and C-terminal domain, our screening uncovered a strong reactivity to the N-terminal epitope VLSHNSYEKTKNNEN (Fig. 3) in serum samples from disease-protected individuals. To our knowledge, our approach resulted in both immunogenic linear peptides reported within our data (ENVETEEIDDVPS; Fig. 3). It has been also shown that antibodies present in the sera of naturally infected individuals recognize a peptide derived from the R2 region of GLURP (54). The peptide used by Ambrosino et al. covers the described antigenic sequence HEIVEVEEI, which is present in the highly repetitive, conserved repeat units of GLURP-R2 (68, 75) and discussed to be responsible for the immunodominant nature of this region. Accordingly, several epitopes identified by our screening overlap with the repetitive sequence motif HEIVEVEEI. Interestingly, as depicted in Fig. 3, our analysis yielded a second GLURP epitope under the most significant hits (QEPVPVTLNENENVT), located within the R2 region, which was not yet described. With respect to LSA-1, beside significantly recognized epitopes originating from the repeat region and C-terminal domain, our screening uncovered a strong reactivity to the N-terminal epitope VLSHNSYEKTKNNEN (Fig. 3) in serum samples from disease-protected individuals. To our knowledge, our approach provides for the first-time insights into the immunogenicity of SEA-1 considering the entire protein as overlapping peptides. Intriguingly, one of the epitopes mapped within the domain SEA-1AA 810–1083 has been identified as one of the strongest recognized epitopes resulted from our approach (KNGNVEVTGNGGNEE; Fig. 3). Novel immunogenic epitopes were discovered N- and C-terminal of SEA-1AA 810–1083 with the N-terminal recognized epitopes resulted from our approach (KNGNVEVTGNGGNEE; Fig. 3). Novel immunogenic epitopes were discovered N- and C-terminal of SEA-1AA 810–1083 with the N-terminal part harboring the most significant epitopes (Fig. 2). Preselecting potential immunogenic malarial peptides by applying bioinformatical algorithms resulted not only in epitopes derived from well-characterized antigens such as CSP, LSA-1 and STARP but also in epitopes from, in terms of antibody responses, previously uncharacterized antigens (PF08_0088 (PFSP3), PFC0330w, and PFL0685w). Recently, a proteomic approach identified SSP3 among others as novel putative surface protein of salivary gland sporozoites derived from both the human malaria parasite P. falciparum and the rodent malaria parasite P. yoelii (108). A subsequent study confirmed the surface localization of SSP3 on salivary gland sporozoites and demonstrated a function of the protein in in vitro gliding motility in P. yoelii (109). PFC0330w, annotated as conserved Plasmodium protein with unknown function, harbors a predicted signal peptide and transmembrane domain (PlasmoDB). Currently available proteome data indicate an expression during intraerythrocytic development (PlasmoDB). Based on sequence homology, PFL0685w (annotated as GPI ethanolamine phosphate transferase 3, also known as PIG-0 (110)) was suggested to be involved in the GPI anchor biosynthesis pathway in P. falciparum and was shown being expressed in late asexual blood stages (110). These novel candidates might be of interest for future studies. Indeed, more patient samples with longitudinal patient data will be necessary to confirm, whether a patient has acquired immunity to malaria, which we so far only derived from the age and parasitemia level.

The complete proteome of P. falciparum comprises more than 4 million amino acids. To cover the whole Pf proteome with overlapping peptides, up to 4 million different 15-mer peptides (with 14 amino acid overlap) would be necessary. Thus, we currently strive to generate peptide arrays with even higher densities, by advancing novel microarray fabrication techniques (122), which will allow us to screen and identify many more immunodominant epitopes in future studies. Moreover, post-translational modifications (PTMs) of proteins, and glycosylation, are of considerable interest, because polysaccharides are known as being recognized by the immune system. In Plasmodium parasites, several PTMs were detected (reviewed in (123–125)) and the generation of antibodies recognizing glycosylated proteins during natural exposure has been described earlier (126, 127). Recent work shed more light into N- and O-glycosylation in malarial parasites. To date, N-glycosylated proteins were only attributed to blood stage parasites synthesizing truncated N-glycans (128). Notably, recently it became possible to discover that CSP and TRAP are O-glycosylated on salivary gland sporozoites (129) and the study by Kupferschmid and colleagues identified target proteins of O-GlcNAcylation in intraerythrocytes stages (130). However, most studies profiling antibody responses do not yet consider PTMs. Indeed, the protein expression system used for protein arrays used to analyze antibody profiles in different settings is based on E. coli lysates (bacterial cell-free expression systems), hence, missing glycosylation and presumably antigens of interest (5, 19–24, 106). Interestingly, a glycopeptide-based microarray was used to study the humoral response against two surface glycoproteins in sera of individuals infected with the apicomplexan parasite Cryptosporidium parvum (131). Technologies allowing the high-throughput analysis of glycosylated proteins/peptides will ultimately provide a more detailed understanding of humoral immune responses and potentially result in novel epitopes.

In summary, the peptide-based approach allows us to distinguish different immunogenic protein epitopes. We observed many highly immunogenic antigens and epitopes, which is in line with published protein array screening data (5, 19–24). However, we postulate that only a few specific epitopes will correlate with an efficient immunity. These distinct peptide reactions could explain the sometimes-contradictory results published on the protective relevance of antibody responses to different immunogenic Pf proteins. In protein arrays, the serum reactivity to a complete protein ignores the differential antibody binding to specific protein domains, which is why current research may so far have missed the correct conclusion toward an effective immunity.

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Thus, we believe that peptide arrays can give a more differential insight into malaria immunity.

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DATA AVAILABILITY

The here presented fluorescence data is available in the supplementary information and is scheduled to be released on http://www.plasmodb.org/.

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