Epitope-specific differential antibody responses to *Plasmodium falciparum* circumsporozoite protein are associated with protection from malaria infection in Malian adults

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INTRODUCTION

Circumsporozoite protein (CSP) coats the *Plasmodium falciparum* sporozoite surface and is the target of multiple vaccines, including RTS,S and R21, and monoclonal antibodies in development (Figure 1).

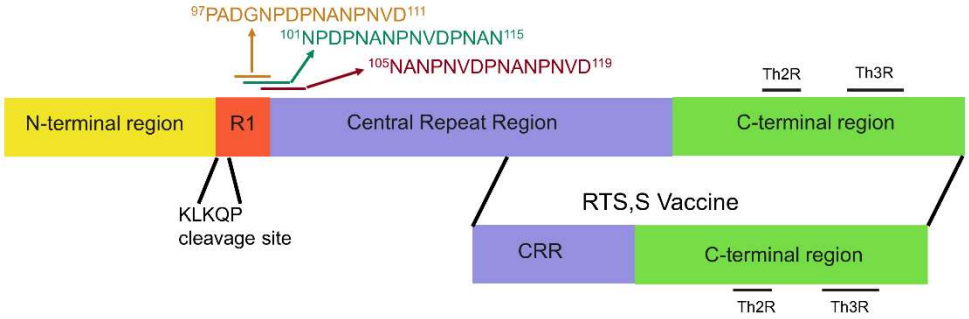

Figure 1: Schematic of Circumsporozoite Protein (adapted from Friedman-Klabanoff et al., *Vaccine*, 2021 Feb 22;39(8):1195-1200.[1]) CSP has four regions: the amino-terminus, Region I, which contains an area that is cleaved to allow for hepatocyte invasion, the central (NANP) repeat region, and the carboxy-terminus. The first and second peptide sequences in gold and green, respectively, are the targets of CIS43[2] and the third peptide is the target of L9.[3] The RTS,S and R21 vaccines target the last 18 NANP repeats and the C-terminal region (excluding the GPI anchor).[4]

In studies of RTS,S, antibodies to the immunodominant central repeat region correlated with protection.[5] However, RTS,S-induced protection remains modest,[6] and monoclonal antibodies targeting the R1-NANP junction were found to be highly efficacious in preventing infection in controlled human malaria infection studies.[7,8]

METHODS

Study Participants

Samples for the study came from a double-blinded randomized, placebo-controlled clinical trial of fully active sporozoites administered with chloroquine prophylaxis (PfSPZ-CVac (CQ)) in Mali

(Table 1).[9] Participants received 204,800 aseptic, purified cryopreserved *P. falciparum* NF54 sporozoites (PfSPZ Challenge) by direct venous injection on days 3, 31, and 59. In addition, participants received 600 mg of chloroquine (CQ) via directly observed therapy on study day 1, then 300 mg CQ weekly for ten additional doses (last dose study day 64). Participants were cleared of existing parasitemia with 200 mg artesunate daily for seven days starting day 71. They were then followed with active surveillance for parasitemia by thick blood smear every 28 days and passive surveillance for any suspected (symptomatic) malaria illness between visits during study days 87-245.

Table 1: Study Interventions and Sampling

Study Day	3	15	31	43	59	71	87-245
PfSPZ Challenge	X		X		X		
Chloroquine	--Weekly until study day 64--						
Artesunate						A	
Follow-up period							F
Study samples	S					S	

We examined CSP antibody responses in vaccinated adults (n = 31) who subsequently developed malaria (infected; could be symptomatic or asymptomatic illness, n = 15) compared to those who did not (uninfected, n = 13) to further define potential correlates of protection. Sera from days 3 (baseline) and 71 were probed on the peptide microarray.

Pre-erythrocytic peptide microarray design and probing

The peptide microarray was designed with coverage of 81 CSP variants from a database containing publicly available sequences and over 400 field samples from Mali, Malawi, Brazil, Cambodia, Myanmar, Thailand, Laos, and Guinea sequenced by our colleagues at the Institute for Genome Sciences at the University of Maryland School of Medicine. Each variant antigen was represented by 16-amino acid peptides with 12-amino acid overlap covering the length of the sequence, but only unique peptides were included on the array. This resulted in 1,405 unique peptides covering the length of the CSP sequence. The peptides were mapped to NF54 (397 amino acids long) for analysis. Further details regarding array synthesis and sample binding and detection can be found in Appendix 1.

Statistical analysis

Fluorescence intensity (FI) was used to approximate the quantity of bound IgG antibodies for each peptide. Raw peptide array FI were $\log_2$ transformed. Because of the high degree of overlap of neighboring peptides, intensities were expected to be correlated and could have contained the same epitope. To increase the signal-to-noise ratio, a sliding-window based average smoothing procedure was applied.[10] Each peptide was represented by its midpoint (end position minus beginning position divided by two) and each amino acid position along the sequence was represented by a smoothed $\log_2$ FI equal to the average FI for all peptides with a midpoint four amino acids before or after the given position (i.e., position 100 had a corresponding smoothed $\log_2$ FI that was the average of all peptides with midpoints between

positions 96-104). The sliding window smoothing procedure was applied to calculate the average FI and average $\log_2$ fold change for each subject and protein variant across overlapping peptides for each respective variant and timepoint (baseline and day 71). The average $\log_2$ fold change was used to calculate the geometric mean of the $\log_2$ fold change for each CSP variant and the geometric mean of the mean of $\log_2$ fold changes across all CSP variants at each position along CSP. Across the protein, 95% bootstrap confidence intervals (CIs) were estimated using 10,000 replicates for each cohort.

Results

Compared to the infected group, the uninfected group had lower mean of the mean $\log_2$ fold change in fluorescence intensity (FI) across CSP variants in the central repeat region at day 71 post-vaccination compared to baseline (Figure 2). Bootstrap CIs across the region did not overlap, suggesting a strong differential signal. In contrast, the uninfected group had higher fold change in FI at the junctional region. CIs did not overlap with the mean antibody signal for the infected group, confirming a differential signal for the junctional region as well, albeit in the opposite direction.

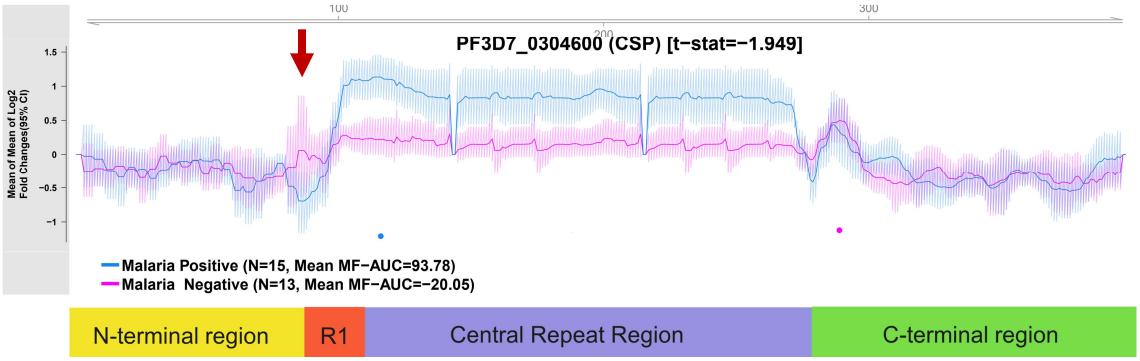

Figure 2: Distribution of smoothed mean of mean of $\log_2$ fold change in IgG fluorescence intensity at Day 71 for circumsporozoite protein (CSP). X-axis: position along CSP. Y-axis: mean of mean of $\log_2$ fold changes. Participants who developed either asymptomatic or symptomatic malaria during follow up are in blue and those who did not are in pink. Shaded areas represent 95% confidence interval based on 10,000 bootstrap replicates.

Discussion/Conclusions

We hypothesize that the immunodominant central repeat region may elicit antibodies that are not as effective as junctional region antibodies. R1 is critical for sporozoite attachment, so antibodies to the adjacent junctional region may be more specific for blocking hepatocyte invasion by sporozoites. It is also possible that responses to the central repeat region may inhibit responses to the junctional region. Future analyses will include assessing cross-reactivity between allelic variants of CSP and analyzing vaccine escape by comparing CSP sequences from infecting parasite strains to CSP antibody responses.

Acknowledgements

We would like to thank the Malian volunteers for graciously consenting to be in this trial, the Division of Microbiology and Infectious Diseases at the NIAID for their invaluable support and advice, and The Emmes Company, LLC for statistical support. Funding: NIAID Vaccine and

Treatment Evaluation Unit (VTEU) contract number HHSN272201300022I (PI: Karen Kotloff, Protocol PI: Matt Laurens), Sanaria, NIAID K23AI155838 (DFK), Burroughs Wellcome Fund/American Society of Tropical Medicine and Hygiene Postdoctoral Fellowship in Tropical Infectious Diseases (DFK), NIAID K23AI125720 (AAB). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Appendix 1

Array Synthesis

The *in situ* peptide synthesis, quality control, serum probing and slide imaging procedures are similar to those have been detailed previously.[11] Microarrays were synthesized by light-directed solid-phase peptide synthesis with a Nimble Therapeutics Maskless Array Synthesizer (MAS) using an amino-functionalized support (Greiner Bio-One) coupled with a 6-aminohexanoic acid linker and amino acid derivatives carrying a photosensitive 2-(2-nitrophenyl) propyloxycarbonyl (NPPOC) protection group (Orgentis Chemicals). Amino acids were pre-mixed for ten minutes to a final concentration of 20 mM in *N,N*-Dimethylformamide (DMF, Sigma Aldrich) with *N,N,N',N'*-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium-hexafluorophosphate (HBTU, Protein Technologies, Inc.; final concentration 20 mM) as an activator, 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt, Protein Technologies, Inc.; final concentration 20 mM) to suppress racemization, and *N,N*-Diisopropylethylamine (DIPEA, Sigma Aldrich; final concentration 31 mM) as base. Activated amino acids were then coupled to the array surface for three minutes. The microarray was then washed with *N*-methyl-2-pyrrolidone (NMP, VWR International), and site-specific cleavage of the NPPOC protection group was accomplished by irradiation of an image created by a Digital Micro-Mirror Device (Texas Instruments), projecting 365 nm wavelength light. These coupling cycles were repeated as necessary to synthesize the full peptide library. All arrays passed proprietary QC metrics including amino acid quality control, synthesis drift, background signal, and signal uniformity.

Sample Binding and Detection

Prior to sample binding, 95% trifluoroacetic acid (TFA, Sigma Aldrich) and 0.5% Triispropylsilane (TIPS, TCI Chemicals) were used for 30 minutes to remove any side chain protecting groups. Arrays were incubated two times in methanol for 30 seconds, rinsed four times with reagent-grade water (Ricca Chemical Co.), washed for one minute in TBST (1× TBS, 0.05% Tween-20), washed two times for one minute in TBS, and washed for 30 seconds in reagent-grade water as a final wash.

Samples were diluted 1:100 in binding buffer (0.01M Tris-Cl, pH 7.4, 1% alkali-soluble casein, 0.05% Tween-20) and bound to arrays overnight at 4°C. The arrays were then washed three times for ten minutes each time in wash buffer (1× TBS, 0.05% Tween-20). Primary sample binding was detected via Alexa Fluor® 647-conjugated goat anti-human IgG secondary antibody (Jackson ImmunoResearch) diluted 1:10,000 (final concentration 0.1 ng/µl) in secondary binding buffer (1x TBS, 1% alkali-soluble casein, 0.05% Tween-20). After incubation with secondary antibody for three hours at room temperature, arrays were washed three times (ten minutes per wash) in wash buffer, washed 30 seconds in reagent-grade water, and dried by spinning in a microcentrifuge equipped with an array holder. Fluorescent signal of the secondary antibody was detected using an InnoScan 1100AL microarray scanner (Innopsys Inc.) by scanning at 635 nm at 2 µm resolution. Proprietary Nimble Therapeutics software was used to extract fluorescence intensity values for each peptide from scanned array images.