Off-target antibody responses induced by RTS,S/AS02A/1B in malaria-naïve adults associated with protection against controlled human malaria infection

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INTRODUCTION

The RTS,S vaccine targets the circumsporozoite protein (CSP), which coats the *Plasmodium falciparum* sporozoite surface and is the target of multiple vaccines and monoclonal antibodies in development (Figure 1).

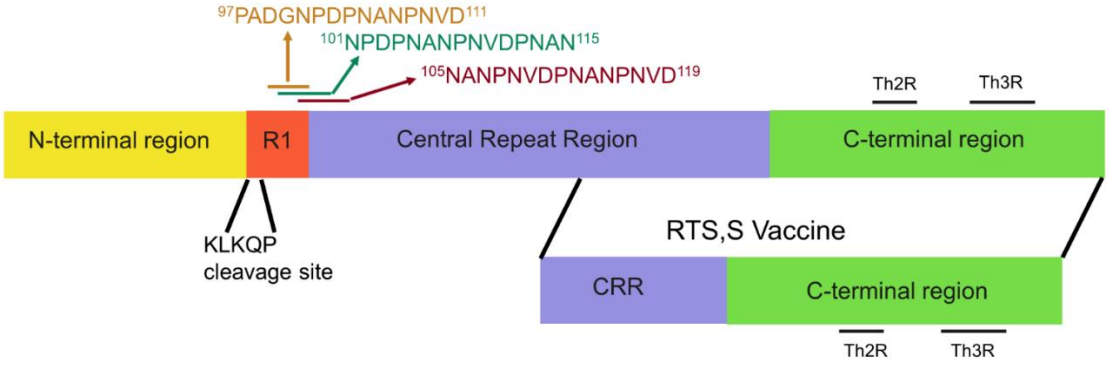

Figure 1: Schematic of Circumsporozoite Protein (adapted from Friedman-Klabanoff et al., *Vaccine*, 2021 Feb 22;39(8):1195-1200).[1] CSP contains three major regions: (i) the amino terminus, which contains Region I, an area cleaved to allow for hepatocyte invasion; (ii) the central (NANP) repeat region, and (iii) the carboxy terminus. The region between Region I and the central repeat region, termed the junctional region, is the area targeted by the monoclonal antibodies CIS43[2] (gold and green) and L9 (maroon).[3] The RTS,S vaccine targets the last 18 NANP repeats and the C-terminal region (excluding the GPI anchor).[4]

RTS,S contains a portion of CSP (Figure 1) and provides modest efficacy (36% efficacy over four years in infants aged 5-17 months).[5] Although some studies have demonstrated an association between anti-CSP repeat region IgG antibody titers and vaccine-induced protection from RTS,S, the relationship does not seem to be linear or suggest an absolute protective threshold.[6-10] Additional CSP epitopes may be important, but whether their association with protection indicates direct action versus an indirect marker of quality or function of antibody responses is unknown.[7,11-17] Our group previously identified a C-terminal epitope, PNDPNRNV, where higher breadth of IgA responses was associated with protection from controlled human

malaria infection (CHMI) after RTS,S vaccination (Friedman-Klabanoff, in prep). New data suggests that off-target/cross-reactive antibody responses may also be associated with protection.[18]

METHODS

We examined IgG responses in malaria-naïve adults vaccinated with RTS,S who were either protected (n=18) or unprotected (n=17) against controlled human malaria infection to examine the association between off-target responses and protection (Table 1). Baseline and post-vaccination (pre-CHMI) sera were probed on a pre-erythrocytic peptide array with representation of diverse variants (2-19 variants each) of five non-CSP proteins previously associated with vaccine-induced immunity after RTS,S[18] or PfSPZ as 16-amino acid peptides overlapping by 15. Further details regarding array synthesis and sample binding and detection can be found in Appendix 1.

Table 1: Study Participants

Study	CHMI Outcome	N (pre-vaccination)	N (Pre-CHMI)
RTS,S	Protected	18	18
RTS,S	Unprotected	17	17

Reference protein sequence alignments and peptide filtering

Proteins from the NF54 strain of *P. falciparum* were used as reference for peptide mapping. For each protein variant, its peptides were mapped against the reference protein sequence using ssearch36 (Version 36.3.8g). Peptides were excluded if the alignment score was less than three standard deviations below the mean, the alignment length was two or more amino acids shorter than the full-length peptide, or if alignments had more than one gap in the reference or the query.

Statistical Analysis

Sliding Window Smoothing Procedure

Fluorescence intensity (FI) was used to approximate the quantity of bound antibodies for each peptide. Raw peptide array FI were $\log_2$ transformed. Because neighboring peptides overlap by 15 amino acid peptides, intensities of neighboring peptides were expected to be similar and could have contained the same epitope. To increase the signal-to-noise ratio, a sliding-window based average smoothing procedure was applied.[19] Each peptide was represented by its midpoint (end minus beginning amino acid 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). For each participant and protein variant, the sliding window smoothing procedure was applied to calculate the average pre-CHMI fluorescence intensity corresponding to each amino acid position on the reference protein sequence.

Intensity of responses

Using the sliding window procedure, the mean $\log_2$ fold change between baseline and pre-CHMI for each group (protected and unprotected) for amino acids along the reference protein sequence was calculated for each protein variant. For each protein at each position, $\log_2$ fold changes for all included protein variants were averaged, and the mean for each group was calculated (mean of the mean of $\log_2$ fold changes, referring to the mean of the protein variants and the mean of the individuals in each cohort). Distribution plots of the geometric mean of the mean of $\log_2$ fold changes and 95% bootstrap confidence intervals using 10,000 replicates for each cohort were then constructed. We also examined for positions where the confidence intervals did not overlap between the groups, implying a significant difference in serorecognition between the groups at those positions.

Breadth of responses

Serorecognition thresholds for each peptide were defined as the mean plus 2.5 times the standard deviation of the $\log_2$ fluorescence intensity at Day 1 (pre-vaccination) from all participants (regardless of protection status). We classified a peptide as serorecognized by an individual participant if its $\log_2$ fluorescence intensity was greater than the serorecognition threshold for that peptide. We then applied a sliding window smoothing procedure so that at a given amino acid position along each protein, the number of serorecognized peptides with mid-points within four amino acids before or after that position were summed. The mean number of serorecognized peptides for each group was captured for each amino acid position along each protein and was used to represent the breadth of responses at that location along the protein. Distribution plots of the mean number of serorecognized peptides and 95% bootstrap confidence intervals using 10,000 replicates for each cohort were then constructed. We also examined for positions where the confidence intervals did not overlap between the groups, implying a significant difference in breadth of responses between the groups at those positions.

Sequence comparison

For epitopes with significant differences in breadth or intensity of antibody responses in protected versus not protected participants, we used BLASTp to find sequence similarities to the CSP 3D7 sequence or hepatitis B surface antigen sequence. The best alignment with an e-value <0.05 was reported.

Software

Data was analyzed using R with R Bioconductor and limma R packages in the Ubuntu (Version 13.04) operating system. The R p.adjust function was used for estimating the FDR based on the Benjamini-Hochberg procedure.

RESULTS

Protected adults were seropositive for more peptide variants in regions of CLAMP (Figure 2A), GSK3, DOC2, and MSP5 compared to unprotected adults. Protected adults also had higher reactivity than unprotected adults to peptide variants in regions of CLAMP (Figure 2B), GSK3, DOC2 (Figure 2C), LRR9, and MSP5, with some peptides of CLAMP, GSK3, and MSP5 being targets of both increased seropositivity and reactivity (Table 1). BLAST between areas of differential responses and CSP identified sequence similarities between a CLAMP peptide and

the CSP central repeat region and a CLAMP peptide and a DOC2 peptide and an area of the CSP C-terminal region upstream of Th2R (Table 1). We previously identified the CSP C-terminal sequence at this location as a potential epitope with higher seropositivity in the RTS,S-protected adults in this study. A different area of DOC2 had sequence similarities to the hepatitis B surface antigen (Table 1 and Figure 3).

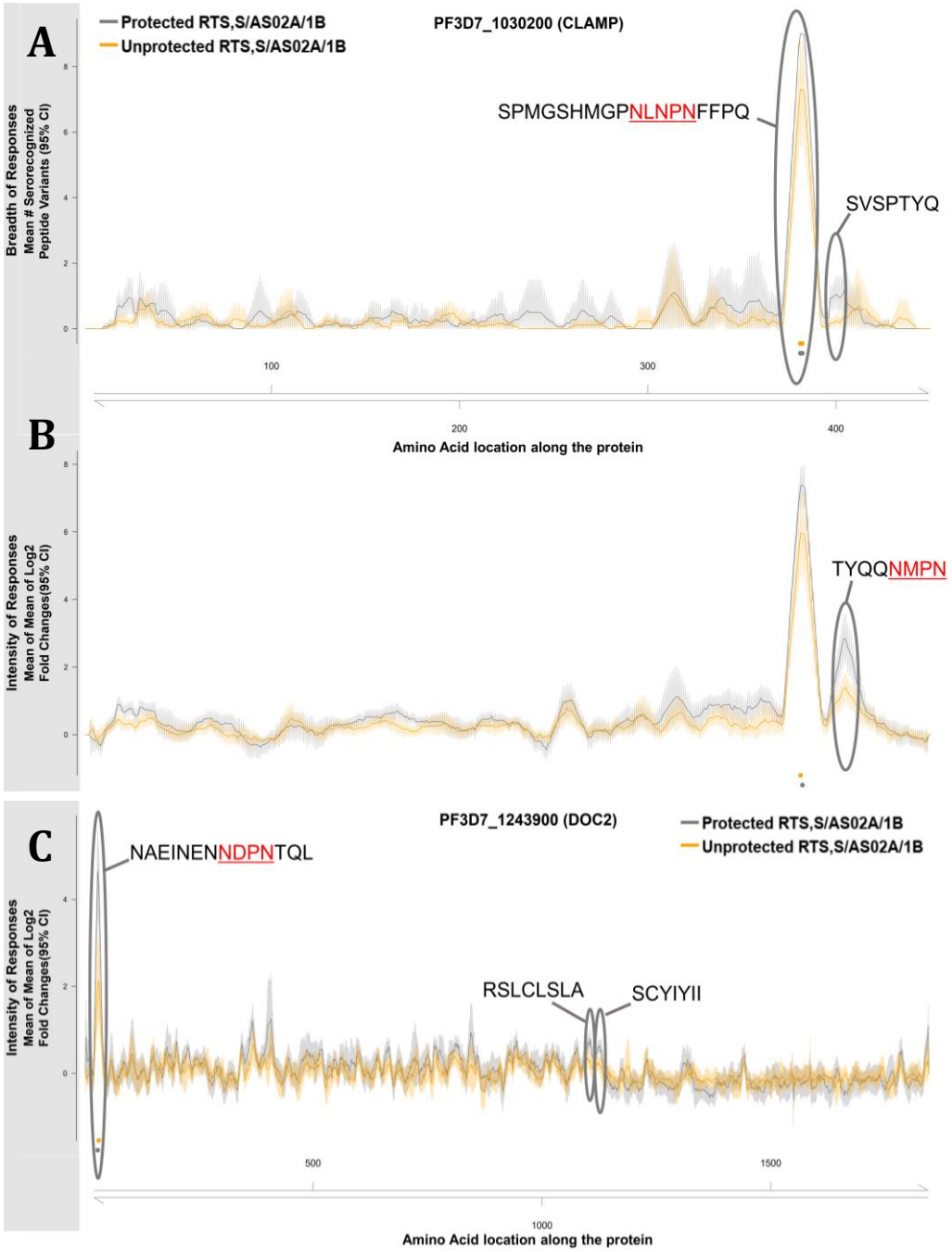

Figure 2: Breadth and intensity of IgG responses post-vaccination for PF3D7_1030200 (CLAMP) (A&B) and intensity of IgG responses post-vaccination for PF3D7_1243900 (DOC2) (C). Areas of non-overlap between 95% CI between the protected (gray) and unprotected

(orange) had sequence similarities to the central repeat region (CLAMP, A) and to a previously identified C-terminal epitope upstream of Th2R (CLAMP, B, and DOC2, C).

Table 1: Areas of greater IgG breadth or intensity in protected versus unprotected RTS,S recipients and sequence similarities to vaccine components

Protein[1]	Epitope location	Epitope sequence	Vaccine component	Aligned location	Aligned sequence
Breadth of responses					
CLAMP	372-389	SPMGSHMGPNLNPNFFPQ	CSP[2]	CRR[3]	PNANPN
CLAMP	397-403	SVSPTYQ	None		
GSK3	290-297	TEDQLKEM	None		
DOC2	536-541	DTYVEV	None		
DOC2	924-934	GPSLPYKTIDL	HBsAg[4]	202-205	GPSL
MSP5	103-120	QKEPEEKENSNPNLDSSE	CSP	375-376	SS
Intensity of responses					
CLAMP	401-408	TYQQNMPN	CSP	283-287	HNMPN
GSK3	56-65	DNEINRSPNK	None		
GSK3	289-298	PTEDQLKEMN	None		
DOC2	24-37	NAEINENNDPNTQL	CSP	287-290	NDPN
DOC2	1102-1109	RSLCLSLA	None		
DOC2	1121-1127	SCYIYII	None		
MSP5	103-111	QKEPEEKEN	None		
MSP5	131-142	DISEHNSNNPET	None		
LRR9	107-116	NMNDINYNPN	None		
LRR9	214-222	LIYFPYISN	None		

[1]CLAMP = PF3D7_1030200, GSK3 = PF3D7_0312400, DOC2 = PF3D7_1243900, MSP5 = PF3D7_0206900, LRR9 = PF3D7_0906700
[2]CSP = circumsporozoite protein = PF3D7_0304600
[3]CRR = central repeat region; PNLNP matches PNANPN at 38 locations within the central repeat region of the 3D7 sequence of CSP
[4]HBsAg = Hepatitis B surface antigen

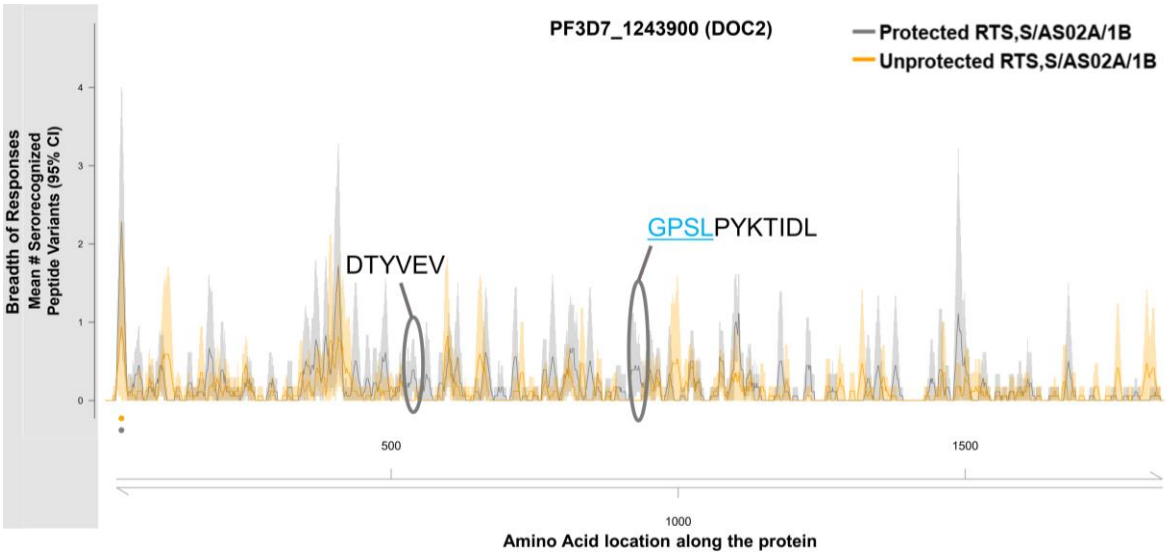

Figure 3: Breadth of responses for IgG post-vaccination for PF3D7_1243900 (DOC2). An area of non-overlap of 95% CI between the protected (gray) and unprotected (orange) RTS,S vaccinated groups has sequence similarities to Hepatitis B surface antigen (blue underlined amino acid residues).

DISCUSSION

Our findings support emerging data that off-target antibody responses induced by RTS,S may be important for protection. Some areas have sequence similarities to central repeat region and a C-terminal epitope **PNDPN**RNV, an epitope we previously found to be associated with RTS,S-induced protection after CHMI. Cross-reactive antibodies could have direct action on off-target proteins or could correlate with avidity or function of CSP antibodies. Further work to isolate cross-reactive antibodies and examine their binding and function is warranted.

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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 that have been detailed previously.[20] Microarrays were synthesized by light-directed solid-phase peptide synthesis with a Nimble Therapeutics Maskless Array Synthesizer 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 (Sigma Aldrich) with *N,N,N',N'*-Tetramethyl-O-(1H- benzotriazol-1-yl)uronium-hexafluorophosphate (Protein Technologies, Inc.; final concentration 20 mM) as an activator, 6-Chloro-1-hydroxybenzotriazole (Protein Technologies, Inc.; final concentration 20 mM) to suppress racemization, and *N,N*-Diisopropylethylamine (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 (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 quality control metrics, including amino acid quality control, synthesis drift, background signal, and signal uniformity.

Sample Binding and Detection

Samples were divided into nine batches in such a way that both samples (baseline and pre-CHMI) for one participant were in the same batch, and study arms and protection status were balanced across batches.

Before sample binding, arrays were treated 95% trifluoroacetic acid (Sigma Aldrich) and 0.5% Triispropylsilane (TCI Chemicals) for 30 minutes to remove any side chain protecting groups. Arrays were then incubated two times in methanol for 30 seconds, rinsed four times with reagent-grade water (Ricca Chemical Co.), washed for one minute in 1× TBS with 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 and Cy3-conjugated goat anti-human IgA 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 captured using an InnoScan 1100AL microarray scanner (Innopsys Inc.) by scanning at 635 nm at 2 µm resolution. Proprietary Nimble Therapeutics software transformed the brightness of each spot into a fluorescence intensity (FI) value for each peptide from the scanned array images.