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Brief communication

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Epitope specificity of antibody-mediated protection induced in mice by the malaria vaccine RTS,S/AS01

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Antibodies induced by the malaria vaccine RTS,S/AS01 neutralize infectivity of transgenic sporozoites expressing *Plasmodium falciparum* CSP (PfCSP). These antibodies recognize the junctional, minor repeats, central repeats, and C-term regions of this antigen. The epitope specificity of antibodies mediating protection in mice was characterized in vivo using transgenic sporozoites expressing restricted antigenic portions of PfCSP. In this model, we found protection is mediated mostly by antibodies specific for the central repeats.

Vaccination against malaria using three doses of RTS,S/AS01 has shown significant protection against clinical disease in young children living in malaria-endemic areas[1]. This protection wanes after 12 months, although a booster dose at 18 months can prolong its efficacy. In 2021, the World Health Organization recommended the widespread use of RTS,S/AS01 in endemic areas experiencing moderate-to-high *Plasmodium falciparum* transmission[2]. This vaccine is a virus-like particle consisting of a portion of the *P. falciparum* circumsporozoite protein (PfCSP) genetically fused to the hepatitis B surface antigen[3]. A new vaccine consisting of the same PfCSP antigenic domains, R21-matrix M (R21MM), has shown significant protective efficacy in an ongoing phase III vaccine trial[4]. In the 3D7 parasite strain, the *Pf*CSP central repeat region contains 1 junctional NPDP repeat, 4 minor NVDP repeats, and ~38 NANP central repeats. The CSP component of RTS,S/AS01 and R21MM comprise 19 NPNA central repeats and the C-term flanking region, residues 273-395 (3D7 strain).

We used the mouse challenge model based on transgenic *P. berghei* sporozoites expressing the *P. falciparum* CSP (tg*Pb-Pf*CSP) to characterize the protective efficacy of RTS,S/AS01 to guide the development of next-generation malaria vaccines[5,6]. Studies with this model indicate that immunization of mice with RTS,S/AS01 induces a strong antibody response that neutralizes infectivity of the sporozoites and confers sterile immunity from challenge with tg*Pb-Pf*CSP sporozoites[6]. We are now using this in vivo model to evaluate promising new vaccine constructs delivered through active immunization, with RTS,S/AS01 as a benchmark, and to identify anti-infection monoclonal antibodies (mAbs) that can be delivered prophylactically via passive transfer[7–9], some of which have now been confirmed in clinical trials[10–12].

The antibodies induced in mice by RTS,S/AS01 immunization recognize synthetic peptides containing the central NPNA repeats and part of the C-terminal flanking region (within residues 311–373) of *P. falciparum* CSP, both of which are contained in this vaccine. These antibodies also bind the junctional region (ADGNPDPNANPNVDP) and the minor repeat region $(NANPNVDP)_2$ even although these epitopes are not present in this vaccine construct (Fig. 1). Antibody responses against the central repeat epitope and the C-term flanking region appear to be larger in magnitude (Fig. 1) when compared to antibodies recognizing junctional or minor repeat epitopes. The central repeats and C-term are relevant to protection, as mAbs specific for these antigenic domains inhibit sporozoite infection[7–9,13]. Defining the functional epitope specificity of vaccine-induced protective antibodies is an important determinant of vaccine immunogenicity and efficacy and is particularly relevant to studies aimed at comparing vaccine constructs targeting the same or closely related antigens.

To define the specificity of protection of RTS,S/AS01 and expand our understanding of this benchmark in this model, we evaluated the neutralizing effect of anti-RTS,S/AS01 hyperimmune mouse sera on the infectivity of *P. berghei* sporozoites expressing chimeric CSP that contains selected antigenic domains of PfCSP. To generate these CSP chimeric parasites, short sequences from the PfCSP gene were used to modify the *P. berghei* CSP (PbCSP) gene. The resulting chimeric genes were incorporated into plasmids that contain a pyrimethamine resistance gene, as described previously. These plasmids were used to transfect *P. berghei* parasites (ANKA Green Fluorescence Protein (GFP)-Luciferase)[14] to replace the wild-type PbCSP gene with the modified chimeric PbPfCSP gene, using standard procedures[15,16]. The PfCSP sequences inserted into the PbCSP gene correspond to the junctional region (tgPbPf-JR), minor repeat epitope (tgPbPf-MR), $NPNA_{11}$ central repeats (tgPbPf-CR), or a truncated C-term region from Pf C-term (tgPbPf-Ct) (Supplementary Table 1). After cloning these knock-in (KI) transgenic parasites, the corresponding chimeric CSPs were confirmed by sequencing. To determine the infectivity of the new transgenic sporozoites compared to that of sporozoites expressing the entire

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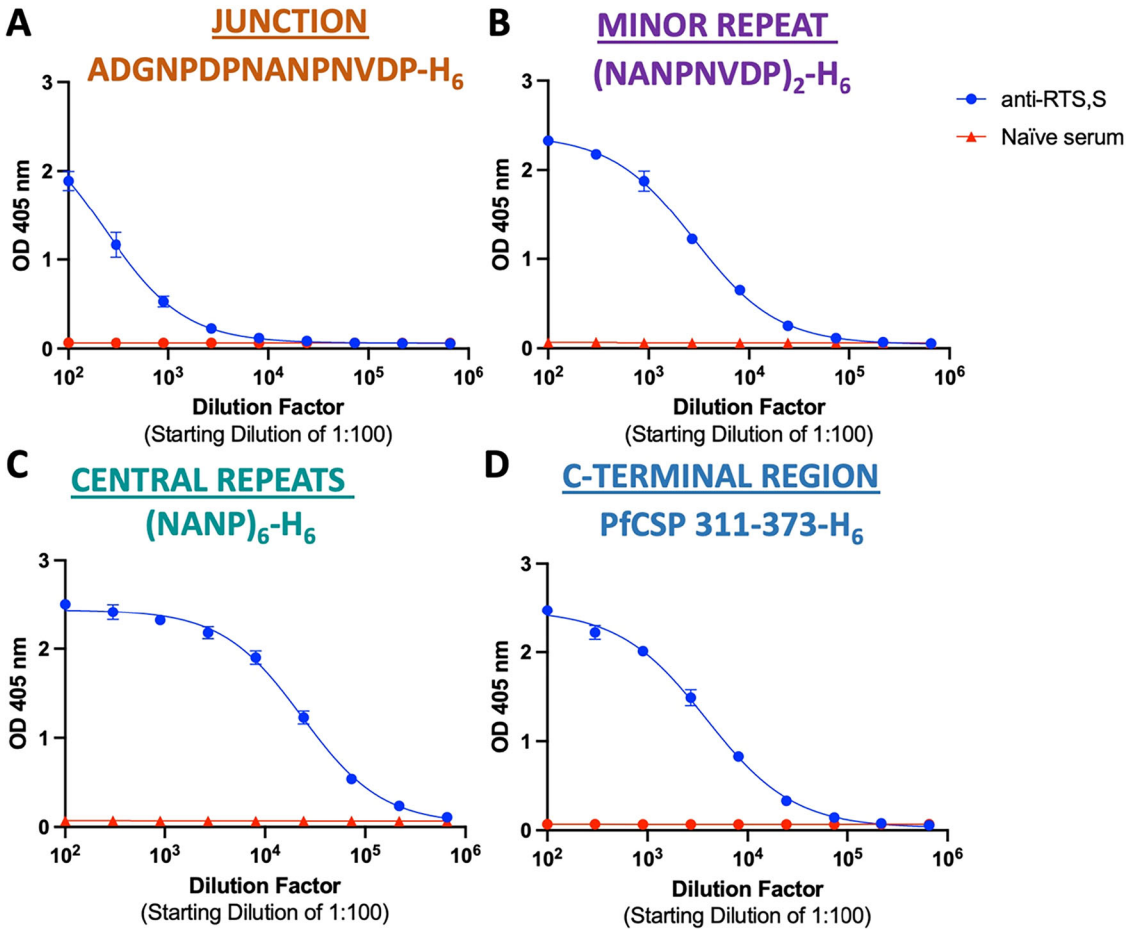

Fig. 1 | Epitope mapping of anti-RTS,S/AS01 antibodies. Binding of anti-RTS,S/AS01 antibodies to specific epitopes of the PfCSP protein by ELISA. Immune sera obtained after 3 immunizations with 5 µg of RTS,S adjuvanted with 10-fold diluted original $AS01_E$ adjuvant dose, representing 2.5 µg of the TLR4 ligand 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL), and 2.5 µg of the QS-21 saponin was assayed. Maxisorp 96 well plates were coated with different peptides at 1 µg/mL, (**A**) Junctional region, (**B**) Minor repeat, (**C**) central repeats and **D** C-term, (S Table 2). Serum dilutions were run in duplicates, starting at 1:100 followed by 3-fold-dilution.

PfCSP, mice were injected intravenously (i.v.) with 2000 sporozoites and the parasite liver burden measured by luciferase activity detected by intravital imaging (IVIS) 42 h later. The results indicate all these strains have comparable infectivity, confirming previous studies[13] (Supplementary Fig. 1B).

Hyperimmune RTS,S/AS01 sera were obtained from C57Bl/6 female mice two weeks following immunization three times at three-week intervals. Mice received 5 µg of RTS,S formulated with 10-fold diluted original $AS01_E$ adjuvant dose, representing 2.5 µg of TLR4 ligand 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL), and 2.5 µg of the QS-21 saponin. Mice received 500 µl of anti-RTS, S/AS01 sera injected i.v., and negative controls received 500 µl of normal mouse sera. Positive control groups received 100 µg of epitope-specific mAb CIS43 for the junctional region[7], mAb L9 for the minor repeat region[8], mAb 317 for the central repeats[17], and mAb 369 for the C-terminal region[18]. Six hours after passive transfer of sera or mAbs, all mice were challenged with 2000 transgenic sporozoites injected i.v. and parasite liver burden was determined 42 h later by measuring luciferase activity using an IVIS imager as described previously[5,19]. 100% infection was measured in mice not receiving sera prior to challenge.

As expected, mice that received RTS,S immune sera and challenged with sporozoites expressing full-length PfCSP showed strong levels of liver burden reduction, 77.8% compared to the 100% infection control, but no reduction was observed in mice that received sera from non-immunized mice (Fig. 2A). No measurable inhibition of parasite infection was observed in mice receiving immune sera prior to challenge with tgPbPf-JR parasites, which was comparable to that of mice receiving sera from non-immunized mice (Fig. 2B). Positive control mice receiving mAb CIS43 specific for the junction region prior to challenge with tgPbPf-JR parasites showed a strong reduction in liver burden, (Fig. 2B). Similarly, mice receiving hyperimmune sera prior to challenge with sporozoites expressing the minor repeat region (tgPbPf-MR) experienced no reduction in parasite liver burden, whereas control mice receiving mAb L9, specific for minor repeats, prior to challenge with tgPbPf-MR had strongly reduced liver burden (Fig. 2C).

In contrast, mice receiving immune sera prior to challenge with sporozoites expressing $(NPNA)_{11}$ (tgPbPf-CR) demonstrated strongly reduced liver infection, to levels similar to those observed in mice challenged with sporozoites expressing full-length PfCSP. As expected, mAb 317 specific for central repeats strongly reduced the infectivity of both $(NPNA)_{11}$ and full-length CSP sporozoites. Finally, when mice receiving anti-RTS,S/AS01 sera were challenged with tgPbPf-Ct sporozoites expressing the Pf C-term flanking region, there was no detectable neutralizing activity by the vaccine-induced immune sera. These sporozoites were bound by mAb 369 and 1512, both specific for this region, by immunofluorescence[18,20] (Supplementary Figure 2). As shown in Fig. 2E, mAb 369 significantly reduced liver burden when transferred to mice before challenge with tgPbPf-Ct sporozoites. Similar results were obtained with mAb 1512 (not shown).

mAbs 369 and 1512, isolated from participants immunized with RTS,S, were shown to have high affinity towards the C-term[18–20]. The characterization of mAbs targeting the PfCSP C-term from both RTS,S-vaccinees and sporozoite immunization reveal dominance of the response towards the polymorphic face of the domain (α-epitope). Whether mAbs induced by mouse immunization with RTS,S/AS01 possess comparable binding kinetics or epitope specificities towards the C-term as those induced by

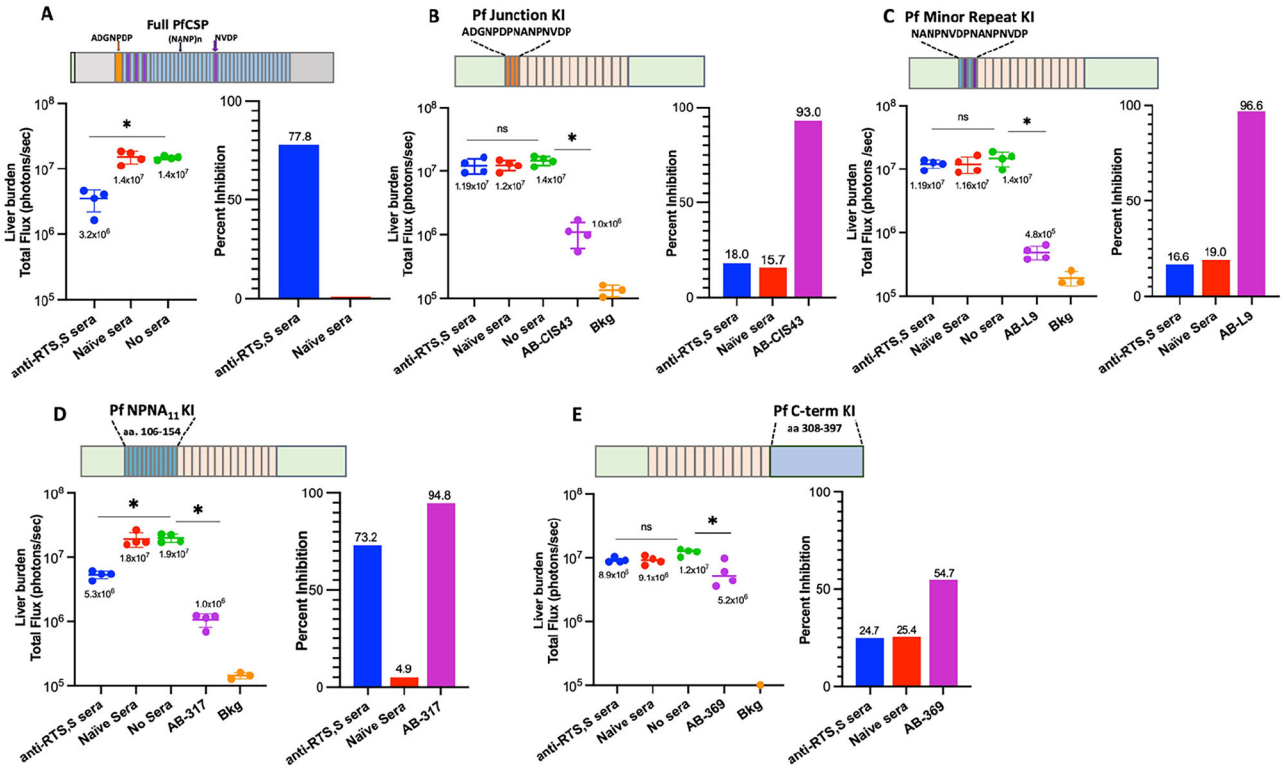

Fig. 2 | Liver burden reduction in mice passively immunized with polyclonal anti-RTS,S/AS01 sera and challenged with knock-in (KI) transgenic sporozoites expressing different epitopes or the full PfCSP. C57Bl/6 mice were passively immunized with anti-RTS,S/AS01 sera, naïve sera or the related positive control monoclonal antibodies, CIS43, L9, 317, 369 or 1512 and 6 h later challenged with 2000 transgenic KI parasites expressing the **A** full PfCSP, **B** PfJR, **C** PfMR, (**D**) PfNPNA11 or (**E**) PfC-term. Results expressed as bioluminescence (photons/sec) and percent reduction, which was calculated using the naïve group as 100% infection. For statistical analysis Mann-Whitney t-test was used. ns: not significant, $p > 0.05$; *$p = 0.02$. These figures show results of a representative experiment. Two independent experiments performed for each transgenic parasite.

human vaccinees is not yet clear. It remains to be determined whether these results are due to lower levels of protective C-term Ab responses in mice, inherent differences in the epitope specificities of the CSP C-term by the murine antibody repertoire, difference in kinetics of monoclonal and polyclonal sera, or because vaccine-induced antibodies recognize specific structural conformations different to those on the sporozoite surface[20]. The role of antibodies against the PfCSP C-terminal region in protection remains inconclusive as different studies disagree regarding their protective efficacy[21,22].

These results indicate anti-RTS,S/AS01 sera, while recognizing different regions of the CSP protein, appears to have highly specific epitope-restricted efficacy in mice driven by responses to the central repeat epitopes. The lack of protective activity of RTS,S-induced anti-central repeats (CR) antibodies against sporozoites expressing only the junctional region or minor repeats is consistent with our previous findings demonstrating that anti-CR mAbs that cross-react with other epitopes of the repeat domain are not necessarily cross-protective[13]. Although antibody levels towards the C-term and central repeat measured by ELISA appear to be similar, the polyclonal antibody response induced by RTS,S/AS01 immunization in mice did not protect against challenge with tgPbPf-Ct parasites.

In these murine models, immunization with RTS,S/AS01 induces protective antibodies with a restricted epitope specificity that exert a strong sporozoite neutralizing effect and confer sterile immunity[6]. Studies using human protective mAbs with minor and major repeat epitope specificities have shown that administration of a combination of antibodies can result in increased protection relative to the major repeat mAb alone[23]. This raises the possibility that vaccines capable of inducing antibody responses with broad protective specificity may exhibit increased efficacy. We have recently shown that plasma from RTS,S-immunized individuals inhibit the infectivity of tgPb-PfCSP sporozoites in mice[6]. Therefore, we expect this mouse model, together with the use of transgenic parasites expressing selected PfCSP antigenic domains, will help the design and evaluation of new vaccine candidates capable of inducing protective antibodies to relevant epitopes.

Methods

Ethics

All animal research reported in this work was approved by the Animal Care and Use Committee of Johns Hopkins University (Approved protocol: MO21H417)[5].

Anti-RTS, S sera and mAbs

Female 6-8-week-old C57Bl/6 mice were used for these studies. Female mice are used as they are more susceptible to infection[5]. Mice were immunized intramuscularly three times at three-week intervals with 5 µg of RTS,S adjuvanted with 10-fold diluted original $AS01_E$ adjuvant dose, representing 2.5 µg of the TLR4 ligand 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL), and 2.5 µg of the QS-21 saponin, as described[6]. Two weeks after the final immunization, mice were terminally bled, and the collected sera was stored at −20 °C. Monoclonal antibodies 317, CIS43, L9, 369, and 1512 used in these experiments were obtained from PATH.

Measuring parasite liver burden

The liver burden assays were performed as previously described in ref. 5. Briefly, for the challenge studies, 6-8-week-old C57Bl/6 mice ($n = 4$ group), were passively immunized i.v. with either mouse sera or mAbs. Six hours later, mice were challenged i.v. with 2000 *P. berghei* KI parasites obtained from salivary glands of parasite-infected *Anopheles stephensi* mosquitoes.

The *P. berghei* KI parasites expressed specific, restricted *P. falciparum* CSP regions consisting of only junctional, minor-repeat, major repeat, or C-term. Forty-two hours later, mice were injected i.p. with 100 µl of d-luciferin (30 mg/mL), anesthetized with isoflurane, and imaged in the IVIS Spectrum Imaging System (Perkin Elmer) to measure luciferase activity in the liver.

Quantitating antibody responses

Serum antibodies against the different peptides (junctional region, minor repeat, central repeats, $NANP_6$, and the C-term) were detected by ELISA (peptides were synthesized at Peptide 2.0 Inc.). ELISA plates (Thermo Fisher Nunc, Cat. 442404, Waltham, MA, USA) were coated with the different antigens at 1 µg/mL, 100 µL/well overnight. The amount of peptide bound to ELISA wells was comparable for all, as indicated by detection of histidine tag using anti-Histidine antibody (Miltenyi Biotec Germany, Cat. 130-092-783). Then, serum dilutions were incubated for 1 h, washed and incubated with the secondary antibody (anti-mouse IgG-HRP, 250 ng/mL Jackson Immunoresearch, Cat. 5220-0341). Finally, plates were developed with ABTS Peroxidase substrate (KPL, Cat. 5120-0032), and the reaction stopped with 1% SDS and read on a plate reader. The peptides used were junctional region epitope: ADGNPDPNANPNVDP, minor repeat epitope: $(NANPNVDP)_2$, major repeat ($NANP_6$), and the C-terminal sequence from Pf, from residues 311 to 373 (3D7 strain).

Generation of transgenic P. berghei knock-in (KI) parasites

P. berghei parasites (strain ANKA, 676m1c11, MRA-868) expressing GFP and luciferase were obtained from The Malaria Research and Reference Reagent Resource Center (MR4) at BEI resources. Pyrimethamine plasmids (pR-CSPFL) encoding the different versions of PbCSP, along with the PbCSP signal sequence, were generated from synthetic genes obtained from Bio Basic Inc. (Markham ON, Canada). Plasmid transfections were performed as previously described to generate different transgenic parasite lines. All plasmids include the hDHFR selection cassette and *csp* 5' and 3' UTRs. pR-CSPFL was excised with XhoI and KasI and transfected into schizont cultures of Pb parasites by electroporation using an Amaxa Nucleofactor (Durham, NC, USA)[16]. Transfected parasites were injected i.v. into Swiss Webster mice and selected with pyrimethamine administered in their drinking water (7 mg/mL). The pyrimethamine-resistant parasites were cloned by limiting dilution in mice, and isolated clones were verified by PCR and sequencing.

Immunofluorescence assay

Immunofluorescence assays were performed using air-dried sporozoites obtained from mosquito salivary glands dissected 21 days after feeding on an infectious blood meal. Briefly, a 5 ul sporozoite suspension (6×105 sporozoites/ml) was air dried at room temperature on poly-L-lysine-coated slides (Tekdon Inc., Myakka City, FL). Fluorescent labeled antibody samples [Alexa Fluor 488 F] were diluted in 1% (wt/vol) bovine serum albumin (BSA) (Sigma, St. Louis, MO) in PBS (1% BSA–PBS) were incubated in slide wells for 30 min at room temperature. Fluorescent sporozoites were visualized under an upright fluorescence microscope (Nikon Eclipse 90i).

Data Availability

The authors confirm the data supporting the findings of this study are available within the article and its supplementary materials.

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Author contributions

Y.F.-G., F.Z., and R.M.; Designed experimental procedures. Y.F.-G., B.S.-J., M.P., and S.M.-T. performed experiments. Y.F.-G., F.Z., R.M., E.L., R.M.G. and I.A.W. analyzed the results. Y.F.-G., F.Z., and R.M. wrote the manuscript. All authors reviewed and approved the present manuscript

Competing interests

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Additional information

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