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Antihomotypic affinity maturation improves human B cell responses against a repetitive epitope

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Affinity maturation selects B cells expressing somatically mutated antibody variants with improved antigen-binding properties to protect from invading pathogens. We determined the molecular mechanism underlying the clonal selection and affinity maturation of human B cells expressing protective antibodies against the circumsporozoite protein of the malaria parasite *Plasmodium falciparum* (PfCSP). We show in molecular detail that the repetitive nature of PICSP facilitates direct homotypic interactions between two PICSP repeat-bound monoclonal antibodies, thereby improving antigen affinity and B cell activation. These data provide a mechanistic explanation for the strong selection of somatic mutations that mediate homotypic antibody interactions after repeated parasite exposure in humans. Our findings demonstrate a different mode of antigen-mediated affinity maturation to improve antibody responses to PICSP and presumably other repetitive antigens.

porozoites of the human malaria parasite *Plasmodium falciparum* (Pf) express a surface protein, circumsporozoite protein (PICSP), with an immunodominant central NANP (Asn-Ala-Asn-Pro) repeat region (1–3). Antibodies against the repeat can mediate protection from *Plasmodium* infection in animal models (4–6). However, anti-Pf antibody-mediated protection is not readily achieved through vaccination. Thus, the induction of protective PICSP NANP antibodies is a major goal in pre-erythrocytic vaccine development (7). We recently showed that the anti-NANP PICSP memory B cell response in Pf-naïve volunteers after infection with live Pf sporozoites under chloroquine prophylaxis (PISPZ-CVac) matured predominantly through the clonal selection and expansion of potent Pf inhibitory *IGHV3-33*- and *IGKV1*-encoded germline antibodies with an 8-amino acid–long immunoglobulin (Ig) light chain κ complementarily-determining region 3 (CDR3) (this 8–amino acid CDR3 is hereafter designated KCDR3:8). (8, 9).

Affinity maturation selects B cells expressing somatically mutated antibody variants with improved antigen-binding properties to protect from invading pathogens. We determined the molecular mechanism underlying the clonal selection and affinity maturation of human B cells expressing protective antibodies against the circumsporozoite protein of the malaria parasite *Plasmodium falciparum* (PfCSP). We show in molecular detail that the repetitive nature of PICSP facilitates direct homotypic interactions between two PICSP repeat-bound monoclonal antibodies, thereby improving antigen affinity and B cell activation. These data provide a mechanistic explanation for the strong selection of somatic mutations that mediate homotypic antibody interactions after repeated parasite exposure in humans. Our findings demonstrate a different mode of antigen-mediated affinity maturation to improve antibody responses to PICSP and presumably other repetitive antigens.

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The majority of NANP-reactive V1210-33–V1210-15–KCDR3:8 B cells belonged to clonally expanded and somatic hypermutation (SHM)–diversified cell clusters with strong selection for replacement mutations in HCDR1 (HS1) and HCDR2 (H.V50 and H.N56), as well as KCDR3 (KCDR3 S93 (K.S93)) (Fig. 1, C and D), single-letter amino acid abbreviations are defined in the legend to Fig. 1).

In contrast, exchanges at H.N56 and K.S93, either alone (in antibodies 1210_H.K56_Nrev, 1210_K.N93_S93, and 2163_H.N56_K(S93)) or in combination (in 1210_NS and 2163_KN), showed no significant effect (Fig. 1, G and H, and table S3). Thus, affinity maturation to the repeat explained the strong selection for only two of the four characteristic replacement mutations in V1210-33–V1210-15–KCDR3:8 anti-NANP antibodies.

We next determined the coecrystal structure of the 1210 antigen-binding fragment (Fab) with NANP15 (Fig. 2, fig. S1A, and tables S4 to S6). The NANP core epitope contained a type I β turn and an elongated conformation (Fig. 2, A and C, and fig. S1B), similar to NANP bound to a chimeric 2140 Ig heavy chain–1210 Ig κ antibody and in line with previous observations (fig. SIC and tables S4 and S7) (10). Main-chain atoms in KCDR3 were optimally positioned to mediate H bonds with the repeat, likely contributing to the strong selection of KCDR3:8 (Fig. 2, B and C, and tables S5 and S10). Two highly selected mutations, H.N56_K and K.S93_N (Fig. 1, E and F), formed H bonds with H.Y52A and H.Y58 in HCDR2, mediated the major antigen contacts (table S5 and fig. S2) (15). Affinity maturation at H.V50 and H.S31 may be explained by strengthened van der Waals interactions with the repeat (Fig. 2C).

Notably, our crystal structure also revealed that two 1210 Fabs (designated 1210 Fab-A and Fab-B) bound to one NANP15 peptide in a head–to-head configuration at a 133° angle (Fig. 2D and fig. S3). This binding mode led to six homotypic antibody-antibody H bonds providing 263 Å2 of buried surface area (BSA) between the two Fabs and an additional ~120 Å2 of BSA between the Fabs and the repeat (Fig. 2, E and F, and tables S5, S6, and S10). Two highly selected mutations, H.N56_K and K.S93_N (Fig. 1, E and F), formed H bonds with H.Y52A and H.Y59 in the opposing Fab, thereby stabilizing the head-to-head configuration (Fig. 2, G and H). KCDR3:8 optimally contacted HCDR3 of the opposite 1210 molecule, providing another explanation for the length restriction in KCDR3.

To investigate homotypic interactions, we next measured the Fab affinities for NANP15 and NANP25 for 1210, 1210_NS (which lacks the selected mutations involved in homotypic binding), a 1210_H.D100_Ymut K.N56_Ymut (1210_YY, designed to disrupt head-to-head binding through steric clashes), and a 1210 germline antibody (1210_GL) (Fig. 2I and fig. S4). Compared with 1210, 1210_YY and 1210_NS showed significantly weakened affinity for NANP15 but not for NANP25, whereas 1210_GL was significantly worse than 1210 at binding both peptides (Fig. 2I and fig. S4) (16). These data suggest that only 1210 efficiently recognized the repeat in a high-affinity homotypic head-to-head binding configuration. An analysis of full-length PICSP with 38 NAP repeats confirmed this hypothesis. Approximately twelve 1210 Fabs bound PCSP and recognized the NANP repeat in a head-to-head binding configuration similar to the 1210 Fab–NANP15 crystal structure (Fig. 2, J and K, and fig. S3D) (11, 17).
Fig. 1. Affinity maturation of high-affinity human PfCSP NAP antibodies. (A) Surface plasmon resonance (SPR) affinity and SHM of selected (labeled) V_{3-3}–V_{1-5}–KCDR3.8 (green) and non-V_{3-3}–V_{1-5}–KCDR3.8 (gray) anti-PfCSP antibodies (9). (B to D) Original and mutated antibodies. (B) and (C) PICSP enzyme-linked immunosorbent assay reactivity. Data in (A), (B), and (C) are from one experiment representative of at least two independent experiments. OD, optical density; Ab, antibody. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (D) Pf liver cell traversal inhibition. Bars represent means from two to four independent experiments (symbols represent results from individual experiments). **P = 0.01 (significant) for two-tailed Student’s t test; ns, not significant. (E) Spr antibody usage compared with a baseline (base) model (22, 23). (G and H) Independent NAP_{3} SPR affinity measurements (dots) and means (gray lines). **P = 0.01 (significant) for Bonferroni multiple-comparisons test; ns, not significant. K_{d}, equilibrium dissociation constant.

Furthermore, 1210_Y.Y IgG, with its restricted ability to engage in homotypic antibody interactions, showed a lower binding avidity to full-length PfCSP than 1210 (fig. S5). Thus, affinity maturation selects for mutations that improve homotypic antibody interactions, thereby indirectly increasing PICSP NAP binding.

To better understand the selection of SHM at the cellular level, we measured the degree of B cell activation in response to NAP_{3} of transgenic B cell lines expressing 1210 or variant B cell receptors (BCRs) (Fig. 3, A to D). BCR signaling was delayed in cells expressing 1210_GL compared with that in cells expressing 1210. This effect was even more pronounced in 1210_Y.Y mutant cells. As expected, 1210_H.V50.1_{mut} (1210 with HCDR2 V30-I), with high repeat affinity, mediated stronger signals than 1210, especially with low antigen concentrations, whereas 1210_NS showed no significant differences (Fig. 3D). Thus, B cell activation is promoted by both direct NAP binding and homotypic antibody interactions. Despite a 2-log difference in NAP_{3} affinities (Fig. 1, G and H) and the varied potential of these antibodies to engage in homotypic interactions, all showed similar capacities to inhibit Pf sporozoite invasion (Fig. 3E and fig. S6). Likewise, all antibodies conferred similar levels of dose-dependent protection from the development of blood-stage parasites after passive immunization in mice, presumably because of strong avidity effects (Fig. 3F). These data provide a mechanistic explanation for the strong in vivo selection of antihomotypic antibody mutants by affinity maturation, independently of their protective efficacy as soluble antibodies.

Table 1. HCDR2 residues encoded by different IGHV3-33, IGHV3-30, IGHV3-30-3, and IGHV3-30-5 alleles. Gene and allele data are from www.imgt.org/igendb/.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele(s)</th>
<th>Residue at position</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGHV3-33</td>
<td>01, 02, 03, 04, 06</td>
<td>50 51 52 52A</td>
</tr>
<tr>
<td>IGHV3-33</td>
<td>05</td>
<td>50 51 52 52A</td>
</tr>
<tr>
<td>IGHV3-30</td>
<td>01, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19</td>
<td>50 51 52 52A</td>
</tr>
<tr>
<td>IGHV3-30-3</td>
<td>01, 02, 03</td>
<td>50 51 52 52A</td>
</tr>
<tr>
<td>IGHV3-30-5</td>
<td>01</td>
<td>50 51 52 52A</td>
</tr>
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V_{3} antibodies dominate the anti-PICSP memory response (9, 11, 14). In addition to V_{3-33}–V_{1-5}–KCDR3.8, we observed a cluster of highly mutated, affinity-matured V_{3-23}–V_{1-5} NAP-reactive memory B cell antibodies in our selection (Fig. 4, A and B) (9). Although the NAP_{3} binding mode of a representative V_{3-23}–V_{1-5} antibody, 1450, was different from that of 1210, it also recognized NAP_{3} in a head-to-head configuration, with HCDR3s in direct juxtaposition.
**Fig. 2. Affinity maturation drives homotypic repeat binding.** (A to H) 1210 Fab-NANP₅ cocrystal structure. (A) Superposition of the four NANP-bound Fab's. (B) Surface representation of the antigen-antibody interaction. (C) Details of core epitope recognition by 1210. Black dashes indicate H bonds. (D) Two 1210 Fab's in complex with NANP₅. (E) and (F) Surface representations of Fab-B (E) and Fab-A (F). Residues involved in homotypic interactions are dark gray. ([G] and [H]) Details of homotypic interactions. Affinity-matured residues are labeled in red. (I) Mean ± SEM Kᵢ, determined by isothermal titration calorimetry (ITC). Dots represent independent measurements. One-tailed Mann-Whitney test: *P < 0.05, **P < 0.01. (J) Results from size exclusion chromatography coupled with multiangle light scattering (SEC-MALS) for the 1210 Fab–PfCSP complex. The red line indicates mean ± SD molar mass from two measurements. RIU, refractive index units. (K) Two-dimensional class averages for the 1210 Fab–PfCSP complex. Red arrows indicate individual Fab's, and red lines indicate the binding angle observed in the crystal structure (D). NF54, Pf strain. Scale bar, 10 nm.

**Fig. 3. B cell activation and parasite inhibition.** (A to D) NANP₅-induced calcium signaling of 1210 and variants. ([A] and [B]) Reaction kinetics and percentages of activated cells (A) and overlay of median signal intensities (B) with 1 µg/ml NANP₅ for one of at least six representative experiments. Indo, calcium indicator. [C] and (D)] Percentages of activated cells and median activation time after the addition of 1 µg/ml (C) (n = 6 or 7 experiments) and 0.1 µg/ml (D) (n = 3 experiments) NANP₅. Symbols indicate results from independent experiments, and lines and error bars indicate mean ± SD. **P = 0.01 (significant) for Bonferroni multiple-comparisons test. (E) and (F) Parasite inhibition. (E) Mean ± SD median inhibitory concentration (IC₅₀) values from at least three independent experiments for 1210 and 2163 antibodies with indicated NANP₅ affinities. We detected no significant differences between IC₅₀ values because of extensively overlapping confidence intervals. (F) Percentages of parasite-free mice after passive immunization with 30 or 100 μg of 1210 or variants 24 hours before subcutaneous injection with *Plasmodium berghei* sporozoites expressing PfCSP (Pb-PfCSP). Data are from one (100 μg) or two (30 μg) independent experiments with five mice per group. We detected no significant differences in survival for 1210 variants (Mantel-Cox test).
and the affinity-matured KN30 residues forming an H bond between Fab-A and Fab-B (Fig. 4, C to E; fig. S7; and tables S4, S8, and S9). Sequence analysis of the VH3-23/Vκ1-5 antibody cluster confirmed enrichment for amino acid exchanges that participate directly in antibody-antigen interactions or antibody-antibody contacts or favor a 1460 paratope conformation optimal for NANP epitope recognition (Fig. 4B).

After the immunization of malaria-naïve individuals with PISPZ-CVac, ~15% of PfCSP-reactive epitope recognition (Fig. 4B).


data and analysis

**REFERENCES AND NOTES**

15. The importance of HY52A and HY5B for repeat reactivity was confirmed by alanine mutations in antibodies 1210, 2340, and 2219 (fig. S2).
16. All antibodies recognized NANP5, and NANP5, with binding stoichiometries of ~2–1, respectively, demonstrating that NANP5 but not the shorter NANP3 enables binding of two FabS.
Government of Saskatchewan, Western Economic Diversification Canada, the National Research Council Canada, and the CIHR. X-ray diffraction experiments were also performed at GM/CA@APS, which has been funded in whole or in part with federal funds from the National Cancer Institute (ACB-12002) and the National Institute of General Medical Sciences (AGM-12006). The Eiger 16M detector was funded by an NIH–Office of Research Infrastructure Programs High-End Instrumentation grant (1S10OD012289-01A1). This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science user facility operated for the DOE Office of Science by Argonne National Laboratory under contract DE-AC02-06CH11357.

Author contributions: K.I., S.W.S., H.J., E.A.L., J.-P.J., and H.W. designed experiments; P.G.K., B.K.L.S., S.L.H., and B.M. provided clinical samples; K.I., S.W.S., G.C., and G.P.M. performed experiments; A.B., G.T., R.M., and V.R. contributed to the experimental work; K.I., S.W.S., G.C., G.P.M., E.A.L., J.-P.J., and H.W. analyzed the data; K.I., S.W.S., J.-P.J., and H.W. wrote the manuscript; and J.-P.J. and H.W. conceived the study. Competing interests: B.K.L.S. and S.L.H. are salaried employees of Sanaria, the owner of the PISPZ-CVac and the sponsor of the clinical trial. B.K.L.S. and S.L.H. have financial interest in Sanaria. All other authors declare no conflicts of interest. Data and materials availability: Structural data are deposited under Protein Data Bank (PDB) IDs 6D01, 6D0X, and 6D11. All other data needed to evaluate the conclusions in this paper are present either in the main text or in the supplementary materials. Materials from the German Cancer Research Center and the Max Planck Institute for Infection Biology will be available upon reasonable request under material transfer agreements (MTAs). Sharing of the NF54 P. falciparum parasite is limited by an MTA with the Radboud University Medical Center; sharing of the P. berghei strain Pb-PfCSP is limited by an MTA with the Leiden University Medical Center.

SUPPLEMENTARY MATERIALS
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Materials and Methods
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Surface antibody maturation

Affinity maturation in B cells generates antibodies with increasingly enhanced antigen-binding properties. Imkeller et al. investigated the maturation of human B cells that express protective antibodies against the circumsporozoite protein of the malaria-causing parasite Plasmodium falciparum (PfCSP). The repetitive structure of PfCSP induces mutations in B cells, facilitating direct interactions between two repeat-bound antibodies against PfCSP, which enhance antigen affinity and B cell activation. Such interactions may optimize binding and promote clustering of surface antibodies in general.

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