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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 PfCSP facilitates direct homotypic interactions between two PfCSP 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 PfCSP and presumably other repetitive antigens.

Porozoites of the human malaria parasite *Plasmodium falciparum* (Pf) express a surface protein, circumsporozoite protein (PfCSP), with an immunodominant central NNP (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-PfCSP antibody-mediated protection is not readily achieved through vaccination. Thus, the induction of protective PfCSP NNP antibodies is a major goal in pre-erythrocytic vaccine development (7). We recently showed that the anti-PfCSP PfCSP memory B cell response in Pf-naïve volunteers after infection with live Pf sporozoites under chloroquine treatment (8) light chain k complementarily-determining region 3 (CDR3) (this 8-amino acid CDR3 is hereafter designated KCDR3-8) (8, 9).

We analyzed five representative germline or low-mutated antibodies with reported affinities for a NNP 5-mer peptide (NNP5) between 10-19 and 10-19 M (Fig. 1A and table S1) (9). Antigen binding was abrogated when the original 1Vx5-1 light chain was replaced by 1Vx2-28 or when the native Ig heavy chains were paired with a Vx5-1 light chain with 9-amino acid-long KCDR3 (Fig. 1B), demonstrating the importance of these specific Ig features in antigen recognition. All 1Vx3-33–Vx1-5–KCDR3-8 antibodies were encoded by the IGKV3-33*01 allele (9). IGKV3-33*01 differs from three otherwise highly similar gene segments (IGKV3-30, IGKV3-30-3, and IGKV3-30-5) at position 52 of heavy-chain CDR2 (HCDR2), which encodes strictly a tryptophan residue and not serine or arginine (Table 1 and table S2). HCDR2 W26–38 (Hx32-28) and H.W32 R mutants of the selected antibodies, as well as an H.W52 A mutant of antibody 2140 and a double mutant (H.V50_F.W52_R) to mimic the IGKV3-30-30*02 and IGKV3-30*02 alleles, all showed reduced PfCSP repeat reactivity associated with reduced in vitro parasite inhibitory activity (Fig. 1, C and D; single-letter amino acid abbreviations are defined in the legend to Fig. 1).

The majority of NNP-reactive 1Vx3-33–Vx1-5–KCDR3-8 B cells belonged to clonally expanded and somatic hypermutation (SHM)-diversified cell clusters with strong selection for replacement mutations in HCDR1 (HS31) and HCDR2 (H.V50 and H.N56), as well as KCDR3 (KCDR3 S83 (K.S93)) (9), likely as a result of affinity maturation (Fig. 1, E and F) (9). The introduction of missing somatic mutations (mut) or reversions (rev) at H.V50 and, to a lesser extent, H.S31 revealed a role in binding to a minimal NNP5 peptide (10, 11), as demonstrated for the germ-line antibody 2163 and the low-mutated antibody 1210 (Fig. 1, G and H, and table S3). In contrast, exchanges at H.N56 and K.S93, either alone (in antibodies 1210_H.Ka6_Nmut, 1210_K.N93_Smut, and 2163_H.N56_Kmut) 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 Vx3-33–Vx1-5–KCDR3-8 anti-NNP5 antibodies.

We next determined the co-crystal structure of the 1210 antibody-binding fragment (Fab) with NNP5 (Fig. 2, fig. S1A, and tables S4 to S6). The PfCSP core epitope contained a type I βturn and an elongated conformation (Fig. 2, A and C, and fig. S1B), similar to NNP5 bound to a chimeric 2140 Ig heavy chain–1210 Ig k antibody and in line with previous observations (fig. SIC and tables S4 and S7) (10–14). 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 S2, S5, and S10). Vx3-33 germline residues, notably H.V50 and H.W52 (the residue encoded only by IGKV3-33 alleles), as well as H.Y52A and H.Y58 in HCDR2, mediated the majority of 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 Fabss (designated 1210 Fab-A and Fab-B) bound to one NNP5 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.N66_K and K.S93_N (Fig. 1E and F), formed H bonds with H.Y52A and H.S99 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 NNP5 and NNP3 for 1210, 1210_NS (which lacks the selected mutations involved in homotypic binding), a 1210_H.D100_Y65*04_K.N93_S*04 (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 NNP5 but not for NNP3, 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 PCSP with 38 NNP repeats confirmed this hypothesis. Approximately twelve 1210 Fabss bound PCSP and recognized the NNP repeat in a head-to-head binding configuration similar to the 1210 Fab–NNP3 crystal structure (Fig. 2, J and K, and fig. S3D) (11, 17).

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Furthermore, 1210_YY IgG, with its restricted ability to engage in homotypic antibody interactions, showed a lower binding avidity compared to full-length PCP3 than 1210 (fig. S5). Thus, affinity maturation selects for mutations that improve homotypic antibody interactions, thereby indirectly increasing PCP3 NAMP binding.

To better understand the selection of SHM at the cellular level, we measured the degree of B cell activation in response to NAMP$_3$ of transgenic B cells expressing 1210 or variant B cell receptors (BCRs) (Fig. 3, A to D). BCR signaling was delayed in cells expressing 1210_GL compared to the parental cell line, 1210, which was delayed in cells expressing 1210. This effect was stronger in cells expressing 1210_GL compared to the parental cell line, 1210, which was delayed in cells expressing 1210. This effect was observed in two-tailed Student’s t test. **P = 0.01 (significant) for two-tailed Student’s t test. (E) Silent (gray) and replacement (red) SHM (bars) in V$_3$,3-33–Vx1–5 antibodies (n = 63). FWR, framework region; aa, amino acid. (F) Expected (obs) amino acid usage compared with a baseline (base) model (22, 23). (G and H) Independent NAMP$_3$ SPR affinity measurements (dots) and means (gray lines). **P = 0.01 (significant) for Bonferroni multiple-comparisons test; ns, not significant. $K_c$, equilibrium dissociation constant.

![Fig. 1. Affinity maturation of high-affinity human PCP3 NAMP antibodies.](image)

(A) Surface plasmon resonance (SPR) affinity and SHM of selected (labeled) V$_3$,3–33–Vx1–5–KCDR3:8 (green) and non–V$_3$,3–33–Vx1–5–KCDR3:8 (gray) anti-PCP3 antibodies (9). (B to D) Original and mutated antibodies. (B) and (C) PICP3 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. (E) Silent (gray) and replacement (red) SHM (bars) in V$_3$,3-33–Vx1–5 antibodies (n = 63). FWR, framework region; aa, amino acid. (F) Expected (obs) amino acid usage compared with a baseline (base) model (22, 23). (G and H) Independent NAMP$_3$ SPR affinity measurements (dots) and means (gray lines). **P = 0.01 (significant) for Bonferroni multiple-comparisons test; ns, not significant. $K_c$, equilibrium dissociation constant.

### Table 1. HCDR2 residues encoded by different IGHV3-33, IGHV3-30, IGHV3-30-3, and IGHV3-30-5 alleles

| Gene    | Allele(s) | Residue at position |
|---------|-----------|---------------------|
| IGHV3-33 | 01, 02, 03, 04, 06 | Y | I | W | Y |
| IGHV3-33 | 01, 03, 04, 05, 06, 07, 08, 09, 10 | V | I | S | Y |
| IGHV3-30 | 11, 12, 13, 14, 15, 16, 17, 18, 19 | V | I | S | Y |
| IGHV3-30-3 | 01, 02, 03 | V | I | S | Y |
| IGHV3-30-5 | 01 | V | I | S | Y |

(V$_3$,3 antibodies dominate the anti-PCP3 memory response (9, 11, 14). In addition to V$_3$,3–33–Vx1–5–KCDR3:8, we observed a cluster of highly mutated, affinity-matured V$_3$,3–23–Vx1–5 NAMP-reactive memory B cell antibodies in our selection (Fig. 4, A and B) (9). Although the NAMP$_3$ binding mode of a representative V$_3$,3–23–Vx1–5 antibody, 1450, was different from that of 1210, it also recognized NAMP$_3$ in a head-to-head configuration, with HCDR3s in direct juxtaposition.
**Fig. 2.** Affinity maturation drives homotypic repeat binding. (A to H) 1210 Fab-NANP<sub>5</sub> cocrystal structure. (A) Superposition of the four NANP-bound Fabs. (B) Surface representation of the antigen-antibody interaction. (C) Details of core epitope recognition by 1210. Black dashes indicate H bonds. (D) Two 1210 Fabs in complex with NANP<sub>5</sub>. (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 K<sub>D</sub> 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 multangle 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 Fabs, 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) NANP<sub>5</sub>-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 NANP<sub>5</sub> 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) NANP<sub>5</sub>. Symbols indicate results from independent experiments, and lines and error bars indicate means ± SD. **P < 0.01 (significant) for Bonferroni multiple-comparisons test. **(E and F) Parasite inhibition. (E) Mean ± SD median inhibitory concentration (IC<sub>50</sub>) values from at least three independent experiments for 1210 and 2163 antibodies with indicated NANP<sub>5</sub> affinities. We detected no significant differences between IC<sub>50</sub> 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 \( V_{H3-33-Vx1-5} \) antibody cluster confirmed enrichment for amino acid exchanges that participate directly in antibody-antigen interactions or antibody-antibody contacts or favor a 1450 paraotope conformation optimal for NANP epitope recognition (Fig. 4B).

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

1450 paratope conformation optimal for NANP affinity and competitive selection of PfCSP-reactive antibodies compared with the nonexpanded population (18).

Indeed, one donor in our study was confirmed enrichment for amino acid exchanges (9) and minimum of the distribution. *** \( p \) = 0.001 (significant) for two-tailed Student’s \( t \) test. (G) Frequency of \( V_{H3-33-Vx1-5-KCDR3:8} \) and \( V_{H3-33-Vx1-5} \) antibodies among clonally expanded versus singlet pooled PB and CSPmem (9).

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SUPPLEMENTARY MATERIALS
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