Naturally acquired blocking human monoclonal antibodies to *Plasmodium vivax* reticulocyte binding protein 2b

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*Plasmodium vivax* preferentially invades reticulocytes and recognition of these cells is mediated by *P. vivax* Reticulocyte Binding Protein 2b (PvRBP2b) binding to human Transferrin receptor 1 (TfR1) and Transferrin (Tf). Longitudinal cohort studies in Papua New Guinea, Thailand and Brazil show that PvRBP2b antibodies are correlated with protection against *P. vivax* infection and disease. Here, we isolate and characterize anti-PvRBP2b human monoclonal antibodies from two individuals in Cambodia with natural *P. vivax* infection. These antibodies bind with high affinities and map to different regions of PvRBP2b. Several human antibodies block PvRBP2b binding to reticulocytes and inhibit complex formation with human TfR1-Tf. We describe different structural mechanisms for functional inhibition, including either steric hindrance with TfR1-Tf or the reticulocyte membrane. These results show that naturally acquired human antibodies against PvRBP2b can inhibit its function which is important for *P. vivax* invasion.
Plasmodium vivax is the most widespread of human malarials causing serious morbidity, occasional mortality, and recurrent relapses even after a single exposure. P. vivax preferentially invades young red blood cells called reticulocytes. The parasite adhesin P. vivax Reticulocyte Binding Protein 2b (PvRBP2b) binds to Transferrin Receptor 1 (TIR1) and Transferrin (Tf) on the surface of reticulocytes, which is critical for red cell invasion. PvRBP2b is a member of the P. vivax Reticulocyte Binding Protein (PvRBP) family of parasite adhesins, which has 10 other members. PvRBP2b is a 326 kDa protein with a signal peptide at the N terminus and putative transmembrane domain at the C terminus. TIR1 mediates the uptake of iron into cells in complex with its human ligand, iron-loaded Tf. On the surface of reticulocytes, TIR1 is abundantly expressed and is shed during reticulocyte maturation into normocytes. TIR1 is critical for P. vivax invasion, as cells expressing a mutant form of TIR1 are completely refractory to P. vivax invasion. Mouse monoclonal antibodies (mAbs) against PvRBP2b inhibit PvRBP2b binding to reticulocytes, block complex formation with TIR1–Tf and inhibit P. vivax invasion by ~50% in Brazilian and Thai clinical isolates. TIR1 is also the entry receptor for several human pathogens including New World hemorrhagic fever arenaviruses and hepatitis C virus.

The PvRBP2b–TfR1–Tf ternary complex has been determined by cryo-electron microscopy (cryo-EM) to 3.7 Å. A TIR1 homodimer (residues 120–760) binds two molecules of Tf (residues 1–679) and two molecules of PvRBP2b (residues 168–633). PvRBP2b interacts with TIR1 through its C-terminal domain (residues 461–633) with a buried surface area of ~1271 Å², and with Tf through its N-terminal domain (residues 168–460) with a buried surface area of ~386 Å². Structure-function analyses of PvRBP2b show that none of the critical residues required for TIR1 interaction overlap with the 11 field polymorphisms identified within the TIR1–Tf binding site of PvRBP2b (residues 168–633). The inhibitory action of PvRBP2b-mouse mAbs has been elucidated using X-ray crystal structures of 3E9, 4F7, and 6H1 binding to PvRBP2b and a small-angle X-ray scattering (SAXS) derived model of 10B12 binding to PvRBP2b. None of these PvRBP2b-mouse mAbs directly bind at the TIR1–Tf interaction sites. Instead, 3E9 causes steric hindrance with TIR1 binding, and 4F7, 6H1, and 10B12 binding result in steric hindrance with the reticulocyte membrane.

Immuno-epidemiological studies in P. vivax endemic areas of Papua New Guinea, Thailand, and Brazil show a strong association between individuals with high levels of antibodies to PvRBP2b and reduced risk of P. vivax infection and disease. However, it is not known if these antibodies are able to inhibit PvRBP2b function. In this study, we isolate and characterize human mAbs against PvRBP2b from peripheral blood mononuclear cells obtained from Cambodians with naturally acquired immunity to P. vivax and high levels of PvRBP2b antibodies, to determine their role in inhibiting either reticulocyte binding or PvRBP2b interaction with recombinant TIR1–Tf. Understanding the human antibody repertoire to PvRBP2b will aid in the discovery of novel inhibitory epitopes with implications for vaccine design and novel therapies such as passive immunization with functional human mAbs.

**Results**

**Isolation and characterization of naturally acquired human mAbs to PvRBP2b.** To generate PvRBP2b human mAbs, we isolated memory B cells from two Cambodian adults whose serum inhibited PvRBP2b binding to reticulocytes, suggesting the presence of functional blocking antibodies (Supplementary Fig. S1). Using a recombinant fragment of PvRBP2b (PvRBP2b<sub>161–1454</sub>) to form fluorescently labeled tetramers, we isolated single PvRBP2b-reactive memory B cells, which were defined as CD19<sup>+</sup>CD20<sup>+</sup>IgG<sup>+</sup>. Subsequent IgG gene amplification and sequencing revealed several distinct clonal groups, with some clonotypes being more abundant than others indicating clonal expansion (Fig. 1a). The majority of B cells were IgG1, and two were IgG3 (Supplementary Data 1). One or two clones were selected for expression from several of the largest clonal groups from both individuals. Cognate antibody heavy-chain and light-chain pairs were cloned into a human IgG1 backbone and expressed as full-length antibodies (Supplementary Data 1). The clonal lineages of chosen human mAbs show varying degrees of somatic hypermutations (SHM) ranging from an average of 17–35 mutations (Fig. 1b). All PvRBP2b human mAbs showed reactivity against PvRBP2b<sub>161–1454</sub> using an enzyme-linked immunosorbent assay (ELISA) (Fig. 1c). The human mAb against tetanus toxoid C fragment (TTCF), 043038, was used as an isotype control and showed negligible detection (Fig. 1c). The human mAbs did not recognize five other PvRBP recombinant proteins, PvRBP1a, PvRBP1b, PvRBP2a, PvRBP2c, and PvRBP2p2, and recombinant protein of the homologous P. falciparum Reticulocyte binding-like homolog (PRb) family member, PRb4, demonstrating their specificity for PvRBP2b (Supplementary Fig. 1). TTGF human mAb 043038 and a human mAb against P. vivax Duffy Binding Protein (PvDBP), 099100, were used to determine background signal levels. All PvRBP2b human antibodies bound to PvRBP2b with >20-fold higher signal compared to 099100 and 043038.

We determined antibody binding affinities to PvRBP2b<sub>161–1454</sub> to be in the low nanomolar to low picomolar range as measured by biolayer interferometry (Fig. 1d and Supplementary Fig. S2). Antibody avidity was measured by ELISA using increasing concentrations of ammonium thiocyanate to determine the concentration at which antibodies would dissociate from PvRBP2b (Fig. 1e). Antibody binding in the absence of ammonium thiocyanate was assigned 100% binding. The highest avidity antibodies showed >75% binding at 2 M ammonium thiocyanate and included 237235, 243244, 281282, 335338, 340341, and 346343 (Fig. 1e(i)). The antibodies with moderate avidity showed >75% binding at 1 M ammonium thiocyanate, but <75% binding at 2 M ammonium thiocyanate and these were 239229, 241242, 250233, 253245, 254255, 260261, 267268, and 326327 (Fig. 1e(ii)). Antibodies with the lowest avidity showed <75% binding at 1 M ammonium thiocyanate, but >75% binding at 2 M ammonium thiocyanate and these were 251249, 252248, 256257, 258259, 262231, 273264, 277278, 279280, and 283284 (Fig. 1e(iii)). The avidity of previously published PvRBP2b inhibitory mouse mAbs 3E9, 6H1, 10B12, and non-inhibitory mouse mAb 8G7 were also measured (Fig. 1e(iv)). 3E9, 6H1, and 10B12 were in the lowest avidity group with <75% binding at 1 M ammonium thiocyanate, while 8G7 showed moderate avidity with <75% binding at 1 M ammonium thiocyanate and <75% binding at 1 M ammonium thiocyanate (Fig. 1e(iv)). All high avidity antibodies showed picomolar affinities except 237235, which showed low nanomolar affinity. There was no correlation in the moderate and low avidity antibodies to the avidity measurements.

Naturally acquired PvRBP2b human mAbs inhibit PvRBP2b binding to reticulocytes and block complex formation with TIR1–Tf. To determine whether PvRBP2b antibodies block PvRBP2b<sub>161–1454</sub> binding to TIR1 on the surface of reticulocytes, we used a well-established flow cytometry-based reticulocyte-binding assay. Antibody inhibition was expressed relative to PvRBP2b binding in the absence of antibody, which was assigned to 100% binding. We show that seven PvRBP2b antibodies, 239229,
Fig. 1 Clonal group analyses and characterization of PvRBP2b human mAbs. a Pie charts representing the repertoire breadth of PvRBP2b161-1454 specific single memory B cell-derived heavy-chain sequences from two Cambodian individuals. The number of variable heavy-chain gene sequences analyzed is indicated in the middle of the pie chart. The colored segments indicate the clonal groups in which at least one human mAb was expressed and their VH genes have been labeled. VH genes for clonal groups that are colored are also shown in Supplementary Data 1. b The frequency of somatic hypermutations (SHM) in IgG heavy-chain variable genes (IgGHV) was determined for the clonal groups of expressed human mAbs from \( n = 2 \) individuals. Human mAbs highlighted with a line underneath are from the same clonal group. Each circle represents a clonal member and the line represents the median number of SHM for each clonal group. c Reactivity against PvRBP2b161-1454 using a dilution series of human mAbs (black solid lines). 043038 was used as isotype control (dashed line). Data are presented as mean values of two independent experiments. d Iso-affinity plot showing the range of dissociation rate constants (\( k_d \)) and association rate constants (\( k_a \)) indicated on the top and right sides of the plot. Numeric values for affinity measurements are shown in Supplementary Fig. S2 for each human mAb. e Avidity measurement of PvRBP2b human mAbs using increasing concentrations of ammonium thiocyanate (NH\(_4\)SCN) by ELISA. Human mAb binding was expressed relative to human mAb binding in the absence of NH\(_4\)SCN. (i) Strong binding antibodies, (ii) moderate binding, (iii) low binding, (iv) avidity of murine mAbs. Data are presented as mean values of two independent experiments. Source data are provided as a Source Data file.
resonance energy transfer (FRET)-based assay. FRET signal in the absence of antibody was designated as 100% FRET signal. PvDBP human mAb 099100 and TTCF human mAb 043038 isotype controls increased the FRET signal by 125% and 156%, respectively, which was also observed for several PvRBP2b human mAbs, and these ranges were considered non-inhibitory (Fig. 2b). As a positive control for inhibition, we utilized PvRBP2b-mouse mAb 3E9 which has been previously shown to inhibit the complex formation and abolish the FRET signal (Fig. 2b). We observed that PvRBP2b antibodies 239229, 241242, 253245, 258259, 260261, 262231, 273264, and 346343, blocked PvRBP2b–TfR1–Tf complex formation as indicated by a >70% decrease in FRET signal (Fig. 2b).

**Fig. 2** *PvRBP2b* human mAbs inhibit *PvRBP2b* binding to TfR1–Tf. **a** *PvRBP2b* binding to reticulocytes in the presence of human mAbs was analyzed by flow cytometry. *PvRBP2b* binding in the absence of mAbs was assigned to 100%. 043038 and 099100 were used as antibody isotype controls. 3E9 is an inhibitory *PvRBP2b*-mouse mAb. *n* = 8 independent experiments and data are presented as mean ± SEM; circles represent independent experiments. Prism (version 8.4.3) was used to perform one-way ANOVA followed by Dunnett’s multiple comparisons test using 043038 as a control to determine *P*-values. **P** ≤ 0.001. **b** The ability of *PvRBP2b* human mAbs to inhibit *PvRBP2b*–TfR1–Tf complex formation was analyzed in the FRET-based assay. The FRET intensity in the absence of mAbs was assigned to 100%. *n* = 5 independent experiments and data are presented as mean ± SEM; circles represent independent experiments. Prism (version 8.4.3) was used to perform one-way ANOVA followed by Dunnett’s multiple comparisons test using 099100 as a control to determine *P*-values. **P** ≤ 0.0001. **c** IP of *PvRBP2b* human antibodies in the presence of TfR1 and Tf were examined by reducing SDS-PAGE. *n* = 1 independent experiment. 2b, *PvRBP2b*; Hc, mAb heavy chain; Lc, mAb light chain; l input; U, unbound; E, eluate; M, molecular weight marker with molecular weights labeled on the left side of the gels in kDa. **d** Summary table of *PvRBP2b* human mAb phenotypes in the reticulocyte-binding, FRET, and IP assays. The different phenotypes indicated in orange, yellow, green, and pink for each assay have been shown in the legend. Source data are provided as a Source Data file.
competition. Inhibition of PvRBP2b161 binding to reticulocytes by 42% and 54% (Fig. 2a). 279280 increased the FRET signal by 274%, and likewise also enhanced PvRBP2b161-1454 binding to reticulocytes (Figs. 2a and 2b). All other PvRBP2b antibodies, 237235, 243244, 250233, 251249, 252248, 255255, 256257, 258259, 260261, 262231, 273264, 277278, 281282, 283284, and 363267, showed no or <10% decrease in FRET signal (Fig. 2b). However, of the aforementioned human mAbs, 326327 has previously been shown to inhibit reticulocyte binding by 86% (Fig. 2a).

We performed co-immunoprecipitation (IP) assays using PvRBP2b human mAbs with PvRBP2b161-1454, TfR1, and Tf to observe if the human mAbs could block complex formation. All PvRBP2b antibodies were able to immuno-precipitate recombinant PvRBP2b161-1454 (Fig. 2c). In the presence of PvRBP2b human mAbs 239229, 241242, 251249, 253245, 256257, 258259, 260261, 262231, 273264, 281282, 363267, 333338, 340341, and 346343, the TfR1–Tf bands were absent from the eluate lanes, suggesting that these antibodies blocked TfR1–Tf binding to PvRBP2b161-1454 (Fig. 2c). In contrast, 237235, 234244, 250233, 252248, 254255, 267268, 277278, 279280, and 283284 immuno-precipitated PvRBP2b161-1454 in complex with TfR1 and Tf, and were therefore non-inhibitory in this assay (Fig. 2c).

Collectively, we have identified eight PvRBP2b human mAbs, 239229, 241242, 253245, 258259, 260261, 262231, 273264, and 346343, that inhibited the binding of PvRBP2b to reticulocytes and blocked complex formation in both the FRET and IP assays (Fig. 2d).

Mapping the binding sites of PvRBP2b human mAbs using PvRBP2b fragments. We utilized a panel of PvRBP2b recombinant fragments to determine the region bound by PvRBP2b human mAbs. Our results show that PvRBP2b human mAbs bind to three different regions. The majority of PvRBP2b human mAbs, 237235, 241242, 243244, 251249, 252248, 254255, 256257, 258259, 260261, 273264, 277278, 281282, 283284, 326327, and 346343, bound epitopes within PvRBP2b161-470 (Fig. 3a and Supplementary Fig. S4). 239229, 262231, 279280, 333338, and 340341 bound epitopes between residues 470–652, and 250233 and 267268 bound epitopes between residues 969–1454 (Fig. 3a). As expected, none of the antibodies showed reactivity to the negative control, PrRh4a161-766 (Supplementary Fig. S4). TTCF human mAb 043038 showed no reactivity against any of the PvRBP2b recombinant fragments or PrRh4a161-766 (Supplementary Fig. S4).

To evaluate whether PvRBP2b human mAbs recognized similar epitopes within PvRBP2b, antibody competition experiments were performed using ELISA plates coated with each human mAb. To identify blocking activity, biotinylated PvRBP2b161-1454 was pre-incubated with individual PvRBP2b human mAbs in solution prior to incubation on the antibody-coated ELISA plates. No competition, partial competition, and full competition were characterized as 0–29%, 30–59%, and 60–100% inhibition of biotinylated PvRBP2b161-1454 binding relative to PvRBP2b161-1454 binding alone, respectively. As expected, all PvRBP2b human mAbs competed with themselves (Fig. 3b). Some PvRBP2b human mAbs showed non-reciprocal responses depending on which antibody was coated and which was in solution, but they were nonetheless considered competing antibodies (Fig. 3b). 241242, 273264, 258259, and 253245 competed with each other and were inhibitory in the reticulocyte-binding, FRET, and IP assays (Figs. 2d and 3b). 260261 and 346343 were also inhibitory across all functional assays and while 260261 competed with all the aforementioned antibodies, 346343

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**Fig. 3 Epitope mapping of PvRBP2b human mAbs.** a) (Left) Surface representation of PvRBP2b from the cryo-EM ternary complex structure (PDB accession code 6D04). PvRBP2b residues S168–E460 and L461–I633 are demarcated to show the domains that mainly interact with Tf (cyan) and TfR1 (green), respectively. (Right) Schematic representation of PvRBP2b from the cryo-EM ternary complex structure (PDB accession code 6D04). PvRBP2b residues S168–E460 and L461–I633 are demarcated to show the domains that mainly interact with Tf (cyan) and TfR1 (green), respectively. (Right) Schematic representation of PvRBP2b human mAbs binding in the presence of antibody in solution was calculated relative to PvRBP2b161-1454 binding in the absence of antibody in solution. A blue to white gradient shows antibodies with the highest levels of competition in dark blue and the lowest in white. Competition (>60% inhibition of PvRBP2b161-1454 binding), partial competition (between 30 and 59%), no competition (<29%). b) Competition ELISA using immobilized PvRBP2b–mouse mAbs incubated with a mixture of human mAbs and PvRBP2b161-1454 at a 20:1 molar ratio. Blue boxes indicate competition.

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**Table 1** Summary of competition ELISA experiments using recombinant protein fragments. Blue boxes represent signals (cyan) and TfR1 (green), respectively. (Bottom) Mapping of PvRBP2b human mAbs binding in the presence of antibody in solution was calculated relative to PvRBP2b161-1454 binding in the absence of antibody in solution. A blue to white gradient shows antibodies with the highest levels of competition in dark blue and the lowest in white. Competition (>60% inhibition of PvRBP2b161-1454 binding), partial competition (between 30 and 59%), no competition (<29%). c) Competition ELISA using immobilized PvRBP2b–mouse mAbs incubated with a mixture of human mAbs and PvRBP2b161-1454 at a 20:1 molar ratio. Blue boxes indicate competition.
competed with 258259 and 253245, but not 241242 and 273264 (Figs. 2d and 3b). Both 260261 and 346343 also competed with 326327, 256257, 251249, 252248, and 281282, which all competed with each other (Fig. 3b). Antibodies 237235, 243244, 254255, and 277278 competed with each other, and all show weaker inhibition (~20–80%) in the reticulocyte-binding assay (except 277278 that showed no inhibition), and none were inhibitory in either the FRET or IP assays (Figs. 2d and 3b). 277278 also competed with 283284, which did not compete with any other antibody and was also not inhibitory in any of the interaction assays (Figs. 2d and 3b). 239229, 262231, 335338, and 340341 that bound between residues 470–652 competed with each other (Fig. 3b). They were inhibitory across all three interaction assays, but 335338 and 340341 were only 20–80% inhibitory in the reticulocyte-binding assay (Fig. 2d). 279280 competed with 239229 and 262231, and 279280 enhanced PvRBP2b 161–1454 binding to reticulocytes and increased the signal intensity in the FRET-based assays (Figs. 2d and 3b). 250233 and 267268 that bound between residues 969–1454 were not inhibitory in any assays and did not compete with each other (Figs. 2d and 3b). In general, the competition ELISA results are consistent with the domain mapping experiments, and human mAbs that compete

Fig. 4 HDX-MS analysis of PvRBP2b regions affected upon PvRBP2b human mAb binding. a Table showing regions of PvRBP2b that are protected from deuteration upon PvRBP2b human mAb binding. Antibodies that show protection in similar regions are grouped by colors. Colored boxes indicate that the antibody shows protection in the region specified in the top row. Black columns indicate regions where no or few peptides could be detected by mass spectrometry. The bottom-most row shows regions that also form part of the TfR1-Tf binding sites. b(i)–(xi) Mapping of PvRBP2b protected regions on X-ray crystal structures. The colored regions correspond to the table in a for different groups of antibodies. The protected regions for human mAbs 250233 and 267268 could not be shown structurally as the cryo-EM structure only extends to 633 residues. Black indicates regions that could not be analyzed by HDX-MS.

competed with 258259 and 253245, but not 241242 and 273264 (Figs. 2d and 3b). Both 260261 and 346343 also competed with 326327, 256257, 251249, 252248, and 281282, which all competed with each other (Fig. 3b). Antibodies 237235, 243244, 254255, and 277278 competed with each other, and all show weaker inhibition (~20–80%) in the reticulocyte-binding assay (except 277278 that showed no inhibition), and none were inhibitory in either the FRET or IP assays (Figs. 2d and 3b). 277278 also competed with 283284, which did not compete with any other antibody and was also not inhibitory in any of the interaction assays (Figs. 2d and 3b). 239229, 262231, 335338, and 340341 that bound between residues 470–652 competed with each other (Fig. 3b). They were inhibitory across all three interaction assays, but 335338 and 340341 were only 20–80% inhibitory in the reticulocyte-binding assay (Fig. 2d). 279280 competed with 239229 and 262231, and 279280 enhanced PvRBP2b 161–1454 binding to reticulocytes and increased the signal intensity in the FRET-based assays (Figs. 2d and 3b). 250233 and 267268 that bound between residues 969–1454 were not inhibitory in any assays and did not compete with each other (Figs. 2d and 3b). In general, the competition ELISA results are consistent with the domain mapping experiments, and human mAbs that compete
with each other share similar phenotypes as observed in the functional assays.

We wanted to determine whether PvRBP2b human mAbs bound similar epitopes compared to PvRBP2b-mouse mAbs. Mouse mAbs 3E9, 6H1, and 10B12 bind PvRBP2b169-470, block PvRBP2b161-1454 binding to reticulocytes and inhibit ex vivo P. vivax invasion by ~50%. 8G7 is a PvRBP2b-mouse mAb that binds residues 813-1454 and has no effect on P. vivax invasion. PvRBP2b human mAbs 241242, 273264, 258259, 253245, 260261, 277278, and 283284 competed with 3E9 (Fig. 3c and Supplementary Fig. S5). 260261, 283284, 346343, 326327, and 256257 competed with 10B12, whereas 251249 and 281282 competed with 6H1 (Fig. 3c). None of the 23 PvRBP2b human mAbs competed with 8G7 (Fig. 3c).
Hydrogen–deuterium exchange epitope mapping. To map antibody epitopes for PvRBP2b human mAbs in a near-native environment, we used hydrogen–deuterium exchange coupled to mass spectrometry (HDX-MS). The method identifies interaction sites by comparing protein dynamics in the absence and presence of a ligand. We determined HDX rates for PvRBP2b, a representative for all three antibodies, and in complex with each human mAb. To identify the best HDX conditions that would allow us to screen all antibodies, we first tested different incubation times for PvRBP2b, both at 0 °C and at room temperature (22 °C) (Supplementary Data 2). These experiments showed that incubation times of 5 min at either 0 or 22 °C were the most appropriate as they showed intermediate levels of deuterium incorporation for a majority of peptides, both for the fast-changing and slow-changing regions of the protein. PvRBP2b was very stable in all tested labeling conditions and we obtained above 85% protein coverage, with limited information for regions 234–322 and 589–686 (Supplementary Fig. S6). Identification of PvRBP2b epitopes for mouse antibodies 3E9, 6H1, and 10B12 highly correlate with the previously published X-ray and SAXS structures, validating our approach to identify human antibody epitopes (Supplementary Fig. S7). Some antibodies can be grouped as they show a similar pattern of HDX changes on RBP2b (Fig. 4a and Supplementary Fig. S7).

A group of four human antibodies, 241242, 273264, 258259, and 253245, all show changes in the 344-373 region, which is located in the N-terminal domain (residues 166–460) next to the junction joining the N-terminal domain to the C-terminal domain (residues 461–633) (Fig. 4b(i) and (v)). Interestingly, antibodies 241242 and 273264 show additional changes in the C-terminally located regions 462–472 and 556–588 (Fig. 4b(i)). Antibodies 260261, 346343, 326327, and 256256 show HDX changes in a loop at the extremity of PvRBP2b, peptide region 405–423, an epitope that overlaps with that of the PbRBP2b inhibitory mouse mAb 10B12 (Fig. 4b(ii) and (vii)). Additionally, antibodies 326327 and 256256 also show changes in a second loop on the same extremity of PbRBP2b, residues 212–232 (Fig. 4b(vii)). The third loop that is sandwiched between the two loops showing HDX changes is unfortunately not amenable to analysis by HDX-MS due to the lack of high-quality peptides that cover this region (Supplementary Fig. S6). Antibodies 251249 and 252248 show HDX changes in a loop that extends from the TF binding interface of PbRBP2b, encompassing residues 178–186 (Fig. 4b(viii)). Antibody 281282 likely binds very similarly to 251249 and 252248 as all three antibodies have the same profile in the competition assay (Fig. 3b), but no HDX changes were observed in the presence of antibody 281282 (Fig. 4a). This may be because the chosen HDX incubation time and temperatures do not permit observable changes in the region where 281282 is binding, or because the antibody is contacting a region that is not covered in the HDX-MS analysis. Antibodies 237235, 243244, and 254256 show multiple regions with HDX changes: PbRBP2b regions 166–177, 186–199, 212–232, 332–343, and 440–450, suggesting that the binding of these antibodies affects multiple sites (Fig. 4b(ix)). The epitope of antibody 277278 partially overlaps with the first three antibodies, with regions 332–343 and 440–450 showing the most significant changes (Fig. 4b(x)). The epitope of antibody 283284 also partially overlaps with 237235, 243244, 254255, and 277278 in the region 332–343 and additionally protects region 202–211 (Fig. 4b(xi)). Of the antibodies that bind to the C-terminal domain, antibodies 239229, 262231, 335338, and 340341 protect regions 494–506 and 521–532, with antibodies 239229 and 262231 showing additional protection of the 545–555 region (Fig. 4b(ii) and (iii)). The latter region is part of the interface that binds TR1. 279280 also shows changes in the C-terminal domain and has a dual-site epitope encompassing residues 477–490 and 539–555 (Fig. 4b(iv)). Antibodies 267268 and 250233 show HDX changes in regions distant from both the TF and TR1 interaction sites (Fig. 4a). None of the antibodies showed increased PbRBP2b, mouse mAbs structures obtained either by X-ray crystallography or SAXS.

Structural characterization of PbRBP2b human mAbs. To further elucidate how human mAbs recognized PbRBP2b, we structurally characterized several antibody Fab fragments in complex with PbRBP2b. We were able to determine eight crystal structures of PbRBP2b bound to antibody Fabs with resolutions ranging from 2.45 to 3.45 Å (Fig. 5a). Superimposing the crystal structures of PbRBP2b, human mAbs structures obtained by X-ray crystallography or PbRBP2b.
PvRBP2b-237264 onto the PvRBP2b-TfR1–Tf cryo-EM ternary complex reveals that these antibodies cause steric hindrance with TfR1–Tf (Fig. 5b). The ability of these antibodies to directly interfere with TfR1–Tf binding is consistent with these antibodies showing inhibition in all interaction assays (Fig. 2d).

251249 contacts PvRBP2b169–470 at helices α3 and α7 and the N-terminal loop between residues D173–N174 and Q191–P194 (Fig. 5a). It binds just adjacent to the Tf binding site. PvRBP2b-251249 when superimposed onto the PvRBP2b-TfR1–Tf complex shows steric occlusion with Tf and its heavy-chain CDR3 loop contacts the region where Tf binds PvRBP2b (Fig. 5b). 251249 is 59% inhibitory in the reticulocyte-binding assay and inhibitory in the IP assay, but not in the FRET assay and appears to act through interference with Tf binding (Fig. 2d).

237235 and 243244 contacts PvRBP2b169–470 helices α2 and α7 and part of the N-terminal loop between residues D173–N174 and Q191–P194 (Fig. 5a). 237235 and 243244 belong to the same clonal group and they bind similarly to their target epitopes with heavy and light-chain CDR loops adopting similar conformations when bound to PvRBP2b (Supplementary Fig. S8). When superimposed onto the PvRBP2b-TfR1–Tf complex, PvRBP2b-237235, and PvRBP2b-243244 show no overlap with TfR1–Tf binding, but they do bind directly opposite the Tf binding site (Fig. 5b). While these antibodies are not inhibitory in the FRET and IP assays, they inhibit PvRBP2b binding to reticulocytes by 47% and 43%, which suggests these antibodies may affect Tf binding from the opposite face of the Tf binding site (Fig. 2d). Finally, 283284 forms interactions with helices α1 and α4 of PvRBP2b169–470. When superimposed onto the PvRBP2b-TfR1–Tf complex, 283284 shows no interference with TfR1–Tf binding (Fig. 5b). This is consistent with 283284 showing no inhibition in any of the interaction assays (Fig. 2d).

We also superimposed the crystal structures of PvRBP2b human mAbs with inhibitory mouse mAbs 3E9 and 6H1 and the SAXS model of 10B12 to determine if there was any overlap. 3E9 shows steric hindrance with 241242, 253245, 258259, 273264, and 283284, while 6H1 shows steric hindrance with 251249 (Fig. 5c). Interestingly, 3E9 and 10B12 shows steric hindrance with 283284 (Fig. 5c). These superimposed structures are consistent with the competition ELISA results between the human and mouse mAbs (Fig. 3c).

The antibody epitopes revealed by X-ray crystal structures corroborate regions that show HDX-MS changes (Fig. 6). HDX-MS also reveals changes in PvRBP2b regions that do not directly form antibody interactions as shown in the crystal structures. In particular, 237235 and 243244 that interact with N-terminal loop regions D173–N174 and Q191–P194 also show HDX-MS changes in the loop between Y186 and Q191, a region that forms interactions with Tf (Fig. 6).

**Discussion**

Longitudinal cohort studies in Papua New Guinea, Thailand, and Brazil show that individuals with higher levels of antibodies to PvRBP2b are associated with a reduced risk of *P. vivax* infection and disease. To understand the putative protective function of human PvRBP2b antibodies, we isolated and characterized mAbs to PvRBP2b from individuals with naturally acquired immunity to *P. vivax*. We show that natural infection elicits PvRBP2b-specific human mAbs that inhibit PvRBP2b binding to reticulocytes and block complex formation between PvRBP2b and TfR1–Tf. X-ray crystallography combined with HDX-MS reveals the epitopes of 22 PvRBP2b antibodies and these results show how naturally acquired human mAbs block PvRBP2b function.

Out of the 23 PvRBP2b human mAbs isolated and examined in detail, eight of these including 239229, 214242, 253245, 258259, 260261, 262231, 273264, and 346343 were inhibitory across all three of our functional assays; reticulocyte-binding, FRET, and IP (Fig. 2d). Crystal structures of PvRBP2b-214242, PvRBP2b-253245, PvRBP2b-258259, and PvRBP2b-273264 show that these antibodies bind to the PvRBP2b-TfR1 interaction interface, and therefore inhibit through steric hindrance with TfR1 (Fig. 5b). While we were unsuccessful in our crystallography efforts with 260261 and 346343, HDX-MS data show that the direct interface lies at the Tf interaction site (Fig. 4b(iii)). These antibodies also show inhibition across all three interaction assays, which strongly suggests that they cause direct steric hindrance with TfR1–Tf

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**Fig. 6 Comparison between HDX protected regions and crystal structures.** Combination of HDX-MS data with crystal structures of PvRBP2b human Fab fragments bound to PvRBP2b. Fabs are shown in transparent surface representation. Regions that show protection by HDX-MS for each mAb are colored and the residue range is labeled. Black indicates the regions where no peptides were detected by HDX-MS.

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Based on our HDX-MS results, we propose that the binding of antibodies 237235, 243244, and 254255 results in allosteric changes in the PvRBP2b regions interacting with Tf (Figs. 4 and 6). Crystal structures of 237235 and 243244 show they bind PvRBP2b on the opposite side of the Tf–Tf binding site (Fig. 5b). Despite this, they are able to inhibit PvRBP2b, which like 251249 show protection in HDX-MS between residues 178–186 (Figs. 3b and 4a). These antibodies show 51% and 46% inhibition in the reticulocyte-binding assay respectively, therefore they may also inhibit complex formation by disrupting PvRBP2b binding to Tf (Fig. 2a).

One possibility is that the overall conformation or structural modes of inhibition, and the fastest association rates. The first set of antibodies includes 239229 or 262231 that bind in the C-terminal domain (L461-1633), resulting in steric hindrance with TfR1 and association rates of 3.78 × 10^5 (Fig. 2d and Supplementary Fig. S2). The fourth combination antibody is 237235, which is proposed to allosterically affect PfRBP2b binding to Tf and has an association rate of 3.57 × 10^5 (Fig. 4 and Supplementary Fig. S2). Although 237235 only inhibits PfRBP2b binding to reticulocytes by 47% (Fig. 2a), it may function similarly to the PfRh5 non-neutralizing antibody R5.011 that acts synergistically with PfRh5 neutralizing antibodies.

279280 enhances PfRBP2b binding to TIR1–Tf by causing a collision with the reticulocyte membrane.

279280 enhances PfRBP2b binding to TIR1–Tf in both the reticulocyte-binding assay and FRET assay. By HDX-MS, 279280 binds PfRBP2b in a region directly opposite the TIR1 binding site and it could stabilize this region to make it favorable for TIR1 binding. These epitopes that elicit enhancing antibodies are important to elucidate as they would be detrimental to a vaccine antigen.

Combining human mAbs that have different inhibitory mechanisms may have either synergistic or additive effects in P. vivax invasion assays. Where inhibitory antibodies compete with each other, choosing the most promising antibody may depend on the antibodies with the fastest association rate. Based on a study of human mAbs targeting the P. falciparum invasion ligand PfRh5, fast-associating antibodies were the most efficacious as they could rapidly bind proteins on the merozoite surface before the merozoite invaded the erythrocytes. Based on our results, we propose a combination of four sets of PvRBP2b human mAbs that bind non-overlapping target epitopes and have different structural modes of inhibition, and the fastest association rates. The first set of antibodies includes 241242 or 273264 which binds the N-terminal domain (S168–E460), resulting in steric hindrance with TfR1 and shows association rates of ~3.26 × 10^5 (Figs. 2d, 3a, 5b and Supplementary Fig. S2). The second set of antibodies includes 239229 or 262231 that bind in the C-terminal domain (L461-1633), resulting in steric hindrance with TfR1 and association rates of 3.80 × 10^5 (Figs. 2d, 3a, 5b and Supplementary Fig. S2). The third combination includes 326327, which causes steric hindrance with the reticulocyte membrane and has an association rate of 3.78 × 10^5 (Fig. 2d and Supplementary Fig. S2). Although 237235 only inhibits PfRBP2b binding to reticulocytes by 47% (Fig. 2a), it may function similarly to the PfRh5 non-neutralizing antibody R5.011 that acts synergistically with PfRh5 neutralizing antibodies.

R5.011 has been shown to slow merozoite invasion and create an opportunity for neutralizing antibodies to bind, albeit its mechanism of slowing invasion is not known. While PfRBP2b and PfRh5 bind different receptors (PfRh5 binds to basigin), they share a similar structural fold. The crystal structure of R5.011 in complex with PfRh5 and a neutralizing antibody, R5.016, shows that R5.011 contacts the N-terminal β-strand and loop at Y155-L160, and similarly, 237235 forms contacts with the N-terminal loop of PfRBP2b at D173–N174 and Q191–P194 (Fig. 5a). This may suggest that antibodies contacting the N-terminal loop of PfRh5 and PfRBP2b can modulate the binding of these ligands to their receptors. In summary, 241242/273264, 239229/262231, 326327, and 237235 could be examined together in P. vivax ex vivo invasion assays to ascertain whether a combination of antibodies is more effective than using single human mAbs for inhibiting parasite invasion.

One of the major challenges in targeting malaria proteins for vaccination is the presence of naturally occurring polymorphisms that may limit the effective use of a vaccine (reviewed in ref. 21). Our human mAbs were characterized using PvRBP2b sequences from the reference P. vivax strain Salvador I isolated from El Salvador and it is, therefore, important to investigate the action of these antibodies in Southeast Asian strains of P. vivax where our antibodies originate and where polymorphisms may affect antibody function. In particular, the N-terminal domain of PfRBP2b encompassing residues S168–E460 is under balancing selection. Targeting multiple PvRBP2b epitopes using a combination of human mAbs may lessen the impact of field polymorphisms that may disrupt antibody binding. Of the four
sets of antibodies that may be useful in combination, structural analyses reveal two field polymorphisms that may affect 241242/273264 binding. 241242/273264 binds in the N-terminal domain where seven polymorphic residues occur and the crystal structures reveal their binding may be affected by K363 and G382 polymorphisms (Supplementary Fig. S9). As the precise epitopes of 239229/262231 and 326327 are unknown, we cannot predict how these polymorphisms may affect their binding. Further mutational studies will be needed to confirm whether these human mAbs are affected by existing field polymorphisms. Of the four antibody sets, only 237235 binds a highly conserved region within the N-terminal domain. The only polymorphism within close proximity is R242, which is located directly adjacent to the 237235 epitope (Supplementary Fig. S9). In future studies, it would be important to isolate and examine more human mAbs that bind within the C-terminal region, which has only four polymorphisms and in particular no polymorphisms that reside in the TIR1 interaction site.11,12

Our structural and functional characterization of PrvRBP2b human mAbs provides us with an improved understanding of how the human immune system recognizes PrvRBP2b during natural infection with P. vivax and the presence of functional blocking antibodies. Furthermore, our interaction assays enable us to distinguish antibodies against different modes of inhibition and prioritize those that may be used in combination to block parasite invasion.

Methods

Study population. Blood samples were obtained from Cambodians with documented P. vivax malaria residing in Pursat Province.16 Large volume blood draws were previously obtained to isolate peripheral blood mononuclear cells (PBMCs) using standard procedures. Plasma samples from individuals with cryopreserved PBMCs were screened for blocking Abs to PrvRBP2b. Institutional review boards from the United States National Institutes of Health (NIAID protocol #09094, Clinicaltrials.gov NCT00663546), Cambodian Ministry of Health, and Universities Hospitals of Cleveland Medical Center approved the protocols (IRB No. 04-14-19) for blood collections. Study protocols were approved by the National Human Research Ethics Committee of the Ministry of Health of Brazil (approval No.551/2010). Written informed consent was obtained from all study participants or their parents/guardians.

Memory B-cell sorting. To generate tetramers, premium-grade allophycocyanin-labeled streptavidin (SA-APC) (Invitrogen Molecular Probes) was mixed on ice with mono-biotinylated PrvRBP2b1414–1422 with a BirA site using a PrvRBP2b1414–1422 SA-APC ratio of 4:1, modified with five additions of SA-APC at 20 min intervals and mixed for 10 min at 4 °C. Aggregates were removed with 17,000 × g centrifugation for 20 min at 4 °C. All tetramers were prepared fresh for each experiment. Single cells were identified and sorted following similar previously described techniques, ex vivo from cryopreserved PBMC without activation.16 Cryopreserved PBMC aliquots were thawed, placed in 10 mL 37 °C RPMI1640 media containing DNase type 1, centrifuged at 400 × g for 5 min, washed twice with 5 mL FACS buffer with 3 mM EDTA and adjusted to a cell density of 1–2 × 10⁶ cells/mL for the sorting. A cell-sorting strategy using for sorting PrvRBP2b-specific memory B cells is summarized in Supplementary Fig. S1. Cells were stained with mouse anti-human CD20 (PE-Cy7 clone G18-145, BD Biosciences) along with SA-APC PrvRBP2b1414–1422 tetramers (Invitrogen, Molecular Probes) and SYTOX Green Dead Cell Stain (ThermoFisher Scientific). Successful transfomants were screened by PCR amplification utilizing a vector-specific primer pair with an insert-specific primer and sequenced and compared against second-round PCR product sequence. Primers used are listed in Supplementary Table S1.

Expression and purification of recombinant proteins. The constructs were derived from the P. vivax strain Salvador 1, obtained from PlasmodB Database (www.plasmodb.org; accession number: PVX_094255, total length 2806 amino acids), PrvRBP2b1414–470 was expressed using E. coli strain Shuffle® T7 (New England BioLabs) and Terrific Broth (TB) supplemented with 50 μg/mL of ampicillin. Flasks containing 1 L of medium were incubated in MultiTorr shaker (Infors HT) at 37 °C at 180 rpm. At OD 600 of around 1.0, IPTG (Astral) was added to a final concentration of 1.0 mg/mL of protein expression was allowed to continue for 20 h at 16 °C. Cells were harvested by centrifugation at 7000 × g for 15 min at 4 °C. Bacterial pellets were suspended in 50 mL 1× PBS-50 mM NaCl, 10 mM EDTA, 0.5% Triton-X100 and 0.1% SDS, vortexed in a cold rack for 10 s before centrifugation at 300 × g for 10 min at 4 °C. The supernatant was removed. The cell debris was washed away using at least 10 column volumes of the buffer. The bound protein was eluted from the column using the same buffer: 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% (v/v) glycerol supplemented with Complete EDTA-free protease inhibitor cocktail (Roche), flash-frozen in liquid nitrogen and stored at −80 °C until further processing. For the purification, the cell pellet was thawed on ice and resuspended in the freezing buffer supplemented with 0.5 mg/mL of DNase and 1.0 mg/mL of lysosome (both from Sigma-Aldrich). Cells were lysed using sonicator Sonopuls UW 3200 (Bandelin) equipped with VS 70 T probe. The resulting crude cell extract was clarified by centrifugation at 30,000 × g for 45 min at 4 °C. The supernatant was applied on a 5 mL HisTrap column (GE Healthcare) pre-equilibrated with the freezing buffer then washed using at least 10 column volumes of the washing buffer. For the first-round of purification, 50 mL of nuclease-free water was used. The bound protein was eluted from the column using the same buffer but containing 300 mM imidazole. The eluted fractions were pooled and dialyzed overnight in the presence of TEV protease into 20 mL HEPES pH 7.0 and 100 mM NaCl. The resulting protein sample was applied on a 5 mL Q-Sepharose HiTrap column (GE Healthcare) pre-equilibrated with the dialysis buffer. Flow-through material was washed away using at least 10 column volumes of the buffer. The protein was eluted from the column using a gradient of 20 mM HEPES pH 7.0 and 1.0 M NaCl. Collected fractions were analyzed on SDS-PAGE and those containing the protein of interest were concentrated using an Amicon Ultra-4 10 kDa molecular weight cut-off centrifugal filter unit (Millipore) and stored at −80 °C. The final yield was calculated from 16/600 size exclusion column (GE Healthcare) pre-equilibrated with 20 mM HEPES pH 7.5 and 150 mM NaCl. The monodisperse peak fractions containing protein were pooled and concentrated using the same type of concentrator and used for crystallization trials. Expression and purification of PrvRBP2b1414–470 and PrvRBP2b1414–722 were performed in a similar manner as described above or previously.13,24

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A construct encoding the soluble ectodomain of human transferrin receptor 1 (TfR1, residues 121–760) was obtained from Addgene (pAcGP67A-TfR-Plasmid #121360). V5 protein expression follows the leader sequence from the human V5-protein GP67, a 6xHis-tag, and a factor X cleavage site in a modified form of the pAcGP67A expression vector (PharMingen) as described before.25, 26 TfR1 was expressed in a lytic baculovirus/insect cell expression system using Sf21 cells (Life Technologies) and Insect-XPRESS Protein-free Insect Cell Medium supplemented with 0.5% glucose, 1.0% bovine serum albumin (BSA), and 1.0% penicillin/streptomycin at 30°C. V5 protein expression was induced by incubating the cell culture at around 1 × 10^6 cells/mL with the third passage stock (P3) of the virus and allowed to progress for three days. The supernatant was separated from the cells by centrifugation at 2000 × g for 20 min and concentrated using an Amicon Ultra-4 30 kDa molecular weight cut-off filtration device equipped in a cassette with 10 kDa molecular weight cut-off (Millipore). Concentrated supernatants were subsequently dialedyzed into a buffer containing 20 mM Tris-HCl pH 7.5 and 300 mM NaCl in order to remove the residuals of the culture media that might interfere with the subsequent steps of purification. The supernatant was applied on a 5 mL HisTrap column (GE Healthcare) pre-equilibrated with the dialysis buffer supplemented with 20 mM imidazole. The protein was eluted using the same buffer but containing 300 mM imidazole, then concentrated using an Amicon Ultra-4 30 kDa molecular weight cut-off concentrator (Millipore) and injected onto S200 Superdex 16/600 size exclusion column (GE Healthcare) pre-equilibrated with 20 mM HEPEs pH 7.5 and 100 mM NaCl and 50 mM NaHCO₃. The final samples were supplemented with 10% (v/v) glycerol, flash-frozen in liquid nitrogen and stored at −80°C.

Apo-transferrin purified from human serum was purchased from Sigma-Aldrich (Catalog Number T4382). The protein powder was resuspended in a buffer containing 100 mM disodium carbonate at pH 5.9. Loading of transferrin with iron was performed in the manufacturer's report by adding a solution of ferrous ammonium sulphate, hexahydrate (Sigma-Aldrich) corresponding to 2% (w/w) of protein mass. The sample was incubated for 1 h at room temperature. The pH was then raised to 8.5 using 1.0 M disodium carbonate and the solution was mixed for an additional 2 h. Holo-transferrin was subsequently purified by gel filtration chromatography on a Superose 12 10/300 column (GE Healthcare) equilibrated with 20 mM NaHEPES pH 7.5, 100 mM NaCl and 50 mM NaHCO₃. The bound protein was eluted using the same buffer but containing 300 mM imidazole, then concentrated using an Amicon Ultra-4 30 kDa molecular weight cut-off concentrator (Millipore) and injected onto S200 Superdex 16/600 size exclusion column (GE Healthcare) equilibrated with 20 mM NaHEPES pH 7.5, 100 mM NaCl and 50 mM NaHCO₃. The final samples were supplemented with 10% (v/v) glycerol, flash-frozen in liquid nitrogen, and stored at −80°C.

Antibody expression and purification. Recombinant human mAbs were expressed in Expi293 HEK cells (Life Technologies), which were maintained in suspension at 100 mM NaCl and 50 mM NaHCO₃ buffer. The exclusion column (GE Healthcare) pre-equilibrated with 20 mM NaHEPES pH 7.5, temperature. The pH was then raised to 8.5 using 1.0 M disodium carbonate and (w/w) of protein mass. The sample was incubated with stirring for 1 h at room temperature. After three washings with PBS (0.05% Tween 20), plates were treated with PBS, 0.5 M, 1 M, and 2 M of NH₄SCN for 20 min at room temperature. Following washing three times with PBS, wells were incubated with HRP labeled anti-human IgG (Fc) antibody (1:1000 dilution, Jackson ImmunoR-esonance energy transfer (FRET) assay. PVRP2b161-1454 and TR1 were labeled with N-hydroxysuccinimide ester-activated DyLight 488 (DL488) and DyLight 594 (DL594) (Life Technologies) respectively. The dyes were dissolved in dimethyl sulfoxide (DMSO) (Sigma) and added at five-fold molar excess to the protein being labeled. After 1 h incubation at room temperature, the un-conjugated dye was removed using a Micro Bio-Spin 6 column (BioRad) at 1000 x g, 2 min. The average dye per protein was ~3.5 dye/protein for PVRP2b161-1454 and ~1.8 dye/protein for TR1. PVRP2b161-1454–DL488, TR1-DL594, and Tf were mixed in a 1:1:1 molar ratio with a 5-fold excess of human mAbs to a final reaction volume of 10 μL in FRET buffer (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 0.01% BSA) read in Corning 384-well plates and each sample was performed in triplicate. 1 μL of 20% SDS was added to one triplicate sample with no human mAbs to measure the background signal. Fluorescence intensity was measured using EnVision plate reader (PerkinElmer Life Sciences). DL488 (donor) fluorescence was measured with a 485/14-nm excitation filter and 535/25-nm emission filter and DL594 (acceptor) fluorescence was measured with a 590/20-nm emission filter. Signal to noise ratio was calculated using a 485/14-nm excitation filter and 615/9-nm emission filter. Fluorescence measurements were analyzed using Prism software (GraphPad; version 8.4.3). To account for bleed-through of dyes into the sensitized emission spectra, standard curves for both dyes were measured and the raw data were transformed by multiplying with the fitting curve of the standards. The y-intercept was subtracted from all binding results. To enable comparisons between biological replicates, the percentage of PVRP2b161-1454–bound cells in the presence of human mAbs was divided by the percentage of PVRP2b161-1454–bound cells with no human mAbs and multiplied by 100 to obtain the percentage of PVRP2b161-1454–bound cells relative to the no human mAbs control.

Fluorescence resonance energy transfer (FRET) assay. PVRP2b161-1454 and TR1 were labeled with N-hydroxysuccinimide ester-activated DyLight 488 (DL488) and DyLight 594 (DL594) (Life Technologies) respectively. The dyes were dissolved in dimethyl sulfoxide (DMSO) (Sigma) and added at five-fold molar excess to the protein being labeled. After 1 h incubation at room temperature, the un-conjugated dye was removed using a Micro Bio-Spin 6 column (BioRad) at 1000 x g, 2 min. The average dye per protein was ~3.5 dye/protein for PVRP2b161-1454 and ~1.8 dye/protein for TR1. PVRP2b161-1454–DL488, TR1-DL594, and Tf were mixed in a 1:1:1 molar ratio with a 5-fold excess of human mAbs in a final reaction volume of 10 μL in FRET buffer (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 0.01% BSA) read in Corning 384-well plates and each sample was performed in triplicate. 1 μL of 20% SDS was added to one triplicate sample with no human mAbs to measure the background signal. Fluorescence intensity was measured using EnVision plate reader (PerkinElmer Life Sciences). DL488 (donor) fluorescence was measured with a 485/14-nm excitation filter and 535/25-nm emission filter and DL594 (acceptor) fluorescence was measured with a 590/20-nm emission filter. The ratio of fluorescence emission of the acceptor dye to that of the donor dye was calculated by dividing the FRET signal with inhibitor by the FRET signal with no inhibitor, multiplied by 100.
Immunoprecipitation assays. Immunoprecipitation assays were performed using equal amounts of PvRBP2b–HRP-α–HRP-β and antibody Fabs overnight. The incubation was terminated by the sequential addition of 20 μl of 100 mM sodium phosphate buffer (pH 6.0) with 0.1 M magnesium chloride, 15 % (w/v) PEG 4,000 at 8 mg/mL of complex. Crystals of 283284 in complex with PvRBP2b–HRP-α–HRP-β were obtained in 0.3 M ammonium nitrate, 0.1 M sodium HEPES pH 7.0, 26 % (w/v) PEG 2000 at 11 mg/mL of complex.

Data collection and structure determination. Crystals were cryo-protected in mother liquor containing 20% (v/v) glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected at the MX2 beamline at the Australian Synchrotron (Clayton, Australia) at 100 K (1.5–0.9 Å) using an Exner X 16 M pixel detector. Diffraction data were processed with the XDS package28 (Version [an 31, 2020 BUILT = 20200131]). The highest resolution shell included for processing was determined based on data completeness of >95% and CC1/2 of >50%. The probable number of molecules in the asymmetric unit was calculated using the program Matthews29. Molecular replacement was performed with Phaser (CCP4 suite; version 7.0.052)30,31 using the structure of PvRBP2b–HRP-α–HRP-β (PDB accession code 5W53) and unrelated Fab antibody fragments as search models. To obtain the best starting search model for antibody Fab, the online tool BLASTp (https://blast.ncbi.nlm.nih.gov/) was used to search the Protein Data Bank (PDB) for antibody structures with the highest sequence similarity to the PvRBP2b human mAbs and the best structural refinement statistics. Heavy and light-chain antibody sequences were queried separately then combined into one Fab to use as a search model. The models used were: 237235/243244—4HWE for both heavy and light chain; 241242–50DB for both heavy and light chain, 251249–6B0S for the heavy chain and 5116–253245 for the light chain; 253245–4RCO for the heavy chain and 4UNU for the light chain; 258259–273264–6AZM for the heavy chain and 5IFH for light chain, and 283284–6MLK for the heavy chain and 5E08 for the light chain. The angle between constant and variable regions of PvRBP2b antibody Fab was different from the model, so to obtain a better molecular replacement solution, antibody constant region structures were reoriented. HDX-MS experiments were performed at the UniGe Protein Platform (University of Geneva, Switzerland). A similar protocol described previously was applied26. HDX reactions were done in 50 μL volumes using 50 pmol of PvRBP2b–HRP-α–HRP-β and a 1.3 fold molar excess of a human antibody. Reaction with every antibody was performed following exactly the same procedure. Briefly, RPB2 was pre-incubated with the antibody on ice for 30–60 min. 7 μL of the RPB2–mAb mixture was pipetted into a 96 well plate to saturate the experimental temperature for 30–60 min. Deuteration reactions were initiated by adding 43 μL of D2O exchange buffer (82.5% D2O, 10 mM Hepes pH 7.5, 150 mM NaCl) to the 7 μL RPB2–mAb mixture. Reactions were carried out for 5 min, either at 0 °C or at 22 °C and terminated by the sequential addition of 20 μL of ice-cold quench buffer (1 M urea/0.1 M NaAc, pHe 4.5/4.5). Samples were immediately frozen in liquid nitrogen and stored at –80 °C for up to 4 weeks. All experiments were repeated in triplicates.

To quantify deutery uptake into the protein, protein samples were thawed and injected into the UPLC system immersed in ice. The protein was digested via two immunoaffinity enrichments (Thermo #22131) and purified by anion exchange chromatography of a 300 Å, 5 μL particle size Fortis Bio column 100 × 2.1 mm. The average standard deviation for percentage deuteration was 3.4%. Changes in deutery were performed following the same protocol. The average standard deviation for percentage deuteration was 3.4%. Changes in deutery incorporation for all peptides can be found in Supplementary Data 2 and 3. Differences in a number of deuterons incorporated and differences in percentage deuteration for PvPBP2b peptides alone and in complex with PvPBP2b human mAbs for all peptides is shown in Supplementary Data 4 and 5.

**Protein crystallization.** Large-scale, sparse-matrix sitting-drop crystallization trials were performed at the CSIRO Collaborative Crystalization Center (CSIRO; Parkville, Australia). Crystals of PvPBP2b–HRP-α–HRP-β were harvested in 0.2 M trimethylamine N-oxide, 0.1 M Tris chloride pH 8.5, 20% (w/v) PEG 3350 at 5 mg/mL of complex. Crystals of PvPBP2b–237235 in complex with HRP-conjugated streptavidin binding to biotinylated PvPBP2b–HRP-α–HRP-β (w/v) PEG 3,350 at 10 mg/mL of complex. Crystals of PvPBP2b–241242 in complex with PvPBP2b–253245, PvPBP2b–258259, and PvPBP2b–251249 and PvPBP2b–273264 were harvested directly from the sitting-drop plates, while PvPBP2b–237235, PvPBP2b–241242, PvPBP2b–243244, PvPBP2b–253245, PvPBP2b–258259, and PvPBP2b–251249 and PvPBP2b–273264 were harvested in 0.2 M sodium dihydrogen phosphate pH 4.0, 16% (w/v) PEG 3350 at 5.5 mg/mL of complex. Crystals of PvPBP2b–243244 in complex with PvPBP2b–253245 were obtained in 0.1 M magnesium formate, 15% (w/v) PEG 3350 at 5 mg/mL of complex. Crystals of PvPBP2b–243244 in complex with PvPBP2b–253245 were extended to 3.48 Å to make maximum use of weak data in the outer shell (3.69–3.48 Å)—within this outer resolution shell, CC1/2 was remained significant at the P = 0.001 level and data completeness remained above >90%. Pseudo-merohedral twinning was detected in the 253245 data set by phenix.xtriage. 253245 was therefore refined in the presence of the twin operator (h, -k, -l), yielding an associated twin fraction of 0.15. Crystallographic data collection and refinement statistics for all structures are summarized in Supplementary Table S2. Interactions between PvPBP2b and antibody Fab was determined using PISA35 (CCP4 suite; version 2.1.0) and summarized in Supplementary Table S3. Figures were prepared using Pymol (http://www.pymol.org).

**Statistical analysis.** Prism (version 8.4.3) was used to perform one-way ANOVA followed by Dunnett’s multiple comparisons test using both 040308 and 089910 as controls.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** *P. vivax* reticulocyte binding protein 2b constructs were derived from the *P. vivax* strain Salvador I from the PlasmoDB Database (www.plasmodb.org; accession number: PVX, 094225). The structure models used in molecular replacement are from the Protein Data Bank under the following accession codes: 6WVE, 6SDB, 6B0S, 5I16, 4RCO, 4UNU, 6AZM, 5IFH, 6MLK, 5E08, 4LLD, and 5NYX. Coordinate and structure factors have been deposited in the Protein Data Bank under accession codes 6W9M (PvRBP2b-237235 complex), 6WN1 (PvRBP2b-241242 complex), 6WNO (PvRBP2b-243244 complex), 6WOZ (PvRBP2b-251249 complex), 6WTY (PvRBP2b-253245 complex), 6WTV (PvRBP2b-258259 complex), 6W TU (PvRBP2b-273264 complex), and 6WQQ (PvRBP2b-283284 complex). Source data are provided with this paper.

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Author contributions
A.G., C.T.F., and L.L.C. sorted B cells, analyzed antibody sequences, and cloned antibody variable regions. A.G. measured antibody avidity and performed competition experiments with PvRP2b human mAbs. L.-J.C. performed the in vitro functional assays, affinity measurements, mapping of PvRP2b human mAbs, and antibody competition experiments with mouse mAbs. L.-J.C. expressed and purified all PvRP2b human mAbs and prepared samples for protein crystallography. L.-J.C. and M.H.D. crystallized PvRP2b Fab complexes, collected diffraction data, and determined the crystal structures. S.M. assisted in the purification of recombinant PvRP2b fragments. O.V. and R.V. performed the HDX-MS sample preparation and acquisition data analysis. D.S.-F., I.M., W.-H.T., and C.L.K. helped design the experiments.

Competing interests
The authors declare no competing interests.

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