Naturally Acquired Human Antibodies Against Reticulocyte-Binding Domains of *Plasmodium vivax* Proteins, PvRBP2c and PvRBP1a, Exhibit Binding-Inhibitory Activity

Enna Dogra Gupta,[1,a,b] Gaurav Anand,[1,a], Hina Singh,[1,a] Kritika Chaddha,[2] Praveen K. Bharti,[4] Neeru Singh,[4] Yagya Dutta Sharma,[3] and Deepak Gaur[1,2]

[1]Malaria Group, International Centre for Genetic Engineering and Biotechnology, [2]Laboratory of Malaria & Vaccine Research, School of Biotechnology, Jawaharlal Nehru University, and [3]Department of Biotechnology, All India Institute of Medical Sciences, New Delhi, and [4]National Institute for Research in Tribal Health, Jabalpur, Madhya Pradesh, India

Background. Crucial gaps in our understanding of *Plasmodium vivax* reticulocyte invasion and protective immunity have hampered development of vivax vaccines. *P. vivax* exclusively invades reticulocytes that is mediated by the *P. vivax* reticulocyte-binding proteins (PvRBPs) specifically PvRBP2c and PvRBP1a. Vivax infections in Duffy-null individuals have suggested the evolution of alternate invasion pathways that may be mediated by the PvRBPs. Thus, PvRBPs appear as potential targets for efficacious *P. vivax* neutralization. However, there are limited data validating their vaccine efficacy. In the absence of vivax invasion assays, binding-inhibitory activity of antibodies has been reported to be associated with protection and a measure of vaccine potential.

Methods. Flow cytometry–based analysis was performed of the PvRBP reticulocyte-binding properties and binding-inhibitory activity of specific anti-PvRBP2c/PvRBP1a human antibodies.

Results. PvRBP2c and PvRBP1a displayed a distinct reticulocyte-binding specificity, and their specific reticulocyte-binding domains were mapped within their N-terminal regions. Importantly, naturally acquired antibodies against the reticulocyte-binding domains efficaciously blocked reticulocyte binding of native PvRBPs, suggesting that the human immune system produced functional binding-inhibitory antibodies through exposure to vivax malaria.

Conclusions. Reticulocyte-binding domains of PvRBP2c/PvRBP1a are targets of naturally acquired binding-inhibitory antibodies, substantiating their promise as candidate antigens against which vaccine-inducible immunity could potentially be boosted through natural infections.

Keywords. vivax malaria; reticulocyte-binding proteins; binding-inhibitory antibodies; naturally acquired immunity; vaccines.

Plasmodium vivax malaria has remained largely neglected despite its being geographically more widespread than falciparum malaria and responsible for enormous morbidity across the tropics [1–4]. To eliminate malaria, it is crucial to develop effective intervention strategies to counter *P. vivax,* and in this regard a potent vaccine would be a highly effective tool in combating the disease. An encouraging aspect for vaccine development is that malaria induces naturally acquired immunity (NAI) that confers disease protection [5, 6]. NAI is known to restrict blood-stage parasite densities leading to lower incidences of clinical and severe malaria [5, 6]. A good understanding of NAI could provide key insights on the immune correlates of protection, which would immensely benefit vaccine development.

Acquisition of NAI against *P. vivax* occurs more rapidly than for *Plasmodium falciparum,* irrespective of the transmission intensity [5]. Mechanisms underlying this rapid NAI acquisition remains poorly understood and warrant further investigations. In this regard, humoral responses against blood-stage antigens constitute a critical component of NAI against vivax malaria [5, 6]. Therefore, parasite proteins involved in erythrocyte invasion are potential targets of protective immunity that need in-depth validation for their development as vaccine candidates.

Unlike *P. falciparum*, *P. vivax* primarily invades only reticulocytes (young immature erythrocytes), and the molecular basis underlying *P. vivax* reticulocyte invasion is not well understood [3, 7]. The central dogma of *P. vivax* merozoite invasion has been its dependence on the essential interaction between the *P. vivax* Duffy-binding protein (PvDBP) and its receptor, Duffy antigen receptor for chemokines (DARC) [8, 9], which does not account for reticulocyte specificity because DARC is present on both normocytes and reticulocytes. *P. vivax* reticulocyte specificity (Belem strain) was first reported to be mediated by 2 *P. vivax* reticulocyte-binding proteins (PvRBPs), PvRBP1a and PvRBP2c [10]. Thereafter, the *P. vivax Sal*I genome revealed a PvRBP family of 11 members (Supplementary Figure 1), of which 2 are pseudogenes [11]. Reports of vivax infection in

Duffy-null individuals strongly suggest that *P. vivax* has evolved Duffy-independent, redundant invasion pathways [12–14]. It is thus critical to elucidate the molecular basis of *P. vivax* merozoite invasion. PvRBPs are implicated to play crucial roles in mediating reticulocyte specificity and may play a role in Duffy-independent invasion. Therefore, PvRBPs are attractive candidate antigens for the development of vivax vaccines and warrant further validation.

The lack of *P. vivax* in vitro culture has deferred in-depth dissection of vivax antigens [3, 7]. In the interim, PvRBP homologues known as reticulocyte binding-like homologous (PfRH) proteins were discovered in *P. falciparum*, where they function as key determinants of erythrocyte invasion [8, 15, 16], and are considered promising vaccine candidates [17–22]. Among the PvRBP family, only native PvRBP1a and PvRBP2c have been demonstrated to specifically bind reticulocytes [10]. However, a functional analysis of the specific reticulocyte-binding domains of PvRBP1a/PvRBP2c correlating with the specific binding activity of the native parasite proteins is lacking. Furthermore, to our knowledge, no previous study has evaluated the generation of functional binding-inhibitory PvRBP antibodies by the human immune system in response to natural vivax infections.

In the present study, we have screened the reticulocyte-specific binding of the PvRBP2c/PvRBP1a proteins and mapped the constructs exhibiting the maximum binding efficiency that correlated with that of native parasite proteins. Importantly, we have demonstrated that PvRBP-specific human antibodies against these receptor-binding domains potently inhibit the reticulocyte-specific binding of the native PvRBP2c/PvRBP1a parasite proteins. In the absence of *P. vivax* invasion inhibition assays, binding inhibition is an important surrogate for evaluating vaccine potential of *vivax* antigens [23]. This is the first report to demonstrate the functionality of naturally acquired human antibodies targeting PvRBPs, which has major implications for the development of vivax malaria vaccines.

MATERIALS AND METHODS

Sample Collection and Ethics Approvals

P. vivax–infected blood samples were collected from the Balaghat district (Madhya Pradesh) by the National Institute of Research in Tribal Health (NIRTH) [24] and at the clinics of the All India Institute of Medical Sciences (AIIMS) [25]. Cross-sectional active door-to-door fever surveys were carried in the study villages of Balaghat (Supplementary Text) [24]. Finger-prick blood smears were collected by NIRTH from all patients with fever or history of fever within the past 14 days. In addition, human subjects from the North Indian belt attending the clinics at AIIMS were screened for *P. vivax* infections.

Peripheral blood samples were collected from human subjects via finger prick after informed consent. *P. vivax* infections were diagnosed using a rapid diagnostic test (SD Bioline

Malaria Antigen Pf/Pv, Bio Standard Diagnostics, India) and confirmed microscopically by examination of Giemsa-stained thin blood smears. Blood samples (2–5 mL) were collected from 66 *P. vivax*–positive subjects identified between the NIRTH and AIIMS studies, with the primary inclusion criterion being a positive rapid diagnostic test results confirmed microscopically. Patients with positive results were treated according to the drug policy of the National Vector Borne Disease Control Programme. Infected *P. vivax* parasites and human plasma samples for our studies were obtained from this source of 66 human blood samples. All approvals were obtained from the institutional ethical review committees, according to the guidelines of the Indian Council of Medical Research (Government of India).

Production of Recombinant Proteins

Codon-optimized genes encoding PvRBP2c/PvRBP1a constructs (Gene Art; Life Technologies) were subcloned in the pET24b expression vector (Novagen). Recombinant proteins were expressed in *Escherichia coli* and purified to homogeneity, as described in the Supplementary Methods. Generation of antibodies [17], preparation of *P. vivax* lysate and culture supernatant [10], enzyme-linked immunosorbent assay (ELISA) [26] and polymerase chain reaction confirmation of *P. vivax* are described in the Supplementary Text.

Affinity Purification of Antigen-Specific Human Antibodies

Affinity purification of human antibodies was performed from ELISA-positive high-responder plasma samples as reported elsewhere [26, 27]. Briefly, recombinant proteins were coupled with cyanogen bromide–activated sepharose (Sigma) and pooled plasma was applied over these affinity columns. Elution was performed using 0.1 mol/L glycine (pH 2.7), and the pH was adjusted with 1 mol/L Tris-HCl (pH 9.0). Eluates were pooled and concentrated using centrifugal filters (Millipore; molecular weight cutoff, 10 kDa), followed by buffer exchange with phosphate-buffered saline (PBS; pH 7.4). Affinity purification was validated with ELISA, which demonstrated that the purified antibodies were enriched compared with individual high-responder plasma samples. Cross-reactivity of the affinity-purified antibodies was analyzed with ELISA.

Reticulocyte Binding and Inhibition Assay

Reticulocyte enrichment [28, 29] and enzymatic treatment [30] were performed as reported elsewhere (described in the Supplementary Text). Reticulocyte binding of native PvRBPs (culture supernatant) and recombinant RBPs (rRBPs) were determined using 2 methods described elsewhere, the overlay assay followed by immunoblotting [18] and a dual-color flow cytometry–based assay [31]. Briefly, culture supernatant or recombinant proteins were incubated with enriched reticulocytes (magnetic sorting or Percoll gradient; Supplementary

Figure 2). Suspension was laid over dibutyl phthalate and centrifuged. Cell-bound proteins were eluted by salt and analyzed by means of immunoblotting.

For the FACS assay, enriched reticulocytes (magnetic sorting) were incubated with recombinant protein or culture supernatant, washed with PBS, and further incubated with anti-PvRBP antibodies. Samples were washed and incubated with anti-mice or anti-rabbit allophycocyanin-conjugated secondary antibody (Molecular Probes), followed by incubation with thiazole orange, as reported elsewhere [31]. A total of 100 000 total events were acquired per sample with a FACSCanto system (Becton Dickinson). Unstained and thiazole orange–stained cells defined the normocyte and reticulocyte populations, respectively [31]. For binding inhibition experiments, recombinant protein/culture supernatant and enriched reticulocytes were incubated at 37°C in the presence of rat or human antibodies. Bound proteins were detected with FACS, as described above.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software, version 5.01 (GraphPad Software). Nonparametric statistical tests were used to analyze data. Medians for the 2 groups were compared using t tests, and differences were considered statistically significant at $P < .05$.

RESULTS

Production of Recombinant Proteins and Generation of Specific Antibodies

Sequence analysis of 8 *P. vivax* worldwide strains showed that while PvRBP2c exhibited numerous antigenic polymorphisms, PvRBP1a was highly conserved (Supplementary Text, alignments 1–2). Sequence alignments of PvRBP2c and PvRBP1a with their phylogenetically close RBL family members showed a homology with their erythrocyte-binding domains (Supplementary Text, alignments 3–6), which framed the basis for mapping the putative reticulocyte-specific binding domains. These conserved regions of the 2 PvRBPs were selected for recombinant protein production (Figure 1A).

PvRBP recombinant proteins—rRBP2.1 (residues 168–524), rRBP2.2 (residues 464–876), and rRBP2.3 (residues 2398–2736) of PvRBP2c (Figure 1A) and rRBP1.1 (residues 30–778) and rRBP1.5 (residues 1956–2315) of PvRBP1a (Figure 1A)—were expressed in *E. coli* with a C-terminal His-tag. To dissect the minimal reticulocyte-binding region within the 82-kDa rRBP1.1 protein, we produced 3 fragments within this protein: rRBP1.2 (residues 30–351), rRBP1.3 (residues 352–778), and rRBP1.4 (residues 352–599) (Figure 1A). The proteins were purified to homogeneity (Supplementary Figure 3), and their identities were confirmed using mass spectrometry (Supplementary Table 1). Specific antibodies were raised in rats and mice against the PvRBP and PvDBP recombinant proteins with end-point titers

in the range of 1:320 000 to 1:640 000 (Supplementary Figures 4–7).

Reticulocyte Specific Binding of Native PvRBP2c and PvRBP1a

We confirmed the specificity of the PvRBP2c/1a antibodies by immunoblotting using *P. vivax* and *P. falciparum* parasite lysates. rRBP1.1 and rRBP2.2 mice antibodies detected native vivax proteins in the range of approximately 280 kDa (Figure 1B), consistent with previous reports [10]. Both antibodies exhibited *P. vivax* specificity, as they failed to cross-react with *P. falciparum* proteins (Figure 1B). Similarly, *P. falciparum* erythrocyte binding antigen 175 (PfEBA-175) and PvDBP antibodies detected only their respective parasite proteins, with no cross-reactivity (Figure 1B). *P. vivax* field samples were confirmed with microscopy and polymerase chain reaction using specific 18S ribosomal RNA primers (Figure 1D and E), as reported elsewhere [32].

The specificity of the native parasite proteins detected by the PvRBP antibodies was further confirmed by binding assays, as reported elsewhere [10]. Native PvRBPs were obtained from culture supernatants of single-cycle matured *P. vivax*–infected human erythrocytes (Supplementary Text). Native *P. vivax* proteins (~250 kDa) detected with the PvRBP2c/PvRBP1a antibodies failed to bind mature erythrocytes (Figure 1C) but specifically bound enriched reticulocytes (Figure 1C), thus confirming them as native PvRBPs, consistent with the previous report [10]. PvDBP bound both mature erythrocytes and enriched reticulocytes (Figure 1C), as both express DARC. Our results confirm the reticulocyte-specific binding of the native PvRBP2c/PvRBP1a proteins and the specificity of our PvRBP antibodies.

Mapping the Reticulocyte-Binding Domain of PvRBP2c

We investigated the reticulocyte-specific binding activity of 2 N-terminal regions of PvRBP2c, rRBP2.1 (residues 168–524) and rRBP2.2 (residues 464–876), which are homologous with the erythrocyte-binding domains of PfRH2 and PkNBPXa, respectively (Supplementary Text, alignments 3 and 4). Reticulocyte binding of the PvRBP2c proteins was analyzed with FACS (Figure 2) [33]. As reported elsewhere, rDBPRII (PvDBP receptor–binding domain) bound both reticulocytes and normocytes [34], whereas the non-binding protein, *P. falciparum* cysteine-rich protective antigen (PfCyRPA) protein [21] failed to exhibit binding (Figure 2A and B). rRBP2.2 exhibited significantly greater reticulocyte-binding activity (34%) than rRBP2.1 (10%) (Figure 2A). rRBP2.3 (C-terminal fragment) exhibited poor reticulocyte-binding activity (0.96%). Results for 3 independent experiments are depicted in the bar graphs (Figure 2B). rRBP2.2 specifically bound enriched reticulocytes and failed to bind unenriched normocytes (Figure 2C). Thus, we demonstrated for the first time that the 413 residue rRBP2.2
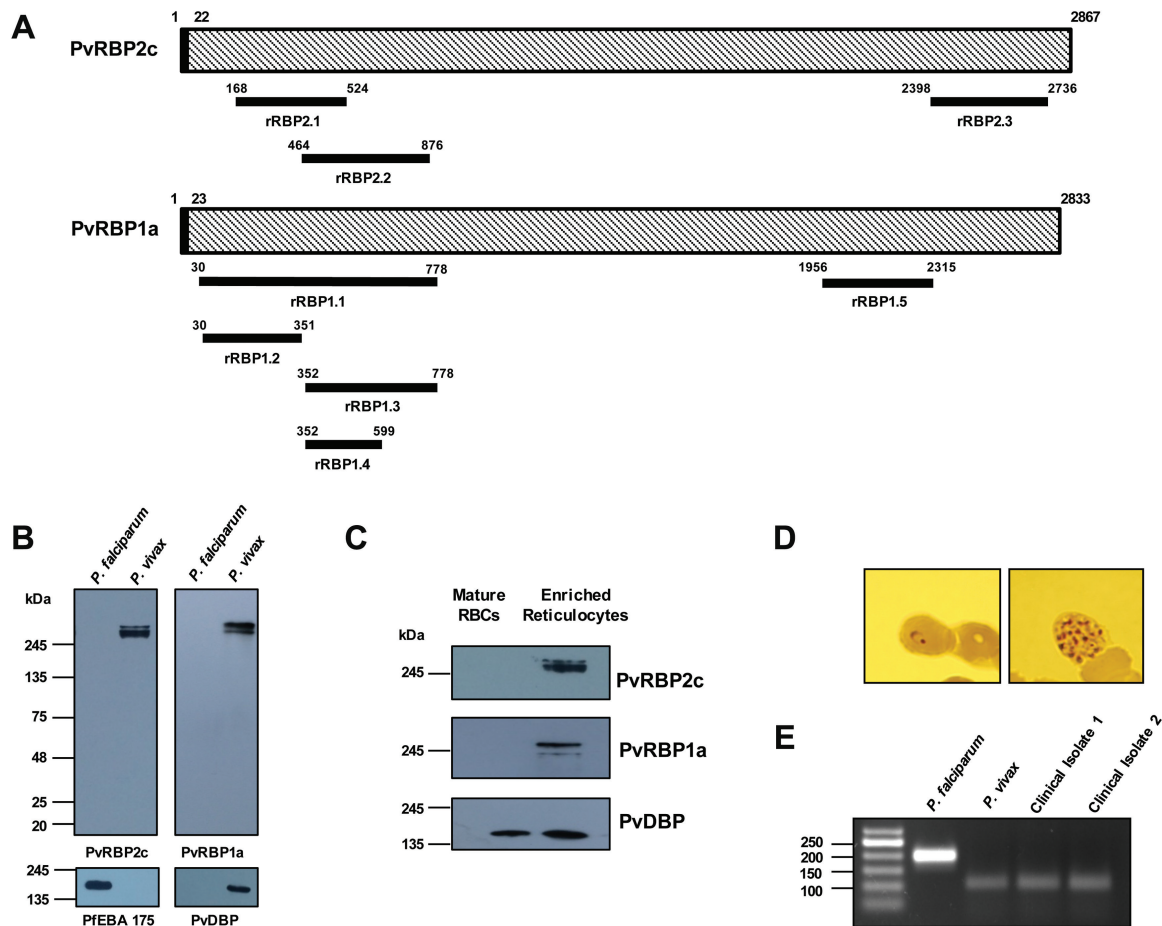
Figure 1. Structure, expression, and reticulocyte-binding activity of the native *Plasmodium vivax* reticulocyte-binding proteins (PvRBPs). *A,* Schematic diagram of the PvRBP2c and PvRBP1a parasite proteins, highlighting the regions against which the recombinant proteins have been produced (*black bars*). *B,* Native PvRBP2c and PvRBP1a parasite proteins were detected by means of immunoblotting only in the *P. vivax* protein extracts by the specific anti-rRBP2.2 and anti-rRBP1.1 mice antibodies. Expression of native PfEBA-175 was detected only in the *Plasmodium falciparum* extract, confirming no cross-contamination of the *P. vivax*–infected samples with *P. falciparum*. Native *P. vivax* Duffy-binding protein (PvDBP) parasite protein was detected only in the *P. vivax* extract, confirming the specificity of the vivax sample. *C,* Binding of native PvRBP2c and PvRBP1a from the *P. vivax* culture supernatant was detected only with enriched reticulocytes and not with normal erythrocytes (red blood cells [RBCs]). Native PvDBP from the same culture supernatant bound with the same set of normal erythrocytes and enriched reticulocytes. Reticulocytes were enriched over a Percoll cushion. *D,* Blood film of the *P. vivax*–infected sample showing the *P. vivax* ring-stage and schizont-stage parasites. *E,* Identities of the *P. vivax*–infected samples were confirmed by means of nested polymerase chain reaction (PCR) amplification of 18S ribosomal RNA sequences, using primers specific for *P. vivax* and *P. falciparum*. The specific PCR-amplified DNA fragments from *P. vivax* and *P. falciparum* correspond to 100 and 200 base pairs (bp), respectively. Two representative *P. vivax*–infected clinical isolates are shown that correspond to the 100-bp size, confirming their identity. Abbreviations: PfEBA-175, *P. falciparum* erythrocyte binding antigen 175; rRBP, recombinant RBP.

region (Lys464-Ile876) constituted the reticulocyte-binding domain of native PvRBP2c.

Mapping the Reticulocyte Binding Domain of PvRBP1a

We analyzed the reticulocyte-specific binding of 4 fragments spanning the 749 amino acid PvRBP1a N-terminal region, which are homologous with the erythrocyte-binding domains of PfRH4 and PfRH1 (Supplementary Text, alignments 5 and 6). rRBP1.1 exhibited the most potent reticulocyte-specific binding activity (37%) (Figure 2A and B). rRBP1.2 and rRBP1.5 failed to bind reticulocytes (Figure 2A and B), and rRBP1.3 and rRBP1.4 exhibited weaker reticulocyte-binding activity (10%–13%) than rRBP1.1 (Figure 2A and B). Thus, large variation in the binding

was observed between rRBP1.1 and its smaller fragments. Like PfRH4, the minimal binding domain of PvRBP1a has been mapped to the 261 amino acid region (352–599) (rRBP1.4). Although the upstream region (residues 30–351) is unable to bind reticulocytes independently, it contributes significantly to the potent reticulocyte-binding activity of rRBP1.1. Thus, efficient reticulocyte-binding activity of PvRBP1a lies within the 749-residue rRBP1.1 region, which specifically bound enriched reticulocytes and failed to bind unenriched normocytes (Figure 2C).

Reticulocyte Binding Specificities of PvRBP2c and PvRBP1a

The nature of the reticulocyte receptor that binds with PvRBP2c/PvRBP1a was determined by analyzing the binding
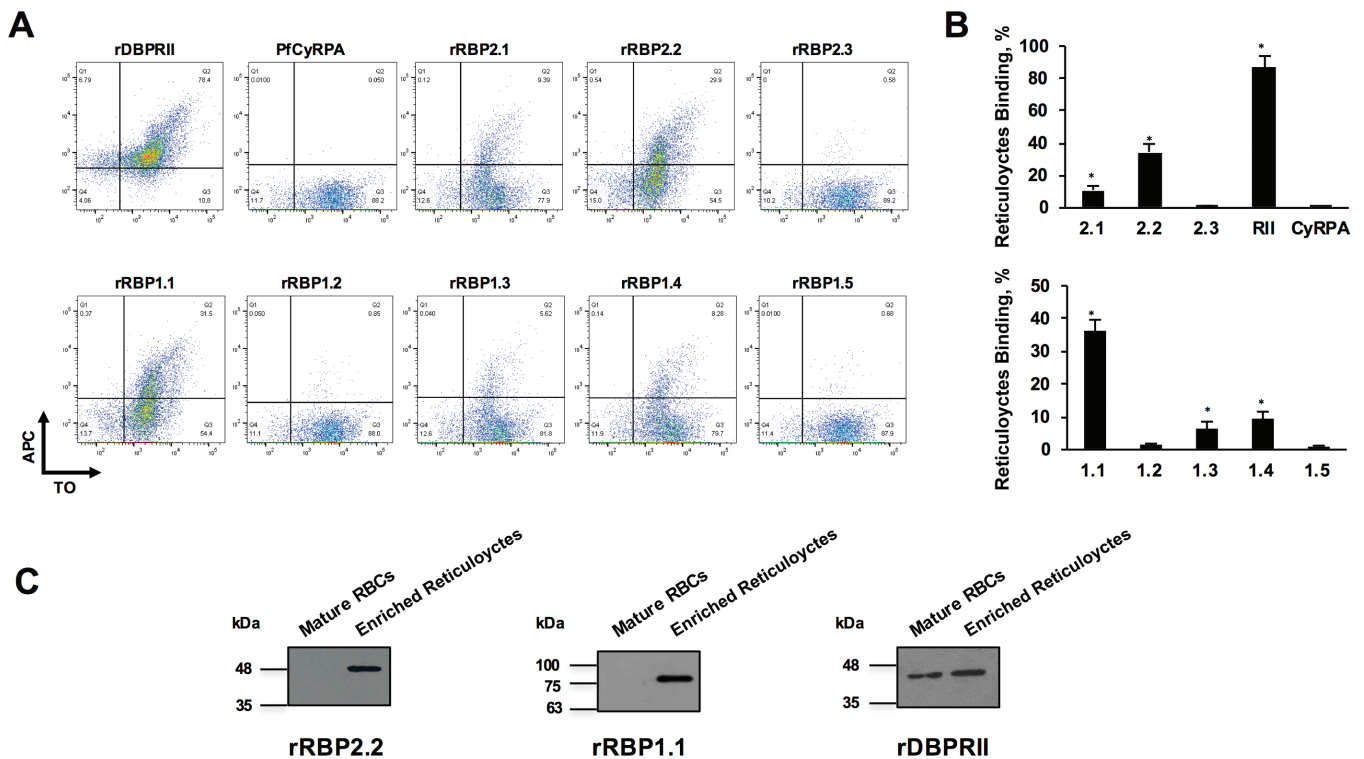
Figure 2. Flow cytometry–based reticulocyte-binding activity of *Plasmodium vivax* reticulocyte-binding protein (PvRBP) 2c and PvRBP1a recombinant fragments. *(A)* Flow cytometry dot plots showing the binding of rRBP2.1, rRBP2.2, rRBP2.3, rDBPRII, and PfCyRPA (*top*) and rRBP1.1, rRBP1.2, rRBP1.3, rRBP1.4, rRBP1.5 (*bottom*) with enriched reticulocytes (thiazole orange [TO] positive). Reticulocyte binding was detected using the antibodies raised against the respective recombinant proteins followed by a secondary allophycocyanin (APC)–conjugated monoclonal antibody. Of the 3 PvRBP2c proteins, reticulocyte binding was observed for rRBP2.2 and rRBP2.1, with no binding observed for rRBP2.3. Of the 5 PvRBP1a protein constructs, reticulocyte binding was observed for rRBP1.1, rRBP1.3, and rRBP1.4, with no binding reported for rRBP1.2 and rRBP1.5. Binding of rDBPRII and PfCyRPA was analyzed as a positive and negative controls, respectively. *B,* Bar charts depicting the percentage reticulocyte binding of rRBP2.1, rRBP2.2, rRBP2.3, rDBPRII, PfCyRPA (*top*) and rRBP1.1, rRBP1.2, rRBP1.3, rRBP1.4, and rRBP1.5 (*bottom*). Error bars represent standard errors of the mean for 3 independent repeats. *$P < .001$. *C,* Binding of recombinant rRBP2.2 and rRBP1.1 was detected only with enriched reticulocytes and not with normal erythrocytes (red blood cells [RBCs]. rDBPRII bound with the same set of normal erythrocytes and enriched reticulocytes. Reticulocytes were enriched by magnetic sorting. Abbreviations: PfCyRPA, *Plasmodium falciparum* cysteine-rich protective antigen; rDBPRII, recombinant Duffy binding protein region II; rRBP, recombinant RBP.

specificity of both recombinant and native parasite proteins with enzymatically treated reticulocytes. rRBP1.1 bound neuraminidase-treated reticulocytes (sialic acid depleted) but failed to bind trypsin- or chymotrypsin-treated reticulocytes, which was detected with FACS (Figure 3A and B) and immunoblotting (Figure 3C). However, rRBP2.2 bound all 3 enzymatically treated reticulocytes (Figure 3A–C). The reticulocyte-binding specificity of native PvRBP2c/PvRBP1a parasite proteins was analyzed from culture supernatants (Figure 3D). Native PvRBP2c and PvRBP1a exhibited binding specificities that appeared to match those of rRBP2.2 and rRBP1.1, respectively (Figure 3A–D). As a control for enzymatic treatments, the binding of PfEBA-175 was analyzed (Figure 3A–D). No protein was detected when PBS was incubated with enriched reticulocytes, confirming no cross-contamination of any reticulocyte protein (Figure 3C and D). Furthermore, no protein was detected when recombinant rRBP2.2/rRBP1.1/rEBA-175RII was incubated with the magnetic beads (Figure 3C).

Reticulocyte Binding Inhibitory Activity of PvRBP Specific Rat Antibodies

To evaluate the binding-inhibitory potential of the PvRBP2c/PvRBP1a specific antibodies against the respective native PvRBPs, we performed a binding assay in which the culture supernatant was incubated with enriched reticulocytes in the presence of purified anti-PvRBP rat antibodies (total immunoglobulin G [IgG]) and the native protein binding was analyzed with FACS (Figure 4). In the presence of the preimmune antibodies (800 µg/mL), 35% reticulocytes were individually bound with either PvRBP2c or PvRBP1a (Figure 4A and B). This reticulocyte-binding efficiency for both native proteins in the presence of anti-PvRBP rat antibodies was significantly reduced, by 74%–76% (400 µg/mL) and 95%–97% (800 µg/mL) (Figure 4A–D). Purified IgG against the non-binders (rRBP2.3, rRBP1.5) did not exhibit any binding inhibition (Figure 4A and B). Thus, antibodies raised against the specific reticulocyte-binding domains of PvRBP2c/PvRBP1a potently neutralized the binding activity of their respective native PvRBP parasite proteins.
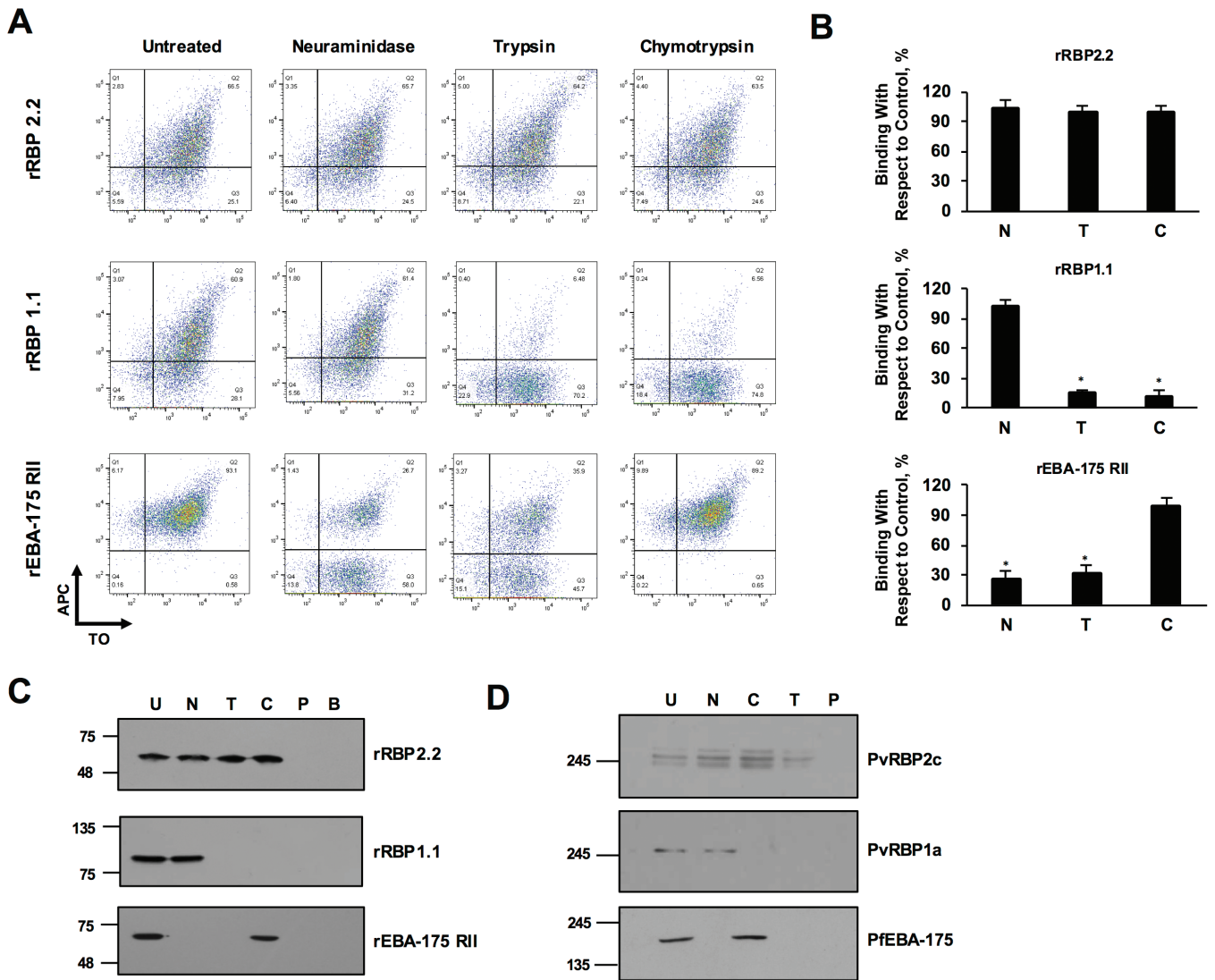
Figure 3. Reticulocyte-binding specificity of rRBP2.2 and rRBP1.1 recombinant proteins. *A,* plots depicting rRBP2.2-binding reticulocytes in a neuraminidase-, chymotrypsin-, and trypsin-resistant manner. rRBP1.1 binding with reticulocytes (thiazole orange [TO] positive) was neuraminidase (N) resistant but sensitive to chymotrypsin (C) and trypsin (T). rEBA-175RII (recombinant receptor–binding domain of PfEBA-175) was analyzed as a control with the same set of enzymatically treated reticulocytes. rPfEBA-175RII bound untreated and chymotrypsin-treated cells but had reduced binding to the neuraminidase- and trypsin-treated cells. APC, allophycocyanin. *B,* Bar charts showing the percentage reticulocyte binding of rRBP2.2, rRBP21.1, and rEBA-175RII. Error bars represent standard errors of the mean for 3 independent repeats. *$P < .001$. *C,* Immunoblots depicting the binding of rRBP2.2, rRBP1.1, and rPfEBA-175RII to enzyme-treated reticulocytes. No protein was detected when cells were incubated with phosphate-buffered saline or when the magnetic beads were incubated with the proteins. B, CD71 magnetic beads control; U, untreated; C, chymotrypsin treated; N, neuraminidase treated; trypsin, P, phosphate-buffered saline control; T, trypsin treated. *D,* Immunoblots depicting the binding of native *Plasmodium vivax* reticulocyte-binding protein (PvRBP) 2c, PvRBP1a, and PfEBA-175 with the enzyme-treated reticulocytes. Reticulocytes were enriched by magnetic sorting. Abbreviations: PfEBA-175, *Plasmodium falciparum* erythrocyte binding antigen 175; rRBP, recombinant RBP.

Detection of Naturally Acquired Humoral Immune Responses Against the PvRBPs

Antibody responses against different PvRBP2c and PvRBP1a recombinant proteins were analyzed among the 66 plasma samples by ELISA (Figure 5). Seven healthy plasma samples from non–malaria-endemic regions with no history of malaria infections were used as controls. High antibody responses against the PvRBP1a/PvRBP2c recombinant proteins were observed among the human plasma samples from endemic regions, compared with the controls (Figure 5),

suggesting that naturally acquired human antibodies were raised against PvRBP2c and PvRBP1a. Human antibody responses against rRBP1.4 and rRBP1.5 were significantly lower than those against rRBP1.1 (Figure 5), suggesting that the entire 749–amino acid N-terminal region of PvRBP1a is immunodominant and exposed to the human immune system during vivax infections. Thus, naturally acquired human antibodies raised against *P. vivax* infections specifically bound our recombinant proteins, confirming their conformational integrity.
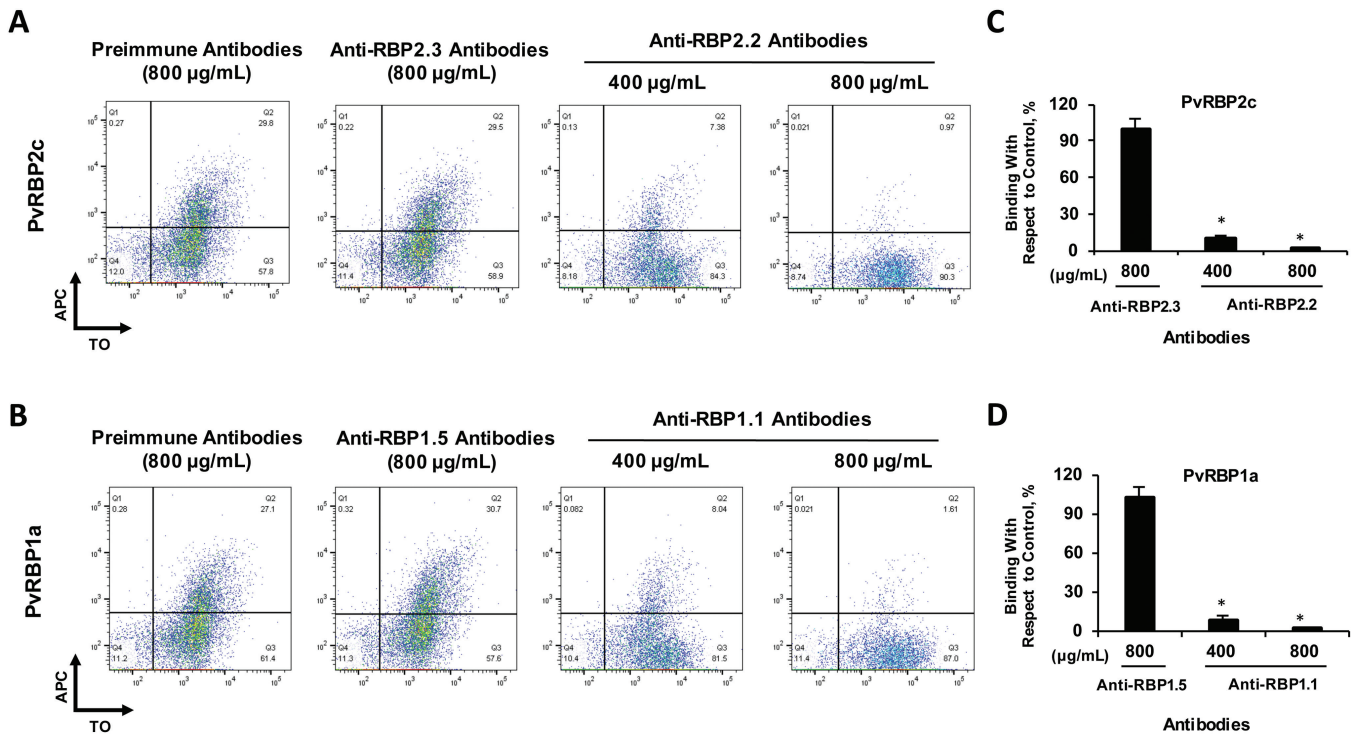
Figure 4. *Plasmodium vivax* reticulocyte-binding protein (PvRBP) rat antibodies block reticulocyte binding of the native PvRBP parasite proteins. Flow cytometry dot plots depicting the inhibition of binding of native PvRBP2c (*A*) and PvRBP1a (*B*) in the presence of the respective anti-rRBP2.2/1.1 total rat immunoglobulin G (IgG; 400 and 800 µg/mL). APC, allophycocyanin; TO, thiazole orange. *C, D,* Bar charts showing the percentage of reticulocyte-binding inhibition of native PvRBP2c (*C*) and PvRBP1a (*D*) by anti-PvRBP IgG compared with preimmune rat IgG. The reticulocyte binding of the native PvRBP2c and PvRBP1a parasite proteins was potently blocked by the rRBP2.2 and rRBP1.1 rat antibodies (total IgG). As controls, reticulocyte binding of native PvRBP2c/PvRBP1a was analyzed in the presence of total rat IgG against the non-binders, rRBP2.3 and rRBP1.5. Anti-rat rRBP2.3/rRBP1.5 IgG failed to block the binding of native PvRBP2c and PvRBP1a proteins. Reticulocytes were enriched by magnetic sorting. *$P < .001$. Abbreviation: rRBP, recombinant RBP.

Binding Inhibitory Activity of Naturally Acquired PvRBP Human Antibodies

Binding inhibition has been reported to correlate with protective malaria immunity [23]. We thus tested the functional ability of affinity-purified human antibodies to inhibit the reticulocyte binding of the native and recombinant PvRBP2c/PvRBP1a proteins.
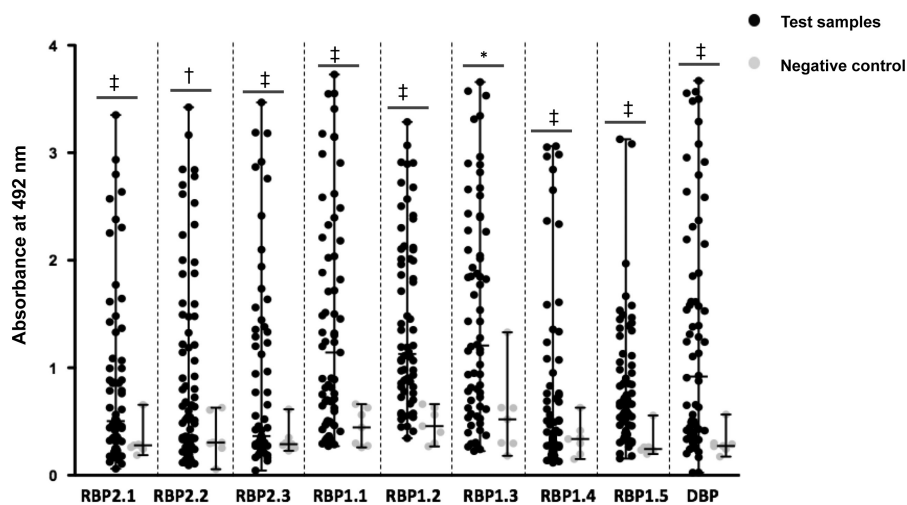

Figure 5. Detection of naturally acquired human antibody responses against *Plasmodium vivax* reticulocyte-binding protein (PvRBP) 2c, and PvRBP1a, and *P. vivax* Duffy-binding protein (PvDBP). Antibody responses were studied among 66 *P. vivax*–infected human plasma samples (dilution 1:200) against 9 recombinant proteins (8 PvRBP and 1 PvDBP). Seven healthy plasma samples from non–malaria-endemic regions with no history of malaria infections were used as controls. Bars indicate medians with ranges. *$P < .05$; †$P < .01$; ‡$P < .001$.
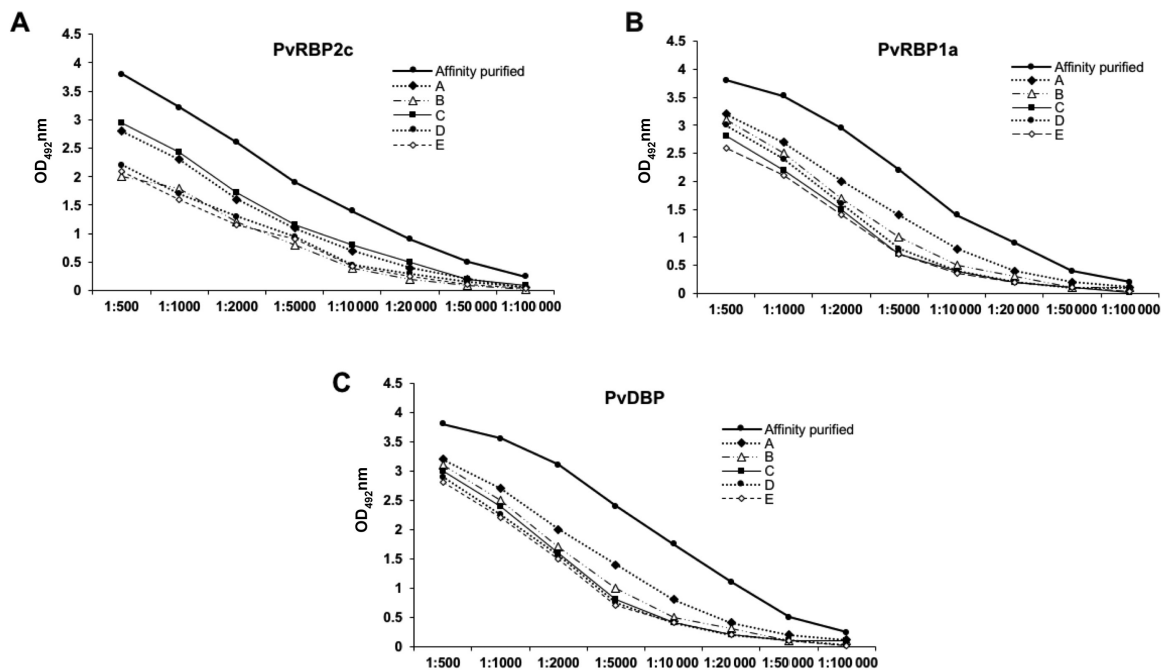
Figure 6. Analysis of the affinity-purified antibodies against *Plasmodium vivax* reticulocyte-binding protein (PvRBP) 2c (*A*), PvRBP1a (*B*), and *P. vivax* Duffy-binding protein (PvDBP) proteins (*C*). The enrichment of antigen-specific immunoglobulin Gs (IgGs) against rRBP2.2, rRBP1.1 and rDBPII were analyzed with enzyme-linked immunosorbent assay (ELISA). The ELISA results are plotted as optical density (OD) at 492 nm (y-axis) to compare concentrations of affinity-purified PvRBP2c (*A*), PvRBP1a (*B*), and PvDBP antibodies *(C)* and the 5 highest responders (plasma samples A–E). Antibody enrichment is reflected by the significantly higher OD values of the antigen-specific IgG in the affinity-purified samples compared with the highest responding individual plasma samples A-E. Abbreviations: DBP, Duffy binding protein; rRBP, recombinant RBP.

Antigen-specific human antibodies against PvRBP2c, PvRBP1a, and PvDBPII were affinity purified from pooled human plasma samples (Figure 6), as reported elsewhere [26, 27]. Antibody enrichment was reflected by the significantly higher optical density values of the antigen-specific, affinity-purified human IgG compared with that of the highest-responding individual plasma samples (Figure 6). The affinity-purified antibodies reacted only with their respective antigens and did not cross-react with the other control antigens (Supplementary Figure 8).

Owing to limitations of affinity-purified human antibodies, we performed the binding inhibition assays against 4 PvRBPs (2 native, 2 recombinant) at only 2 concentrations. PvRBP1.1- and PvRBP2.2-specific human antibodies inhibited the reticulocyte binding of the respective parasite proteins by 72%–80% (100 µg/mL) (Figure 7A and C), which was augmented at 200 µg/mL such that the reticulocyte binding of the native PvRBPs was almost completely inhibited (Figure 7A and C). Similar results were observed for rRBP2.2 and rRBP1.1. The binding inhibition of the recombinant proteins by the human antibodies was 56%–60% at 100 µg/mL, which further increased to 76%–89% at 200 µg/mL (Figure 7B and D). PvDBP-specific human antibodies had no effect on the binding of the PvRBP2c/PvRBP1a parasite proteins (Figure 7A and C) or recombinant rRBP2.2/rRBP1.1 (Figure 7B and D). Thus, our results clearly demonstrate that affinity-purified anti-PvRBP2c and anti-PvRBP1a human antibodies exhibited a specific binding-inhibitory activity.

DISCUSSION

P. vivax merozoite invasion was believed to be completely dependent on the essential PvDBP-DARC molecular interaction; thus, vivax vaccine development efforts have focused on PvDBP. However, reports of vivax infections in Duffy-null individuals [12–14], which could not be attributed to PvDBP polymorphisms [32], suggest the evolution of alternate invasion pathways. The significance of PvRBPs has risen as potential mediators of these alternate invasion pathways [14, 32]. Thus, it is evident that *P. vivax* neutralization would require targeting multiple antigens, including PvDBP and PvRBPs. Precise inhibitory targets within the PvRBP antigens remain unknown.

The discovery of PvRBPs (PvRBP1a/PvRBP2c) provided an important lead toward understanding the molecular mechanisms underlying *P. vivax* reticulocyte invasion [10]. Owing to the lack of *P. vivax* in vitro culture, it has been challenging to screen the vaccine potential of vivax antigens, including PvRBPs. In this regard, binding-inhibitory PvDBP antibodies have been reported to be associated with malaria protection [23]. Therefore, binding inhibition (ie, the ability of antibodies to block the reticulocyte binding of key *P. vivax* adhesins) is of prime significance for assessing the vaccine potential of vivax antigens.

Our study examines the functional ability of naturally acquired human antibodies against the receptor-binding domains of the PvRBPs to inhibit the specific reticulocyte
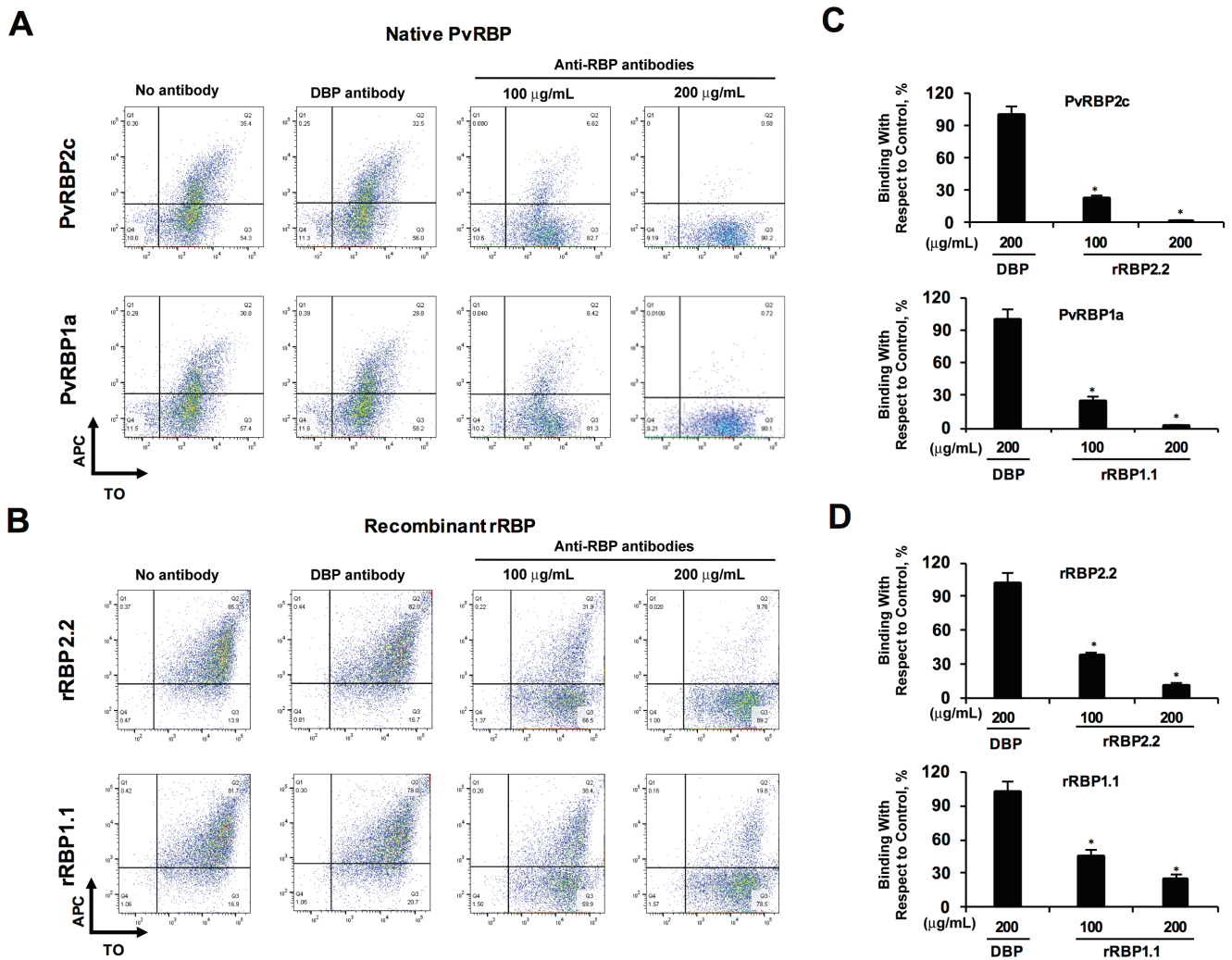
Figure 7. Purified *Plasmodium vivax* reticulocyte-binding protein (PvRBP)–specific human antibodies block reticulocyte binding of the native and recombinant PvRBPs. *A, B,* Flow cytometry dot plots depicting the inhibition of binding of native PvRBP parasite (*A*) proteins and rRBP2.2/rRBP1.1 (*B*) proteins in the presence of the respective anti-PvRBP2c/anti-PvRBP1a human immunoglobulin G (IgG; 100 and 200 μg/mL, respectively). APC, allophycocyanin; TO, thiazole orange. *C, D,* Bar charts showing the percentage inhibition of reticulocyte binding of native PvRBPs (*C*) and rRBPs (*D*) by the anti-PvRBP/*P. vivax* Duffy-binding protein (PvDBP) IgG compared with binding in the absence of IgG. The reticulocyte binding of the native PvRBP2c/PvRBP1a and recombinant rRBP2.2/rRBP1.1 were potently blocked by the respective PvRBP2c/PvRBP1a-specific human IgGs. As controls, reticulocyte binding of PvRBP2c/PvRBP1a was analyzed in the presence of PvDBP-specific purified human antibodies. Anti-PvDBP human IgG failed to block the reticulocyte binding of native PvRBP2c and PvRBP1a proteins. Reticulocytes were enriched by magnetic sorting. *$P < .001$. Abbreviation: rRBP, recombinant RBP.

binding of the native parasite proteins, PvRBP2c and PvRBP1a [10]. We have demonstrated the receptor-binding specificity of the native PvRBP2c and PvRBP1a parasite proteins, which was sialic acid independent. However, trypsin/chymotrypsin abolished PvRBP1a binding with no effect on PvRBP2c binding, suggesting that only the PvRBP1a receptor was trypsin/chymotrypsin sensitive. Thus, the distinct binding specificity of the PvRBPs suggests that 2 binding sites, on the same or on different receptors, may be involved in reticulocyte invasion. The identity of the reticulocyte-specific receptors remains a major gap in vivax biology.

We have mapped the receptor-binding domains of both PvRBP2c (rRBP2.2) and PvRBP1a (rRBP1.1), which exhibit potent reticulocyte-binding activity with a specificity that appears to match that of native PvRBPs. We compared 3 PvRBP2c constructs and demonstrated that rRBP2.2 (residues 464–876) exhibited the most potent reticulocyte-binding activity. This is the first report for the identification of the reticulocyte-binding domain of PvRBP2c. A study reported last year noted the binding activity of several PvRBP recombinant proteins with no direct data on PvRBP2c [35]. The percentage of reticulocyte binding reported for the recombinant constructs was modestly low, with no correlation shown with the binding of native PvRBPs [35]. Similarly, we analyzed 5 PvRBP1a constructs and showed that rRBP1.1 (residues 30–778) exhibited the highest reticulocyte-binding activity. Also last year, a

248–amino acid region of PvRBP1a/1b homologous with the PfRH4 binding region was reported to bind reticulocytes [36], which is consistent with our results. However, we show that the binding of this 248–amino acid stretch was significantly weaker than that of the 749–amino acid rRBP1.1 construct, which suggests that the larger region favors a stronger interaction with its receptor.

Previous reports produced recombinant reticulocyte-binding proteins with N-terminal His-tags [35, 36], whereas our study has produced C-terminal His-tag proteins. It is plausible that the different His-tag locations may influence binding efficiency or specificity. To address this possibility, we demonstrated that the recombinant rRBP2.2, rRBP1.1 bound reticulocytes with a specificity that appears similar to that of native PvRBPs in their monomeric form. Native PvRBP1a/PvRBP2c have been suggested to be part of homogeneous or heterogeneous protein complexes [10, 37]. Thus, further studies are crucial to investigate the potential protein-protein interactions involving PvRBP2c/PvRBP1a and their effect on the reticulocyte-binding specificity during merozoite invasion.

Importantly, we demonstrated that specific human antibodies against the reticulocyte-binding domains of the 2 PvRBPs raised through natural *P. vivax* exposure efficiently block the binding of native parasite proteins. To the best of our knowledge, ours is the first report demonstrating the functional binding-inhibitory activity of naturally acquired human antibodies targeting any PvRBP. Binding-inhibitory antibodies suggest that the recombinant proteins had a structure resembling the native 3-D configuration, in which the critical binding epitopes were exposed in the correct conformation. PvRBP1a/PvRBP2c elicited strong humoral responses among humans residing in endemic areas, consistent with findings of previous studies [35, 36, 38]. Significantly, affinity-purified naturally acquired PvRBP2c/PvRBP1a-specific human antibodies potently inhibited the reticulocyte-binding activity of the respective native proteins.

We have thus demonstrated that the human immune system generates functional binding-inhibitory antibodies against the reticulocyte-binding domains of the PvRBPs, which, like PvDBP antibodies [23], may have the potential to neutralize *P. vivax*. Therefore, PvRBPs seem to be promising candidate antigens for vivax vaccines. Importantly, PvRBPs are immunogenic in humans, suggesting that immunity induced by a PvRBP-based vaccine could further be boosted through natural exposure, potentially leading to an augmentation in immunity and protection against vivax malaria.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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