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Inhibitory Humoral Responses to the *Plasmodium falciparum* Vaccine Candidate EBA-175 Are Independent of the Erythrocyte Invasion Pathway

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***Plasmodium falciparum* utilizes multiple ligand-receptor interactions for invasion. The invasion ligand EBA-175 is being developed as a major blood-stage vaccine candidate. EBA-175 mediates parasite invasion of host erythrocytes in a sialic acid-dependent manner through its binding to the erythrocyte receptor glycophorin A. In this study, we addressed the ability of naturally acquired human antibodies against the EBA-175 RII erythrocyte-binding domain to inhibit parasite invasion of *ex vivo* isolates, in relationship to the sialic acid dependence of these parasites. We have determined the presence of antibodies to the EBA-175 RII domain by enzyme-linked immunosorbent assay (ELISA) in individuals from areas of Senegal where malaria is endemic with high and low transmission. Using affinity-purified human antibodies to the EBA-175 RII domain from pooled patient plasma, we have measured the invasion pathway as well as the invasion inhibition of clinical isolates from Senegalese patients in *ex vivo* assays. Our results suggest that naturally acquired anti-EBA-175 RII antibodies significantly inhibit invasion of Senegalese parasites and that these responses can be significantly enhanced through limiting other ligand-receptor interactions. However, the extent of this functional inhibition by EBA-175 antibodies is not associated with the sialic acid dependence of the parasite strain, suggesting that erythrocyte invasion pathway usage by parasite strains is not driven by antibodies targeting the EBA-175/glycophorin A interaction. This work has implications for vaccine design based on the RII domain of EBA-175 in the context of alternative invasion pathways.**

Erythrocyte (RBC) invasion is an essential step of the *Plasmodium falciparum* life cycle involving multiple specific interactions between parasite ligands and erythrocyte receptors, termed invasion pathways. *Plasmodium falciparum* uses different invasion pathways to invade human erythrocytes, relying on two primary families of invasion ligands: the erythrocyte binding antigen (EBA) family and the reticulocyte binding protein homolog (PfRH) family (1–3).

EBA-175 is located in the apical micronemes of merozoites and mediates parasite invasion of host erythrocytes in a sialic acid-dependent manner (4, 5) EBA-175 is divided into several regions, annotated I to VII; region II of the protein (RII) has a cysteine-rich motif that is also present in the Duffy-binding proteins of *Plasmodium vivax* and *Plasmodium knowlesi* (6, 7). EBA-175 RII has two subdomains, F1 and F2. The F2 domain has been shown biochemically to bind to red blood cells (8, 9); this binding is dependent on sialic acid on glycophorin A (Gly A) (4, 5). The crystal structure of EBA-175 RII has confirmed both the requirement for sialic acid and the necessary dimerization of glycophorin A (10). In addition to the RII binding domain, there is a large dimorphic domain in region III known as the F/C segment (containing the F and C segments [F-seg and C-seg]). The RII and the dimorphic F-seg, and C-seg domains of EBA-175 have been shown to be under diversifying selection by the human immune response in global populations (11–13). Previous studies have shown that antibodies recognize all of these domains (14), although the functional impact of these human antibodies on invasion is unknown.

The EBA-175/glycophin A pathway is one of the dominant invasion pathways used by *P. falciparum* parasites to invade the red blood cells in a sialic acid-dependent fashion (4, 5). Genetic disruption of EBA-175 results in a change in invasion pathway for sialic acid-dependent parasite strains (15).

Several studies have shown that a humoral response against EBA-175 is generated in subjects living in areas of endemicity (13, 14, 16–22). Some studies have reported that antibodies against EBA-175 domains correlate with protection from symptomatic malaria but not reinfection (22), and others show marginal, but not significant, protection (14). While antibodies induced in experimental animals against EBA-175 RII have invasion-inhibitory activity *in vitro* (17, 23, 24), few studies have measured EBA-175-based protection against clinical malaria in humans.

The RII binding domain of EBA-175 is currently being pursued as a vaccine candidate antigen (25, 26) because of its high level of sequence conservation, its expression among laboratory and patient parasite isolates (8, 27), and the observation that there is an age-dependent acquisition of antibodies in endemic populations

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(14, 20). In animal studies, the EBA-175 RII vaccine was shown to be safe and immunogenic, producing antibodies that inhibit invasion, with protection of 1 of 3 vaccinated *Aotus* monkeys from disease (26). It has been observed that antibodies raised against EBA-175 RII in rabbits inhibit invasion regardless of the invasion pathway utilized (23). Prior experiments show that total IgG acquired by malaria-exposed individuals has the ability to inhibit erythrocyte invasion in an invasion pathway-dependent manner (19). In this study, we demonstrated that antibodies against EBA-175 RII from naturally exposed humans can inhibit invasion by *P. falciparum* clinical isolates and analyzed the dependence of inhibition on the invasion pathway.

MATERIALS AND METHODS

Study sites and samples. Approval for this study was granted by the Institutional Review Board of the Harvard School of Public Health and by the Ethics Committee of the Ministry of Health in Senegal. Whole blood was collected in EDTA Vacutainers (for separation of plasma) from Senegalese consenting patients with uncomplicated malaria during the transmission season (September to December 2004 and 2005 in Velingara and in the years 2009 to 2011 in Thies). Individual patient plasma samples were collected from Thies ($n = 133$), an area of low endemicity in Senegal located 70 km from Dakar (entomological inoculation rate [EIR] = 1 to 10), and Velingara ($n = 94$), a region of hyperendemicity situated in the southeast, 570 km from Dakar (EIR > 100). In Thies, the median patient age is 21 years, whereas in Velingara, the median patient age is 7 years. In Velingara, 38% of the patients were female and 62% of the patients were male, whereas in Thies, 34% of the patients were female and 66% of the patients were male. Plasma from 75 unexposed individuals living in an area of nonendemicity (Boston, MA) were collected as negative controls.

***Plasmodium falciparum* patient isolates.** Five milliliters of parasitized blood was collected in EDTA Vacutainers from patients with uncomplicated malaria from Thies during the transmission seasons (October to November) from 2009 to 2011. Patients with a parasitemia level of ≥0.7% were included in invasion and inhibition experiments. The diagnosis of malaria was done by thick smear and rapid diagnosis test (RDT), and parasitemia was assessed by thin smear counted using a Miller reticle. Parasitized cells were washed 3 times with unsupplemented RPMI medium prior to assay plating.

Recombinant EBA-175 RII protein. Recombinant RII was produced by oxidative refolding optimized for Duffy-binding-like (DBL) domains as has been described for other DBL domain proteins (28–32). Briefly, amino acids 145 to 761 (RII) from EBA-175 strain 3D7 were expressed in *E. coli* BL21 cells using a pET-28 vector. Denatured protein was extracted from inclusion bodies using 6 M guanidine hydrochloride, 50 mM Tris (pH 8), 100 mM NaCl, and 5 mM dithiothreitol (DTT) and refolded via rapid dilution in 400 mM L-arginine, 50 mM Tris (pH 8.0), 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM reduced glutathione, and 2 mM oxidized glutathione. The refolded RII was concentrated using Amicon concentrators and then purified by ion-exchange chromatography using a Mono S column followed by gel filtration chromatography using a Superdex 200 16/60 column.

ELISA. Individual patient plasma and pooled Velingara plasma and affinity purification fractions were tested for the presence of antibodies against EBA-175 RII by enzyme-linked immunosorbent assay (ELISA). The recombinant proteins were coated in 96-well plates (Dynex Technologies; Immulon 1B) in phosphate-buffered saline (PBS) and incubated overnight at 4°C. The plates were washed three times with PBS plus Tween 20 (PBST), then blocked with 1% milk in PBST, and incubated at room temperature for 2 h. Plates were washed 3 times with PBST, individual Senegalese sample plasma was added to the plates in duplicate (1/800), and plates were again incubated at room temperature for 2 h. Plates were washed 3 times with PBST before a 1/8,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG secondary antibody (Southern Biotech) was added, and plates were incubated at room temperature for 2 h. Plates were washed 5 times with PBST, and 100 μl of Sureblue TMB substrate (KPL) was added to each well and allowed to develop for 10 min; the reaction were stopped by adding an equal volume of 1 N filtered HCl in the same room as the plate reader to avoid delay causing precipitation. The optical densities (ODs) were read at 450 nm on an ELISA plate reader. Positive cutoffs for the RII recombinant protein were determined as an OD of 3 times the standard deviation of the mean for 72 plasma samples from unexposed individuals from Boston.

Affinity-purified antibodies from pooled patient plasma. A total of 200 pooled patient plasma from an area of high endemicity (Velingara) were used to affinity purify the antibodies to RII protein coupled to CNBr Sepharose (Sigma). Column chromatography was performed on an AktaFPLC (GE Healthcare) using standard techniques. Briefly, pooled plasma was diluted 1:10 with PBS and loaded onto the affinity column at 1 ml/min. The column was washed with PBS until a stable A_{280} baseline was reached. Antibodies were eluted with 100 mM glycine (pH 2.5) in 500-μl fractions in wells containing 50 μl of 1 M Tris HCl (pH 8.0) to limit the amount of time the antibodies were exposed to low pH. Fractions containing the peaks were pooled, dialyzed into 1× PBS, and concentrated. Antibodies to EBA-175 recombinant RII protein were tested for enrichment and specificity by ELISA.

Invasion assay. The invasion assay was carried as previously described (15). Infected erythrocytes were incubated with enzyme-treated RBCs in a final volume of 50 μl at 2% hematocrit for approximately 48 h (or until reinvasion as assessed by microscopy). The erythrocytes were treated with the following: neuraminidase (66.7 mU/ml; Roche), trypsin (1.0 mg/ml; Sigma), chymotrypsin (1.0 mg/ml; Worthington), low trypsin (66.7 μg/ml)-chymotrypsin, neuraminidase-trypsin-chymotrypsin (NTC; negative control), and RPMI medium only (positive control). Upon reinvasion, assays were analyzed by both microscopy and staining with SYBR green I (Invitrogen), as previously described (33). Flow data were analyzed using FlowJo 8.8.6 (Tree Star). Inclusion criteria for further analysis include a parasite multiplication rate (PMR) of >1 and invasion in positive-control samples (RPMI medium) >2-fold higher than in the negative control (NTC). Invasion for each enzyme treatment is displayed as a percentage of invasion of RPMI medium-treated cells.

Invasion inhibition assay. Affinity-purified antibodies were incubated with clinical isolates at a final concentration of 10 to 50 μg/ml in one round of invasion inhibition assays, and invasion was measured by flow cytometry. The starting parasitemia was 0.7% to 1%. Untreated red blood cells or chymotrypsin-treated red blood cells at 4% hematocrit were incubated with infected erythrocytes to obtain a parasitemia of 0.3 to 0.5% in 25 μl per well, in duplicate. Parasites were incubated for 48 h (or until reinvasion as assessed by microscopic examination of each assay). Invasion (or inhibition) is displayed as percentage of invasion (or inhibition) of Boston unexposed plasma.

Statistical analysis. Comparison of EBA-175 RII titers by age group and RII inhibition stratified by invasion pathway were assessed using a Mann-Whitney U test. Comparisons between EBA-175 RII positivity by ELISA and age group were assessed using the Fisher exact test. Comparisons between inhibition into RPMI medium-treated cells and chymotrypsin-treated cells were conducted using the Mann-Whitney U test. Comparison of inhibition of invasion into RPMI medium-treated cells versus receptor-restricted erythrocytes was conducted using a Wilcoxon ranked-pair test. Statistical analyses were performed using GraphPad Prism 5 software.

RESULTS

Immune recognition of EBA-175 domains in different regions of endemicity in Senegal. We determined the immune reactivity to different domains of EBA-175 by measuring levels in plasma of IgG to region II (RII) (Fig. 1A). Total IgG reactivity was determined for 133 plasma samples from Thies and 94 from Velingara and was found to be 35% positive in both sites overall. As the

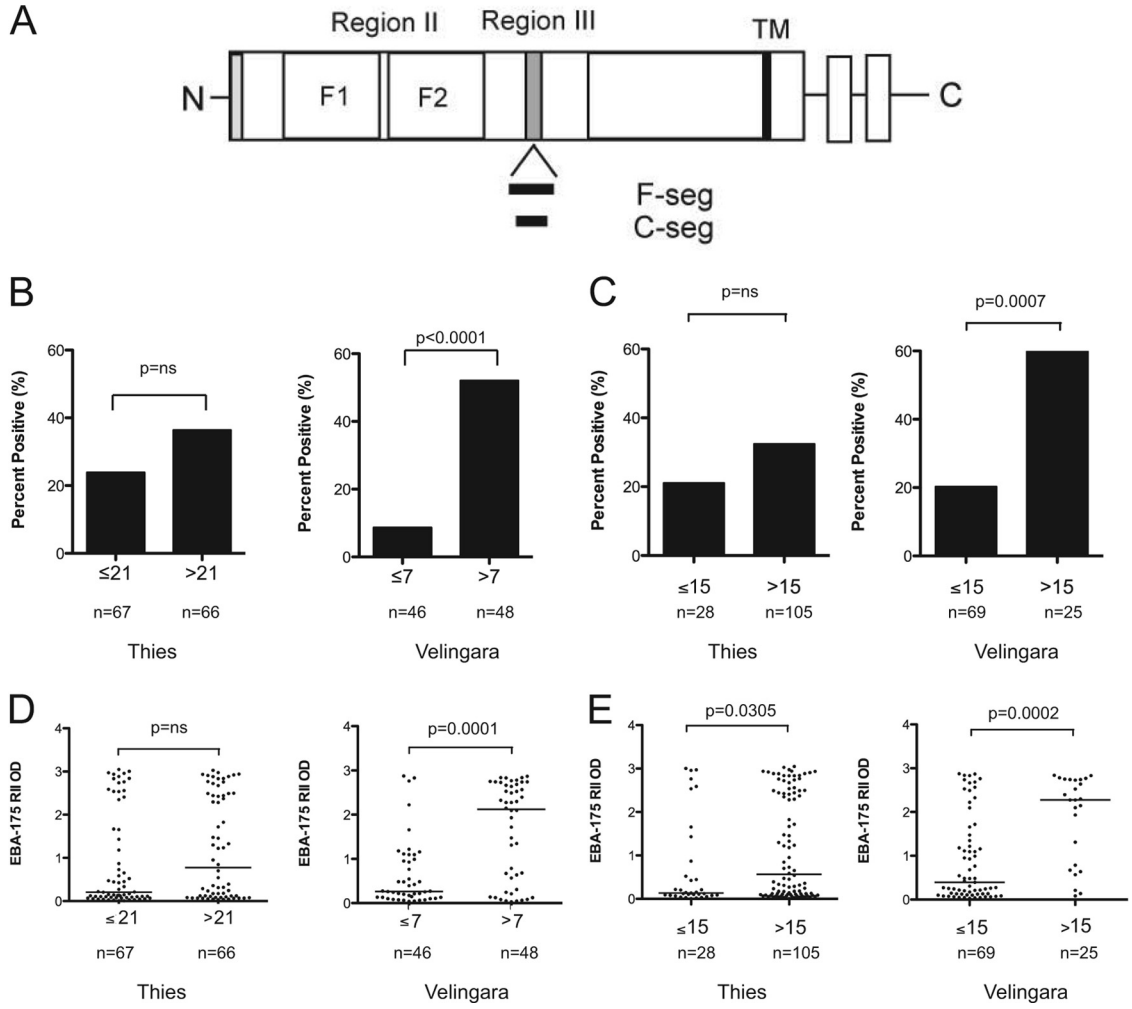

FIG 1 IgG response to EBA-175 RII increases with age in Senegal. (A) Schematic of EBA-175 illustrating the RII domain of interest. TM, transmembrane domain; F-seg, F segment; C-seg, C segment. (B) IgG responses measured by ELISA in Thies ($n = 133$), where the median age in the study population was 21 years, and Velingara ($n = 94$), where the median age in the study population was 7 years. Positive RII responses are not correlated with age in Thies but are positively correlated with age in Velingara. ns, not significant. (C) IgG responses measured by ELISA in Thies and Velingara, stratified by the intermediate age group of 15 years. Positive RII responses are not correlated with age in Thies but are positively correlated with age in Velingara. *P* values for the Fisher exact test associating ELISA positivity with age are shown in panels B and C. (D) IgG levels (OD) were measured by ELISA in Thies and Velingara and stratified by region-specific age group (21 years in Thies and 7 years in Velingara). ELISA OD significantly increased with age in Velingara but not in Thies (D). (E) IgG levels (OD) measured by ELISA in Thies and Velingara, stratified by the intermediate age group of 15 years. Positive RII responses are correlated with age in both Thies and in Velingara. *P* values displayed were derived from Mann-Whitney U tests for panels D and E. Median ELISA titers are shown.

endemicities at these sites are dramatically different, we assessed the age-dependent acquisition of anti-RII antibodies in a site-dependent manner (Fig. 1B and D). When associating EBA-175 RII positivity with age group, positivity was not significantly correlated with age in Thies (Fisher exact test, $P = 0.133$), whereas positivity was associated with increased age in Velingara (Fisher exact test, $P < 0.0001$) (Fig. 1B). Similarly, titers of antibody to EBA-175 RII did not increase significantly with age in Thies (Mann-Whitney U test, $P = 0.0894$), whereas an increase in titer with age was significant in Velingara (Mann-Whitney U test, $P = 0.0001$) (Fig. 1D). We further compared the two areas of endemicity using the same age group for both sites. Because of the low number of young children among the patients from Thies, we used the intermediate cutoff of 15 years of age; we observed similar trends. When associating EBA-175 RII positivity with age group, positivity was not significantly correlated with age in Thies (Fisher exact test, $P = 0.3547$), whereas positivity was associated with increased age in Velingara (Fisher exact test, $P = 0.0007$) (Fig. 1C). Similarly, titers of antibody to EBA-175 RII marginally increased with age in Thies (Mann-Whitney U test, $P = 0.0305$), whereas a significant increase in titer with age was observed in Velingara (Mann-Whitney U test, $P = 0.0002$) (Fig. 1E). Together, these data demonstrate the presence of antibodies to EBA-175 domains in the Senegalese population that are acquired in an age-dependent manner.

Inhibition of invasion for lab and field isolates with RII antibodies. We next addressed the functional activity of these antibodies in inhibiting *P. falciparum* invasion. We affinity purified immunoglobulin against the RII domain from 200 pooled plasma samples from Velingara, a region of hyperendemicity, that showed increased RII reactivity by ELISA (Fig. 2A). These affinity-purified antibodies show 1,000-fold enrichment of RII-specific IgG rela-

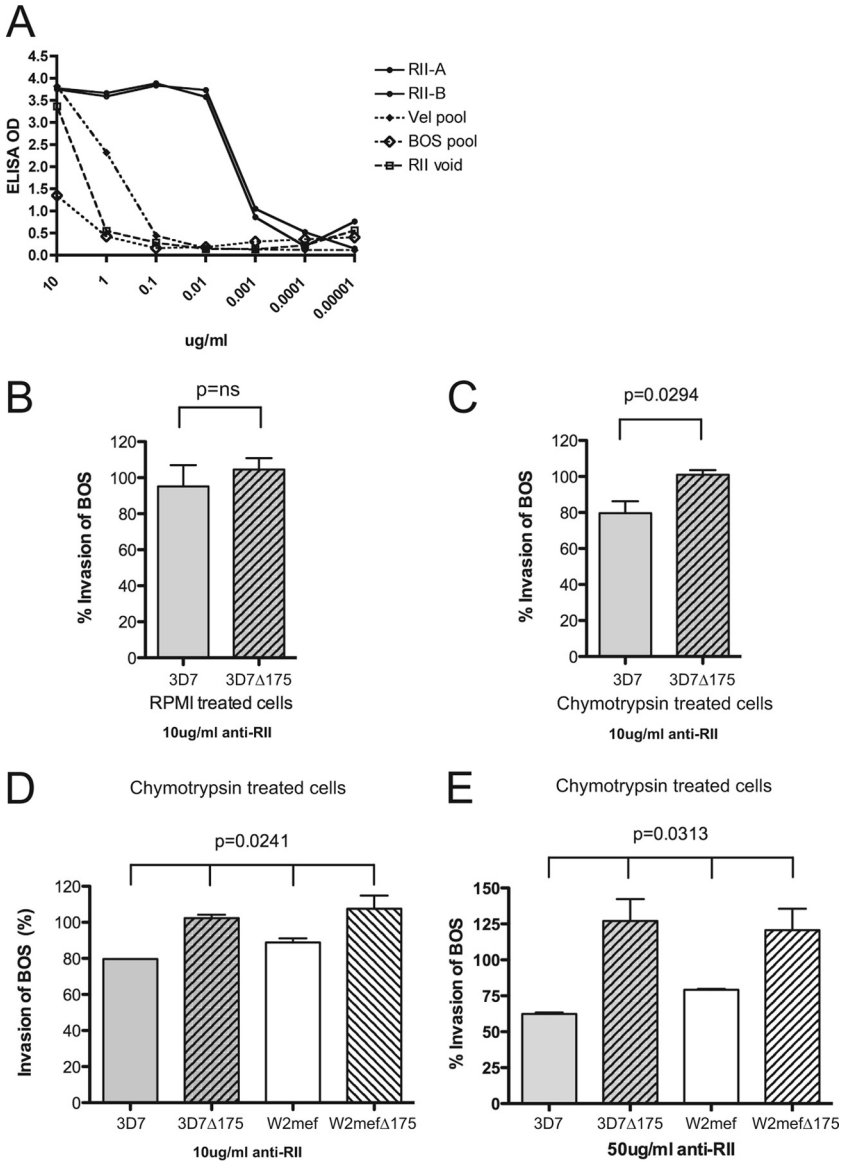

FIG 2 Receptor restriction increases inhibition of EBA-175-expressing parasites by human anti-RII antibodies. (A) Fold enrichment of affinity-purified human antibodies to EBA-175 RII. RII-A and RII-B are two different purifications of the same pool from Velingara patients (Vel Pool). BOS pool represents 75 unexposed plasma samples from Boston, pooled. RII void represents the flowthrough (IgG depleted of RII antibodies by affinity purification). At 10 μg/ml, inhibition levels of 3D7 and 3D7 EBA-175 knockout parasites were equivalent when measured in RPMI medium-treated cells (B); however, a significant increase in inhibition was observed when receptors were restricted by chymotrypsin (C). Inhibition of 3D7 and W2mef parasites (but not EBA-175 knockouts) was observed in chymotrypsin-treated (receptor-restricted) erythrocytes at the physiological concentrations of 10 μg/ml (D) and 50 μg/ml (E). Error bars correspond to median invasion and interquartile range.

tive to the Velingara pool as measured by ELISA (Fig. 2A). We used these affinity-purified antibodies to test invasion inhibition of laboratory isolates that utilize alternative invasion pathways (3D7, sialic acid independent; W2mef, sialic acid dependent). We tested the specificity of inhibition using EBA-175 knockout parasite lines as controls (3D7Δ175 and W2mefΔ175). To more closely investigate the role of EBA-175, we also performed invasion assays using red blood cells treated with chymotrypsin, which cleaves many receptors but does not cleave the EBA-175 receptor, glycophorin A. We observed minimal inhibition of invasion into RPMI medium-treated red blood cells for wild-type 3D7 and EBA-175 knockout lines (Fig. 2B). Treatment with chymotrypsin revealed significant inhibition of wild-type 3D7 but not the EBA-175 knockout (Fig. 2C), demonstrating the specificity of the affinity-purified RII antibodies. We performed these experiments with receptor-restricted cells at the physiological concentrations of 10 μg/ml and 50 μg/ml of RII antibody (Fig. 2D and E). At these concentrations, W2mef and 3D7 were equally inhibited, despite alternative invasion pathway utilization, as has been observed by others (23), and inhibition increased with antibody concentration (Fig. 2D and E). These data show inhibition specific to RII but not necessarily dependent on invasion pathway utilization in laboratory isolates. We tested the inhibitory re-

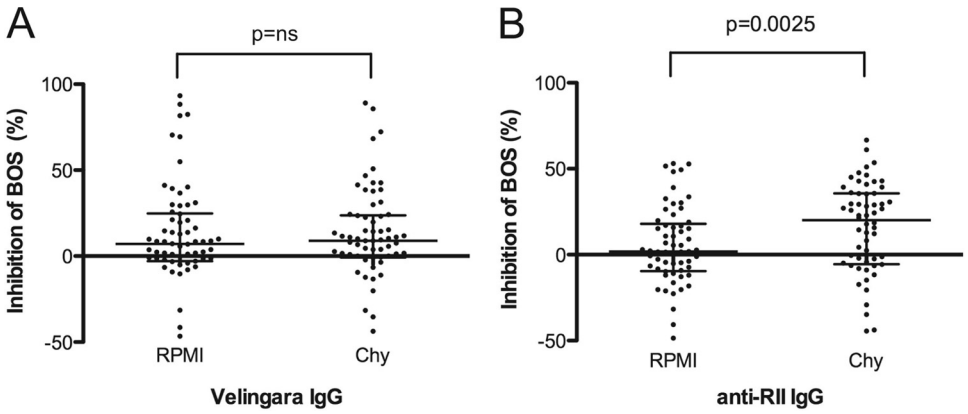

FIG 3 Receptor restriction increases inhibition of *ex vivo* Senegalese isolates by human anti-RII antibodies but not total IgG. While limiting the receptor repertoire by treatment with chymotrypsin does not affect the inhibition of total Velingara immunoglobulin (A), it does increase the inhibition with anti-RII human affinity-purified antibodies (B). Wilcoxon ranked-pair tests were performed with a significance level of 0.05. Error bars correspond to median invasion and interquartile range.

sponse to affinity-purified RII antibodies in a larger number of clinical isolates using the validated concentration of 10 μg/ml.

Association between invasion pathway utilization and RII inhibition. To determine the role of the invasion pathway in inhibition with RII antibodies, we expanded our analyses to study inhibition in a large number ($n = 66$) of *ex vivo* Senegalese parasite isolates spanning three transmission seasons (2009 to 2011). During the 3 years of collection, 66 patient samples met our inclusion criteria (see Materials and Methods): 27 samples in 2009, 22 samples in 2010, and 17 samples in 2011.

Invasion inhibition assays were performed with RPMI medium-treated red blood cells and chymotrypsin-treated cells at a 10-μg/ml concentration of affinity purified anti-RII antibodies. We observed variation in inhibition of the pooled Velingara immunoglobulin; however, this inhibition did not significantly increase when erythrocytes were treated with chymotrypsin (Wilcoxon matched-pair test, $P = 0.3815$) (Fig. 3A). In contrast, while inhibition with affinity-purified RII antibodies was observed with both RPMI medium- and chymotrypsin-treated cells, significantly higher inhibition was observed with chymotrypsin-treated cells (Wilcoxon matched-pair test, $P = 0.0025$) (Fig. 3B). We addressed whether this variation could be due to alternative invasion pathway utilization by measuring relative utilization of invasion pathway by enzymatic treatment and associating the levels of invasion with the levels of anti-RII inhibition (Fig. 4).

We observed variation in invasion pathways for *ex vivo P. falciparum* isolates from Thies, as we have previously reported (34, 35) (Fig. 4A). Interestingly, we saw no significant differences in invasion pathway utilization year to year (Kruskal-Wallis: neuraminidase, $P = 0.5837$; chymotrypsin, $P = 0.3205$), implying that invasion pathways in a given region may be stable over time (Fig. 4B). Based on the invasion pathway data, 61% ($n = 40$) of the strains were sialic acid dependent as defined by invasion of less than 50% into neuraminidase-treated erythrocytes, measured by microscopy. When we compared RII inhibition of sialic acid-dependent versus independent strains (using the 50% cutoff), we observed no difference in the median level of RII inhibition (Mann-Whitney U test, $P = 0.3338$) (Fig. 4C). Similarly, we observed no difference in the median level of RII inhibition of chymotrypsin-sensitive versus chymotrypsin-resistant strains (using the 50% cutoff) (Mann-Whitney U test, $P = 0.6677$) (Fig. 4D). To determine whether there is a difference in dependent versus independent isolates, we divided the data into quartiles and assessed the 25th percentile (dependent) and the 75th percentile (independent) for their levels of RII inhibition. With both RPMI medium- and chymotrypsin-treated cells, we observed no significant difference in the level of RII inhibition (as determined by the Mann-Whitney U test) (data not shown).

Association between antibody reactivity and RII inhibition. We assessed individual patient RII titers correlation with either invasion pathway utilization or RII inhibition by measuring RII reactivity by ELISA (Fig. 1D and E) and comparing ELISA OD with percent invasion and percent RII inhibition for patients from Thies for which we had both invasion and inhibition data. There was no significant association by Spearman rank test for either RII titer and invasion pathway (neuraminidase or chymotrpysin) or RII titer and RII inhibition (data not shown).

DISCUSSION

In this study, we found that antibodies against EBA-175 are acquired in areas of both low endemicity and hyperendemicity and that IgG antibodies to EBA-175 RII are acquired in an age-dependent manner. In Thies, the majority of the patients are adults with a median age of 21 years, while in Velingara, the median age is 7 years. In Velingara, the antibody acquisition occurs at an earlier age and is more dramatic than in Thies, which is what would be expected given the intensity of exposure (Veligara EIR = 100; Thies EIR < 10).

Despite the difference in endemicities, the level of EBA-175 ELISA positivity observed in Senegal (35%) is different from what has been reported by others (14, 20). In fact, in a study done in an area of holoendemicity in Kenya, the antibody positivity against the RII domain was high 98.7% (20), whereas in Gambian children and Nigerian adults, the positivities were found to be 43% and 70%, respectively (14). Our results show much lower antibody prevalence for RII in both sites (35%), despite the difference in endemicities. Our results could be explained by the decreasing malaria pressure in Senegal or differences in study populations, as our patients had uncomplicated malaria at the time plasma antibodies were isolated. Occasionally, differences in apparent anti-

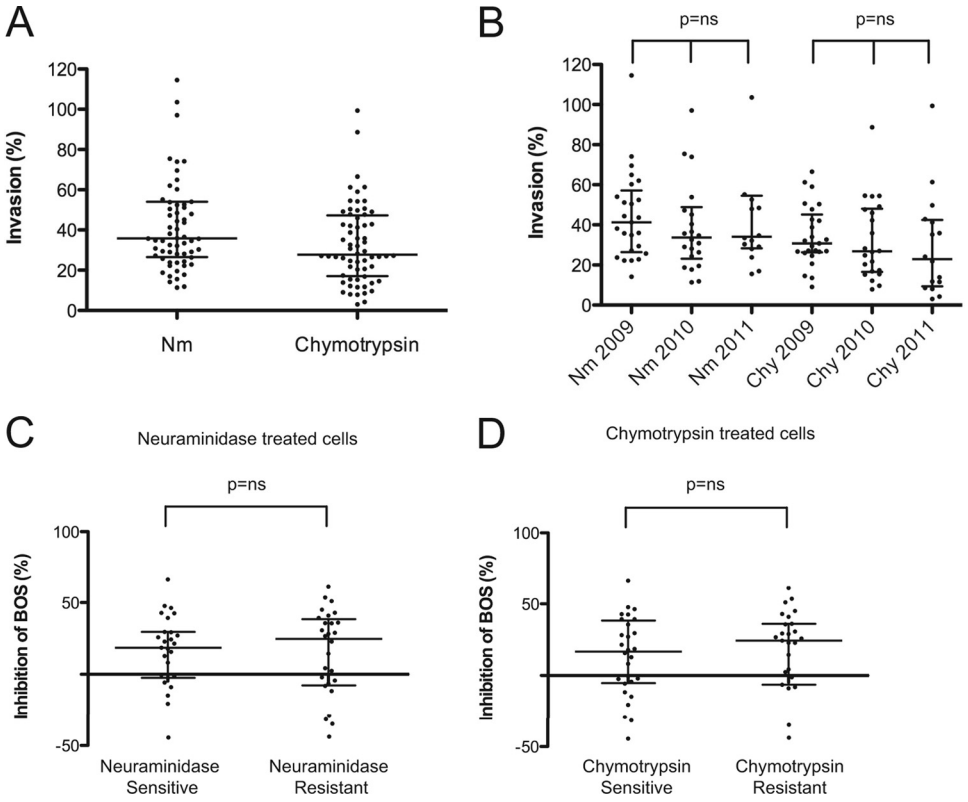

FIG 4 Variation in inhibition is not linked to *ex vivo* invasion pathways. (A) Invasion pathway was measured for 66 *ex vivo P. falciparum* isolates from Thies, Senegal, from 2009 to 2011. (B) Invasion pathways remain stable over time (Kruskal-Wallis; P = NS). Antibody inhibition (anti-RII antibodies) into chymotrpysin (Chy)-treated cells was compared for parasites invading via either neuraminidase (Nm)-sensitive and -resistant pathways (C) or chymotrypsin-sensitive and -resistant pathways (D). Cutoffs shown represent the median inhibition into both neuraminidase-treated (C) and chymotrypsin-treated (D) cells; however, data were analyzed using the cutoff of 50% as well, and no significant differences were observed (Mann-Whitney U test, $P = 0.05$ [data not shown]). Error bars represent median and interquartile range.

body reactivity are due to the quality of protein used for ELISA; misfolded bacterially expressed protein can display epitopes that are not physiologically relevant, resulting in spuriously high ELISA positivity. We note that the RII protein we used was validated for correct folding and was the same as that used to solve the crystal structure of EBA-175 RII (28, 29).

Since vaccines should be designed to protect exposed individuals from diverse field strains, it is imperative to assess the invasion efficiency of field isolates in the presence of anti-RII EBA-175 antibodies. While a few studies have been performed using affinity-purified human antibodies to assess merozoite inhibition, our study has performed this analysis using affinity-purified human antibodies to EBA-175 RII using *ex vivo* parasite isolates from patients. In this study, we tested the strains with two concentrations of affinity-purified human anti-RII antibodies: 10 and 50 μg/ml, which are in the range of the physiological concentrations 10 to 100 μg/ml (36). Others have used affinity-purified human antibodies to other invasion ligands at similar concentrations and have observed various degrees of inhibition. We show for the first time increased inhibition of the field isolates in receptor-restricted erythrocytes. This finding supports the hypothesis that interrupting the EBA-175/glycophorin A interaction in conjunction with other invasion pathways could be a viable strategy to significantly inhibit blood-stage proliferation (23, 24, 37, 38).

Others have also observed a lack of correlation between invasion pathway and inhibition in studies using higher concentrations of antibodies and laboratory lines (23, 24, 39). We found similar levels of inhibition of the laboratory strains 3D7 and W2mef with anti-RII antibodies when invasion into receptor-restricted chymotrypsin-treated erythrocytes was measured, whereas both EBA-175 knockouts are not inhibited by anti-RII antibodies. While this is an important observation with diverse laboratory isolates, we sought to determine whether this observation was true in the context of naturally acquired antibodies and *ex vivo* parasites from clinical malaria cases invading via naturally divergent invasion pathways.

We found that the invasion pathways utilized by the Senegalese population of parasites were stable over the 3 years of our study. Further, similar to the observations with laboratory isolates and knockouts, the sialic acid dependence of the field isolates, associated with the EBA-175/GPA pathway, did not correlate with functional inhibition by anti-RII antibodies. This is most likely due to the sialic acid-dependent pathway being a complex phenotype resulting from the contribution of multiple ligand-receptor interactions, challenging the prevailing dogma that the EBA-175/GPA pairing is at the top of the ligand-receptor hierarchy (40). While the EBA-175/GPA pathway is an important invasion pathway in Senegal (61% of the strains being sialic acid dependent), it is clearly not the only pathway being used by parasites. The ability to inhibit invasion with the pre-affinity-purified Velingara pool im-

plies that other non-RII antibodies also play an important role in invasion inhibition, and ideally, a vaccine targeting multiple invasion pathways would be designed and employed, as targeting a dominant pathway alone will be insufficient for protection.

There is a critical need for an effective and strain-transcendent malaria vaccine. RTS,S is the most advanced malaria vaccine to date; it targets the sporozoite stage and is in phase III clinical trials. In a recent study conducted with children, the vaccine showed an efficacy of 30% in an intention-to-treat analysis (41). While such results are promising, they are far from the goal of sterile protective immunity. In the face of such a challenge, there has been a recent renewed interest in developing blood-stage vaccines for malaria, specifically strain-transcending vaccines based on a combination of blood-stage antigens (24, 38, 42). Although a recombinant EBA-175 vaccine is currently in phase I clinical trials (43), few other blood-stage vaccine candidates have progressed beyond phase I clinical trials, largely due to low levels of protection and allele-specific immunity (42, 44–48).

Here, we show that naturally acquired human antibodies against EBA-175 RII can inhibit invasion of clinical isolates of *P. falciparum* parasites to various degrees; however, this inhibition is not complete. While we conclude that invasion pathway differences are not the primary reason for differential inhibition, it will be important to determine whether variation in expression or sequence of RII in these clinical isolates or other factors can influence the functional activity of anti-RII antibodies. To our knowledge, this is the first report of invasion-inhibitory activity of naturally acquired antibodies to EBA-175 using *ex vivo P. falciparum* parasite isolates. Our data suggest that the RII region of EBA-175 elicits invasion-inhibitory antibodies in humans and that this region may be an effective antigen as a component of a multisubunit blood-stage vaccine (19, 23, 24, 38).

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