January 2007

Variant merozoite protein expression is associated with erythrocyte invasion phenotypes in Plasmodium falciparum isolates from Tanzania

A.K. Bei
Harvard School of Public Health

C.D. Membi
Muhimbili University College of Health Sciences

J.C. Rayner
University of Alabama at Birmingham

M. Mubi
Muhimbili University College of Health Sciences

B. Ngasala
Muhimbili University College of Health Sciences

See next page for additional authors

Recommended Citation

Authors
A.K. Bei, C.D. Membi, J.C. Rayner, M. Mubi, B. Ngasala, A.A. Sultan, Zul Premji, and M.T. Duraisingh

Brief report

Variant merozoite protein expression is associated with erythrocyte invasion phenotypes in *Plasmodium falciparum* isolates from Tanzania

Amy K. Bei [a,b], Christopher D. Membi [b], Julian C. Rayner [c], Marycelina Mubi [b], Billy Ngasala [b], Ali A. Sultan [a,d], Zul Premji [b], Manoj T. Duraisingh [a,*]

[a] *Department of Immunology & Infectious Diseases, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA*
[b] *Department of Parasitology and Medical Entomology, Muhimbili University College of Health Sciences, Dar-es-Salaam, Tanzania*
[c] *William C. Gorgas Center for Geographic Medicine, University of Alabama at Birmingham, 845 19th St South, Birmingham, AL 35294, USA*
[d] *Weill Cornell Medical College in Qatar, Qatar Foundation, P.O. Box 24144, Doha, Qatar*

Infection with the protozoan parasite *Plasmodium falciparum* results in the severest form of human malaria. The symptoms and pathology of malaria occur during the intra-erythrocytic stage of the parasite life-cycle, and virulence has been shown to be associated with parasite multiplication and the selectivity of erythrocyte invasion [1]. Understanding the parasite molecules that mediate erythrocyte selectivity may lead to new interventions aimed at limiting virulence.

Invasion of erythrocytes by *P. falciparum* merozoites involves multiple ligand–receptor interactions, known as invasion pathways [2]. Several alternate erythrocyte surface receptors have been identified, including the major glycoproteins A and B, as well as the unrelated glycophorin C. Others have been described by their sensitivity to enzymes such as neuraminidase, chymotrypsin and trypsin, but the specific erythrocyte proteins involved are not known. Laboratory strains of *P. falciparum* vary in their dependence on these alternate receptors for invasion.

Recognition of alternate receptors on the erythrocyte surface by *P. falciparum* merozoites is thought to be mediated by members of the reticulocyte-binding homolog (PfRh) and erythrocyte binding antigen (PfEBA) ligand families; each family contains five paralogous proteins [2]. Importantly, distinct laboratory lines express different members of the PfRh family [3–8]. This variant expression could explain the use of alternative invasion pathways by different parasite lines. Minimal quantitative variation has been noted in the expression of PfEBA paralogs, and all appear to be expressed in most laboratory lines [9,10].

Antibody inhibition studies and genetic studies have yielded a wealth of information implicating members of the PfRh and PfEBA families in the invasion process in vitro [6,9–11]. The ability to knockout various parasite ligands implies a degree of redundancy among the alternative ligands. Strikingly, some knockouts lead to a shift in dependence on other ligands—an observation found to be dependent on the parasite genetic background.

Our current understanding of alternative erythrocyte invasion patterns relies on either laboratory adapted strains that have been passaged for many generations or on wild isolates that have been recently adapted to in vitro culture. Since selection pressures for efficient growth in vitro may differ from those in vivo, first-round invasion assays on fresh field isolates are crucial to our understanding of the dynamics of invasion in the natural context. Previous studies examining alternative invasion pathways using recently adapted or first-round invasion isolates have been conducted in The Gambia, India, and Brazil [12–14]. This study was initiated to determine the use of alternative invasion pathways by a natural population of Tanzanian parasites, and to investigate whether any correlation existed between specific pathways and the differential expression of the PfRh and PfEBA ligands, in order to determine the role that these ligands play in defining invasion pathway utilization in vivo.

* Corresponding author at: Harvard School of Public Health, 665 Huntington Avenue, Building 1, Room 715, Boston, MA 02115, USA.
Tel.: +1 617 432 2675; fax: +1 617 436 4766.
E-mail address: mduraisi@hsph.harvard.edu (M.T. Duraisingh).

1. Variation in invasion pathway utilization

Invasion assays were performed as previously described [6] to determine invasion efficiencies of Tanzanian isolates into erythrocytes that had been treated with enzymes to expose alternative invasion pathways—neuraminidase removes sialic-acid from erythrocyte receptors, while trypsin and chymotrypsin cleave non-overlapping sets of protein receptors. Of the 89 total invasion assays performed, 27 developed to schizonts in in vitro culture. Of these 27, 11 *P. falciparum* isolates (41%) successfully reinvaded treated donor cells—a success rate comparable to that observed in other field studies.

We observed significant variation in invasion pathway utilization, measured as the percent invasion into enzyme-treated erythrocytes compared to untreated controls (Table 1). A significant association was observed in the use of trypsin-sensitive and chymotrypsin-sensitive receptors between isolates (Rs = 0.70, $p = 0.017$, Spearman's) (Fig. 1C). We observed no association between the use of these pathways and neuraminidase-sensitive receptors (Fig. 1A and B).

Nested PCR amplifying *MSP-1* and *MSP-2* was performed to determine parasite clonality [15]; all isolates were multiclonal with a mean clonality of 3.5 parasite genotypes per patient.

2. Polarized expression of invasion ligands

To determine the extent of variant expression of PfRh and PfEBA invasion ligands, we performed Western blots on parasite proteins using polyclonal antibodies (Fig. 2A). Supernatants have been shown to reflect the levels of invasion molecules expressed in schizonts and are therefore useful in determining protein expression and secretion during invasion [16] (data not shown).

Importantly, we used two different schizont stage controls to normalize loading volumes and expression levels: PfSERA5 and PfAMA-1. PfSERA5 is released during schizont rupture and is unlikely to be functionally linked to the expression of the PfRh and PfEBA proteins. PfAMA-1 is essential and is unlikely to vary in expression level and is secreted during invasion in a similar manner to PfRh and PfEBA proteins. The correlation coefficients obtained when controlling with either PfSERA5 or PfAMA-1 were virtually identical, legitimizing the use of these proteins as controls to estimate the expression levels of PfRh and PfEBA proteins (data not shown). PfAMA-1 expression values were used in subsequent analysis to normalize expression.

Significant variation in the expression level of individual PfRh and PfEBA proteins was observed (Fig. 2A), with clear evidence of coordinate expression between some sets of ligands (Fig. 2B). PfRh2a and PfRh2b expression, for example, was tightly correlated (Rs = 0.94, $p = 0.0001$, Spearman's), suggesting coordinate expression of these proteins. In contrast, expression of PfRh1 was inversely correlated with that of PfRh2a and PfRh2b (Rs = −0.92, $p = 0.0002$, Spearman's). A significant positive correlation was observed between expression of EBA-181 and PfRh2b (Rs = 0.98, $p < 0.00001$, Spearman's). Similarly, a significant positive correlation was observed between EBA-140 and EBA-175 levels (Rs = 0.85, $p = 0.002$, Spearman's). No other correlations between the different invasion ligands were significant.

The polarized expression that we observe between PfRh1 and PfRh2a/PfRh2b in multiclonal isolates, is similar to the patterns observed in clonal laboratory isolates [16]. This can be interpreted in two ways—firstly that there are dominant clones within each isolate, which alternately express PfRh1 and PfRh2a/PfRh2b, and/or secondly that there are several clones in each isolate, all which possess similar expression

Table 1
Invasion of red blood cells treated with neuraminidase, trypsin, and chymotrypsin by 11 Tanzanian *P. falciparum* field isolates

Sample	Pre-assay: % parasitemia[a]		In vitro invasion assay: % parasitemia ($\pm$ S.D.)[b]				In vitro invasion assay: % invasion[c]		
	In patient	Adjusted	Control	Nm-treated	Trypsin-treated	Chymo-treated	Nm-treated	Trypsin-treated	Chymo-treated
MLD 013	1.60	0.80	1.20 ± 0.13	0.46 ± 0.085	0.11 ± 0.056	0.17 ± 0.00	38.46	9.23	13.85
MLD 018	2.10	1.05	2.30 ± 0.37	1.70 ± 0.032	0.29 ± 0.032	0.56 ± 0.15	73.90	12.85	24.10
MLD 019	1.70	0.85	1.03 ± 0.37	0.46 ± 0.085	0.33 ± 0.096	0.09 ± 0.032	44.64	32.14	8.93
MLD 021	2.00	1.00	1.13 ± 0.13	0.48 ± 0.085	0.20 ± 0.032	0.07 ± 0.032	42.62	18.03	6.56
MLD 022	1.30	0.65	0.57 ± 0.085	0.39 ± 0.056	0.15 ± 0.064	0.07 ± 0.032	67.74	25.81	12.90
MLD 052	7.50	1.00	0.48 ± 0.064	0.28 ± 0.056	0.46 ± 0.064	0.24 ± 0.032	57.69	96.15	50.00
MLD 054	1.80	0.90	1.09 ± 0.032	0.02 ± 0.056	0.57 ± 0.056	0.42 ± 0.064	13.43	58.21	43.28
MLD 056	4.20	1.00	0.48 ± 0.056	0.28 ± 0.056	0.31 ± 0.085	0.22 ± 0.00	57.69	61.54	46.15
MLD 080	1.70	0.85	0.60 ± 0.14	0.33 ± 0.056	0.43 ± 0.064	0.17 ± 0.00	55.38	70.77	27.69
MLD 081	1.80	0.90	0.99 ± 0.23	0.47 ± 0.032	0.19 ± 0.032	0.07 ± 0.032	46.30	18.52	7.41
MLD 082	1.00	0.50	0.65 ± 0.078	0.22 ± 0.056	0.33 ± 0.056	0.11 ± 0.00	34.48	51.72	17.24

Blood samples were collected from 100 patients with clinically mild malaria and no reported prior antimalarial treatment from the Mlandizi Health Centre in Tanzania (January to June, 2004). if peripheral blood parasitemia exceeded 1.0%, following informed consent from patients and their guardians. Acceptor erythrocytes from a single local O + donor were washed and treated with neuraminidase (66.7 mU/ml), trypsin (1 mg/ml) or chymotrypsin (1 mg/ml) for 1 hour at 37 °C. Positive control cells were treated with RPMI and negative control cells with neuraminidase and chymotrypsin. Infected donor blood was adjusted to 1–2% parasitemia and treated with neuraminidase and chymotrypsin to prevent reinvasion. Equal volumes of infected donor cells and treated acceptor cells were plated in triplicate in 96-well plates resulting in a final parasitemia between 0.5–1 and 4% hematocrit. Plates were cultured in a candle jar at 37 °C for one invasion cycle. Successful reinvasion was defined as greater than 0.5% parasitemia post-invasion. Percent invasion into enzyme-treated cells compared to control cells is shown for 11 isolates.

  [a] Parasitemia is calculated as the number of parasitized red cells per 1800 uninfected red cells × 100.
  [b] Standard deviation of invasion parasitemia is calculated between triplicate wells in the assay.
  [c] Percent invasion is calculated as (parasitemia of enzyme-treated cells)/(parasitemia of control cells) × 100.
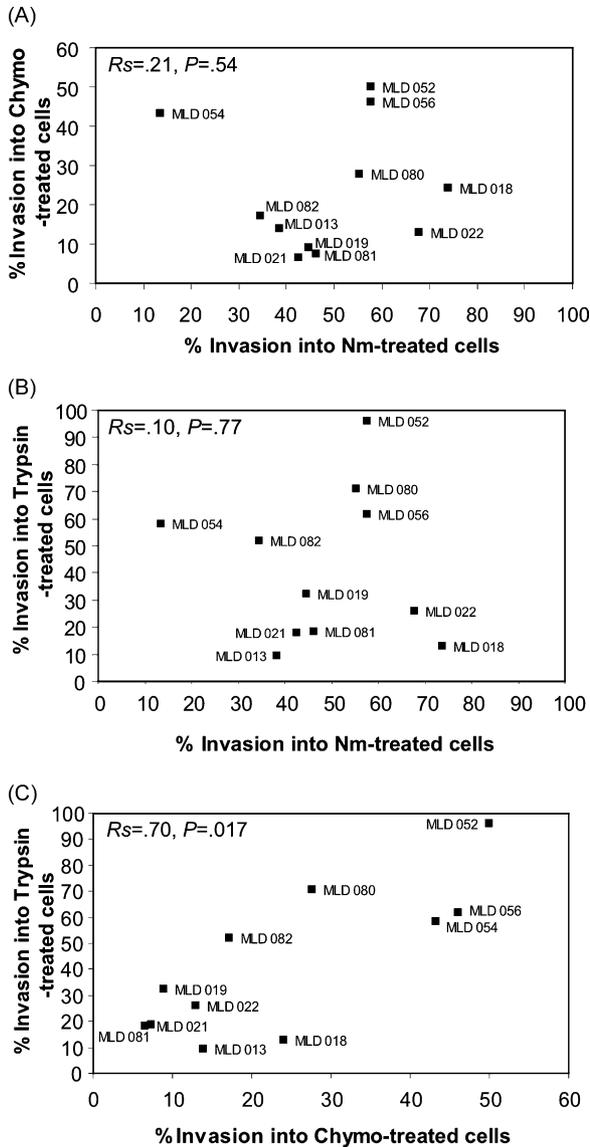
Fig. 1. Variation in utilization of invasion pathways in Tanzanian field isolates. (A–C) Correlations of invasion efficiencies into enzyme-treated erythrocytes for the series of isolates. Rs(*p*) and *p*-values shown are derived from Spearman's Rank Correlation. (A) Percent invasion into neuraminidase-treated cells compared to chymotrypsin-treated cells. (B) Percent invasion into neuraminidase-treated cells compared to trypsin-treated cells. (C) Percent invasion into chymotrypsin-treated cells compared to trypsin-treated cells.

phenotypes, expressing either PfRh1 or PfRh2a/PfRh2b, with polarized expression resulting from selection for parasites with similar expression profiles in each isolate. The latter explanation is consistent with the notion that invasion ligands such as the PfRh and PfEBA are under frequency-dependent selection. Similar correlations have been made with regards PfRh and PfEBA transcript levels in multiclonal isolates from Kenya [17].

3. Correlation of PfRh and PfEBA protein expression with invasion pathway utilization

We next determined whether correlations existed between individual invasion pathways and variant expression of PfRh and PfEBA proteins (Fig. 2C). Parasite populations in

which PfRh1 was highly expressed invaded in a trypsin-resistant (Rs = 0.73, *p* = 0.016, Spearman's), chymotrypsin-resistant manner (Rs = 0.71, *p* = 0.022, Spearman's). In contrast, parasite populations in which PfRh2b was highly expressed invaded in a trypsin-sensitive (Rs = −0.87, *p* = 0.0012, Spearman's), chymotrypsin-sensitive manner (Rs = −0.78, *p* = 0.0075). Expression of PfRh2a and PfEBA-181 also strongly correlated with the use of a trypsin- and chymotrypsin-sensitive receptor (data not shown). No significant associations were evident between the expression level of any of the invasion ligands and the use of sialic acid-containing receptors. There was no correlation observed between the time post-schizont rupture at which supernatant was collected and the expression profile of any individual invasion ligand (for all comparisons, *p* > 0.23, Spearman's), ruling out variation in supernatant protein stability as an explanation for our results.

4. Implications for our understanding of alternative invasion pathways

In these studies, we have examined the extent to which *P. falciparum* parasites isolated from individuals within a single Tanzanian population utilize alternative pathways for the erythrocyte invasion. Invasion assays were conducted without culturing to avoid potential bias resulting from culture-adaptation. We found considerable variation in the use of alternative invasion pathways, in contrast to a previous study in The Gambia in which most isolates utilized sialic acid-independent and trypsin-sensitive receptors [13]. Isolates from India and Brazil have demonstrated a greater reliance on trypsin-resistant receptors [12,14]. The strong association between the use of trypsin-sensitive and chymotrypsin-sensitive receptors in some of our isolates (Fig. 1C) is suggestive of the use of a common dominant receptor. In contrast, the lack of association between the use of sialic acid-containing receptors and trypsin- and chymotrypsin-sensitive receptors, suggests a more complex relationship.

Our study represents the first analysis of protein expression levels of multiple merozoite proteins from a series of fresh isolates from a single population. We found considerable variation in the expression levels of PfEBA and PfRh proteins. While variation in PfRh protein expression is consistent with what we and others have observed in laboratory isolates [5,6,16], the dramatic degree of variant expression of PfEBA paralogs has not previously been observed using laboratory-adapted strains. This result highlights the importance of conducting these studies using first-round invasion isolates from the field.

Despite our small sample size, there were significant correlations in the expression levels of certain invasion ligands, suggesting a strong molecular basis for these associations (Fig. 2B). The positive correlation between PfRh2a and PfRh2b is not surprising, as they share similar promoter sequences, lie head to tail on chromosome 13, and are coordinately expressed in laboratory isolates [6]. More intriguing are the associations between the expression levels of PfRh2b and EBA-181, and between EBA-175 and EBA-140, which may reflect the use of shared mechanisms for coordinate expression. Interestingly,
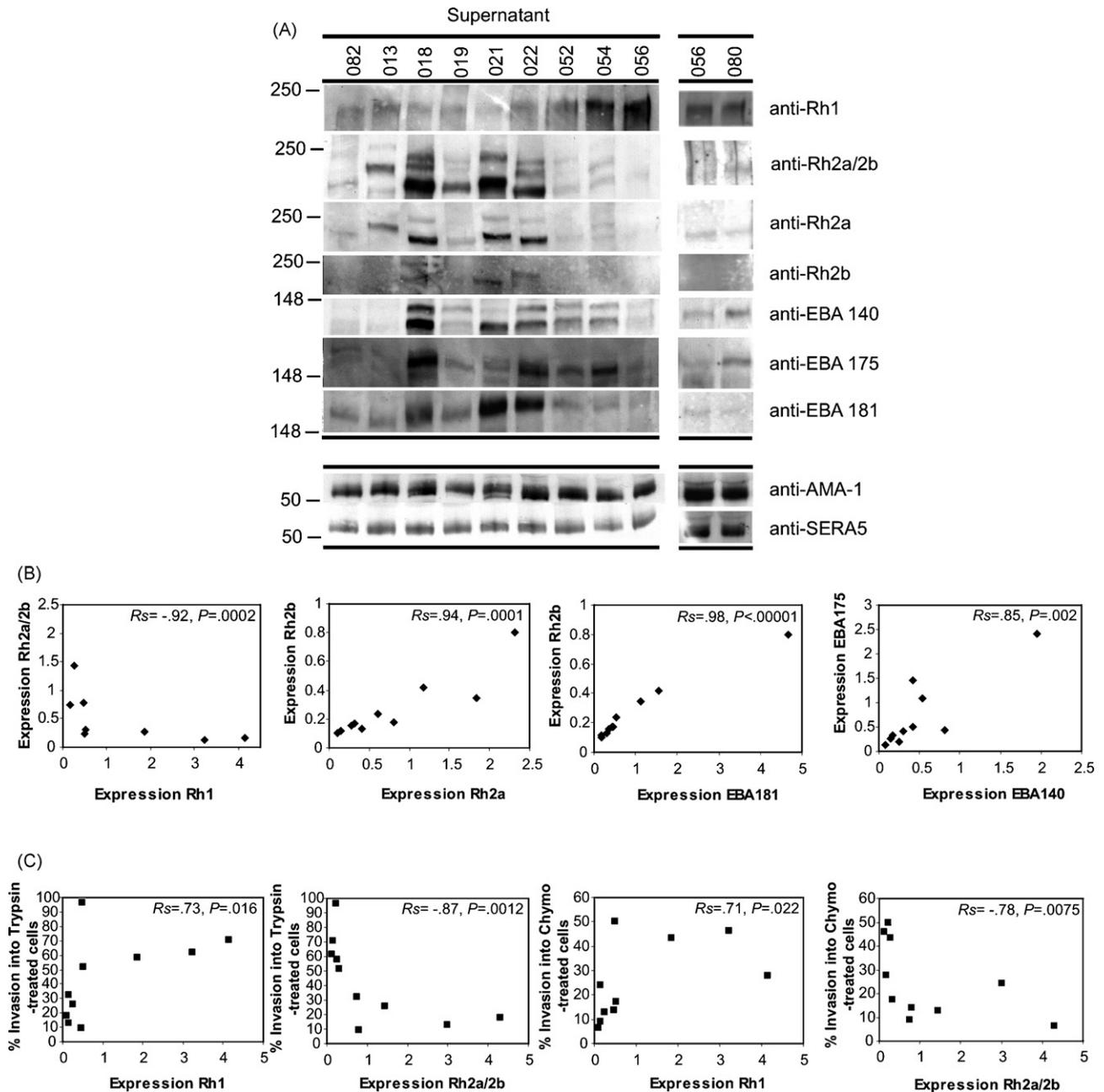
Fig. 2. Polarized expression of invasion ligands correlates with invasion pathway. (A) Expression profile of invasion ligands in the PfRh and PfEBA families. Parasite protein was collected after short-term culture of infected blood treated with neuraminidase and chymotrypsin. Between 10 and 24 h following schizont rupture, supernatant containing processed forms of secreted invasion ligands was collected and stored at −80 °C. Invasion supernatants were run on 5% SDS-PAGE gels for maximum separation of PfRh and PfEBA proteins. Polyclonal antibodies against PfEBA-140, PfEBA-175, PfEBA-181, PfAMA-1, and PfSERA5 were kindly provided by Prof. Alan Cowman and Dr. Brendan Crabb (WEHI, Australia). Antibodies against PfRh1, PfRh2a and PfRh2b have been described previously [3,4]. All samples were controlled with PfAMA-1 and PfSERA-5. Quantification of bands corresponding to processed forms of invasion proteins [3,4,5–10] was performed using UN-SCAN-IT™ software (Silk Scientific Inc., V. 5.1). (B) Quantitation reveals polarized expression of invasion ligands with positive correlations for Rh2a and Rh2b, EBA181 and Rh2b, and EBA140 and EBA 175; and negative correlations for Rh1 and Rh2a/2b. (C) Correlation of expression profile with invasion pathway utilization. Positive associations are observed between expression of Rh1 with trypsin-resistant, chymotrypsin-resistant invasion and expression of Rh2a/2b with trypsin-sensitive, chymotrypsin-sensitive invasion. Rs(p) and p-values shown are derived from Spearman's Rank Correlation. Expression is represented in arbitrary units.

both PfRh2b and EBA-181 are thought to bind chymotrypsin-sensitive receptors [6,9,18] and it is possible that they may both be present within a common invasion complex that engages the same receptor. Significant correlations were observed between PfRh2a and PfRh2b transcript levels in multiclonal Kenyan isolates, with inverse correlations between PfRh1 and PfRh2b [17],

corroborating our findings. However, in contrast to our study, a strong inverse correlation was observed between PfRh2b and EBA-175. Future studies are required to determine whether this reflects genuine differences between Kenyan and Tanzanian parasites or represents a lack of concordance between transcript and protein levels for the merozoite proteins.

This study, which used multiclonal isolates, reflects the reality of *P. falciparum* infections in many areas of sub-Saharan Africa. However, because of this multiclonality a caveat needs to be raised. PfRh and PfEBA levels measured in culture supernatants represent the total expression profiles for all of the clones within an isolate, while only a subset of specific clones within that pool actually successfully invade in the invasion assays. By making associations between expression and invasion, we may be comparing two different, albeit overlapping, groups of parasites. Comparing PfRh and PfEBA expression both before and after invasion would be a powerful way to address this problem in future studies. Nevertheless, the correlations we have observed are very similar to those found in previous studies using laboratory-adapted isolates [4,6,16].

Reverse genetic studies previously implicated PfRh1 in binding a trypsin/chymotrypsin-resistant receptor termed Receptor Y [16]. Increased PfRh1 expression in these Tanzanian isolates was positively correlated with invasion by a trypsin/chymotrypsin-resistant pathway. Interestingly, we found no association between PfRh1 expression and neuraminidase-sensitivity, as was previously observed in laboratory strains [16], suggesting that additional ligands also contribute to sialic acid-dependence. We previously found the receptor for PfRh2b to be chymotrypsin-sensitive and trypsin-resistant [11]; however, here we report that the level of PfRh2b is correlated with a chymotrypsin-sensitive, but trypsin-sensitive invasion pathway. Interestingly, we have recently found that targeted deletion of PfRh2b in the W2mef parasite line, which expresses both PfRh2b and PfRh1, results in an increased reliance on a chymotrypsin-resistant, trypsin-resistant invasion pathway, consistent with our field data (Jennings et al., manuscript in preparation). Therefore, in the absence of PfRh2b expression, parasites may become more reliant on the trypsin-resistant and chymotrypsin-resistant PfRh1-associated invasion pathway.

It is striking that we see such polarized expression of merozoite proteins in the multiclonal Tanzanian isolates. These expression patterns may represent frequency-dependent selection either by polymorphic host receptors or by immune responses, supporting the consideration of these proteins as vaccine candidates. While sequence polymorphisms have been identified in *PfRh* and *PfEBA* genes that may influence invasion pathways [3–5,19], their analysis in our study is precluded due to the multiclonality of the isolates. Larger studies, focused on the expression levels of the PfRh and PfEBA proteins and the invasion pathways they define, may reveal a role in disease severity and immune evasion.

Conflict of interest statement

The authors do not have a commercial or other association that might pose a conflict of interest.

Acknowledgements

References

[1] Chotivanich K, Udomsangpetch R, Simpson JA, et al. Parasite multiplication potential and the severity of Falciparum malaria. J Infect Dis 2000;181:1206–9.

[2] Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell 2006;124:755–66.

[3] Rayner JC, Galinski MR, Ingravallo P, Barnwell JW. Two *Plasmodium falciparum* genes express merozoite proteins that are related to *Plasmodium vivax* and *Plasmodium yoelii* adhesive proteins involved in host cell selection and invasion. Proc Natl Acad Sci USA 2000;97:9648–53.

[4] Rayner JC, Vargas-Serrato E, Huber CS, Galinski MR, Barnwell JW. A Plasmodium falciparum homologue of *Plasmodium vivax* reticulocyte binding protein (PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. J Exp Med 2001;194:1571–81.

[5] Taylor HM, Grainger M, Holder AA. Variation in the expression of a *Plasmodium falciparum* protein family implicated in erythrocyte invasion. Infect Immun 2002;70:5779–89.

[6] Duraisingh MT, Triglia T, Ralph SA, et al. Phenotypic variation of *Plasmodium falciparum* merozoite proteins directs receptor targeting for invasion of human erythrocytes. EMBO J 2003;22:1047–57.

[7] Gaur D, Furuya T, Mu J, Jiang LB, Su XZ, Miller LH. Upregulation of expression of the reticulocyte homology gene 4 in the *Plasmodium falciparum* clone Dd2 is associated with a switch in the erythrocyte invasion pathway. Mol Biochem Parasitol 2006;145:205–15.

[8] Stubbs J, Simpson KM, Triglia T, et al. Molecular mechanism for switching of *P. falciparum* invasion pathways into human erythrocytes. Science 2005;309:1384–7.

[9] Gilberger TW, Thompson JK, Triglia T, Good RT, Duraisingh MT, Cowman AF. A novel erythrocyte binding antigen-175 paralogue from *Plasmodium falciparum* defines a new trypsin-resistant receptor on human erythrocytes. J Biol Chem 2003;278:14480–6.

[10] Maier AG, Duraisingh MT, Reeder JC, et al. Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med 2003;9:87–92.

[11] Duraisingh MT, Maier AG, Triglia T, Cowman AF. Erythrocyte-binding antigen 175 mediates invasion in *Plasmodium falciparum* utilizing sialic acid-dependent and -independent pathways. Proc Natl Acad Sci USA 2003;100:4796–801.

[12] Lobo CA, de Frazao K, Rodriguez M, Reid M, Zalis M, Lustigman S. Invasion profiles of Brazilian field isolates of *Plasmodium falciparum*: phenotypic and genotypic analyses. Infect Immun 2004;72:5886–91.

[13] Baum J, Pinder M, Conway DJ. Erythrocyte invasion phenotypes of *Plasmodium falciparum* in The Gambia. Infect Immun 2003;71:1856–63.

[14] Okoyeh JN, Pillai CR, Chitnis CE. *Plasmodium falciparum* field isolates commonly use erythrocyte invasion pathways that are independent of sialic acid residues of glycophorin A. Infect Immun 1999;67:5784–91.

[15] Viriyakosol S, Siripoon N, Zhu XP, et al. *Plasmodium falciparum*: selective growth of subpopulations from field samples following in vitro culture, as detected by the polymerase chain reaction. Exp Parasitol 1994;79:517–25.

[16] Triglia T, Duraisingh MT, Good RT, Cowman AF. Reticulocyte-binding protein homologue 1 is required for sialic acid-dependent invasion into human erythrocytes by *Plasmodium falciparum*. Mol Microbiol 2005;55:162–74.

[17] Nery S, Deans AM, Mosobo M, Marsh K, Rowe JA, Conway DJ. Expression of *Plasmodium falciparum* genes involved in erythrocyte invasion varies among isolates cultured directly from patients. Mol Biochem Parasitol 2006.

[18] Camus D, Hadley TJ. A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. Science 1985;230:553–6.

[19] Lobo CA, Rodriguez M, Struchiner CJ, Zalis MG, Lustigman S. Associations between defined polymorphic variants in the PfRH ligand family and the invasion pathways used by *P. falciparum* field isolates from Brazil. Mol Biochem Parasitol 2006;149:246–51.