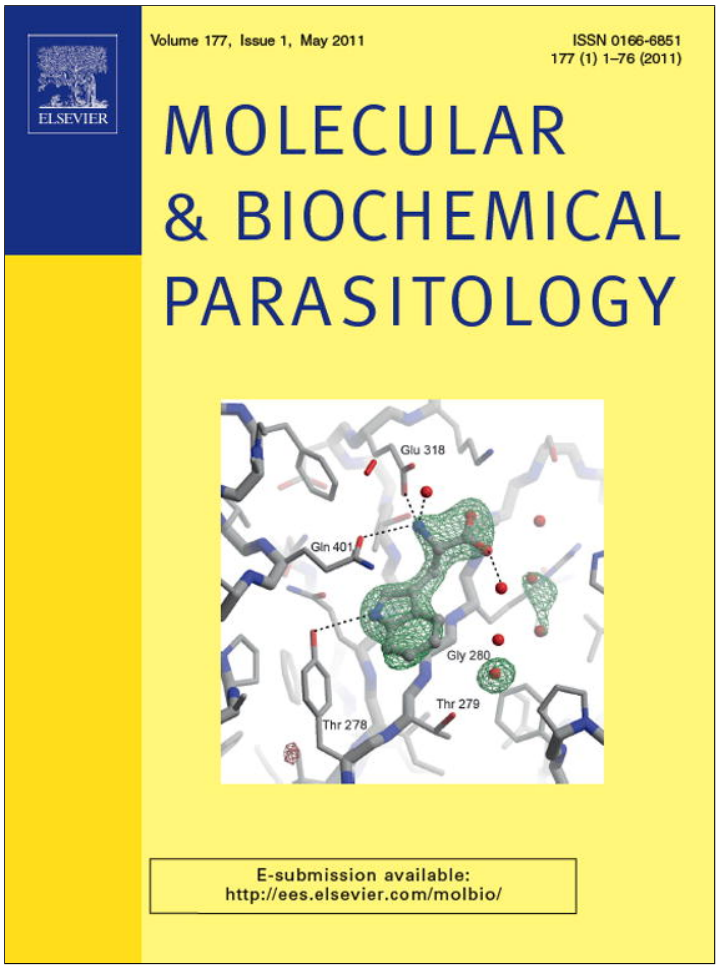

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Short communication

# *Plasmodium falciparum* field isolates use complement receptor 1 (CR1) as a receptor for invasion of erythrocytes

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ABSTRACT

A majority of *Plasmodium falciparum* strains invade erythrocytes through interactions with sialic acid (SA) on glycophorins. However, we recently reported that complement receptor 1 (CR1) is a SA-independent invasion receptor of many laboratory strains of *P. falciparum*. To determine the role of CR1 in erythrocyte invasion among *P. falciparum* field isolates, we tested eight isolates obtained from children in Kenya. All the parasites examined were capable of invading in a SA-independent manner, and invasion of neuraminidase-treated erythrocytes was nearly completely blocked by anti-CR1 and soluble CR1 (sCR1). In addition, anti-CR1 and sCR1 partially inhibited invasion of intact erythrocytes in a majority of isolates tested. Sequencing of the hypervariable region of *P. falciparum* AMA-1 showed considerable diversity among all the isolates. These data demonstrate that CR1 mediates SA-independent erythrocyte invasion in *P. falciparum* field isolates.



*Plasmodium falciparum* infections are the leading cause of malaria-related morbidity and mortality in sub-Saharan Africa [1]. An integral part of the parasite's life cycle in humans is the blood stage, during which parasites repeatedly invade and multiply in erythrocytes. This part of the life cycle is responsible for all the morbidity and mortality. Therefore, vaccines targeting this stage could potentially be effective in preventing disease. However, the development of a blood stage vaccine is hampered by a lack of adequate understanding of the molecular mechanisms through which the parasite invades erythrocytes. Furthermore, it is important that studies examining erythrocyte invasion pathways include field parasites since laboratory-adapted strains often differ significantly from clinical isolates.

It is known that sialic acid (SA) residues on glycophorins are an important receptor for the invasion of erythrocytes by *P. falciparum* [2,3]. However, a significant number of laboratory-adapted and field strains of *P. falciparum* are capable of invading erythrocytes depleted of SA after treatment with neuraminidase [4–8], indicating the existence of one or more SA-independent invasion pathways. In addition, studies of *P. falciparum* field isolates from Kenya demonstrated that the vast majority (~74%) of these parasites relied on an unknown trypsin-sensitive receptor for SA-independent invasion of erythrocytes [5,9].

Our recent investigations [10], now confirmed by others [11], have identified complement receptor 1 (CR1, CD35) as the major neuraminidase-resistant, trypsin-sensitive receptor used for SA-independent invasion of erythrocytes by laboratory strains of *P. falciparum*, including 3D7, 7G8, Dd2NM, and HB3. CR1 is a complement regulatory protein that serves as co-factor in the factor I-mediated cleavage of C3b to C3bi and also accelerates the decay of C3 and C5 convertases. Our previous studies and those of others have also shown that CR1 expression levels [12,13], as well as genetic polymorphisms that influence CR1 expression [14–16], are associated with susceptibility to severe malaria in children.

To extend our earlier findings with laboratory strains [10], we investigated the role of CR1 in mediating erythrocyte invasion by field isolates of *P. falciparum* by examining the ability of these isolates to invade erythrocytes in the presence of CR1 inhibitors in vitro. Parasite isolates used in this study were collected from children diagnosed with acute malaria at hospitals in western Kenya, under protocols approved by the Kenya National Ethical Committee and the Human Subjects Research Committee at the Walter

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**Table 1**
CR1-dependent invasion of erythrocytes by *P. falciparum* clinical isolates.

| Parasites | Control | Anti-CR1 | IgY | sCR1 | α-2M/Fetuin |
|---|---|---|---|---|---|
| *Invasion of untreated erythrocytes expressed as % control (SD)* | | | | | |
| 3D7 ($n=3$) | 100.0 | 102.0 (3.5) | 101.8 (5.2) | 98.6 (4.7) | 101.3 (3.6) |
| JASC8-19 ($n=5$) | 100.0 | 82.6 (6.7)* | 93.1 (4.6) | 73.7 (7.1)* | 100.2 (3.4) |
| SA005 ($n=3$) | 100.0 | 90.2 (2.4) | 93.5 (5.9) | 69.4 (2.1)* | 92.9 (3.8) |
| SA154 ($n=3$) | 100.0 | 82.2 (7.0) | 93.6 (1.9) | 75.9 (8.3) | 95.4 (3.4) |
| SA162 ($n=4$) | 100.0 | 98.7 (3.8) | 101.7 (3.6) | 79.6 (2.9)* | 99.5 (4.0) |
| SA222 ($n=5$) | 100.0 | 84.7 (7.8)* | 97.4 (4.5) | 64.9 (3.9)* | 100.9 (2.4) |
| SA250 ($n=3$) | 100.0 | 82.8 (5.3) | 92.9 (2.1) | 72.9 (1.5)* | 91.4 (1.4) |
| CM028 ($n=5$) | 100.0 | 90.6 (8.9)* | 99.9 (8.0) | 70.5 (7.9)* | 98.2 (5.3) |
| CM033 ($n=3$) | 100.0 | 86.1 (4.9)* | 99.0 (3.1) | 78.9 (5.0)* | 98.7 (5.4) |
| *Invasion of neuraminidase-treated erythrocytes expressed as % untreated control (SD)* | | | | | |
| 3D7 ($n=3$) | 62.1 (5.5) | 15.8 (2.6)* | 62.1 (4.7) | 8.4 (2.3)* | 60.6 (4.8) |
| JASC8-19 ($n=3$) | 45.2 (2.2) | 10.8 (1.6)* | 43.1 (2.8) | 11.3 (1.8)* | 44.0 (3.7) |
| SA005 ($n=3$) | 42.8 (7.3) | 11.4 (1.7)* | 38.3 (2.6) | 8.2 (1.8)* | 38.4 (5.7) |
| SA154 ($n=3$) | 39.7 (5.6) | 14.9 (8.7)* | 37.7 (5.5) | 13.5 (7.7)* | 40.0 (2.6) |
| SA162 ($n=3$) | 49.1 (2.1) | 8.5 (1.4)* | 30.1 (0.8) | 6.0 (0.6)* | 31.2 (4.0) |
| SA222 ($n=5$) | 36.8 (2.7) | 13.2 (0.7)* | 37.9 (4.7) | 14.0 (2.4)* | 34.4 (3.9) |
| SA250 ($n=3$) | 36.0 (6.8) | 15.8 (6.7)* | 31.3 (5.7) | 13.0 (5.8)* | 33.4 (5.6) |
| CM028 ($n=5$) | 43.6 (7.3) | 9.4 (5.2)* | 39.4 (6.5) | 9.6 (5.9)* | 40.4 (5.6) |
| CM033 ($n=3$) | 37.5 (2.4) | 10.8 (3.5)* | 38.9 (4.0) | 10.0 (3.7)* | 36.5 (4.4) |

*P. falciparum* isolates SA005, SA154, SA162, SA222, SA250, JASC8-19, CM028, and CM033 were collected from children with malaria in western Kenya, while 3D7 is laboratory-adapted strain. Parasites were cultivated on RPMI containing 0.2% $NaHCO_3$, 0.1 mM hypoxanthine and 10% normal serum (type O+), maintained at 37 °C and a gas mixture of 90% $N_2$, 5% $O_2$ and 5% $CO_2$. Invasion assays were set up in duplicates in 96-well plates with 100 μL/well of culture media. Uninfected untreated erythrocytes or neuraminidase-treated erythrocytes at 2% hematocrit were inoculated with purified schizonts to obtain 1–2% parasitemia. CR1-mediated invasion was inhibited with either 50 μg/mL of sCR1 or 8 μg/mL of polyclonal chicken IgY raised against sCR1 (Gallus Immunotech Inc., Fergus, Canada). Fetuin and apha-2-macroglobulin (α-2-M) were used as control proteins, and chicken IgY as control antibody. After overnight incubation, erythrocytes were stained with Hoechst 33342 and invasion rates were calculated as the percentage of ring-infected erythrocytes as determined by flow cytometry using a LSR-II system (Becton-Dickinson, San Diego, CA). "*n*" denotes the number of experiments. Data are presented as means (SD) of three to five separate experiments for each parasite strain.

* Indicates that invasion inhibition by anti-CR1 or sCR1 was statistically significant compared to the effects of IgY or α-2-M and fetuin, respectively ($P<0.05$ vs respective control proteins, paired Student's *t*-test).

Reed Army Institute of Research. The parasites were cultured for 1–2 weeks to adapt and synchronize before invasion assays were performed. Schizonts were purified using Percoll gradient centrifugation and used to inoculate uninfected erythrocytes at 1–2%. Each experiment was set up in duplicate wells, and invasion rates were averaged between the 2 wells. Three to five separate invasion experiments were performed at 2-day intervals for each isolate. Treatment of erythrocytes with the anti-CR1 polyclonal antibody decreased invasion by 10–18% in all the parasites tested (Table 1). The inhibitory effects elicited by anti-CR1 were statistically significant compared to the changes induced by control IgY antibodies in four out of eight isolates tested ($P<0.05$, Table 1). In addition, invasion of untreated erythrocytes by all parasite isolates tested was inhibited by as much as 20–35% in the presence of sCR1. Compared to the effects of control proteins α-2-macroglobulin (α-2-M) or fetuin, inhibition of invasion by sCR1 was statistically significant in seven out of eight parasite strains examined ($P<0.05$, Table 1). In contrast, invasion of untreated erythrocytes by laboratory strains such as 3D7 were not significantly affected by the presence of CR1 inhibitors (Table 1 and [10]), demonstrating the important contribution that CR1 makes to invasion of the intact red cells by field parasites.

All parasite isolates were capable of invading red cells in a SA-independent manner, retaining 36–49% of their normal invasion levels after erythrocytes were treated with neuraminidase (Table 1). For comparison, 3D7 on the average retained 62% of its invasion after treatment of erythrocytes with neuraminidase (Table 1). Anti-CR1 significantly inhibited invasion of neuraminidase-treated erythrocytes in all parasites ($P<0.01$ compared to IgY for all isolates; Table 1). These effects of anti-CR1 represented a 60–85% inhibition of SA-independent invasion in the eight field parasites, suggesting that CR1 plays an important role in SA-independent erythrocyte invasion. Furthermore, sCR1 similarly decreased invasion of neuraminidase-treated erythrocytes in the clinical parasites by 65–90% ($P<0.01$ compared to control for all isolates). The relatively lower dependence of these parasites on CR1 for invasion of untreated erythrocytes compared to neuraminidase-treated erythrocytes is consistent with the existing notion that glycophorins are the primary or preferred receptors for *P. falciparum*. Consistent with our observations in laboratory strains [10], these results indicate that CR1 is the previously unknown SA-independent receptor used by a majority of wild *P. falciparum* isolates.

To assess the extent of genetic diversity among the parasite isolates that use CR1 as an invasion receptor, a sequence analysis of the highly polymorphic C1 cluster of *P. falciparum* AMA-1 domain 1 was performed. AMA-1 plays a key role in erythrocyte invasion [17], and amino acid substitutions within the C1 cluster have been shown to confer antigenic escape from invasion inhibitory antibodies [18]. As controls, two clones of JASC8-19 (JASC8-8 and JASC8-10), as well as the laboratory strain 3D7 (GenBank accession number U65407.1) were also analyzed. These analyses revealed that seven isolates, JASC8-19, SA005, SA154, SA162, SA222, SA250, and CM028, were genetically distinct with >25% differences in amino acid sequences between some of them (Fig. 1). The eighth parasite, CM033, had a sequence that was identical to SA154 at the same locus, suggesting that these could be the same parasite. Furthermore, multiple AMA1 sequences were detected in SA162 and CM028, indicating that these isolates were mixtures of at least two different parasites. Within the 30-amino acids spanning the C1 cluster, the sequences of the seven distinct isolates differed from 3D7 by three to nine amino acid residues (Table 2). Therefore, the use of CR1 as a receptor for SA-independent invasion of erythrocytes was conserved across a genetically diverse group of parasites. These data argue for a broad reliance on CR1 as the major SA-independent receptor in widely diverse field strains.

Our previous investigations demonstrated that CR1 is a major receptor used by many laboratory strains of *P. falciparum* for SA-independent invasion of erythrocytes [10]. Those findings marked an important step towards the understanding of erythrocyte inva-

**Table 2**
Amino acid substitutions within the AMA-1 C1 clusters of the field isolates.

| | AMA-1 C1 cluster sequence (amino acids 181–210) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3D7 | F | A | F | P | P | T | E | P | L | M | S | P | M | T | L | D | E | M | R | H | F | Y | K | D | N | K | Y | V | K | N |
| SA005 | - | - | - | - | - | - | K | - | - | - | - | - | - | - | - | - | D | - | - | D | L | - | - | N | - | E | - | - | - | - |
| SA154 | - | - | - | - | - | - | N | - | - | I | - | - | - | - | - | N | G | - | K | D | - | - | - | - | - | E | D | - | - | - |
| SA162-A | - | - | - | - | - | - | N | - | - | - | - | - | - | - | - | N | G | - | - | - | - | - | - | N | - | E | - | - | - | - |
| SA162-B | - | - | - | - | - | - | N | - | - | - | - | - | - | - | - | N | H | - | - | D | - | - | - | - | - | E | - | - | - | - |
| SA222 | - | - | - | - | - | - | K | - | - | - | - | - | - | - | - | - | D | - | - | L | L | - | - | - | - | E | D | - | - | - |
| SA250 | - | - | - | - | - | - | N | - | P | - | - | - | - | - | - | N | G | - | - | D | L | - | - | N | - | E | D | - | - | - |
| JASC8-8 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | D | - | - | R | - | - | - | - | - | E | - | - | - | - |
| JASC8-10 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | D | - | - | R | - | - | - | - | - | E | - | - | - | - |
| JASC8-19 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | D | - | - | R | - | - | - | - | - | E | - | - | - | - |
| CM028-A | - | - | - | - | - | - | K | - | - | I | - | - | - | - | - | - | - | - | - | D | L | - | - | N | - | E | - | - | - | - |
| CM028-B | - | - | - | - | - | - | K | - | - | I | - | - | - | - | - | - | H | - | - | D | L | - | - | N | - | E | - | - | - | - |
| CM033 | - | - | - | - | - | - | N | - | - | I | - | - | - | - | - | N | G | - | K | D | - | - | - | - | - | E | D | - | - | - |

The parasites were analyzed for the C1 cluster of the *P. falciparum* apical membrane protein 1 (AMA-1) gene [18]. A 510-bp fragment spanning the hypervariable region of AMA-1 was amplified by PCR using flanking primers (forward: 5′-GGAACTCAATATAGA CTTCC-3′ and reverse: R: 5′-AAATTCTTCTAGGGCAA AC-3′) [20] and the product was sequenced on an ABI Prism 3730 DNA analyzer. The sequences were truncated from amino acid 181–210 that represent the C1 polymorphic cluster of the AMA1 gene [18] and aligned by CLUSTAL software. *P. falciparum* isolates SA005, SA154, SA162, SA222, SA250, JASC8-19, CM028, and CM033 were collected from children with malaria in western Kenya. JASC8-8 and JASC8-10 were cloned from JASC8-19 and included as controls, as well as the common laboratory strain 3D7. AMA-1 C1 cluster sequence data are presented relative to the 3D7 sequence. Positions at which amino acids are in agreement with 3D7 are indicated by "-". Multiple AMA-1 sequences were detected in SA162 and CM028 and two possible haplotypes were presented for each of these isolates.




**Fig. 1.** AMA-1 C1 cluster sequences show diversity among field isolates. *P. falciparum* isolates SA005, SA154, SA162, SA222, SA250, JASC8-19, CM028, and CM033 were collected from children with malaria in western Kenya. JASC8-8 and JASC8-10 were cloned from JASC8-19 and included as controls, as well as the common laboratory strain 3D7. The genetic diversity among the isolates is illustrated by a phylogenetic tree showing interrelationships according to the percentage differences in amino acid sequences of the polymorphic AMA-1 C1 cluster. Multiple AMA-1 sequences were detected in SA162 and CM028 and two possible haplotypes were presented for each of these isolates.

sion by *P. falciparum* and brought us closer to achieving the goal of developing an effective blood stage vaccine. In this study we extended our studies to determine the relevance of our observations in field isolates which ultimately will be the target of any potential malaria vaccine.

Unlike laboratory strains, where the contribution of CR1 to invasion of untreated erythrocytes was small [10], nearly all the clinical isolates tested here demonstrated a significant utilization of CR1 for invasion of intact erythrocytes. The reasons for this difference are unclear. However, it is possible that CR1 loss from red cells during in vitro culture may lead to selection of parasites with less reliance on CR1. In malaria-endemic areas the use of CR1 as an invasion receptor may become more significant due to immunological pressure from antibodies targeting the EBA proteins which mediate the SA-dependent pathway, and field parasites could switch from complete or modest SA-dependency to partial or more SA-independence. This hypothesis is supported by evidence that children exposed to endemic *P. falciparum* transmission acquire immunity to the SA-dependent pathway before resistance to SA-independent invasion is developed [19]. This suggests that the role of CR1 in mediating erythrocyte invasion in exposed individuals will increase as they develop antibodies to the SA-dependent ligands EBA-175 and EBA-140.

Taken together, our studies show that the use of CR1 as an invasion receptor is common among Kenyan strains of *P. falciparum*. Although a recent study showed that PfRh4 is a ligand for CR1 [11], additional parasite ligands may exist. Therefore, characterization of all the parasite ligand(s) that bind CR1 will be critical to increase our understanding of the interaction of CR1 with the parasite.

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## Conflict of interest

The authors have no conflict of interest to report.

## Disclaimer

The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense. The

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