

A Receptor for the Malarial Parasite *Plasmodium vivax*: The Erythrocyte Chemokine Receptor

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Plasmodium vivax and *P. falciparum* are the major causes of human malaria, except in sub-Saharan Africa where people lack the Duffy blood group antigen, the erythrocyte receptor for *P. vivax*. Duffy negative human erythrocytes are resistant to invasion by *P. vivax* and the related monkey malaria, *P. knowlesi*. Several lines of evidence in the present study indicate that the Duffy blood group antigen is the erythrocyte receptor for the chemokines interleukin-8 (IL-8) and melanoma growth stimulatory activity (MGSA). First, IL-8 binds minimally to Duffy negative erythrocytes. Second, a monoclonal antibody to the Duffy blood group antigen blocked binding of IL-8 and other chemokines to Duffy positive erythrocytes. Third, both MGSA and IL-8 blocked the binding of the parasite ligand and the invasion of human erythrocytes by *P. knowlesi*, suggesting the possibility of receptor blockade for anti-malarial therapy.

The erythrocyte chemokine receptor binds a family of chemotactic and proinflammatory soluble peptides (1-3), including IL-8, MGSA, monocyte chemotactic protein 1 (MCP-1), and regulated on activation, normal T expressed and secreted (RANTES) (4). We have previously shown that the erythrocyte chemokine receptor differs from the IL-8 receptors, IL-8RA and IL-8RB, on neutrophils (1-3, 5, 6). Whereas the neutrophil IL-8 receptors bind only IL-8 and MGSA (5, 6), the erythrocyte receptor binds IL-8, MGSA, RANTES, and MCP-1 (1-3). In the course of characterizing the erythrocyte chemokine receptor, we noted that erythrocytes from the majority of African Americans studied did not bind IL-8 (7). A high percentage of African Americans have the Duffy blood group negative phenotype (Duffy negative), which is rare in Caucasians (8). In this study we found that African Americans whose erythrocytes did not bind IL-8 lacked the Duffy blood group antigen. The Duffy blood group antigen has previously been shown to be required for the invasion of human erythrocytes by the human malarial parasite *Plasmodium vivax* (9) and the related monkey malaria *P. knowlesi* (10). We now show that IL-8 and MGSA block the binding of the *P. knowlesi* protein that binds to the Duffy blood group antigen and also block invasion of human erythrocytes by *P. knowlesi*.

Erythrocytes from African American and Caucasian blood donors were classified as Duffy positive or Duffy negative, and

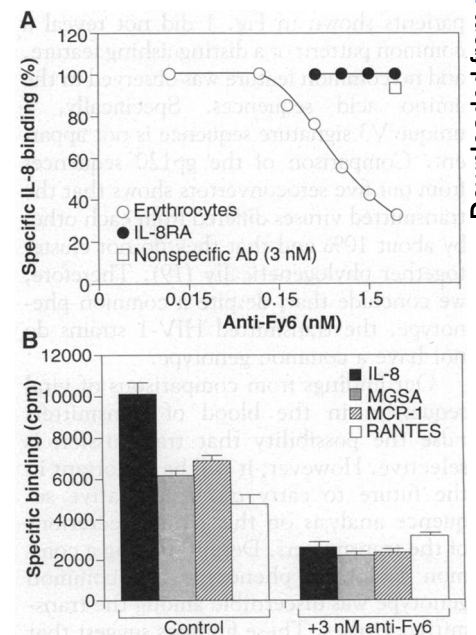
their ability to bind ^{125}I -labeled IL-8 was determined (Table 1). There was an absolute association of IL-8 binding and the Duffy positive phenotype, indicating that the erythrocyte chemokine receptor is the Duffy blood group antigen or that they are closely linked.

There are a number of similarities in the biochemical properties of the erythrocyte chemokine receptor and the Duffy blood group antigen. First, both proteins have a molecular size of 35 to 45 kD (2, 3, 11). Second, both proteins aggregate to a high molecular weight complex in SDS-polyacrylamide gels when boiled in SDS-2-mercapto-

Fig. 1. Inhibition of chemokine binding to the erythrocyte chemokine receptor by anti-Fy6, a monoclonal antibody to the human Duffy blood group antigen. **(A)** Inhibition of specific ^{125}I -labeled IL-8 binding. Erythrocytes (2×10^8 cells in $500 \mu\text{l}$) were preincubated with increasing concentrations of anti-Fy6 for 2 hours at 4°C . The cells were then incubated for a further hour with 0.5 nM ^{125}I -labeled IL-8 in a final volume of $600 \mu\text{l}$. The binding reactions were terminated as described in Table 1. Nonspecific binding was determined by addition of 100 nM unlabeled IL-8 to the binding reactions. This value was subtracted from the total binding to determine the specific binding of IL-8. Parallel binding experiments to examine the effect of anti-Fy6 on the binding of ^{125}I -labeled IL-8 to human kidney cells transfected with one of the leukocyte IL-8 receptors, IL-8RA (5), were carried out as described for the erythrocytes. The effect of an isotype-matched antibody to gp120 on IL-8 binding to erythrocytes was also determined. **(B)** Inhibition of specific binding of radiolabeled IL-8, MGSA, MCP-1, and RANTES. ^{125}I -labeled IL-8 (specific activity 874 Ci/mmol) and ^{125}I -labeled MGSA (specific activity 322 Ci/mmol) were labeled as previously described (21); ^{125}I -labeled MCP-1 and ^{125}I -labeled RANTES (specific activity 1100 Ci/mmol) were from New England Nuclear. Experiments were carried out as described above except that cells were preincubated with anti-Fy6 at a final concentration of 3 nM for 2 hours at 4°C . The cells were then incubated for a further hour with 0.5 nM radiolabeled chemokines. Data are represented as the mean \pm SEM (22).

ethanol (11, 12). Third, the number of chemokine receptor binding sites detected by radiolabeled IL-8 binding is about the same as the number of Duffy blood group antigens detected by antibody (5,000 to 10,000 per cell) (1, 2, 13). Fourth, both proteins are resistant to degradation by treatment of intact erythrocytes with trypsin; both are degraded by treatment of erythrocytes with chymotrypsin (1, 11).

A monoclonal antibody to the Duffy blood group antigen, anti-Fy6 (13), which inhibits the binding of the *P. knowlesi* ligand (14), was tested for its ability to inhibit the binding of radioactively labeled IL-8 to Duffy positive erythrocytes. Preincubation of erythrocytes with anti-Fy6 inhibited the binding of IL-8 in a dose-dependent manner, with 70% inhibition at an antibody concentration of 3 nM (Fig. 1A). An irrelevant, isotype-matched antibody had no effect at a similar concentration. The inhibition of IL-8 binding to the Duffy blood group antigen was specific because anti-Fy6 had no effect on IL-8 binding to cells transfected with one of the cloned IL-8 receptors, IL-8RA (Fig. 1A). Because the erythrocyte chemokine receptor binds MGSA, MCP-1, and RANTES in addition to IL-8 (2), we examined the ability of anti-Fy6 to inhibit the binding of these chemokines to erythrocytes. Preincubation of Duffy positive erythrocytes with anti-Fy6 at a final concentration of 3 nM inhibited the binding of each of these chemokines (Fig. 1B).



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Human Duffy negative erythrocytes are refractory to invasion by the human malarial parasite *P. vivax* (9) and the simian parasite *P. knowlesi* (10). A 135-kD parasite protein that binds the Duffy blood group antigen on human erythrocytes has been identified from *P. knowlesi* culture supernatants (14). We examined whether the chemokines MGSA and IL-8 could block the binding of this protein to the Duffy blood group antigen. Preincubation of Duffy positive erythrocytes with MGSA inhibited the binding of the 135-kD Duffy antigen binding protein, with greater than 97% inhibition at MGSA concentrations of 100 nM or higher (Fig. 2). IL-8 also blocked binding, with 72% inhibition at a concentration of 100 nM and complete blocking at concentrations of 1000 nM or higher.

Both MGSA and IL-8 inhibited the invasion of human Duffy positive erythrocytes by *P. knowlesi* (Table 2). With MGSA, the inhibition of invasion reached

saturation at around 100 nM, with greater than 94% inhibition (Table 2). Inhibition with IL-8 reached saturation at higher concentrations, with about 95% blocking at 1000 nM. The affinity constants (K_d 's) of MGSA and IL-8 binding to the erythrocyte chemokine receptor are very similar, 5 nM (1, 2). We cannot explain why an almost 10-fold higher concentration of IL-8 than MGSA is required to inhibit invasion by *P. knowlesi*.

Control studies excluded the possibilities that the chemokines had nonspecific effects on the parasites or on the susceptibility of human erythrocytes to invasion. The chemokines had no effect on *P. knowlesi* invasion of rhesus erythrocytes and trypsin-treated Duffy negative erythrocytes (Table 2), both of which are invaded independently of the Duffy blood group antigen (14). This indicated that the chemokines had no toxic effect on the parasites. To exclude nonspecific effects of the binding of the chemokines to human erythrocytes, we

studied invasion by *P. falciparum*, which does not bind to the Duffy blood group antigen (15). Invasion by *P. falciparum* was not affected by incubation in the presence of 1000 nM MGSA or IL-8, indicating that these chemokines were specifically blocking invasion by *P. knowlesi* at the binding step.

The data presented indicate that the Duffy blood group antigen and the erythrocyte chemokine receptor are the same protein. Further evidence would be provided by the demonstration that purified Duffy antigen could bind IL-8. However, the methods developed for the purification of the Duffy blood group antigen utilize SDS-polyacrylamide gel electrophoresis (PAGE) (16), which denatures the chemokine receptor, as do a variety of other detergents

Table 1. Association between Duffy blood group antigen and IL-8 binding to human erythrocytes. Blood samples were collected from 33 Caucasian and African American donors of known Duffy type. Erythrocytes were isolated from human blood as described (1) and incubated (2×10^8 cells per 600 μ l) with 0.5 nM 125 I-labeled IL-8 at 4°C for 1 hour. The binding reactions were terminated by centrifugation of erythrocytes through a paraffin-oil mixture as described (2). Nonspecific binding was determined in the presence of 100 nM unlabeled IL-8 and was less than 10% of total binding. Specific IL-8 binding was determined by subtraction of nonspecific binding from total binding.

Donors	Duffy type*	Number tested	Specific IL-8 binding (cpm \pm SEM)
Caucasians	Positive	11	9705 \pm 695
African Americans	Positive	5	7075 \pm 1079
African Americans	Negative	17	162 \pm 46

*The Duffy phenotypes of erythrocytes were determined by standard blood banking techniques with the use of two antisera (anti-Fya and anti-Fyb). See (8) for a description of the Duffy blood group system. Duffy negative, Fy(a⁻b⁻) erythrocytes did not react with either antiserum. Duffy positive erythrocytes included the phenotypes Fy(a⁺b⁻), Fy(a⁻b⁺), and Fy(a⁺b⁺). There was no significant difference in specific 125 I-labeled IL-8 binding to erythrocytes of the three Duffy positive phenotypes.

Table 2. Erythrocyte invasion efficiencies for *P. knowlesi* in the presence of MGSA and IL-8. To study the effect of MGSA and IL-8 on invasion, we preincubated erythrocytes [2×10^7 in a volume of 870 μ l of RPMI containing 22 mM glucose, 29 mM HEPES (pH 7.4), and 10% fetal calf serum, per invasion] with increasing concentrations of these chemokines for 1 hour at room temperature. Percoll-purified *P. knowlesi* schizont-infected erythrocytes (2×10^6 in 100 μ l) and 30 μ l of 7.5% sodium bicarbonate were added to these erythrocytes in the presence of the chemokines for 6 to 7 hours, during which time the infected erythrocytes ruptured, releasing merozoites that were able to invade other erythrocytes. The cells were centrifuged through Percoll to separate the ring-infected and normal erythrocytes from free merozoites. A thin smear of the resuspended erythrocytes was stained with Giemsa, and the percentage of erythrocytes infected with ring-stage parasites was determined. The invasion rates are expressed as a percentage of the rate of invasion in the absence of chemokines. Values for IL-8 are given in parentheses.

MGSA (or IL-8) (nM)	Percent invasion			
	Fy(a ⁺ b ⁺)	Fy(a ⁻ b ⁺)	Rhesus	Trypsin-treated Duffy negative
0	100	100 (100)	100	100
0.1	87	109 (99)	110	84
1	90	75 (87)	110	100
10	38	45 (96)	92	87
100	3	6 (29)	89	100
1000	3	1 (5)	117	97

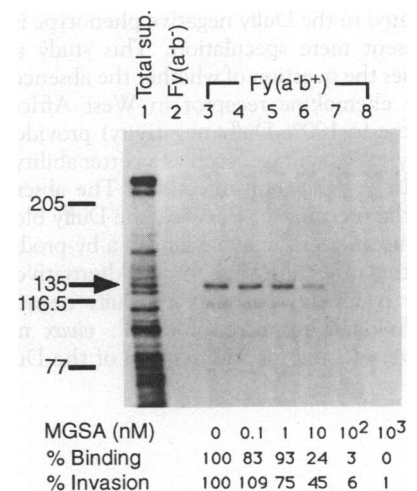


Fig. 2. Inhibition of the binding of a *P. knowlesi* protein to the Duffy blood group antigen with MGSA. *Plasmodium knowlesi* schizonts were metabolically labeled with [35 S]methionine and [35 S]cysteine, and the labeled culture supernatants were used in erythrocyte binding assays as described (14). Briefly, 1×10^9 human Duffy positive, Fy(a⁻b⁺), or human Duffy negative, Fy(a⁻b⁻), erythrocytes were incubated with 200 μ l of labeled culture supernatant for 1 hour at room temperature and centrifuged through silicone oil. Proteins bound to the erythrocytes were eluted with 300 mM NaCl. The eluted proteins were visualized by SDS-PAGE on 7.5% acrylamide gels followed by fluorography. Lane 1, total parasite supernatant; lane 2, Fy(a⁻b⁻) erythrocytes, no MGSA; lanes 3 to 8, Fy(a⁻b⁺) erythrocytes, preincubated in the presence of increasing concentrations of MGSA as shown. The autoradiograph was scanned with a densitometer to quantify the decrease in binding in the presence of increasing concentrations of MGSA. Binding of the 135-kD *P. knowlesi* protein was expressed as a percentage of binding in the absence of MGSA and is shown below each lane in the figure. The figure also shows *P. knowlesi* invasion rates (data from Table 2) in the presence of MGSA as a percentage of the invasion rate in the absence of MGSA. Molecular size markers (BRL) are indicated on the left (in kilodaltons).

(3). Thus, it would not be possible to demonstrate IL-8 binding to the Duffy antigen purified by these routes. Direct evidence in support of the idea that the Duffy blood group antigen and the chemokine receptor are the same protein will have to await their molecular cloning.

The physiological role of the erythrocyte chemokine receptor is not yet known. It has been postulated to act as a scavenger for certain inflammatory mediators, including the leukocyte chemotaxin IL-8 (1). If this hypothesis is correct, then there might be physiologic differences between Duffy negative humans, who lack the receptor, and Duffy positive humans. In this connection it has been observed that Africans and African Americans have a lower peripheral neutrophil count than Caucasians (17). Whether this lowered white cell count is causally related to the Duffy negative phenotype is at present mere speculation. This study also raises the question of whether the absence of the chemokine receptor in West Africans (close to 100% Duffy negativity) provides a survival advantage, such as a better ability to resist *P. falciparum* infections. The absence of the receptor for *P. vivax*, the Duffy blood group antigen, may have been a by-product of this other selective force. Alternatively, the complete resistance of Duffy negative individuals to infection by *P. vivax* may have selected for the fixation of the Duffy

negative phenotype in West Africa.

Parasites use host cell surface determinants for invasion, and in some cases these are receptors involved in receptor-ligand interactions of importance to the host (18). The finding that the erythrocyte chemokine receptor is also a receptor for the malaria parasite *P. vivax* suggests that receptor blockade therapy using drugs modeled on the structure of IL-8 and MGSA could be useful therapeutically. Such therapy may become important in the future with the spread of chloroquine-resistant strains of *P. vivax* (19). This is particularly important in Asia and in Central America, where *P. vivax*-associated malaria predominates (20).

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22. Input counts and nonspecific binding cpm for the experiment shown in Fig. 1B were as follows: ¹²⁵I-labeled IL-8 (67,649 and 787 cpm), ¹²⁵I-labeled MGSA (48,935 and 854 cpm), ¹²⁵I-labeled MCP-1 (44,067 and 461 cpm), and ¹²⁵I-labeled RANTES (42,752 and 297 cpm).
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