Review

Roles of the Duffy antigen and glycophorin A in malaria infection and erythrocyte

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ABSTRACT: We constructed gene knockout mice lacking either the Duffy antigen (*Dfy*) or glycophorin A (*GPA*), major glycoproteins that are expressed on erythrocyte membranes, to examine the role of these proteins in malaria infection and erythrocyte. All of the rodent malarias examined proliferated in the erythrocytes of these knockout mice, indicating that neither the Duffy antigen nor GPA has an essential role as a receptor for malaria parasites. Duffy antigen knockout mice infected by *Plasmodium yoelii* 17XL exhibited autotherapy. At the early stage of the infection, the parasite proliferated exponentially, whereas at the late stage, parasitemia decreased to a level at which the mice were considered cured. The results of depletion experiments with anti-CD4 antibodies suggested that CD4-positive cells in the Duffy antigen knockout mice were responsible for the autotherapy effect. The Duffy antigen is a chemokine receptor. Compared to wild-type mice, chemokines which have affinities for the Duffy antigen injected intravenously more rapidly disappeared from the Duffy antigen knockout mice. Stimulation of the immune response by the increase of leukocytes might lead to the suppression of parasitemia in the Duffy antigen knockout mice. The absence of GPA decreased the amount of *O*-linked oligosaccharides on the erythrocyte membranes. The erythrocyte membranes of the GPA knockout mice decreased several *O*-linked glycoproteins and TER-119 protein. GPA has an essential role in the expression of *O*-linked antigens on erythrocyte membranes, but these proteins are not important for malaria parasite invasion of erythrocytes.

Keywords: Duffy antigen, Glycophorin A, Malaria, Knockout mouse

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Introduction

The initial step in the life cycle of malaria parasites in blood, is their invasion of the erythrocyte membranes (*1-3*). There have been several efforts to identify the malaria parasite receptors on the erythrocyte membranes (*4,5*). In humans, the Duffy antigen (*6-9*) and glycophorin A (GPA) (*10,11*) are thought to have roles as malaria receptors (*12*). In mice, however, little is known about the malaria receptors. To understand the roles of the Duffy antigen and GPA in malaria infection, we constructed mice with gene knockout mice lacking these proteins (*13,14*). In this review, we describe some characteristic features of the knockout mice to elucidate the physiologic functions of the Duffy antigen and glycophorin A proteins on erythrocyte and their roles on malarial infection.

Construction of gene knockout mice lacking either the Duffy antigen or glycophorin A

The gene encoding the Duffy antigen contains two exons (*15*). We deleted the chromosomal region of embryonic stem cells encoding the protein by homologous recombination followed by germ line transmission to construct the gene knockout mice (*13*). Inter-crossing of heterozygote mice resulted in the appearance of a homozygous deletion of the *Dfy* gene, according to Mendelian laws of inheritance (Table 1). The knockout mice appeared normal, and had no reproductive problems. These results demonstrated that the Duffy antigen is not essential for mouse development.

GPA contains a transmembrane domain (*16,17*). The *N*-terminal region of the protein is modified by *O*-linked oligosaccharides (*18,19*). To construct of gene knockout mice, we attempted to delete exons 4, 5, 6, and 7, which encode the transmembrane region of GPA (*14*). Inter-crossing of mice with a heterozygous deletion of the GPA gene resulted in he appearance of a homozygous deletion according to Mendelian law, indicating that

GPA is also not essential for mouse development (Table 1).

Growth of rodent malaria parasites in erythrocyte at the early stage of infection

Both the Duffy antigen knockout mice and the GPA knockout mice were susceptible to all types of rodent malaria examined; *Plasmodium berghei* NK65, *Plasmodium chabaudi*, *Plasmodium vinckei*, and *Plasmodium yoelii* 17XL. These results suggest that neither the Duffy antigen nor GPA do not function as receptors for these malarias (*20*). However, the Duffy antigen knockout mice infected with *Plasmodium yoelii* 17XL exhibited autotherapy (Figure 1). In wild-type mice, parasitemia increased to more than 80% within 5 days after infection, and the mice began to die. In the Duffy antigen knockout mice, parasitemia did not increase to more than 50%, and was maintained at a similar level for 2 weeks, then decreased to zero 3 weeks after infection.

Effect of the Duffy antigen on immunity

Because *Plasmodium yoelii* 17XL proliferated in the erythrocytes of Duffy antigen-knockout mice in the early stage of infection, we assumed that the autotherapy was due to an immunologic event in the host. The number of leukocytes in the blood of the Duffy antigen knockout mice increased twice as much as that in wild-type mice 5 days after infection (Figure 2A). The numbers of neutrophils, monocytes, and lymphocytes increased were much higher in Duffy antigen knockout mice than in wild-type mice, whereas the number of eosinophilic leukocytes was not different (Figure 2B).

We then examined the types of leukocytes responsible for the autotherapy depletion experiments by using carrageenan and CD4 and CD8 antibodies. The decrease in parasitemia in the Duffy antigen knockout mice was not affected by carrageenan treatment, which depletes phagocytosis-active lymphocytes (Figure 3). When CD4-positive cells were depleted with antibodies against CD4, the decrease in parasitemia was blocked (Figure 4). When the mice were treated with anti-CD8 antibody to deplete CD8-positive cells, the decrease in parasitemia resumed. These results suggest that CD4-positive cells, but not CD8-positive cells or macrophages, were responsible for the autotherapy in the Duffy antigen knockout mice infected with *Plasmodium yoelii* 17XL.

A role for the Duffy antigen in maintaining the plasma concentration of chemokines

The Duffy antigen has affinities for various types of chemokines (*15,21,22*). The Duffy antigen selectively

Table 1. Genotype of glycophorin A and the Duffy antigen knockout mouse heterozygote intercrossed pups

Glycophorin A	genotype		
	+/+	+/–	–/–
Number of pups	18	37	22

Duffy antigen	genotype		
	+/+	+/–	–/–
Number of pups	106	201	77

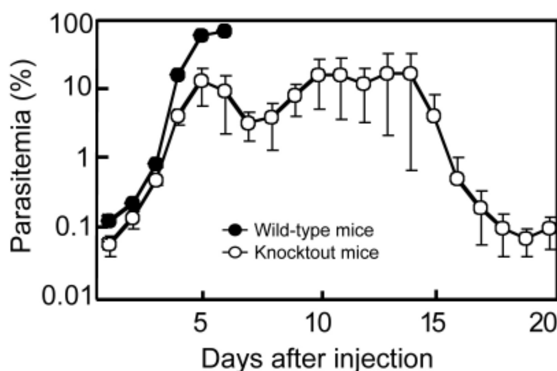

Figure 1. Resistance and invasion of *P. yoelii* 17XL infection in the Duffy antigen knockout mice. Mice were intravenously injected with erythrocyte suspension containing 105 erythrocytes parasitized with *P. yoelii* 17XL.

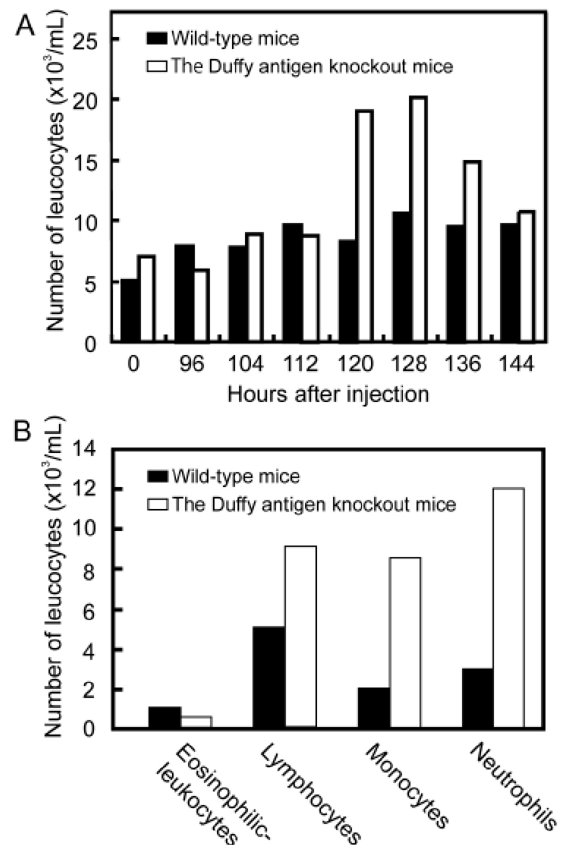

Figure 2. Increase of leukocytes in the Duffy antigen knockout mice 5 days after infection of *P. yoelii* 17XL. A: Blood was collected from mice at the indicated periods and stained with Turk's stain solution, and the number of leukocytes was counted with a hemocytometer. B: Tail blood smears were prepared 5 d after infection followed by staining with the Write-Giemsa method. Each type of leukocyte was determined based on the staining pattern and the number of leukocytes in the blood was calculated.

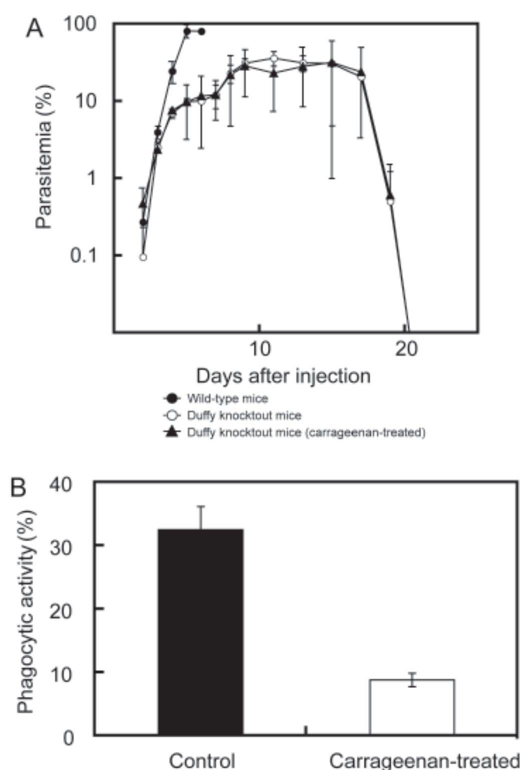

Figure 3. Effect of carrageenan on infection by *P. yoelii* 17XL in erythrocytes in the Duffy antigen knockout mice. A: Mice were intravenously injected with an erythrocyte suspension containing 105 erythrocytes parasitized with *P. yoelii* 17XL. Parasitemia was assessed by microscopic examination of Giemsa-stained smears of tail blood. B: Inhibition of phagocytotic activity of adherent spleen cells by carrageenan. Adherent spleen cells from carrageenan-treated or control mice were incubated with fluorescein isothiocyanate-conjugated beads at 37°C for 30 min. Phagocytic activity was assayed by FACScan analysis with gating on a Mac-1 high population. Data are mean ± SD.

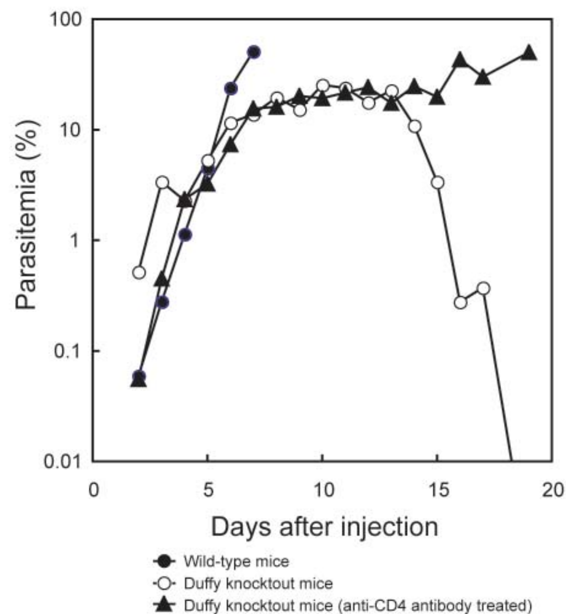

Figure 4. Effect of injection with anti-CD4 antibody or anti-CD8 antibody to the Duffy antigen knockout mice infected with *P. yoelii* 17XL. Mice were intravenously injected with an erythrocyte suspension containing 105 erythrocytes parasitized with *P. yoelii* 17XL. Parasitemia was assessed by microscopic examination of Giemsa-stained tail blood smears.

Table 2. Plasma concentrations of chemokines in wild-type and the Duffy antigen knockout mice

Chemokine	Genotype of the *Dfy* gene	
	+/+	–/–
Eotaxin (pg/mL)	4,200 ± 1,800	930 ± 30
MCP-1 (pg/mL)	110 ± 40	< 40

binds distinct members of the pro-inflammatory chemokines such as CXCL1, CXCL5, CCL2, CCL5, and CCL7, but not lymphoid chemokines such as CCL21, CCL19, CXCL12, and CXCL13 (*23*). The Duffy antigen is suggested to act as a chemokine “sink”, thereby excluding plasma chemokines from being reutilized (*22,24*). We examined the plasma concentrations of eotaxin and MCP-1, which have affinities for the Duffy antigen. The plasma concentrations of these chemokines were much lower in Duffy antigen-knockout mice than in wild-type mice (Table 2). We also the examined clearance rate of these chemokines in both the Duffy antigen knockout mice and wild-type mice. In this experiment, we injected intravenously recombinant chemokines such as eotaxin, hMGSA, and MCP-1, which bind to the Duffy antigen, and hIP-10 and interferon-γ, which do not bind the Duffy antigen (*25-28*), then determined the plasma concentrations by enzyme-linked immunosorbent assay. The results indicated that concentration of eotaxin, hMGSA, and MCP-1 in blood were less stable in the plasma of the Duffy antigen knockout mice than in that of the wild-type mice (Figure 5) (*13,29*). A recent study also demonstrated rapidly vanishing of chemokines in the Duffy antigen knockout mice (*30*). We propose that the Duffy antigen acts as a "reservoir" to regulate plasma chemokine concentrations (*13*).

Therefore, a lack of the Duffy antigen should negatively affect the plasma concentrations of a set of chemokines. Interleukin-27 acts as a negative regulator of inflammatory T-cell responses against parasitic infections (*31*). Based on these findings, we propose a model to explain autotherapy in the Duffy antigen knockout mice infected with *P. yoelii* 17XL (Figure 6). At the initial infection, *P. yoelii* 17XL induces certain cytokines, which function to repress CD4-positive cell activity and whose plasma concentrations are maintained by the Duffy antigen. The plasma concentrations of these cytokines were lower in the Duffy antigen knockout mice than in wild-type mice; thus CD4-positive cell activity was enhanced and the autotherapeutic phenotype was observed.

Characterization of GPA knockout mice

GPA is a major glycoprotein on erythrocyte membranes. This protein contains *O*-linked oligosaccharides on the *N*-terminal regions locating in the external space of the erythrocytes. We extracted the oligosaccharides from erythrocyte membranes of the GPA knockout

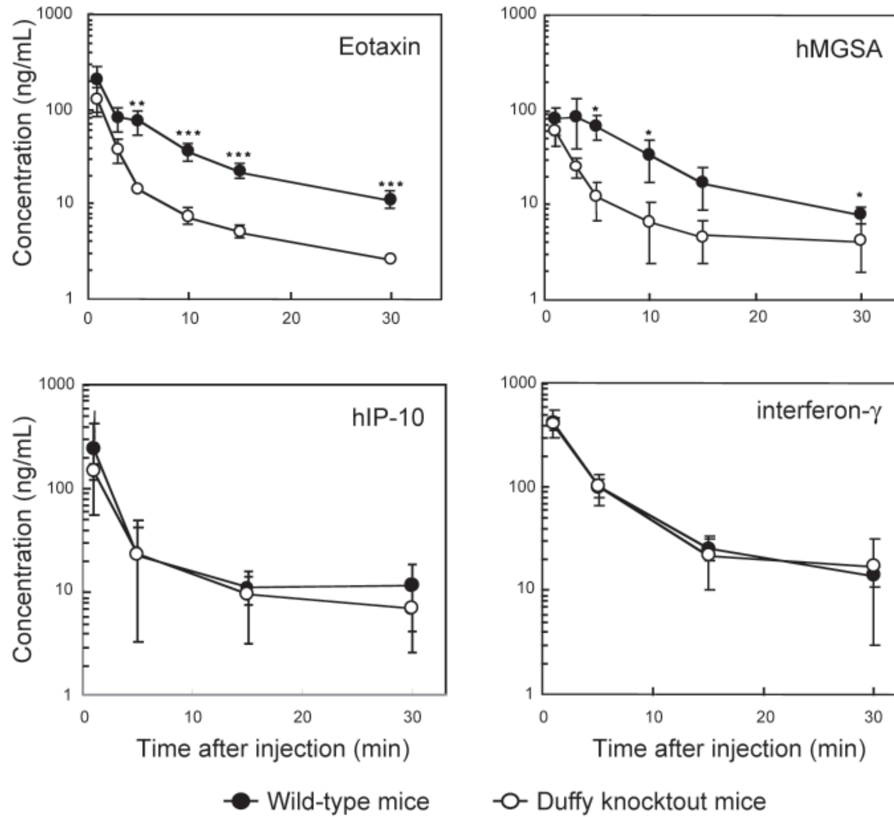

Figure 5. Clearance of eotaxin, hMGSA, hIP-10, and interferon-g from plasma. After intravenous injection of these chemokines and IFN-γ, blood samples were collected from tails using heparinized capillary tubes. Chemokines in the plasma concentration were determined by enzyme-linked immunosorbent assay. *** $P < 0.005$, ** $P < 0.02$, * $P < 0.05$, $n = 3$-5, mean ± SD.

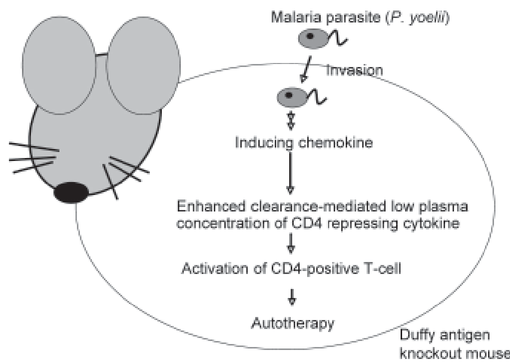

Figure 6. Scheme of autotherapy in the Duffy antigen knockout mice infected with *P. yoelii* 17XL.

Table 3. Comparison of monosaccharides amount on the erythrocyte membrane of wild-type and GPA knockout mouse

Monosaccharides	Amount of monosaccharides (nmol/mg protein)	
	WT	KO
Fucose	17 ± 4	17 ± 6
GalNAc	23 ± 3*	14 ± 3*
GlcNAc	97 ± 29	112 ± 34
Galactose	82 ± 22	75 ± 22
Mannose	68 ± 6	94 ± 38
NeuAc	182 ± 33	135 ± 22

(* $P < 0.01$)

mice, followed by acid-hydrolysis of the materials, and analysis by high performance liquid chromatography with an anion-exchange column. The amounts of all of three different species of oligosaccharide chains were decreased in the erythrocyte membranes of the GPA mice compared to the wild-type mice (Figure 7).

We then analyzed the monosaccharide composition of the oligosaccharides. Isolated oligosaccharides were further hydrolyzed, followed by analysis on high performance liquid chromatography. The amount of *N*-acetylgalactosamine (GalNAc) was decreased in the fraction from the GPA knockout mice (Table 3). Because GalNAc is a characteristic of *O*-linked sugars (*32*), the results suggest that the amount of *O*-linked sugars in the erythrocyte membrane was decreased in the GPA knockout mice.

TER-119 antigens are localized on the erythrocyte membrane in close relation to GPA (*33*). Therefore, we tested for the presence of TER-119 antigens on the erythrocyte membranes of the GPA knockout mice. Immunofluorescence staining using an antibody against TER-119 antigens demonstrated the presence of TER-119 antigens on the erythrocyte membranes of wild-type mice. TER-119 antigens were not present, however, in the erythrocyte membranes of GPA knockout mice (Figure 8A). Fluorescence-activated cell-sorting analysis using the antibody also revealed

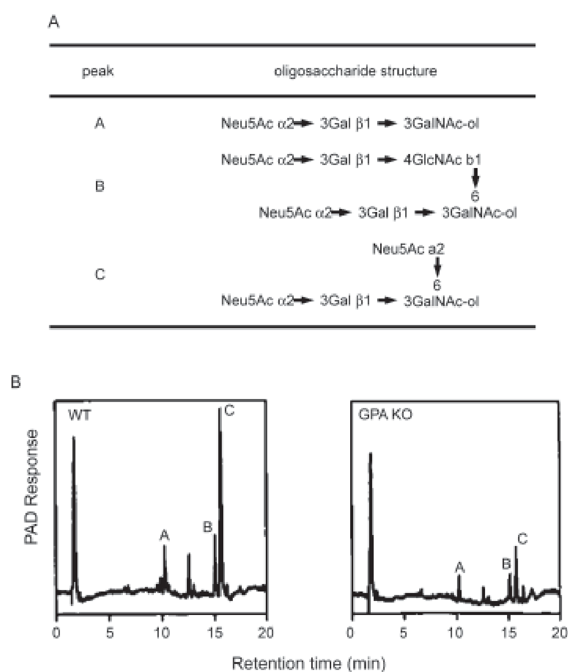

Figure 7. Decrease of *O*-glycans on the erythrocytes of GPA knockout mice. A: Schematics of *O*-glycans on the murine erythrocyte. B: Analysis of *O*-glycans by high-performance anion-exchange chromatography with pulsed amperometric detection.

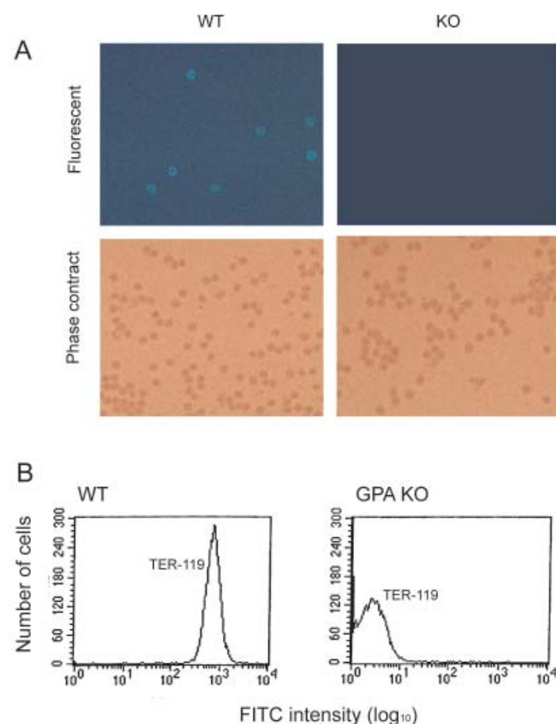

Figure 8. Disappearance of TER-119 antigens on the erythrocytes of GPA knockout mice. Blood cells isolated from wild-type and GPA knockout mice were stained with TER-119 antibody, followed by staining with fluorescein isothiocyanate-conjugated secondary antibody (A). Samples were analyzed by FACScan (B).

the absence of TER-119-positive erythrocytes in the knockout mice (Figure 8B) (*14*). The absence of the TER-119 antigen on the erythrocyte membranes was further confirmed by Western blot analysis using the

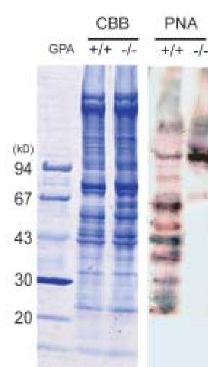

Figure 9. Lectin stain of GPA knockout mouse. Erythrocyte membranes prepared from wild-type and GPA knockout mice were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, After transferring, glycoproteins were detected using PNA.

anti-TER-119 antibody. Two bands stained with the anti-TER-119 antigen in the sample from wild-type mice were not present in the sample from the GPA knockout mice (*14*). Because the TER-119 antigens are distinct from GPA, the results indicate that the amounts of proteins other than GPA were also decreased in the erythrocyte membranes of GPA knockout mice.

Peanut agglutinin is a lectin that has affinities for *O*-linked sugars (*34*). Western blot analysis probing with Peanut agglutinin demonstrated the lack of some glycoproteins with *O*-linked sugars in the erythrocyte membranes of GPA knockout mice (Figure 9). GPA seems to have an essential role in the expression of those glycoproteins with *O*-linked sugars onto the red blood cell surface. The results considering with the sugar analysis suggest that not only GPA, but also some of other glycoproteins with *O*-linked sugars are lost in the erythrocyte membrane of GPA knockout mice. Because the rodent malaria parasites initially proliferated in the GPA knockout mice, the present results suggest neither GPA nor glycoproteins with *O*-linked sugars have a role as receptors for rodent malarias.

In this review, we discussed about our research findings regarding the functions of two erythrocyte membrane proteins, GPA and the Duffy antigen, in malaria infection and in the physiology. We hope to further clarify the function of the erythrocyte membranes, which comprise 40% to 50% of the blood.

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