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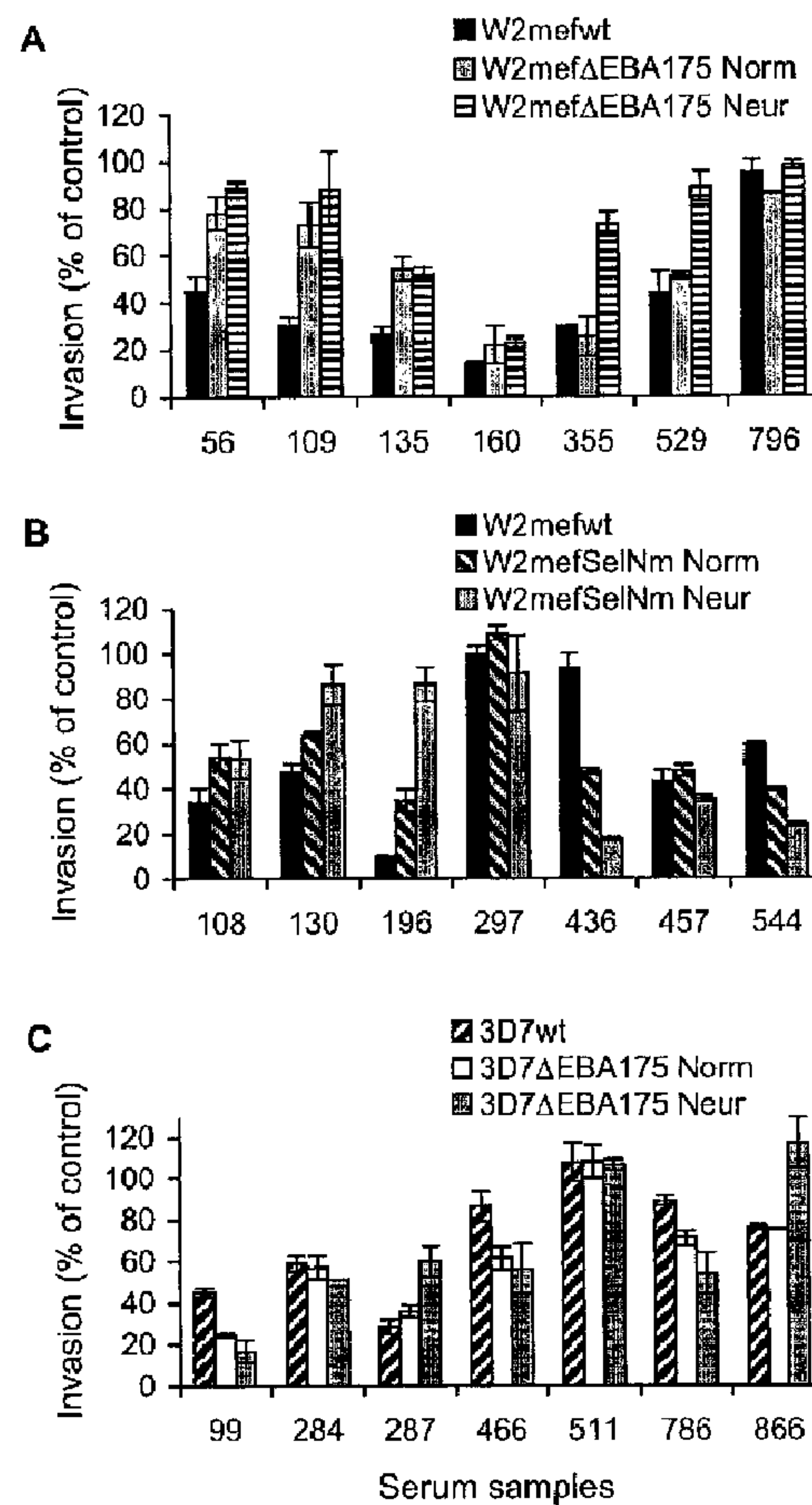
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(72) **Inventeurs/Inventors:**
COWMAN, ALAN, AU;
BEESON, JAMES, AU;
MAIER, ALEXANDER GERD, AU;
PERSSON, KRISTINA E.M., AU;
RICHARDS, JONATHAN S., AU;
LOPATICKI, SASH, AU

(73) **Propriétaire/Owner:**

(54) **Titre : METHODES ET COMPOSITIONS POUR LE TRAITEMENT ET LA PREVENTION DE LA MALARIA (2)**

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(57) **Abrégé/Abstract:**

Vaccines, in particular, antigens capable of eliciting antibodies capable of preventing invasion of Plasmodium parasite into erythrocytes, and use of said vaccines for the treatment and/or prevention of malaria. In one embodiment, the antigen is an

(73) **Propriétaires(suite)/Owners(continued):**THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH, AU

(74) **Agent:** RIDOUT & MAYBEE LLP

(57) **Abrégé(suite)/Abstract(continued):**

immunogenic molecule comprising a contiguous amino acid sequence of an erythrocyte binding antigen (EBA) protein of a strain of Plasmodium falciparum, wherein when administered to a subject the molecule is capable of inducing an invasion- inhibitory immune response to the strain. Also disclosed is a method of screening for the presence of a Plasmodium falciparum invasion-inhibitory antibody directed against an erythrocyte binding antigen (EBA) of a strain of Plasmodium falciparum in a subject.

Abstract

Vaccines, in particular, antigens capable of eliciting antibodies capable of preventing invasion of Plasmodium parasite into erythrocytes, and use of said vaccines for the treatment and/or prevention of malaria. In one embodiment, the antigen is an immunogenic molecule comprising a contiguous amino acid sequence of an erythrocyte binding antigen (EBA) protein of a strain of Plasmodium falciparum, wherein when administered to a subject the molecule is capable of inducing an invasion-inhibitory immune response to the strain. Also disclosed is a method of screening for the presence of a Plasmodium falciparum invasion-inhibitory antibody directed against an erythrocyte binding antigen (EBA) of a strain of Plasmodium falciparum in a subject.

METHODS AND COMPOSITIONS FOR TREATING AND PREVENTING MALARIA (2)

FIELD OF THE INVENTION

The present invention relates to vaccines for the treatment and prevention of malaria. In particular the invention provides antigens capable of eliciting antibodies capable of preventing invasion of Plasmodium parasite into erythrocytes.

BACKGROUND

Human malaria is caused by infection with protozoan parasites of the genus Plasmodium. Four species are known to cause human disease: Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale and Plasmodium vivax. However, Plasmodium falciparum is responsible for the majority of severe disease and death. Recent estimates of the annual number of clinical malaria cases worldwide range from 214 to 397 million (World Health Organization. The world health report 2002: reducing risks, promoting healthy life. Geneva: World Health Organization, 2002; Breman et al (2004) American Journal of Tropical Medicine and Hygiene 71 Suppl 2:1–15.), although a higher estimate of 515 million (range 300 to 660 million) clinical cases of Plasmodium falciparum in 2002 has been proposed (Snow et al. (2004) American Journal of Tropical Medicine and Hygiene 71(Suppl 2):16–24). Annual mortality (nearly all from Plasmodium falciparum malaria) is thought to be around 1.1 million (World Health Organization. The world health report 2002: reducing risks, promoting healthy life. Geneva: World Health Organization, 2002; Breman et al (2004) American Journal of Tropical Medicine and Hygiene 71 Suppl 2:1–15.). Malaria also significantly increases the risk of childhood death from other causes (Snow et al. (2004) American Journal of Tropical Medicine and Hygiene 71 Suppl 2:16–24). Almost half of the world's population lives in areas where they are exposed to risk of malaria (Hay et al (2004) Lancet Infectious Diseases 4(6):327–36), and the increasing numbers of visitors to endemic areas are also at risk. Despite continued efforts to control malaria, it remains a major health problem in many regions of the world, and new ways to prevent and/or treat the disease are urgently needed.

Early optimism for vaccines based on malarial proteins (so called subunit vaccines) has been tempered over the last two decades as the problems caused by allelic polymorphism and antigenic variation, original antigenic sin, and the difficulty of generating high levels of durable immunity emerged, and with the notable failures of

many promising subunit vaccines (such as SPf66) have led to calls for a change in approach towards a malaria vaccine. Consequently, this growing sense of frustration has led to the pursuit of different approaches that focus on attenuated strains of malaria parasite or irradiated *Plasmodium falciparum* sporozoites (Hoffmann et al. (2002) *J Infect Dis* 185(8):1155-64). Similarly, both the limited success achieved to date with protein-based vaccines and the recognition that cell mediated immunity may be critical to protection against hepatic and perhaps blood stages of the parasite has led to a push for DNA and vectored vaccines, which generate relatively strong cell mediated immunity. To date DNA vaccines have demonstrated poor efficacy in humans with respect to antibody induction (Wang et al. (2001) *PNAS* 98: 10817-10822).

To be effective, a malaria vaccine could prevent infection altogether or mitigate against severe disease and death in those who become infected despite vaccination. Four stages of the malaria parasite's life cycle have been the targets of vaccine development efforts. The first two stages are often grouped as 'pre-erythrocytic stages' (i.e. before the parasite invades the human red blood cells): these are the sporozoites inoculated by the mosquito into the human bloodstream, and the parasites developing inside human liver cells (hepatocytes). The other two targets are the stage when the parasite is invading or growing in the red blood cells (the asexual stage); and the gametocyte stage, when the parasites emerge from red blood cells and fuse to form a zygote inside the mosquito vector (gametocyte, gamete, or sexual stage). Vaccines based on the pre-erythrocytic stages usually aim to completely prevent infection. For asexual, blood stage vaccines, because the level of parasitaemia is in general proportional to the severity of disease (Miller, et al. (1994) *Science* 264, 1878–1883), vaccines aim to reduce or eliminate (e.g. induce sterile immunity) the parasite load once a person has been infected. However, most adults in malaria-endemic settings are clinically immune (e.g. do not suffer symptoms associated with malaria), but have parasites at low density in their blood. Gametocyte vaccines aim towards preventing the parasite being transmitted to others through mosquitoes. Ideally, a vaccine effective at all these parasite stages is desirable (Richie and Saul, *Nature*. (2002) 415(6872):694-701).

The SPf66 vaccine (Patorroyo et al. (1988) *Nature* 332:158–161) is a synthetic hybrid peptide polymer containing amino acid sequences derived from three *Plasmodium falciparum* asexual blood stage proteins (83, 55, and 35 kilodaltons; the 83 kD protein

corresponding to merozoite surface protein (MSP)-1) linked by repeat sequences from a protein found on the *Plasmodium falciparum* sporozoite surface (circumsporozoite protein). Therefore it is technically a multistage vaccine. SPf66 was one of the first types of vaccine to be tested in randomized controlled trials in endemic areas and is the vaccine that has undergone the most extensive field testing to date. While having marginal efficacy in four trials in South America (Valero et al. (1993) *Lancet* 341(8847):705–10. Valero et al. (1996) *Lancet* 348(9029):701–7; Sempertegui et al. (1994) *Vaccine* 12(4):337–42; Urdaneta et al. (1998) *American Journal of Tropical Medicine and Hygiene* 58(3):378–85.), these trials suggested a slightly elevated incidence of *Plasmodium vivax* in the vaccine groups. The vaccine has also been demonstrated to be ineffective for reducing new malaria episodes, malaria prevalence, or serious outcomes (severe morbidity and mortality) in Africa (Alonso et al. *Lancet* 1994;344(8931):1175–81 and Alonso et al *Vaccine* 12(2):181–6); D'Alessandro et al. (1995) *Lancet* 346(8973):462–7.; Leach et al. (1995) *Parasite Immunology* 1995;17(8): 441–4.; Masinde et al. (1998) *American Journal of Tropical Medicine and Hygiene* 59(4):600–5; Acosta 1999 *Tropical Medicine and International Health* 1999;4(5):368–76) and Asia (Nosten et al. (1996) *Lancet*; 348(9029):701–7.), and is consequently no longer being tested.

Four types of pre-erythrocytic vaccines (CS-NANP; CS102; RTS,S; and ME-TRAP) have been trialed. The CS-NANP-based pre-erythrocytic vaccines were the first to be tested, beginning in the 1980s. The vaccines used in the first trials comprised three different formulations of the four amino acid B cell epitope NANP, which is present as multiple repeats in the circumsporozoite protein covering the surface of the sporozoites of *Plasmodium falciparum*. The number of NANP repeats in these vaccines varied from three to 19, and three different carrier proteins were used. The CS-NANP epitope alone appears to be ineffective in a vaccine, with no evidence for effectiveness of CS-NANP vaccines in three trials (Guiguemde et al. (1990) *Bulletin de la Societe de Pathologie Exotique* 83(2):217–27; Brown et al. (1994) *Vaccine* 12(2):102–7; Sherwood et al. (1996) *Vaccine* 14(8):817–27).

The CS102 vaccine is also based on the sporozoite CS protein, but it does not include the NANP epitope. It is a synthetic peptide consisting of a stretch of 102 amino acids containing T-cell epitopes from the C-terminal end of the molecule. All 14 participants in

this small trial of non-immune individuals had malaria infection as detectable by PCR (Genton et al. (2005) *Acta Tropica Suppl* 95:84).

The RTS,S recombinant vaccine also includes the NANP epitope. It contains 19 NANP repeats plus the C terminus of the CS protein fused to hepatitis B surface antigen (HBsAg), expressed together with un-fused HBsAg in yeast. The resulting construct is formulated with the adjuvant ASO2/A. Thus the vaccine contains a large portion of the CS protein in addition to the NANP region, as well as the hepatitis B carrier. The RTS,S pre-erythrocytic vaccine has shown some modest efficacy, in particular with regard to prevention of severe malaria in children and duration of protection of 18 months (Kester et al. (2001) *Journal of Infectious Diseases* 2001;183(4):640–7.1; Bojang et al. (2001) *Lancet* 358(9297):1927–34; Alonso et al. (2005) *Lancet* 366(9502):2012 Alonso et al. (2005) *Lancet* 366(9502):2012–8), Bojang et al. (2005) *Vaccine* 23(32):4148–57). In four trials, it was effective in preventing a significant number of clinical malaria episodes, including good protection against severe malaria in children, with no serious adverse effects (Graves et al. (2006) *Cochrane Database of Systematic Reviews* 4: CD006199). The RTS,S vaccine has shown significant efficacy against both experimental challenge (in non-immunes) and natural challenge (in participants living in endemic areas) with malaria. Although no evidence was found for efficacy of RTS,S against clinical malaria in adults in The Gambia in the first year of follow up, efficacy was observed in the second year after immunization, after a booster dose. However, there was no reduction in parasite densities (which positively associate with pathology). Nonetheless, in a recent study in Mozambique, the vaccine appeared to have efficacy in infants (Aponte et al. (2007) 370(9598) 1543-1551).

The ME-TRAP pre-erythrocytic vaccine is a DNA vaccine that uses the prime boost approach to immunization. It uses a malaria DNA sequence known as ME (multiple epitope)-TRAP (thrombospondin-related protein). The ME string contains 15 T-cell epitopes, 14 of which stimulate CD8 T-cells and the other of which stimulates CD4 T-cells, plus two B-cell epitopes from six pre-erythrocytic antigens of *Plasmodium falciparum*. It also contains two non-malarial CD4 T-cell epitopes and is fused in frame to the TRAP sequence. This sequence is given first as DNA (two doses) followed by one dose of the same DNA sequence in the viral vector MVA (modified vaccinia virus Ankara). There was no evidence for effectiveness of ME-TRAP vaccine in preventing

new infections or clinical malaria episodes, and the vaccine did not reduce the density of parasites or increase mean packed cell volume (a measure of anaemia) in semi-immune adult males (Moorthy et al. (2004) *Nature* 363(9403):150–6).

The first blood-stage vaccine to be tested in challenge trials is Combination B, which is a mixture of three recombinant asexual blood-stage antigens: parts of two merozoite surface proteins (MSP-1 and MSP-2) together with a part of the ring-infected erythrocyte surface antigen (RESA), which is found on the inner surface of the infected red cell membrane. The MSP-1 antigen is a 175 amino acid fragment of the relatively conserved blocks 3 and 4 of the K1 parasite line; it also includes a T-cell epitope from the *Plasmodium falciparum* circumsporozoite (CS) protein as part of the MSP1 fusion protein. The MSP2 protein includes the nearly complete sequence from one allelic form (3D7) of the polymorphic MSP-2 protein. The RESA antigen consists of 70% of the native protein from the C-terminal end of the molecule. A small efficacy trial of Combination B in non-immune adults with experimental challenge showed no effect (Lawrence (2000) *Vaccine* 18(18):1925–31). In the single natural-challenge efficacy trial of in semi-immune children (Genton (2002) *Journal of Infectious Diseases* 185(6):820–7), no effect on clinical malaria infections was detected. In this trial, significant efficacy (measure by reduction in parasite density) was only observable in the group who were not pretreated with sulfadoxine-pyrimethamine. Also, in these children there was a reduction in the proportion of children with medium and high parasitaemia levels. Vaccinees in the Genton et al. (2002) trial had a lower incidence and prevalence of parasites with the 3D7 type of MSP2 (the type included in the vaccine) than the placebo group, and a higher incidence of malaria episodes were associated with the FC27 type of MSP2, suggesting specific immunity. Importantly, there was no statistically significant change in prevalence of parasitemia, nor was there evidence for an effect of combination B against episodes of clinical malaria in either the group pretreated with the antimalarial or the group with no antimalarial, in fact the results for these subgroups tended in the opposite direction. Furthermore, the relative role of the three vaccine constituents cannot be assessed when based on the trials that have been carried out to date.

In addition to the asexual-stage components of Combination B, many other potential asexual stage vaccines have been under preclinical evaluation, such as regions of apical

membrane antigen 1 (AMA1), the merozoite surface proteins MSP1, MSP2, MSP3, MSP4, and MSP5, glutamate-rich protein (GLURP), rhoptry associated protein-2 (RAP2), EBA-175, EBP2, MAEBL, and DBP, and *Plasmodium falciparum* (erythrocyte membrane protein-1 (PfEMP1). Importantly however, a recent examination of the vaccine candidate still under consideration (Moran et al. (2007) The Malaria Product Pipeline, The George Institute for International Health, September 2007) has shown that many preclinical vaccine projects are inactive; in particular vaccine projects using the F1 domain of EBA-175 (e.g. by ICGB), EBA-140 (also known as BAEBL), and RAP-2 are inactive. The inactivity of these projects highlights that much work is needed to find blood stage antigens that will afford a protective immune response.

There are many problems faced in the selection of antigens for malaria vaccine development, including antigenic variation, antigen polymorphism, and original antigenic sin, and further problems such as MHC-limited non-responsiveness to malarial antigens, inhibition of antigen presentation, and the influence of maternal antibodies on the development of the immune system in infants.

Many blood stage vaccine candidates, such as MSP-1, MSP-2, MSP-3 and AMA-1, have substantial polymorphisms that may have an impact on both immunogenicity and protective effects, and in the case of MSP-1, and MSP-2, immune responses to particular allelic forms has been observed in vaccine trials (and also for MSP-3 and AMA-1 in mice). Molecular epidemiological studies can guide antigen selection and vaccine design as well as provide information that is needed to measure and interpret population responses to vaccines, both during efficacy trials and after introduction of vaccines into the population. They also may provide insight into the selective forces acting on antigen genes and potential implications of allele specific immunity. Consequently the different allelic forms would need to be included in any vaccine to counter the affect of antigenic polymorphism at immunogenic residues.

The cyclical recrudescences of malaria parasites in humans is thought to be due to the selective pressure placed upon parasitized red cells by antibodies to variant antigens, such as PfEMP1. *Plasmodium falciparum* possesses about 50 variant copies of PfEMP1 which are expressed clonally such that only one is expressed at a time, and the development of antibodies against the expanding clonal type then reduce this clone from the affected individual, and subsequently a different variant, not recognized by

antibodies, emerges and cycling continues. This antigenic variation also poses a problem for vaccines containing clonally expressed antigens, and immunization studies with recombinant conserved CD36-binding portion of PfEMP1 failed to confer protection in Aotus monkeys (Makobongo et al. (2006) JID 193:731-740.

A third problem confounding malaria vaccine initiatives is original antigenic sin; a phenomenon in which individuals tend to make antibodies only to epitopes expressed on antigenic types to which they have been exposed (or cross-reactive antigens), even in subsequent infections carrying additional, highly immunogenic epitopes (Good, et al. (1993) *Parasite Immunol.* 15, 187-193. Taylor et al. (1996) *Int. Immunol.* 8, 905-915, Riley, (1996) *Parasitology* 112, S39-S51 (1996))

It has also been proposed that immunity to malaria relies on maintaining high levels of immune effector cells, rather than in the generation of effectors from resting memory cells (Struck and Riley (2004) *Immunological Reviews* 201: 268-290). Consequently, the time taken to generate sufficient levels of effector cells may be crucial in determining whether a protective memory response can be mounted to prevent disease. Also, malaria parasites may interfere directly with memory responses by interfering with antigen presentation by dendritic cells (Urban et al. (1999) *Nature* 400:73-77, Urban et al.(2001) *PNAS* 98:8750-8755), and premature apoptosis of memory cells (Toure-Balde et al.(1996) *Infection and Immunity* 64: 744-750, Balde et al. (2000) *Parasite Immunology* 22:307-318).

Furthermore, it has been demonstrated that antibodies to particular malarial antigens (such as MSP-1) may inhibit the activity of malaria-protective antibodies (Holder et al (1999) *Parassitologica* 41:409-14), and that there may be MHC-limited non-responsiveness to malarial antigens (Tian et al (1996) *J Immunol* 157:1176-1183, Stanisic et al. (2003) *Infection and Immunity* 71: 5700-5713). Maternally derived antibodies have also been shown to interfere with the development of antibody responses in infants, and has been implicated for malaria in mice (Hirunpetcharat and Good (1998) *PNAS* 95:1715-1720), consequently these problems need to be addressed for vaccination of children against malaria.

As will be apparent from the foregoing review of the prior art, there remained significant problems to be overcome in the design of an efficacious vaccine against malaria. It is an

aspect of the present invention to overcome or ameliorate a problem of the prior art by providing antigens capable of eliciting antibodies that can treat or prevent malaria.

A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

SUMMARY OF THE INVENTION

The present invention provides an immunogenic molecule comprising a contiguous amino acid sequence of an erythrocyte binding antigen (EBA) protein of a strain of *Plasmodium falciparum*, wherein when administered to a subject the molecule is capable of inducing an invasion-inhibitory immune response to the strain. The EBA protein may be EBA175, EBA140, or EBA181. The contiguous amino acid sequence comprises about 5, 8, 10, 20, 50 or 100 or more amino acids.

The contiguous amino acid sequence may be found in the region between the F2 domain and the transmembrane domain of the EBA protein. The contiguous amino acid sequence may be found in the region from about residue 746 to about residue 1339 of the EBA protein. In one form of the immunogenic molecule the EBA is EBA140. In one form the contiguous amino acid sequence is found in the region between the F2 domain and the transmembrane domain of EBA140, or in the region from about residue 746 to about residue 1045 of EBA140.

In another form of the immunogenic molecule the EBA is EBA175. In one form of the immunogenic molecule the contiguous amino acid sequence is found in the region between the F2 domain and the transmembrane domain of EBA175. The contiguous amino acid sequence may be found in the region from about residue 761 to about residue 1271 of EBA175.

In another form of the invention the EBA is EBA181. In one form of the invention the

contiguous amino acid sequence may be found in the region between the F2 domain and the transmembrane domain of EBA181. In another form of the immunogenic molecule the contiguous amino acid sequence is found in the region from about residue 755 to about residue 1339 of EBA181.

Another aspect of the present invention provides a composition comprising an immunogenic molecule as described herein and a pharmaceutically acceptable excipient and optionally a vaccine adjuvant.

Yet a further aspect of the present invention provides a composition comprising a contiguous amino acid sequence of an invasion ligand of a strain of *Plasmodium falciparum* involved in sialic-acid-dependant invasion of red cells further comprising a contiguous amino acid sequence of an invasion ligand of a strain of *Plasmodium falciparum* involved in sialic-acid-independent invasion of red cells wherein when administered to a subject the composition is capable of inducing an invasion-inhibitory immune response to the strain.

The composition may comprise an immunogenic molecule comprising a contiguous amino acid sequence of a reticulocyte-binding protein homologue (Rh) protein of the strain of *Plasmodium falciparum*, wherein when administered to a subject the Rh protein is capable of inducing an invasion-inhibitory immune response to the strain. The Rh may be Rh1, Rh2a, Rh2b or Rh4.

In one form of the composition the Rh is Rh2b. In another form of the immunogenic molecule the contiguous amino acid sequence is found in the region between about 31 amino acids N-terminal of the Prodom PD006364 homology region to about the transmembrane domain of Rh2b.

In one form of the composition the contiguous amino acid sequence is found in the region from about residue 2027 to 3115 of Rh2b, or the region from about residue 2027 to about residue 2533 of Rh2b, or the region from about residue 2098 to about residue 2597 of Rh2b, or the region from about residue 2616 to about residue 3115 of Rh2b.

In one form of the composition the Rh is Rh2a. In one form of the composition the

contiguous amino acid sequence is found in the region between about 31 amino acids N-terminal of the Prodom PD006364 homology region to about the transmembrane domain of Rh2a.

In one form of the invention the contiguous amino acid sequence is found in the region from about residue 2027 to 3115 of Rh2a, or the region from about residue 2027 to about residue 2533 of Rh2a, or the region from about residue 2098 to about residue 2597 of Rh2a, or the region from about residue 2616 to about residue 3115 of Rh2a.

In one form of the composition the Rh is Rh1. In one form of the composition the contiguous amino acid sequence is found in the region between about residue 1 to about the transmembrane domain of Rh1, or the region from about residue 1 to about residue 2897 of Rh1.

In one form of the composition the Rh is Rh4. In another form of the composition the contiguous amino acid sequence is found in the region from about the MTH1187/YkoF-like superfamily domain to about the transmembrane domain of Rh4. In a further form of the composition the contiguous amino acid sequence is found in the region from about residue 1160 to about residue 1370 of Rh4.

The contiguous amino acid sequence may comprise about 5, 8, 10, 20, 50 or 100 or more amino acids. The strain of *Plasmodium falciparum* may be a wild type strain.

In another aspect the present invention provides a method of treating or preventing a condition caused by or associated with infection by *Plasmodium falciparum* comprising administering to a subject in need thereof an effective amount of a composition as disclosed herein.

A further aspect provides use of a composition as described herein in the manufacture of a medicament for the treatment or prevention of a condition caused by or associated with infection by *Plasmodium falciparum*.

A further aspect of the present invention provides a method of screening for the presence of a *Plasmodium falciparum* invasion-inhibitory antibody directed against an

erythrocyte binding antigen (EBA) of a strain of *Plasmodium falciparum* in a subject, comprising obtaining a biological sample from the subject and identifying the presence or absence of an antibody capable of binding to an immunogenic molecule as described herein.

A further aspect of the present invention provides a method of screening for the presence of a *Plasmodium falciparum* invasion-inhibitory antibody directed against a reticulocyte-binding homologue protein (Rh) of a strain of *Plasmodium falciparum* in a subject, comprising obtaining a biological sample from the subject and identifying the presence or absence of an antibody capable of binding to an immunogenic molecule as described herein. The method may further comprise identifying the presence of a *Plasmodium falciparum* invasion-inhibitory antibody directed against an erythrocyte binding antigen (EBA) of a strain of *Plasmodium falciparum* in a subject comprising identifying the presence or absence of an antibody capable of binding to an immunogenic molecule as described herein.

Throughout the description and the claims of this specification the word "comprise" and variations of the word, such as "comprising" and "comprises" is not intended to exclude other additives, components, integers or steps.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Inhibition of different *Plasmodium falciparum* lines by serum antibodies from malaria exposed Kenyan children and adults.

Results shown were selected to demonstrate representative examples of the inhibitory activities observed. Values are expressed as a percentage of invasion using non-exposed donors. All samples were tested in duplicate; values represent mean $\pm$ range. **A)** Inhibition of W2mef-wt compared to W2mef Δ EBA175 cultured with normal or neuraminidase-treated erythrocytes. **B)** Inhibition of W2mef-wt compared to W2mef-SeINm cultured with normal or neuraminidase-treated erythrocytes. **C)** Inhibition of 3D7-wt compared to 3D7 Δ EBA175 cultured with normal or neuraminidase-treated erythrocytes. Numbers on the X-axis are study codes for individual serum samples. Norm, cultured with normal erythrocytes. Neur, cultured with neuraminidase-treated erythrocytes.

Figure 2. Differential inhibition of W2mef *Plasmodium falciparum* lines by serum antibodies from malaria-exposed Kenyan children and adults.

Results show the proportion of sera (n=80) that differentially inhibited the two parasite lines tested for each comparison shown. Grey bars show the proportion of samples that inhibited the parental wild-type parasite line more than the W2mef Δ EBA175 line or W2mefselNm line (type-A response). Black bars show the proportion of samples that inhibited the W2mef Δ EBA175 line or W2mefselNm line more than the corresponding parental line (type-B response). The proportion with differential inhibitory activity is shown for all samples and separately by age groups (≤ 5 , 6-14, and >14 years of age). **A, B)** W2mef-wt compared to W2mef Δ EBA175 cultured with normal (A) or neuraminidase-treated (B) erythrocytes. **C, D)** W2mef-wt compared to W2mefSelNm cultured with normal (C) or neuraminidase-treated (D) erythrocytes. W2mef-wt was cultured with normal erythrocytes in all assays. Differences between the age groups were not statistically significant.

Figure 3. Differential inhibition of 3D7 *Plasmodium falciparum* lines by serum antibodies from malaria-exposed Kenyan children and adults.

Results show the proportion of sera (n=80) that differentially inhibited the two parasite lines tested for each comparison shown. Grey bars show the proportion of samples that inhibited parental 3D7 (3D7-wt) more than 3D7 Δ EBA175 (type-A response). Black bars show the proportion of samples that inhibited 3D7 Δ EBA175 more than the parental 3D7 (type-B response). The proportion with differential inhibitory activity is shown for all samples and separately by age groups (≤ 5 , 6-14, and >14 years of age). Comparisons are shown with 3D7 Δ EBA175 cultured with normal (**A**) or neuraminidase-treated (**B**) erythrocytes. 3D7-wt was cultured with normal erythrocytes in all assays. Differences between the age groups were not statistically significant.

Figure 4. The effect of serum antibodies from Kenyan donors on erythrocyte invasion by different *Plasmodium falciparum* lines.

Results represent the mean from testing 80 Kenyan serum antibody samples; error bars

represent 95% confidence intervals. Values are expressed relative to control samples from non-exposed donors. Samples were not tested for inhibition of 3D7-wt or W2mef-wt invasion into neuraminidase-treated erythrocytes (NT, not tested). Numbers on the X-axis are study codes for individual serum samples. Norm, invasion into normal erythrocytes. Neur, invasion into neuraminidase-treated erythrocyte.

Figure 5. Differential inhibition of 3D7-wt and 3D7 Δ EBA175 by serum antibodies.

A selection of individual samples is shown that inhibit 3D7-wt to a greater extent than 3D7 Δ EBA175. This suggests the presence of inhibitory antibodies against EBA175. Values are expressed as a percentage of invasion using non-exposed donors. All samples were tested in duplicate; values represent mean $\pm$ range.

Figure 6. Age-associated acquisition of antibodies to recombinant EBA and Rh proteins measured by ELISA.

Results (n=150) are grouped by age and show mean $\pm$ SEM absorbance expressed relative to the levels for adults (>14 years). $P < 0.001$ for comparisons between age groups for all antigens. Donors were residents of a malaria endemic region of Kenya (Kilifi District). The relative absorbance using sera from non-exposed donors (n=10) is also shown (Contr).

Figure 7. Recombinant Rh4 binds the surface of erythrocytes

A. Schematic representation of *Plasmodium falciparum* Rh4 and recombinant Rh4 proteins. Rh4 is a two exon gene with a transmembrane domain (green) at the C-terminal end. Amino acids 28-766 and amino acids 853-1163 of Rh4 are fused to a hexa-histidine tag (orange) to generate Rh4₈₈ and Rh4₄₂ respectively. Diagram is not drawn to scale. B. Purification of Rh4₈₈. Rh4₈₈ was purified using Ni-NTA agarose beads and eluted with 250 mM imidazole buffer. Lane 1 and 2 are expression levels of purified Rh4₈₈ obtained from two separate bacteria clones. C. Rh4₈₈ binds to the surface of erythrocytes. The PBS lane represents the control lane in which proteins were eluted in a binding assay performed in the presence of PBS with no fusion protein. Eluted Rh4₈₈ is detected using a penta-histidine antibody upon binding to erythrocytes, spun through

oil and also upon washing the bound erythrocytes with PBS. D. Rh4₄₂ does not bind the surface of erythrocytes. No detection of Rh4₄₂ could be observed in the erythrocyte binding assay using a penta-histidine antibody.

Figure 8. Age-associated acquisition of antibodies to Rh2 measured by ELISA

Results (n=150) are grouped by age and show mean $\pm$ SEM absorbance expressed relative to the levels for adults (>14 years). $P < 0.001$ for comparisons between age groups for both antigens. Donors were residents of a malaria endemic region of Kenya (Kilifi District). The relative absorbance using sera from non-exposed donors (n=10) is also shown (Melbourne). Antibodies to both amino acids 2098 to 2597 of Rh2 and 2616 to 3115 of Rh2 were detected and acquired in an age-dependent manner.

Figure 9. Antibodies to Rh2 are associated with protection from malaria among a cohort of 206 children in Madang Province, Papua New Guinea

Graphs are Kaplan Meier survival curves. The cumulative proportion (Y-axis) of individuals with symptomatic *Plasmodium falciparum* malaria over time (X-axis) is plotted. Children were classified into three groups on the basis of their antibody response to Rh2: 0 = highest tercile of responders (i.e. these children had the highest antibody levels; 1= middle tercile of responders; and 2= lowest tercile of responders (i.e. these children had the lowest levels of antibodies).

A. Children with the highest level of antibodies to PfRh2-A9 (amino acids 2098 to 2597 of Rh2) had the lowest risk of malaria ($p < 0.01$). B. Children with the highest level of antibodies to PfRh2-A11 (amino acids 2616 to 3115 of Rh2) had the lowest risk of malaria ($p < 0.01$).

Figure 10. Antibodies to EBA are associated with protection from malaria

Graphs are Kaplan Meier survival curves. The cumulative proportion (Y-axis) of individuals with symptomatic *Plasmodium falciparum* malaria over time (x-axis) is plotted.

A. Antibodies to EBA proteins (EBA175, EBA140, and EBA181) by ELISA were associated with reduced risk of clinical malaria. Children were classified into three groups (high, medium, low) on the basis of their antibody response to all three EBAs. Highest responders show lowest risk of malaria, indicating that the breadth and level of antibodies is associated with protection ($P < 0.01$). B, C, D. Children were classified as having high (red) or low (blue) antibody levels and plotted against time to first clinical episode. Those with high antibody levels had a significantly lower risk of malaria ($P < 0.01$).

Figure 11. Differential inhibition of 3D7-wt and 3D7 Δ EBA140 by serum antibodies.

A selection of individual samples is shown that inhibit 3D7-wt to a greater extent than 3D7 Δ EBA140. This suggests the presence of inhibitory antibodies against EBA140. Values are expressed as a percentage of invasion using non-exposed donors. All samples were tested in duplicate and values represent means.

Figure 12. Inhibition of both SA-dependent and SA-independent invasion pathways (e.g. EBA175, EBA140, EBA181 and Rh2) acts synergistically to inhibit invasion of human erythrocytes.

Antibodies were generated to specific domains of EBA175, EBA140, EBA181 and PfRh2b in rabbits. Protein G purified antibodies (IgG) from these sera were obtained and used to test inhibition of merozoite invasion at 1 mg/ml in wild type 3D7 as well as lines in which the gene encoding different ligands had been disrupted i.e. 3D7 Δ 175, 3D7 Δ 140, 3D7 Δ 175/140 and 3D7 Δ 181. Anti-EBA140 antibodies inhibited parental 3D7 approximately 20% and this disappears in 3D7 Δ 140 as would be expected for specific inhibition of function. Similarly, antibodies to EBA175 inhibit 3D7 merozoite invasion approximately 18% and this does not occur for 3D7 Δ 175 again showing that function of this ligand is specifically inhibited. Importantly, antibodies targeting Rh2 inhibit invasion of parasites lacking EBA175, or EBA174 and EBA140, to a greater extent than inhibition of wild-type parasites indicating that the SA-dependent and SA-independent invasion pathways are the major pathways of invasion into human erythrocytes, and that inhibition of these two pathways acts to synergistically inhibit invasion.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated on the finding that antibodies raised against erythrocyte binding antigen (EBA) proteins of *Plasmodium falciparum* are capable of inhibiting invasion of the parasite into human red blood cells. The invasion of red blood cells is a key event in the infection of a subject with the malaria parasite, and it is therefore proposed that EBA proteins may be used as antigens in the formulation of a vaccine against malaria. Accordingly, in one aspect, the present invention provides an immunogenic molecule comprising a contiguous amino acid sequence of a erythrocyte binding antigen (EBA) of a strain of *Plasmodium falciparum*, wherein when administered to a subject the molecule is capable of inducing an invasion-inhibitory immune response to the strain. This approach to formulating a vaccine for malaria is distinguished from approaches of the prior art, and is indeed contrary to the general teaching of the prior art prior to the present invention.

Previous work characterizing the function of EBA proteins (and also the reticulocyte-binding protein homologue (Rh) proteins) in human red cell (erythrocyte) invasion by the malaria parasite *Plasmodium falciparum* has demonstrated that these molecules are *not* essential for red cell invasion since the genes encoding these molecules (e.g. EBA175, EBA140, EBA181, Rh1, Rh2a, Rh2b and Rh4) can be disrupted in different *Plasmodium falciparum* lines without an obvious effect on blood stage growth rates. Also, antibodies raised in rabbits and mice to Rh2a and Rh2b are unable to inhibit invasion of *Plasmodium falciparum* into untreated red cells in vitro, again suggesting that these molecules are not essential for invasion of red cells. Furthermore, recent work examining Rh4 (Gaur et al. (2007) PNAS in press) has identified that while antibodies to both native Rh4 and a recombinant protein encoding a region of Rh4 (rRH4₃₀) inhibited the binding of these proteins to red cells, the same antibodies failed to block invasion of red cells, causing the authors to conclude that Rh4 is inaccessible for antibody-mediated inhibition of the invasion process. In contrast, the applicants proposal that Rh proteins are capable of eliciting a protective immune response suggests that reticulocyte-binding protein homologues are accessible to the human immune system, that human antibodies to these proteins inhibit invasion of *Plasmodium falciparum* into red cells, and that antibodies to these proteins result in immunity to malaria in humans.

A study by Duraisingh et al. (2003; EMBO J 22:1047) demonstrated that antibodies to

Rh2a and Rh2b are unable to inhibit invasion of *Plasmodium falciparum* into untreated red cells. This work also demonstrated that Rh2a is not expressed by MCAMP, FCB1, T994 or FCR3, and Rh2b, in addition to being absent from D10, is not expressed by MCAMP, FCB1, T994 or FCR3, further suggesting that Rh2a and Rh2b are not essential for invasion of red cells. The growth and merozoite invasion rate of *Plasmodium falciparum* parasites in which the Rh2a and Rh2b genes have been disrupted is unchanged relative to their wild-type parent lines, suggesting that these molecules are not essential for invasion of wild-type parasites into normal erythrocytes. Therefore therapeutics targeting these non-essential invasion pathways would not be expected to be invasion-inhibitory (Duraisingh et al. (2003) EMBO J 22:1047).

In complete contrast to the findings of the aforementioned authors, the present invention demonstrates that human antibodies to Rh proteins inhibit invasion. Figure 1 shows that human antibodies to Rh proteins inhibit invasion. The inhibitory activity of serum antibodies from children and adults resident in a malaria endemic region of coastal Kenya was compared against different W2mef and 3D7 parasite lines with different invasion phenotypes. While EBA and Rh proteins are not essential for invasion as discussed above, these molecules play a role in invasion of enzyme treated red cells. In particular, neuraminidase removes sialic acid residues from the erythrocyte surface and blocks invasion pathways dependent on sialic acid present on both glycophorin A and other receptors, trypsin treatment cleaves proteins such as glycophorin A and C, but does not affect glycophorin B, and chymotrypsin cleaves a non-overlapping set of proteins including glycophorin B and band 3 on the erythrocyte surface. Using this approach, invasion phenotypes can be broadly classified into two main groups: i) sialic acid (SA)-dependent invasion, demonstrated by poor invasion of neuraminidase-treated erythrocytes (neuraminidase cleaves SA on the erythrocyte surface), and ii) SA-independent invasion, demonstrated by efficient invasion of neuraminidase-treated erythrocytes, involves Rh2 and Rh4. SA-dependent (neuraminidase-sensitive) invasion of enzyme treated cells involves the three known EBAs (EBA175, EBA181, EBA140), Rh1. EBA175 and EBA140 bind to glycophorin A and C, respectively. EBA181 binds to SA on the erythrocyte surface and to band 4.1 protein. W2mef-wt uses SA-dependent invasion mechanisms (EBA- and Rh1-dependent), whereas invasion of W2mef Δ EBA175 is largely SA-independent (Rh2 and Rh4-dependent). In comparative inhibition assays (Figure 1), 27% of samples differentially inhibited the two lines (e.g. samples 56, 109,

and 135 in Figure 1A), indicating that the inhibitory activity of acquired antibodies is influenced by the invasion pathway being used (Figure 1 and 2). Although W2mef Δ EBA175 has switched to use a largely SA-independent invasion pathway, it remains possible that other ligands involved in SA-dependent invasion (e.g. EBA140, EBA181, Rh1) may still be functional to some extent in W2mef Δ EBA175, despite the switch in phenotype. To inhibit these interactions, and more clearly compare antibodies against SA-dependent versus SA-independent invasion pathways, antibody inhibition assays were performed using W2mef Δ EBA175 and neuraminidase-treated erythrocytes, in comparison to inhibition of W2mef-wt with normal erythrocytes (Figure 2B). This approach further emphasizes differences in antibody activity linked to variation in invasion phenotype. The proportion of samples showing differential inhibition of the two lines was 48% versus 27% when using normal erythrocytes with both lines. The extent of differences in inhibitory activity was strongly increased for some individual samples (e.g. sample 355 in Figure 3A). This indicates that the inhibitory activity of antibodies against ligands of SA-independent invasion (e.g. Rh2 and Rh4) was enhanced once the residual activity of SA-dependent ligands (e.g. EBA175, EBA140, EBA181 and Rh1) is inhibited by neuraminidase treatment of erythrocytes.

Differential inhibition by samples was also observed with W2mef-wt compared to W2mefSelNm (Figure 1B and 2C). The latter isolate is genetically intact and its phenotype was generated by selection for invasion of neuraminidase-treated erythrocytes. Like W2mef Δ EBA175, it uses an alternate SA-independent invasion pathway and has upregulated expression of Rh4. It still expresses EBA175 but does not depend on this ligand for invasion. 35% of samples from children and adults were found to differentially inhibit the two lines (e.g. samples 196 and 436, Figure 1B), confirming that a change in invasion phenotype, or pathway, can substantially alter the efficacy of inhibitory antibodies. As expected, the inhibition of W2mefSelNm and W2mef Δ EBA175 by samples was highly correlated ($r=0.61$; $n=80$; $p<0.001$) as these isolates invade via the same pathway and only differ by the presence of EBA175. Antibody dependent inhibition of W2mefSelNm invasion into neuraminidase-treated erythrocytes (Figure 2D), compared to W2mef-wt in normal erythrocytes, was tested to more clearly evaluate antibodies against SA-independent (Rh2- and Rh4-dependent) versus SA-dependent invasion (EBA- and Rh1-dependent pathways). Overall, 45% of samples differentially inhibited the two lines. Some samples showed greater differences in the inhibition of

W2mef-wt and W2mefSelNm than when normal erythrocytes were used (e.g. samples 196 and 436 in Figure 1B), indicating that human antibodies to SA independent ligands (e.g. Rh2 and Rh4) inhibit invasion. Differential antibody inhibition of 3D7 lines with different invasion phenotypes further confirmed that variation in invasion phenotypes influences the activity of inhibitory antibodies (Figure 1C and Figure 3, A and B). The proportion of samples that differentially inhibited parental 3D7 versus 3D7 Δ EBA175 was 26% when using normal erythrocytes and 37% when using neuraminidase-treated erythrocytes with 3D7 Δ EBA175. These combined results with W2mef and 3D7 lines clearly established that the availability of alternate pathways for erythrocyte invasion is immunologically important and a mechanism for evasion of acquired invasion-inhibitory antibodies of SA-independent invasion ligands (e.g. Rh2 and Rh4) and SA-dependent ligands (e.g. EBA175, EBA140, EBA181 and Rh1).

Of those samples that differentially inhibited W2mef-wt versus W2mef Δ EBA175 (cultured with normal erythrocytes), 26 of 27 inhibited the parental W2mef more than W2mef Δ EBA175 ($P < 0.001$; Figure 2) indicating inhibitory antibodies targeting EBA175 and other ligands of SA-dependent invasion (e.g. EBA140, EBA181 and Rh1). Overall, the mean inhibition of W2mef-wt by all samples (39.4%) was significantly greater than W2mef Δ EBA175 (29.4%; $p < 0.01$) (Figure 2). When W2mef Δ EBA175 was cultured with neuraminidase-treated erythrocytes to inhibit any residual SA-dependent interactions, there was an increase in the difference in the mean inhibition of W2mef-wt versus W2mef Δ EBA175 by samples (a difference of 18.9% versus 10% using untreated erythrocytes; $p < 0.01$; Figure 4). Antibodies from 60% of children ≤ 5 years inhibited W2mef-wt to a greater extent than W2mef Δ EBA175 (Figure 2B) (e.g. inhibiting EBA175, EBA140, EBA181 and Rh1), whereas among adults, 22% showed this pattern of inhibition ($p = 0.019$). Similar to results from assays using W2mef Δ EBA175, 31% of samples inhibited W2mef-wt more than W2mefSelNm (Type A response; Figure 4C), whereas only 4% inhibited W2mefSelNm more than W2mef-wt ($p < 0.001$). Additionally, the mean inhibition of W2mef-wt (39.4%) by all samples was greater than W2mefSelNm (20%; $p < 0.01$) (Figure 4). Furthermore, serum samples inhibited the invasion of 3D7-wt into normal erythrocytes more than 3D7 Δ EBA175 using neuraminidase-treated erythrocytes (Figure 5B). This indicates the presence of antibodies against the ligands of SA-dependent invasion (EBA175, EBA140, EBA181 and Rh1). In contrast to W2mef, disruption of EBA175 in 3D7 does not lead to a major switch in invasion phenotype.

3D7 Δ EBA175 shows slightly greater resistance to the effect of neuraminidase-treatment of erythrocytes compared to 3D7-wt, and increased sensitivity to inhibition by chymotrypsin-treatment of erythrocytes, consistent with the loss of function of EBA175.

Invasion-inhibitory antibodies to SA-independent invasion ligands (e.g. Rh2 and Rh4) were examined by identifying human serum samples that inhibited W2mef Δ EBA175 or 3D7 Δ EBA175 more than the corresponding parental parasites. Invasion of W2mef Δ EBA175 or 3D7 Δ EBA175 into neuraminidase-treated erythrocytes is dependent on ligands of the SA-independent invasion pathway (e.g. Rh2 and Rh4). Using the W2mef line, 5% of samples (Figure 2B) showed inhibition of ligands of the SA-independent invasion pathway (e.g. Rh2 and Rh4) and inhibited invasion of W2mef Δ EBA175 into neuraminidase-treated erythrocytes more effectively than W2mef-wt. Furthermore, 13% inhibited W2mefselNm more than W2mef-wt (e.g. sample 436, Fig 1B). Inhibition of ligands of the SA-independent invasion pathway (e.g. Rh2 and Rh4) was more prevalent with 3D7 parasite lines than W2mef ($p < 0.001$). A substantial number of samples inhibited 3D7 Δ EBA175 more than 3D7-wt (18% of samples when using normal erythrocytes and 16% when using neuraminidase-treated erythrocytes; Figure 5, A and B). No children ≤ 5 years inhibited W2mef Δ EBA175 more than W2mef-wt (Figure 2, A and B).

Furthermore, antibodies to SA-dependent (EBA175, EBA140, EBA181 and Rh1) and SA-independent invasion pathway ligands (e.g. Rh2 and Rh4) are acquired in an age dependant manner. Figure 6 shows that antibody levels to EBA175 (both 3D7 and W2mef alleles), EBA140, EBA181, Rh2 and Rh4 were positively associated with increasing age.

As discussed *supra* the present invention is predicated on the finding that EBA proteins of *Plasmodium falciparum* are capable of eliciting invasion-inhibitory immune responses in humans. The Duffy-binding like (DBL) proteins include erythrocyte-binding antigen (EBA)175, EBA140 (also known as BAEBL) and EBA181 (also known as JSEBL). Another DBL gene family member, eba165 (also known as PEBL) of *Plasmodium falciparum*, appears not to be expressed as a functional protein. These proteins are orthologs of DBL proteins identified in *Plasmodium vivax*. The cysteine-rich dual DBL domains found toward the N-terminus of EBA175 (called F1 and F2 domains) mediates

binding to its cognate receptor, and it is likely that similar domains in EBA140 and EBA181 also play receptor-binding roles. C-terminal of a transmembrane domain, is a cytoplasmic tail of the DBL proteins that does not appear to be directly linked to the actin-myosin motor. The structure of *Plasmodium falciparum* EBA175 is disclosed herein as SEQ ID NOs; 5 and 6. The F1 and F2 domains of EBA175 are at amino acids 158 to 397, and 462 to 710, respectively. The transmembrane domain of EBA175 is located at amino acids 1425 to 1442. The structure of *Plasmodium falciparum* EBA181 is disclosed herein as SEQ ID NOs; 7 and 8. The F1 and F2 domains of EBA181 are at amino acids 129 to 371, and 433 to 697, respectively. The transmembrane domain of EBA181 is located at amino acids 1488 to 1510. The structure of *Plasmodium falciparum* EBA140 is disclosed herein as SEQ ID NOs; 9 and 10. The F1 and F2 domains of EBA140 are at amino acids 154 to 405, and 456 to 706, respectively. The transmembrane domain of EBA140 is located at amino acids 1134 to 1153.

As discussed *supra* the present invention is predicated on the finding that Rh proteins of *Plasmodium falciparum* are capable of eliciting invasion-inhibitory immune responses in humans. The reticulocyte-binding protein (RBP) proteins were identified as homologs of rhoptry proteins in *Plasmodium yoelii* and *Plasmodium vivax*. *Plasmodium vivax* is a parasite of humans that preferentially invades reticulocytes, and it expresses two homologs of the Py235 family, PvRBP1 and PvRBP2. These proteins bind to reticulocytes but not normocytes (i.e. erythrocytes), suggesting that they are responsible for the host-cell preference of this species. A 500-amino-acid region that showed homology between the Py235 and PvRBP-2 protein families was used to search the *Plasmodium falciparum* (3D7 parasite) genome sequence databases, identifying five reticulocyte-binding protein homologue (Rh) genes containing the homologous region; Rh1 (RBP1), Rh2a (RBP2a), PfRh2b (RBP2b), and Rh4 (RBP4); a fifth, Rh3 (RBP3), does not appear to be expressed as a protein. Rh2a and Rh2b have a putative signal sequence at the N terminus and a potential transmembrane domain followed by a short cytoplasmic tail at the C terminus, similar to the structures of Py235, PvRBP-1, and PvRBP-2. The structure of *Plasmodium falciparum* Rh2b is disclosed herein as SEQ ID NOs; 1 and 2. The structure of *Plasmodium falciparum* Rh2a is disclosed herein as SEQ ID NOs; 11 and 12. Analysis of Rh2a and Rh2b has identified a region showing homology to the "0045457 Spectrin repeat" domain (SUPERFAMILY Accession: SSF46966) at amino acids 1735 to 1833, and a region showing homology to the

"UPF0103 YJR008W C21ORF19-LIKE- CEREVISIAE P47085 SACCHAROMYCES CHROMOSOME C2ORF4 PA5G0009 IPF893" domain (PRODOM Accession: PD006364) at amino acids 2133 to 2259 of Rh2a and amino acids 2058 to 2184 of Rh2b. The transmembrane domain of Rh2a is located at amino acids 3066 to 3088. The transmembrane domain of Rh2b is located at amino acids 3113 to 3135. The structure of Plasmodium falciparum Rh4 is disclosed herein as SEQ ID NOs; 3 and 4. Analysis of Rh4 has identified a region showing homology to the "0044828 MTH1187/YkoF-like" domain (SUPERFAMILY Accession: SSF89957) at amino acids 1031 to 1141. The transmembrane domain of Rh4 is located at amino acids 1627 to 1649. The structure of Plasmodium falciparum Rh1 is disclosed herein as SEQ ID NOs; 13 and 14. The transmembrane domain of Rh4 is located at amino acids 2898 to 2920.

As discussed *supra*, enzyme treatment of red cells has allowed examination of the receptors to which the Rh and DBL proteins bind. In particular, DBL proteins bind erythrocytes in a sialic-acid-dependent manner as neuraminidase treatment of the host cell ablates binding. EBA175 and EBA140 bind to glycophorin A and C, respectively, and while sialic acid on these receptors is essential for binding, the protein backbone is also important for specificity. EBA181 and Rh1 also bind to glycosylated erythrocyte receptors, although their identity is currently unknown. In contrast, there is no evidence that Rh2a directly binds to erythrocytes. Rh2b and Rh4 have been implicated in merozoite invasion since disruption of the corresponding gene causes these parasites to change the receptor they use for invasion on enzyme-treated red cells.

Antibodies that inhibit the growth of blood stage Plasmodium falciparum parasites in vitro are found in the sera of some, but not all, individuals living in malaria endemic regions (Marsh, et al (1989) Trans. R. Soc. Trop. Med. Hyg. 83:293, Brown, et al (1982) Nature. 297:591, Brown, et al. (1983) Infect. Immun. 39:1228, Bouharoun-Tayoun, et al. (1990) J. Exp. Med. 172:1633-1641). Inhibitory antibodies are likely to contribute to the clinical immunity observed in highly exposed individuals but their overall significance to protection remains unclear. Inhibitory antibodies may act in a manner that is independent of complement or other cellular mediators and function by preventing invasion of erythrocytes by the extracellular merozoite form of the parasite. A role for invasion-inhibitory antibodies in immunity to malaria has not been previously demonstrated. One practical reason for this is that there has been a lack of robust in

vitro inhibition assays that account for confounding factors present in serum that can cause non-specific inhibitory, or indeed growth-promoting, effects. Although in vitro inhibition assays have been used for some time to assess antibodies to *Plasmodium falciparum* merozoite antigens and have provided a useful guide as to the inhibitory activity of a particular serum or monoclonal antibody, the problems associated with accurate quantification of this activity, especially in whole serum, are well recognized in the field. This problem has now been overcome with the development of an assay that allows accurate quantification of molecule-specific inhibitory antibodies in whole serum. This assay involves a comparison of the inhibitory effect of a given serum on two isogenic parasite lines that differ only in the gene (or genes) of interest. Using this assay, the invasion-inhibitory activity of antibodies present in serum obtained from adults that are clinically immune to malaria may be determined.

The present invention requires that the immunogenic molecule is capable of inducing an invasion-inhibitory immune response in the subject. As used herein, the term "invasion-inhibitory" is intended to include the complete prevention of invasion of an invasion-competent erythrocyte for the life-span of the subject. The term is also intended to include the partial prevention of invasion, as measured by for example, the proportion of a population of invasion-competent erythrocytes that are invaded, the number of attempts by which it is necessary for a given parasite to invade an erythrocyte, the time taken for a parasite to invade an erythrocyte, and the number of parasites required to ensure that a single erythrocyte is invaded. The complete or partial inhibition of invasion may be for a short period of time (such as several hours), an intermediate period of time (such as weeks, or months), or a protracted period of time (such as years or decades). The inhibition of invasion may be measured in vivo or in vitro.

For the avoidance of doubt, the term "invasion" is intended to include the entire invasion process such that the complete parasite enters the cytoplasm, and is completely encircled by the cytoplasm. The term also includes components of the entire invasion process such as the binding of the parasite to the surface of the erythrocyte, the reorientation of the apical end of the parasite to contact the erythrocyte surface, entry of the parasite into a parasitophorous vacuole, release of protein from apical organelles, and the shedding of parasite surface protein by proteases. Furthermore, the term "invasion" includes both SA dependent and SA-independent invasion pathways. Immune

responses to these pathways are known as type-A and type-B inhibitory responses, respectively.

The present invention includes immunogenic molecules capable of eliciting an immune response against a wild-type strain of *P. falciparum*, or any of the following strains: 3D7, W2MEF, GHANA1, V1_S, RO-33, PREICH, HB3, SANTALUCIA, 7G8, SENEGAL3404, FCC-2, K1, RO-33, D6, DD2, or D10, or any other known or newly isolated strain of *Plasmodium falciparum*. An isolate or strain of *Plasmodium falciparum* is a sample of parasites taken from an infected individual on a unique occasion. Typically, an isolate is uncloned, and may therefore contain more than one genetically distinct parasite clone. A *Plasmodium falciparum* line is a lineage of parasites derived from a single isolate, not necessarily cloned, which have some common phenotype (e.g. drug-resistance, ability to invade enzyme treated red cells etc.). A *Plasmodium falciparum* clone is the progeny of a single parasite, normally obtained by manipulation or serial dilution of uncloned parasites and then maintained in the laboratory. All the members of a clone have been classically defined as genetically identical, but this is not necessarily the case, since members of the clone may undergo mutations, chromosomal rearrangements, etc, which may survive in in vitro culture conditions. While the immunogenic molecule will typically include amino acid sequences found in an Rh protein of the strain for which protection is desired, this is not necessarily required.

Typically, the immunogenic molecule is a polypeptide, or includes a polypeptide region. As used herein, the term "polypeptide" refers to amino acid polymers of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. Polypeptides can occur as single chains or associated chains.

In one form of the immunogenic molecule, the EBA molecule is EBA175 (PlasmoDB Accession No: MAL7P1.176), and the contiguous amino acid sequence is found in SEQ

ID NO: 5:

MKCNISITYFFASFFVLYFAKARNEYDIKENEFKFLDVYKEKFNELDKKKYGNVQKTDKKIF
 TFIENKLDILNNSKFNKRWKSYPGTPDNIDKNMSLINKHNNEEMFNNNYQSFLSTSSLIKQ
 NKYVPINAVRVSRLSFLDSRINNGRNTSSNNEVLSNCREKRKGMKWDCCKKKNDRSNYVC
 IPDRRIQLCIVNLSIIKTYTKETMKDHFIEASKKESQLLLKKNDNKYNSKFCNDLKNSFL
 DYGHLAMGNDMDFGGYSTKAENKIQEVFKGAHGEISEHKIKNFRKKWWNEFREKLWEAML
 SEHKNNINNCNIPQEELQITQWIKewHGEFLERDNRSKLPKSKCKNNTLYEACEKECI
 DPCMKEYRDWIIIRSKFEWHTLSKEYETQKVPKENAENYLIKISENKNDKVSLLLNNCDAE
 YSKYCDCKHTTTTLVKSVLNGNDNTIKEKREHIDLDDFSKFGCDKNSVDNTTKVWECKKPY
 KLSTKDVCPVPRRQELCLGNIDRIYDKNLLMIKEHILAIAYESRILKRKYKNKDDKEVC
 KIINKTFADIRDIIGGTDYWNDLSNRKLVGKINTNSNYVHRNKQNDKLFREDEWWKVIKCD
 VWNVISWVFKDKTVCKEDDIENIPQFFRWFSEWGDDYCQDKTKMIETLKVECKEPCEDD
 NCKRKCNSYKEWISKKKEEYNKQAKQYQYQKGNMYSEFKSIKPEVYLKKYSEKCSN
 LNFEDFKEELHSDYKNKCTMCPEVKDVPISIIIRNNEQTSQEAPEESTETIAHRTETRTD
 ERKNQEPANKDLKNPQQSVGENGTCDLLQEDLGGSSEDEVTQEFVNHGIPKGEDQTLG
 KSDAIPNIGEPETGISTTEESRHEEGHNKQALSTSVDEPELSDTLQLHEDTKENDKLPLE
 SSTITSPTESGSSDTEETPSISEGPKGNEQKKRDDDSLKISVSPENSRPETDAKDTSNL
 LKLKGDVDISMPKAVIGSSPNDNINVTEQGDNISGVNSKPLSDDVRPDKNHEEVKEHTSN
 SDNVQQSGGIVNMNVEKELKDTLENPSSSLDEGKAHEELSEPNLSSDQDMSNTPGPLDNT
 SEETTERISNNEYKVNREGERTLTKEYEDIVLKSHMNRESDDGELYDENSDDLSTVNDES
 EDAEAKMKGNDTSEMSHNSQHIESDQQKNDMKTVDLGTTHVQNEISVPVTGEIDEKLR
 ESKEKIHKAEEERLSHTDIHKINPEDRNSNTLHLKDIRNEENERHLTNQNNINISQERDL
 QKHGFHTMNNLHGDGVSESRQINSHHGHNQRDRGGNSGNVLMRSNNNNNFNNIPSRYNLY
 DKKLDLDLYENRNDSTTKELIKKLAIEINKCENEISVKYCDHMIHEEIPKKTCTKEKTRNL
 CCAVSDYCMSYFTYDSEEYNNCTKREFDDPSYTCFRKEAFSSMPYYAGAGVLFIIILVILG
 ASQAKYQRLEKINKNKIEKNVN

The nucleotide sequence of EBA181 (PlasmoDB Accession No: PFA0125c) is given
 below (SEQ ID NO: 7)

MKGKMMCLFFFYSLYVVLCTYVLGISEEYLKERPOGLNVETNNNNNNNNNNNSNSNDA
 MSFVNEVIRFIEKEDDKEDKKVKIISRPVENTLHRYPVSSFLNIKKYGRKGEYLNRSF
 VQRSYIRGCKGKRSTHTWICENKGNNNICIPDRRVQLCITALQDLKNSGSETTDRKLLRD
 KVFDSDAMYETDLLWNKYGFRGFDDFCDDVKNSYLDYKDVIFGTDLDKNNISKLVEESLKR
 FFKKDSSVLNPTAWWRRYGTRLWKTMIQPYAHLGCRKPDENEPQINRWILEWGKYNCRLM
 KEKEKLLTGECSVNRKSDCSTGCNNECYTYRSLINRQRYEVSIKGKYYIKVVRYTIFRR
 KIVQPDNALDFLKLNCSECKDIDFKPFFEFYGYEEKCMCQSYIDLKIQFKNNDICSFN
 AQTDTVSSDKRFCLEKKEFKPWKCDKNSFETVHHKGVCVSPRRQGFCLGNLNYLLNDDIY
 NVHNSQLLIEIIMASKQEGKLLWKKHGTILDNQNACKYINDSYVDYKDIDVIGNDLWNDNN
 SIKVQNNLNLIFERNFGYKVGRNKLFTIKELKNVWILNRNKVWESMRCGIDEVDQRRK
 TCERIDELENMPQFFRWFSQWAHFFCCKEKEYWELKLNKCTGNNGKSLCQDKTCQNVCTN
 MNYWYTRKLAYEIQSVKYDKDRKLFSLAKDKNVTTFLENKAKNCSNIDFTKIFDQLDKL
 FKERCSCMDTQVLEVKNKEMLSIDSNSDATDISEKNGEEELYVNHNSVSVASGNKEIEK
 SKDEKQPEKEAKQTNGTLTVRTDKSDRNKGKDTATDTKNPENLKVQEHGTNGETIKEE
 PPKLPESSETLQSQEQLEAEAKQKQKEEEPPKKKQEEEPKKKQEEEQKREQEOKQEQE
 QKQEEEQQIQDQSQSGLDQSSKVGVASEQNEISSGQEQNVKSSSPEVVPQETTSNGSSQ
 DTKISSTEPNENSVDNRATDSMNLDPEKVHNENMSDPNTNTEPDASLKDDKKEVDDAKKE
 LQSTVSRIESNEQDVQSTPPEDTPTVEGKVGDKAEMLTSPHATDNSESESGLNPTDDIKT

TDGVVKEQEILGGGESATETSKSNLEKPKDVEPSHEISEPVLSGTTGKEESELLKSKSIE
 TKGETDPRSNDQEDATDDVVENSRRDDNNSLSNSVDNQSNVLNREDPIASETEVVSEPEDS
 SRIITTEVPSTTVKPPDEKRSEEVGEKEAKEIKVEPVVPRAIGEPMENSVSQSPPNVED
 VEKETLISENGLHNDTHRGNISEKDLIDIHLRNEAGSTILDDSRNGEMTEGSESDVG
 ELQEHNFSTQOKDEKDFDQIASDREKEEIQKLLNIGHEEDEDVLKMDRTEDSMSDGVNSH
 LYNNLSSEEKMEQYNNRDASKDREEILNRSNTNTCSNEHSLKYCQYMERNKDLLETCE
 DKRLHLCCEISDYCLKFFNPKSIEYFDCTQKEFDDPTYNCFRKQRFSTSMHYIAGGGIIAL
 LLFILGSASYRKNLDDEKGFYDSNLNDSAFEYNNNKYNKLPYMFDDQQINVVNSDLYSEGI
 YDDTTTF

The sequence of EBA 140 (PlasmoDB Accession No: MAL13P1.60) is provided below
 (SEQ ID NO: 9)

MKGYFNIYFLIPLIFLYNVIRINESIIGRTLNRQDESSDISRVNSPELNNNHKTNIYDS
 DYEDVNNKLINSFVENKSVKKRSLSFINNKTYSYDIIPPSYSYRNDKFNSLSENEEDNSG
 NTNSNNFANTSEISIGKDNKQYTFIQKRTHLFACGIKRKSIKWICRENSEKITVCVPDRK
 IQLCIANFLNSRLETMEKFKEIFLISVNTAEKLLYNKNEGKDPSIFCNELRNSFSDFRNS
 FIGDDMDFGGNTDRVKGYNKKFSDYYKEKNVEKLNNIKKEWWEKNKANLWNHMIIVNHKG
 NISKECAIIPAEFPQINLWIKEWNNENFLMEKKRFLNIDKDCVENKKYEACFGGCRLPCS
 SYTSFMKSKTQMEVLTNLYKKKNSGVDKNNFLNDLFKNNKNDLDDFFKNEKEYDDLCD
 CRYTATIISFLNGPAKNDVDIASQINVNDLRGFGCNYKSNNEKSWNCTGTFTNKFPGTC
 EPPRRQTLCLGRTYLLHRGHEEDYKEHLLGASIYEAQLLYKYKYKEKDENALCSIIQNSYA
 DLADIIGSDI IKDYYGKKMEENLNKVNKDKKRNEESLKFREKWWDENKENVWKVMSAV
 LKNKETCKDYDKFQKIPQFLRWFKEWGDDFCEKRKEKIYSFESFKVECKKKDCDENTCKN
 KCSEYKKWIDLKKSEYEKQVDKYTKDKNKKMYDNIDEVKNKEANVYLKEKSKECKDVNFD
 DKIFNESPNEYEDMCKKCDEIKYLNEIKYPKTKHDIYDIDTFSDTFGDGTPISINANINE
 QQSGKDTSENTGNETSDSPVSHEPESDAAINVEKLSGDESSSETRGILDINDPSVTNNVN
 EVHDASNTQGSVSNTSDITNGHSESSLNRTTNAQDIKIGRSGNEQSDNQENSSHSSDNSG
 SLTIGQVPSEDNTQNTYDSQNPHRDTPNALASLPSSDKINEIEGFDSSRDSENGRGDTTS
 NTHDVRRTNIVSERRVNSHDFIRNGMANNNAHHQYITQIENNGIIRGOEESAGNSVNYKD
 NPKRSNFSSENDHKKNIQEYNSRDTKRVREEIIKLSKQNKCNNEYSMEYCTYSDERNSSP
 GPCSREERKKLCCQISDYCLKYFNFYSIEYNCIKSEIKSPEYKCFKSEGQSSIPYFAAG
 GILVVIVLLLSSASRMGKSNEEYDIGESNIEATFEENNYLNKLSRIFNQEVQETNISDYS
 EYNYNEKNMY

In one form of the composition, where the EBA is EBA175 the contiguous amino acid sequence is found in SEQ ID NO: 5. The scope of the invention includes mutations of the sequence described in SEQ ID NO: 5. Preferably, mutations for EBA175 include N at amino acid 157 replaced with S, E at amino acid 274 replaced with K, K at amino acid 279 replaced with E, K at amino acid 286 replaced with E, D at amino acid 336 replaced with Y, K at amino acid 388 replaced with N, P at amino acid 390 replaced with S, E at amino acid 403 replaced with K, K at amino acid 448 replaced with E, K at amino acid 478 replaced with N K at amino acid 481 replaced with I, N at amino acid 577 replaced with K, Q at amino acid 584 replaced with K, R at amino acid 664 replaced with S, S at

amino acid 768 replaced with N, E at amino acid 923 replaced with K, K at amino acid 932 replaced with E, E at amino acid 1058 replaced with V, or G at amino acid 1100 replaced with D.

In one form of the composition, where the EBA is EBA181 the contiguous amino acid sequence is found in SEQ ID NO: 7. The scope of the invention includes mutations of the sequence described in SEQ ID NO: 7. Preferably, mutations for EBA181 include the V at amino acid 64 replaced with L, Q at amino acid 364 replaced with H, V at amino acid 363 replaced with D, R at amino acid 358 replaced with K, N at amino acid 414 replaced with I, K at amino acid 443 replaced with Q, P at amino acid 878 replaced with Q, E at amino acid 884 replaced with Q, E at amino acid 1885 replaced with K, Q at amino acid 890 replaced with E, P at amino acid 1197 replaced with L, K at amino acid 1219 replaced with N, D at amino acid 1433 replaced with Y or N, or K at amino acid 1518 replaced with E.

In one form of the composition where the EBA is EBA140 the contiguous amino acid sequence is found in SEQ ID NO:9. Preferably, mutations for EBA140 include the V at amino acid 19 replaced with I, L at amino acid 112 replaced with F, I at amino acid 185 replaced with V, N at amino acid 239 replaced with S, K at amino acid 261 replaced with T.

In another form of the composition, where the contiguous amino acid sequence of the EBA protein is found in the region between the F2 domain and the transmembrane domain of the EBA protein. More particularly, the contiguous amino acid sequence may be found in the region from about residue 746 to about residue 1339 of the EBA protein.

In one form of the composition, where the EBA is EBA140 the contiguous amino acid sequence is found in the region from about residue 746 to about residue 1045 of EBA140.

Where the EBA is EBA175 the contiguous amino acid sequence is found in the region from about residue 761 to about residue 1271 of EBA175. Where the EBA is EBA181 the contiguous amino acid sequence is found in the region from about residue 755 to about residue 1339 of EBA181.

As for the EBA protein, in one form of the immunogenic molecule, the contiguous amino acid sequence of the EBA protein comprises about 5 or more amino acids. In another form, the contiguous amino acid sequence molecule comprises about 8, 10, 20, 50, or 100 amino acids. The skilled person is capable of routine experimentation designed to identify the shortest efficacious sequence, or the length of sequence that provides the greatest or most effective invasion-inhibitory response in the subject.

Applicant proposes that Rh4 may be involved in invasion using the SA-independent pathway in enzyme treated red cells, and that inhibiting Rh4-mediated invasion is important in treating or preventing infection. However, recent data (Gaur et al. PNAS in press) has suggested that a region of Rh4 known as rRh4₃₀ (comprising amino acids 328 to 588 of Rh4) and native Rh4 bind strongly to neuraminidase treated erythrocytes. Importantly this work demonstrated that while antibodies to the region of Rh4 encoded by amino acids 328 to 588 block binding of the native protein to red cells, these antibodies fail to block invasion, leading the authors to conclude that Rh4 is inaccessible for antibody-mediated inhibition of the invasion process. The authors propose that this inaccessibility may be explained by Rh4 being released after formation of the tight junction during invasion, and consequently antibodies have no access, or the receptor may form the junction too rapidly following merozoite attachment such that interaction with the antibody is not possible. The authors conclude that Rh4 will probably not be an effective candidate in vaccine development.

In contrast, the present invention provides that Rh4 is an effective target of the immune response in humans. Figure 7C shows that an 88 kDa region of Rh4 of *Plasmodium falciparum* strain 3D7 binds to erythrocytes (amino acids 28 to 766 of Rh4; e.g. amino acids 28 to 766 of SEQ ID NO: 3), whereas a 42kDa region of Rh4 (amino acids 853 to 1163) is unable to bind erythrocytes. This is consistent with recent work demonstrating that a region of Rh4 (rRh4₃₀; amino acids 328 to 588) is able to bind to enzyme treated red cells (Gaur et al. PNAS in press). However, Gaur et al demonstrate that while rRh4₃₀ is able to block invasion of *Plasmodium falciparum* strain Dd2 into neuraminidase treated red cells, rRh4₃₀ does not block invasion of *Plasmodium falciparum* strain 3D7 into neuraminidase treated red cells. Furthermore, rRh4₃₀ is unable to block invasion of untreated red cells, and antibodies to Rh4 fail to block red cell invasion. In contrast, the

present invention demonstrates that Rh proteins (including Rh4) are targets of acquired antibodies that inhibit invasion (Figure 1 B, and C, Figure 2 A and B, Figure 3 A and B, Figure 6E). In combination with the differential inhibition of parasite lines that vary in their invasion phenotype, but not genotype, suggests that members of the EBA and Rh proteins may therefore be effective candidates for vaccine development.

To examine the acquisition of invasion-inhibitory antibodies observed in serum samples from children and adults resident in the Kilifi District, Kenya, in 1998 (a year that was preceded with a relatively high incidence of malaria in the region) (EXAMPLES 1 to 7) (Figures 1 to 5), recombinant Rh and EBA proteins were utilized (Figure 6). Human antibodies to EBA175 were detected (Figure 6A and B) in the serum samples used to identify invasion-inhibitory antibodies (Figures 1 to 5). In particular, antibodies recognizing the region of EBA175 between from about the F2 domain to about the transmembrane domain of EBA175 were detected and acquired in an age-dependent manner. In particular, antibodies recognizing the region of EBA175 from amino acid 760 to 1271 of EBA175 (e.g. SEQ ID NO: 5) were detected and acquired in an age-dependent manner.

Comparison of inhibition of 3D7 and 3D7 Δ EBA175 allows examination of human invasion-inhibitory antibodies specifically targeting EBA175. 15% of children and 17% of adults inhibited 3D7-wt more than 3D7 Δ EBA175 (Figure 3A), indicating individuals in the population have invasion-inhibitory antibodies against EBA175. These antibodies were responsible for up to 47% of the total inhibitory activity measured in some individuals (Figure 5), indicating that EBA175 is an important target of invasion-inhibitory antibodies. In particular, invasion-inhibitory antibodies recognized the region of EBA175 from amino acid 760 to 1271 of EBA175.

To further examine the role of invasion-inhibitory antibodies to EBA175 in protection from malaria, the association of antibodies to EBA175 with time to first infection following drug treatment was examined in the same cohort of children in northern Papua New Guinea (Figure 10 A and B). In particular, antibodies recognizing the region of EBA175 between from about the F2 domain to about the transmembrane domain of EBA175 were associated with reduced risk of clinical malaria. In particular, antibodies recognizing the region of EBA175 from amino acid 760 to 1271 of EBA175 (e.g. SEQ ID NO: 5) were

associated with reduced risk of clinical malaria.

Human antibodies to EBA181 were detected (Figure 6D) in the serum samples used to identify invasion-inhibitory antibodies (Figures 1 to 5). In particular, antibodies recognizing the region of EBA181 between from about the F2 domain to about the transmembrane domain of EBA181 were detected and acquired in an age-dependent manner. In particular, antibodies recognizing the region of EBA181 from amino acid 755 to 1339 of EBA181 (e.g. SEQ ID NO: 7) were detected and acquired in an age-dependent manner.

To further examine the role of invasion-inhibitory antibodies to EBA181 in protection from malaria, the association of antibodies to EBA181 with time to first infection following drug treatment was examined in the same cohort of children in northern Papua New Guinea (Figure 10 A and C). In particular, antibodies recognizing the region of EBA181 between from about the F2 domain to about the transmembrane domain of EBA181 were associated with reduced risk of clinical malaria. In particular, antibodies recognizing the region of EBA181 from amino acid 755 to 1339 of EBA181 (e.g. SEQ ID NO: 7) were associated with reduced risk of clinical malaria.

Human antibodies to EBA140 were detected (Figure 6C) in the serum samples used to identify invasion-inhibitory antibodies (Figures 1 to 5). In particular, antibodies recognizing the region of EBA140 between from about the F2 domain to about the transmembrane domain of EBA140 were detected and acquired in an age-dependent manner. In particular, antibodies recognizing the region of EBA140 from amino acid 746 to 1045 of EBA140 (e.g. SEQ ID NO: 9) were detected and acquired in an age-dependent manner.

To further examine the role of invasion-inhibitory antibodies to EBA140 in protection from malaria, the association of antibodies to EBA140 with time to first infection following drug treatment was examined in the same cohort of children in northern Papua New Guinea (Figure 10 A and D). In particular, antibodies recognizing the region of EBA140 between from about the F2 domain to about the transmembrane domain of EBA140 were associated with reduced risk of clinical malaria. In particular, antibodies recognizing the region of EBA140 from amino acid 746 to 1045 of EBA140 (e.g. SEQ ID NO: 9) were

associated with reduced risk of clinical malaria.

Comparison of inhibition of 3D7 and 3D7 Δ EBA140 allows examination of human invasion-inhibitory antibodies specifically targeting EBA140. Serum samples from children and adults inhibited 3D7-wt more than 3D7 Δ EBA140 (Figure 11), indicating individuals in the population have invasion-inhibitory antibodies against EBA140, indicating that EBA175 is an important target of invasion-inhibitory antibodies. In particular, invasion-inhibitory antibodies recognized the region of EBA140 from amino acid 746 to 1045 of EBA140.

Human antibodies to Rh2 were detected (Figure 6F) in the serum samples used to identify invasion-inhibitory antibodies (Figures 1 to 5). In particular, antibodies recognizing the region of Rh2 between about 31 amino acids N-terminal of the Prodom PD006364 homology region to about the transmembrane domain of Rh2 were detected and acquired in an age-dependent manner. In particular, antibodies recognizing the region of Rh2 from amino acid 2027 to 2533 of Rh2 (e.g. SEQ ID NO: 1) were detected and acquired in an age-dependent manner. Also, antibodies recognizing the region of Rh2 from amino acid 2098 to 2597 of Rh2 were detected and acquired in an age-dependent manner (Figure 8A). Also, antibodies recognizing the region of Rh2 from amino acid 2616 to 3115 of Rh2 were detected and acquired in an age-dependent manner (Figure 8B).

To further examine the role of invasion-inhibitory antibodies to Rh2 in protection from malaria, the association of antibodies to Rh2 with time to first infection following drug treatment was examined in children in northern Papua New Guinea (Figure 9). In particular, human antibodies recognizing Rh and EBA proteins were associated with reduced risk of clinical malaria. In particular, antibodies recognizing the region of Rh2 between about 31 amino acids N-terminal of the Prodom PD006364 homology region to about the transmembrane domain of Rh2 were associated with reduced risk of clinical malaria. In particular, antibodies recognizing the region of Rh2 from amino acid 2027 to 3115 of Rh2 (e.g. SEQ ID NO: 1) were associated with reduced risk of clinical malaria (Figure 9). In particular, antibodies recognizing the region of Rh2 from amino acid 2098 to 2597 of Rh2 were associated with reduced risk of clinical malaria (Figure 9A). Also, antibodies recognizing the region of Rh2 from amino acid 2616 to 3115 of Rh2 were

associated with reduced risk of clinical malaria (Figure 9B).

Human antibodies to Rh4 were detected (Figure 6E) in the serum samples used to identify invasion-inhibitory antibodies (Figures 1 to 5). In particular, antibodies recognizing the region of Rh4 between from about the MTH1187/YkoF-like superfamily domain to about the transmembrane domain of Rh4 were detected and acquired in an age-dependent manner. In particular, antibodies recognizing the region of Rh4 from amino acid 1160 to 1370 of Rh4 (e.g. SEQ ID NO: 3) were detected and acquired in an age-dependent manner.

To further examine the role of invasion-inhibitory antibodies to Rh4 in protection from malaria, the association of antibodies to Rh4 with time to first infection following drug treatment was examined in the same cohort of children in northern Papua New Guinea. In particular, antibodies recognizing the region of Rh4 from about the MTH1187/YkoF-like superfamily domain to about the transmembrane domain of Rh4 were associated with reduced risk of clinical malaria. In particular, antibodies recognizing the region of Rh4 from amino acid 1160 to 1370 of Rh4 (e.g. SEQ ID NO: 3) were associated with reduced risk of clinical malaria.

In one form of the immunogenic molecule, the contiguous amino acid sequence comprises about 5 or more amino acids. In another form, the contiguous amino acid sequence molecule comprises about 8, 10, 20, 50, or 100 amino acids. The skilled person is capable of routine experimentation designed to identify the shortest efficacious sequence, or the length of sequence that provides the greatest or most effective invasion-inhibitory response in the subject.

Similarly, the skilled person understands that strict compliance with any amino acid sequence disclosed herein is not necessarily required, and he or she could decide by a matter of routine whether any further mutation is deleterious or preferred. Thus, the immunogenic molecules of the present invention include sequences having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to any protein disclosed herein. The immunogenic molecules also include variants (e.g. allelic variants, homologs, orthologs, paralog, mutants, etc.). The molecules may lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7,

8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus.

Expression of the immunogenic molecules of the invention may take place in *Plasmodium*, however other heterologous hosts may be utilised. The heterologous host may be prokaryotic (e.g. a bacterium) or eukaryotic. It is preferably *E. coli*, but other suitable hosts include *Bacillus subtilis*, *Vibrio cholerae*, *Salmonella typhi*, *Salmonella typhimurium*, *Neisseria lactamica*, *Neisseria cinerea*, *Mycobacteria* (e.g. *M. tuberculosis*), yeasts, etc. The immunogenic molecules of the present invention may be present in the composition as individual separate polypeptides. Generally, the recombinant fusion proteins of the present invention are prepared as a GST-fusion protein and/or a His-tagged fusion protein.

Polypeptides of the invention can be prepared by various means (e.g. recombinant expression, purification from cell culture, chemical synthesis, etc.) and in various forms (e.g. native, fusions, non-glycosylated, lipidated, etc.). They are preferably prepared in substantially pure form (i.e. substantially free from other Plasmodial or host cell proteins).

While the immunogenic molecule may comprise a single antigenic region, by the use of well-known recombinant DNA methods, more than one antigenic region may be included in a single immunogenic molecule. At least two (i.e. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) antigens can be expressed as a single polypeptide chain (a 'hybrid' polypeptide). Hybrid polypeptides offer two principal advantages: first, a polypeptide that may be unstable or poorly expressed on its own can be assisted by adding a suitable hybrid partner that overcomes the problem; second, commercial manufacture is simplified as only one expression and purification need be employed in order to produce two polypeptides which are both antigenically useful.

Hybrid polypeptides can be represented by the formula $\text{NH}_2\text{-A-}(-\text{X-L-})_n\text{-B-COOH}$, wherein: X is an amino acid sequence of a *Plasmodium falciparum* antigen as defined herein; L is an optional linker amino acid sequence; A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; and n is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15.

If a -X- moiety has a leader peptide sequence in its wild-type form, this may be included or omitted in the hybrid protein. In some embodiments, leader peptides (if present) will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein i.e. a leader peptide of X_i will be retained, but the leader peptides of $X_2 \dots X_n$ will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

For each n instances of (-X-L-), linker amino acid sequence -L- may be present or absent. For instance, when $n=2$ the hybrid may be $\text{NH}_2\text{-X}_1\text{-L}_1\text{-X}_2\text{-L}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-X}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-L}_1\text{-X}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-X}_2\text{-L}_2\text{-COOH}$, etc. Linker amino acid sequence(s) -L- will typically be short (e.g. 20 or fewer amino acids i.e. 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short peptide sequences which facilitate cloning, poly-glycine linkers (i.e. comprising Gly_n where $n = 2, 3, 4, 5, 6, 7, 8, 9, 10$ or more), and histidine tags (i.e. His_n where $n = 3, 4, 5, 6, 7, 8, 9, 10$ or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG, with the Gly-Ser dipeptide being formed from a BamHI restriction site, thus aiding cloning and manipulation, and the $(\text{Gly})_4$ tetrapeptide being a typical poly-glycine linker. The same variants apply to (-Y-L-). Therefore, for each m instances of (-Y-L-), linker amino acid sequence -L- may be present or absent.

-A- is an optional N-terminal amino acid sequence. This will typically be short (e.g. 40 or fewer amino acids i.e. 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (e.g. histidine tags i.e. His_n where $n = 3, 4, 5, 6, 7, 8, 9, 10$ or more). Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X_1 lacks its own N-terminus methionine, -A- is preferably an oligopeptide (e.g. with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides an N-terminus methionine.

-B- is an optional C-terminal amino acid sequence. This will typically be short (e.g. 40 or fewer amino acids i.e. 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples

include sequences to direct protein trafficking, short peptide sequences which facilitate cloning or purification (e.g. comprising histidine tags i.e. His_n, where n = 3, 4, 5, 6, 7, 8, 9, 10 or more), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art. Most preferably, n is 2 or 3.

The invention provides a process for producing an immunogenic molecule of the invention, comprising the step of synthesising at least part of the immunogenic molecule by chemical means.

Polypeptides used with the invention can be prepared by various means (e.g. recombinant expression, purification from cell culture, chemical synthesis, etc.). Recombinantly-expressed proteins are preferred, particularly for hybrid polypeptides.

Polypeptides used with the invention are preferably provided in purified or substantially purified form i.e. substantially free from other polypeptides (e.g. free from naturally-occurring polypeptides), particularly from other Plasmodium or host cell polypeptides, and are generally at least about 50% pure (by weight), and usually at least about 90% pure i.e. less than about 50%, and more preferably less than about 10% (e.g. 5%) of a composition is made up of other expressed polypeptides. Thus the antigens in the compositions are separated from the whole organism with which the molecule is expressed.

The present invention provides compositions comprising an immunogenic molecule as described herein. Compositions of the invention can be combined with pharmaceutically acceptable excipient. Such excipients include any excipient that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, sucrose, trehalose, lactose, and lipid aggregates (such as oil droplets or liposomes). Such carriers are well known to those of ordinary skill in the art. The vaccines may also contain diluents, such as water, saline, glycerol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present. Sterile pyrogen-free, phosphate-buffered physiologic saline

is a typical carrier.

The pH of the composition is preferably between 6 and 8, preferably about 7. The pH may be maintained by the use of a buffer. A phosphate buffer is typical. The composition may be sterile and/or pyrogen-free. The composition may be isotonic with respect to humans. Compositions may include sodium salts (e.g. sodium chloride) to give tonicity. A concentration of 10+/-2 mg/ml NaCl is typical. Compositions may also comprise a detergent e.g. a Tween (polysorbate), such as Tween 80. Detergents are generally present at low levels e.g. <0.01%.

Compositions may comprise a sugar alcohol (e.g. mannitol) or a disaccharide (e.g. sucrose or trehalose) e.g. at around 15-30 mg/ml (e.g. 25 mg/ml), particularly if they are to be lyophilised or if they include material which has been reconstituted from lyophilised material. The pH of a composition for lyophilisation may be adjusted to around 6.1 prior to lyophilisation.

The composition may further comprise an antimalarial that is useful for the treatment of Plasmodial infection. Preferred antimalarials for use in the compositions include the chloroquine phosphate, proguanil, primaquine, doxycycline, mefloquine, clindamycin, halofantrine, quinine sulphate, quinine dihydrochloride, gluconate, primaquine phosphate and sulfadoxine.

The compositions of the invention may also comprise one or more immunoregulatory agents. Preferably, one or more of the immunoregulatory agents include(s) an adjuvant. The adjuvant may be selected from one or more of the group consisting of a TH1 adjuvant and TH2 adjuvant, further discussed below.

Adjuvants which may be used in compositions of the invention include, but are not limited to those described in the following passages.

Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminium salts and calcium salts. The invention includes mineral salts such as hydroxides (e.g. oxyhydroxides), phosphates (e.g. hydroxyphosphates, orthophosphates), sulphates, etc. (e.g. see chapters 8 & 9 of Powell & Newman (eds.)

Vaccine Design (1995) Plenum), or mixtures of different mineral compounds, with the compounds taking any suitable form (e.g. gel, crystalline, amorphous, etc.), and with adsorption being preferred. The mineral containing compositions may also be formulated as a particle of metal salt (WO00/23105).

A typical aluminium phosphate adjuvant is amorphous aluminium hydroxyphosphate with PO_4/Al molar ratio between 0.84 and 0.92, included at 0.6 mg Al^{3+}/ml . Adsorption with a low dose of aluminium phosphate may be used e.g. between 50 and 100 $\mu\text{g Al}^{3+}$ per conjugate per dose. Where an aluminium phosphate is used and it is desired not to adsorb an antigen to the adjuvant, this is favoured by including free phosphate ions in solution (e.g. by the use of a phosphate buffer).

Oil emulsion compositions suitable for use as adjuvants in the invention include oil-in-water emulsions and water-in-oil emulsions.

A submicron oil-in-water emulsion may include squalene, Tween 80, and Span 85 e.g. with a composition by volume of about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85 (in weight terms, 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85), known as 'MF595' (57-59 chapter 10 of Powell & Newman (eds.) Vaccine Design (1995) Plenum; chapter 12 of O'Hagen (ed.) Vaccine Adjuvants: Preparation Methods and Research Protocols (Volume 42 of Methods in Molecular Medicine series)). The MF59 emulsion advantageously includes citrate ions e.g. 10 mM sodium citrate buffer.

An emulsion of squalene, a tocopherol, and Tween 80 can be used. The emulsion may include phosphate buffered saline. It may also include Span 85 (e.g. at 1%) and/or lecithin. These emulsions may have from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% Tween 80, and the weight ratio of squalene tocopherol is preferably <1 as this provides a more stable emulsion. One such emulsion can be made by dissolving Tween 80 in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5 g of DL- α -tocopherol and 5ml squalene), then microfluidising the mixture. The resulting emulsion may have submicron oil droplets e.g. with an average diameter of between 100 and 250nm, preferably about 180nm.

An emulsion of squalene, a tocopherol, and a Triton detergent (e.g. Triton X-100) can be used.

An emulsion of squalane, polysorbate 80 and poloxamer 401 ("Pluronic™ L 121") can be used. The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle for muramyl dipeptides, and has been used with threonyl-MDP in the "SAF-I" adjuvant, (0.05-1% Thr-MDP, 5% squalane, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the "AF" adjuvant (Hariharan et al. (1995) Cancer Res 55:3486-9) (5% squalane, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.

Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) may also be used.

Saponin formulations may also be used as adjuvants in the invention (see for example Chapter 22 of Powell & Newman (eds.) Vaccine Design (1995) Plenum). Saponins are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponin from the bark of the Quillaia saponaria Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from Smilax ornata (sarsapilla), Gypsophilla paniculata (brides veil), and Saponaria officianalis (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs. QS21 is marketed as Stimulon™.

Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS 17, QSI 8, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 63. Saponin formulations may also comprise a sterol, such as cholesterol (WO96/33739).

As discussed *supra*, combinations of saponins and cholesterol can be used to form unique particles called immunostimulating complexes (ISCOMs) (see for example Chapter 23 of Powell & Newman (eds.) Vaccine Design (1995) Plenum). ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or

phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA and QHC. ISCOMs are further described in WO96/33739, EP-A-0109942, WO96/11711). Optionally, the ISCOMS may be devoid of additional detergent WO00/07621.

A review of the development of saponin based adjuvants can be found in Barr et al. (1998) *Advanced Drug Delivery Reviews* 32:247-271 and Sjolander et al. (1998) *Advanced Drug Delivery Reviews* 32:321-338.

Virosomes and virus-like particles (VLPs) can also be used as adjuvants in the invention. These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins may be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, Q β -phage (such as coat proteins), GA-phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein pi). VLPs are discussed further in (Niikura et al. (2002) *Virology* 293:273-280, Lenz et al. (2001) *J Immunol* 166:5346-5355, Pinto et al. (2003) *J Infect Dis* 188:327-338, Gerber et al. (2001) *Viol* 75:4752-4760, WO03/024480 and WO03/024481). Virosomes are discussed further in, for example, Gluck et al. (2002) *Vaccine* 20:B10-B16.

Adjuvants suitable for use in the invention include bacterial or microbial derivatives such as non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), Lipid A derivatives, immunostimulatory oligonucleotides and ADP-ribosylating toxins and detoxified derivatives thereof.

Non-toxic derivatives of LPS include monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3dMPL). 3dMPL is a mixture of 3 de-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred "small particle" form of 3 De-O-acylated monophosphoryl lipid A is disclosed in ref. 77. Such "small particles" of 3dMPL are small

enough to be sterile filtered through a 0.22 μ m membrane (EP-A-0689454v). Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosamine de phosphate derivatives e.g. RC-529 (Johnson et al (1999) Bioorg Med Chem Lett 9:2273-2278, Evans et al. (2003) Expert Rev Vaccines 2:219-229).

Lipid A derivatives include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in Meraldi et al. (2003) Vaccine 21:2485-2491, Pajak et al. (2003) Vaccine 21:836-842.

Immunostimulatory oligonucleotides suitable for use as adjuvants in the invention include nucleotide sequences containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine linked by a phosphate bond to a guanosine). Double-stranded RNAs and oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. Kandimalla et al (2003) Nucleic Acids Research 31: 2393-2400, WO02/26757 and WO99/62923 disclose possible analog substitutions e.g. replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in Krieg (2003) Nature Medicine 9:831-835, McCluskie et al. (2002) FEMS Immunology and Medical Microbiology 32:179-185, WO98/40100, US patent 6,207,646, US patent 6,239,116 and US patent 6,429,199.

The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT (Kandimalla et al. (2003) Biochemical Society Transactions 31 (part 3):654-658). The CpG sequence may be specific for inducing a TH1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. Blackwell et al. (2003) J Immunol 170:4061-4068, Krieg (2002) Trends Immunol 23:64-65. Preferably, the CpG is a CpG-A ODN.

Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at

their 3' ends to form "immunomers". See, for example, Kandimalla et al. (2003) *Biochemical Society Transactions* 31 (part 3):654-658, Kandimalla et al (2003), *BBRC* 306:948-953, Bhagat et al. (2003) *BBRC* 300:853-861 and WO03/035836.

Other immunostimulatory oligonucleotides include a double-stranded RNA or an oligonucleotide containing a palindromic sequence, or an oligonucleotide containing a poly(dG) sequence.

Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the invention. Preferably, the protein is derived from *E.coli* (*E.coli* heat labile enterotoxin "LT"), cholera ("CT"), or pertussis ("PT"). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in WO95/17211 and as parenteral adjuvants in WO98/42375. The toxin or toxoid is preferably in the form of a holotoxin, comprising both A and B subunits. Preferably, the A subunit contains a detoxifying mutation; preferably the B subunit is not mutated. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LT-G192. The use of ADP-ribosylating toxins and detoxified derivatives thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in Beignon et al. (2002) *Infect Immun* 70:3012-3019, Pizza et al. (2001) *Vaccine* 19:2534-2541, Pizza et al. (2000) *Int J Med Microbiol* 290:455-461, Scharton-Kersten et al. (2000) *Infect Immun* 68:5306-5313, Ryan et al. (1999) *Infect Immun* 67:6270-6280, Partidos et al. (1999) *Immunol Lett* 67:209-216, Peppoloni et al. (2003) *Expert Rev Vaccines* 2:285-293, Pine et al. (2002) *J Control Release* 85:263-270. Numerical reference for amino acid substitutions is preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in Domenighini et al. (1995) *Mol Microbiol* 15:1165-1167.

Human immunomodulators suitable for use as adjuvants in the invention include cytokines, such as interleukins (e.g. IL-15 IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-17, IL-18 (WO99/40936), IL-23, IL27 (Matsui M. et al. (2004) *J. Virol* 78: 9093) etc.) (WO99/44636), interferons (e.g. interferon- γ), macrophage colony stimulating factor, tumor necrosis factor and macrophage inflammatory protein-1 alpha (MIP-1 alpha) and MIP-1 beta (Lillard JW et al, (2003) *Blood* 101(3):807-14).

Bioadhesives and mucoadhesives may also be used as adjuvants in the invention.

Suitable bioadhesives include esterified hyaluronic acid microspheres (Singh et al) (2001) JCont Release 70:267-276) or mucoadhesives such as cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrrolidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof may also be used as adjuvants in the invention (WO99/27960).

Microparticles may also be used as adjuvants in the invention. Microparticles (i.e. a particle of ~100nm to ~150µm in diameter, more preferably ~200nm to ~30µm in diameter, and most preferably ~500nm to ~10µm in diameter) formed from materials that are biodegradable and non-toxic (e.g. a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, etc.), with poly(lactide-co-glycolide) are preferred, optionally treated to have a negatively-charged surface (e.g. with SDS) or a positively-charged surface (e.g. with a cationic detergent, such as CTAB).

Examples of liposome formulations suitable for use as adjuvants are described in US patent 6,090,406, US patent 5,916,588, EP-A-0626169.

Adjuvants suitable for use in the invention include polyoxyethylene ethers and polyoxyethylene esters (WO99/52549). Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol (WO01/21207) as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol (WO01/21152). Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

Phosphazene adjuvants include poly(di(carboxylatophenoxy)phosphazene) ("PCPP") as described, for example, in references Andrianov et al. (1998) Biomaterials 19:109-115 and Payne et al. (1998) Adv Drug Delivery Review 31:185-196.

Examples of muramyl peptides suitable for use as adjuvants in the invention include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-

2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE).

Imidazoquinoline adjuvants include Imiquimod ("R-837") (US 4,680,338 and US 4,988,815), Resiquimod ("R-848") (WO92/15582), and their analogs; and salts thereof (e.g. the hydrochloride salts). Further details about immunostimulatory imidazoquinolines can be found in references Stanley (2002) Clin Exp Dermatol 27:571-577, Wu et al. (2004) Antiviral Res. 64(2):79-83, Vasilakos et al. (2000) Cell Immunol. 204(I):64-74, US patents 4689338, 4929624, 5238944, 5266575, 5268376, 5346905, 5352784, 5389640, 5395937, 5482936, 5494916, 5525612, 6083505, 6440992, 6627640, 6656938, 6660735, 6660747, 6664260, 6664264, 6664265, 6667312, 6670372, 6677347, 6677348, 6677349, 6683088, 6703402, 6743920, 6800624, 6809203, 6888000 and 6924293 and Jones (2003) Curr Opin Investig Drugs 4:214-218.

Thiosemicarbazone adjuvants include those disclosed in WO2004/060308. Methods of formulating, manufacturing, and screening for active compounds are also described in WO2004/060308. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- α .

Tryptanthrin adjuvants include those disclosed in WO2004/064759. Methods of formulating, manufacturing, and screening for active compounds are also described in WO2004/064759. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- α .

Various nucleoside analogs can be used as adjuvants, such as (a) Isatorabine (ANA-245; 7-thia- 8-oxoguanosine) and prodrugs thereof; (b) ANA975; (c) ANA-025-1; (d) ANA380; (e) the compounds disclosed in US 6,924,271, US2005/0070556 and US 5,658,731, or (f) a pharmaceutically acceptable salt of any of (a) to (g), a tautomer of any of (a) to (g), or a pharmaceutically acceptable salt of the tautomer.

Q. Lipids linked to a phosphate-containing acyclic backbone Adjuvants containing lipids linked to a phosphate-containing acyclic backbone include the TLR4 antagonist E5564 (Wong et al. (2003) J Clin Pharmacol 43(7):735-42 and US2005/0215517).

Small molecule immunopotentiators useful ad adjuvants include N2-methyl-1-(2-methylpropyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2,N2-dimethyl-1-(2-methylpropyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-ethyl-N2-methyl-1-(2-methylpropyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-methyl-1-(2-methylpropyl)-N2-propyl-1H-imidazo(4,5-c)quinoline-2,4-diamine; 1-(2-methylpropyl)-N2-propyl-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-butyl-1-(2-methylpropyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-butyl-N2-methyl-1-(2-methylpropyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-methyl-1-(2-methylpropyl)-N2-pentyl-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-methyl-1-(2-methylpropyl)-N2-prop-2-enyl-1H-imidazo(4,5-c)quinoline-2,4-diamine; 1-(2-methylpropyl)-2-((phenylmethyl)thio)-1H-imidazo(4,5-c)quinolin-4-amine; 1-(2-methylpropyl)-2-(propylthio)-1H-imidazo(4,5-c)quinolin-4-amine; 2-((4-amino-1-(2-methylpropyl)-1H-imidazo(4,5-c)quinolin-2-yl)(methyl)amino)ethanol; 2-((4-amino-1-(2-methylpropyl)-1H-imidazo(4,5-c)quinolin-2-yl)(methyl)amino)ethyl acetate; 4-amino-1-(2-methylpropyl)-1,3-dihydro-2H-imidazo(4,5-c)quinolin-2-one; N2-butyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-butyl-N2-methyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2-methyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; N2,N2-dimethyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo(4,5-c)quinoline-2,4-diamine; 1-(4-amino-2-(methyl(propyl)amino)-1H-imidazo(4,5-c)quinolin-1-yl)-2-methylpropan-2-ol; 1-(4-amino-2-(propylamino)-1H-imidazo(4,5-c)quinolin-1-yl)-2-methylpropan-2-ol; N4,N4-dibenzyl-1-(2-methoxy-2-methylpropyl)-N2-propyl-1H-imidazo(4,5-c)quinoline-2,4-diamine.

One potentially useful adjuvant is an outer membrane protein proteosome preparation prepared from a first Gram-negative bacterium in combination with a liposaccharide preparation derived from a second Gram-negative bacterium, wherein the outer membrane protein proteosome and liposaccharide preparations form a stable non-covalent adjuvant complex. Such complexes include "IVX-908", a complex comprised of *Neisseria meningitidis* outer membrane and lipopolysaccharides. They have been used as adjuvants for influenza vaccines (WO02/072012).

Other substances that act as immunostimulating agents are disclosed in Vaccine Design

((1995) eds. Powell & Newman. ISBN: 030644867X. Plenum) and Vaccine Adjuvants: Preparation Methods and Research Protocols (Volume 42 of Methods in Molecular Medicine series) (ISBN: 1-59259-083-7. Ed. O'Hagan). Further useful adjuvant substances include: Methyl inosine 5'-monophosphate ("MIMP") Signorelli & Hadden (2003) *Int Immunopharmacol* 3(8):1177; a polyhydroxylated pyrrolizidine compound (WO2004/064715), examples include, but are not limited to: casuarine, casuarine-6- α -D-glucopyranose, 3-epz-casuarine, 7-epz-casuarine, 3,7-diepz-casuarine, etc; a gamma inulin (Cooper (1995) *Phar Biotechnol* 6:559) or derivative thereof, such as algammulin; compounds disclosed in PCT/US2005/022769; compounds disclosed in WO2004/87153, including: Acylpiperazine compounds, Indoleione compounds, Tetrahydroisoquinoline (THIQ) compounds, Benzocycloione compounds, Aminoazavinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds (US6,606,617, WO02/018383), Hydrapthalamide compounds, Benzophenone compounds, Isoxazole compounds, Sterol compounds, Quinazolinone compounds, Pyrrole compounds (WO/04/018455), Anthraquinone compounds, Quinoxaline compounds, Triazine compounds, Pyrazalopyrimidine compounds, and Benzazole compounds (WO03/082272); loxoribine (7-allyl-8-oxoguanosine) (US 5,011,828); a formulation of a cationic lipid and a (usually neutral) co-lipid, such as aminopropyl- dimethyl-myristolexyloxy-propanaminium bromide-diphytanoylphosphatidyl- ethanolamine ("Vaxfectin™") or aminopropyl-dimethyl-bis-dodecyloxy-propanaminium bromide-dioleoylphosphatidyl-ethanolamine ("GAP-DLRIE:DOPE"). Formulations containing ($\pm$)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(syn-9-tetradeceneyloxy)-l- propanaminium salts are preferred (US6,586,409).

The invention may also comprise combinations of aspects of one or more of the adjuvants identified above. For example, the following adjuvant compositions may be used in the invention: (1) a saponin and an oil-in-water emulsion (WO99/11241); (2) a saponin (e.g. QS21) + a nontoxic LPS derivative (e.g. 3dMPL) (WO94/00153); (3) a saponin (e.g. QS21) + a non-toxic LPS derivative (e.g. 3dMPL) + a cholesterol; (4) a saponin (e.g. QS21) + 3dMPL + IL-12 (optionally + a sterol) (WO98/57659); (5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions (EP0835318, EP0735898, EP0761231); (6) Ribi™ adjuvant system (RAS), (Ribi Immunochern) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); and

(7) one or more mineral salts (such as an aluminum salt) + a non-toxic derivative of LPS (such as 3dMPL).

In one embodiment of the invention the composition comprises a *Plasmodium falciparum* invasion protein of the EBA family. As discussed *supra*, EBA proteins have also been found by the Applicants to be capable of eliciting an invasion-inhibitory immune response. The use of compositions containing combinations of Rh and EBA proteins relates to the Applicants further discovery that the *Plasmodium falciparum* parasite is capable of evading the host immune response by switching from the use of one invasion protein to another. For example, if the parasite initially utilised a Rh-mediated invasion pathway the host will generate antibodies capable of blocking the method of entry. The parasite is capable of then using an alternative pathway (such as an EBA-mediated pathway) in order to evade the host immune response.

Data provided herein establishes that the invasion-inhibitory activity of naturally acquired antimalarial antibodies is influenced by phenotypic variation in erythrocyte invasion pathways and suggests that the use of alternative invasion pathways may act as a mechanism of immune evasion. These findings indicate members of the EBA and Rh invasion ligand families are key targets of inhibitory antibodies. This knowledge is highly significant for understanding the acquisition of malarial immunity and the capacity of *Plasmodium falciparum* to cause repeated infections, and will aid the prioritisation and validation of candidate vaccine antigens. This establishes, in *Plasmodium falciparum*, the presence of a novel mechanism of immune evasion among microbial pathogens, which may be relevant to other organisms.

Comparing antibody inhibition of W2mef lines with different invasion phenotypes enabled a clear evaluation of the effect of variation in invasion pathway use on the efficacy of inhibitory antibodies (EXAMPLES 1 to 6). W2mef Δ EBA175 uses an alternate SA-independent invasion pathway compared to the parental W2mef-wt (SA-dependent). Many samples that inhibited the parental W2mef lost their inhibitory activity against W2mef Δ EBA175, providing evidence that a switch in invasion pathway use can facilitate immune evasion. These results were confirmed using W2mefSelNm, a line that is genetically intact and uses a SA-independent pathway following selection for invasion into neuraminidase-treated erythrocytes. The present invention also demonstrates that

invasion pathway use alters susceptibility to inhibitory antibodies using a genetically different isolate, 3D7. By varying the use of different members of the EBA and Rh ligand families, *Plasmodium falciparum* appears to evade invasion-inhibitory antibodies targeting specific EBA and Rh proteins. In addition to explaining the lack of phenotype observed for *Plasmodium falciparum* lines disrupted for these molecules on untreated red cells, this surprising result explains the lack of invasion inhibition observed using antibodies to the Rh family of molecules on untreated cells (e.g. Rh2, as discussed *supra*). The present invention demonstrates that effective immunity may depend on the presence of antibodies against a broad range of invasion ligands, in particular antibodies against both SA-dependent invasion and SA-independent invasion ligands, and defines the ligands responsible for this immune invasion (e.g. Rh1, Rh2, Rh4, EBA175, EBA181, and EBA140).

Greater inhibition of W2mef-wt compared to W2mef Δ EBA175 or W2mefSelNm by samples from exposed donors points to antibodies targeting the EBAs and Rh1, which define the SA-dependent pathway. EBA175 is the major target of these antibodies as it is essential for utilisation of the SA-dependent invasion pathway in enzyme treated red cells. Disruption of EBA175 in W2mef results in a switch to an alternative SA-independent invasion pathway in enzyme treated red cells that is Rh4-dependent. This indicates that EBA175 is the major determinant of erythrocyte SA-dependent invasion in W2mef-wt parasites. On the other hand, greater inhibition of W2mef Δ EBA175 or W2mefSelNm compared to W2mef-wt indicates the presence of human invasion-inhibitory antibodies against Rh4 and Rh2, as discussed *supra* and demonstrated in Figures 1, 2, and 3. Furthermore, Figure 12 demonstrates antibodies to Rh2 inhibit *Plasmodium falciparum* lines in which SA-dependent invasion has been reduced by disruption of EBA175 or EBA140 into normal untreated red cells. This demonstrates that there is synergy between the inhibition of invasion by targeting both major pathways of invasion (SA-dependent and SA-independent) into untreated red cells. By targeting the ligands that mediate invasion via these pathways (e.g. Rh1, Rh2, Rh4, EBA175, EBA140 and EBA181), invasion-inhibition on untreated cells is achieved. In particular, rabbit antibodies recognizing the region of Rh2 between about 31 amino acids N-terminal of the Prodom PD006364 homology region to about the transmembrane domain of Rh2 inhibited invasion of *Plasmodium falciparum* parasites on untreated red cells in which EBA175 or EBA140 or EBA175 and EBA140 has been disrupted relative to wild-

type *Plasmodium falciparum* (Figure 12). In particular, antibodies recognizing the region of Rh2 from amino acid 2027 to 3115 of Rh2 (e.g. SEQ ID NO: 1) inhibited invasion on untreated red cells. In particular, antibodies recognizing the region of Rh2 from amino acid 2616 to 3115 of Rh2 inhibited invasion on untreated red cells.

Given the demonstration of the advantages gained by targeting two discrete invasion pathways, one embodiment of the composition comprises a contiguous amino acid sequence of a reticulocyte-binding protein homologue (Rh) protein of the strain of *Plasmodium falciparum*, wherein when administered to a subject the Rh protein is capable of inducing an invasion-inhibitory immune response to the strain. The Rh may be Rh1, Rh2a, Rh2b or Rh4. In one form of the immunogenic molecule, the Rh molecule is Rh2b, and the contiguous amino acid sequence is found in SEQ ID NO: 1:

SEQ ID NO: 1 Amino acid sequence of Rh2b (PlasmoDB Accession No: MAL13P1.176)

MKRSLINLENDLFRLEPISYIQRYYYKKNINRSDIFHNKKERGSKVYSNVSSFHSHFIQEGK
 EEVEVFSIWGSNSVLDHIDVLRDNGTVVFSVQPYLDIYTCKEAILFTTSFYKDLDKSSI
 TKINEDIEKFNEEIIKNEEQCLVGGKTDFDNLLIVLENAEKANVRKTLFDNTFNDYKNKK
 SSFYNCLKNKKNDYDKKIKNIKNEITKLLKNIESTGNMCKTESYVMNNNLYLLRVNEVKS
 TPIDLYLNRAKELLESSSKLVNPIKMKLGDNKNMYSIGYIHDEIKDIIKRYNFHLKHIEK
 GKEYIKRITQANNIADKMKKDELIKKIFESSKHFASFYSNEMISKLDSLFIKNEEILNN
 LFNNIFNIFKKKYETYVDMKTIESKYTTVMTLSEHLLEYAMDVLKANPQKPIDPKANLDS
 EVVKLQIKINEKSNELDNAISQVKTLIIIMKSFYDIIISEKASMEDEMEKKELSLNNYIEK
 TDYILQTYNIFKSKSNIINNNSKNISSKYITIEGLKNDIDELNSLISYFKDSQETLIKDD
 ELKKNMKT DYLN NVKYIEENVTHINEIILLKDSITQRIADIDELNSLNLININDFINEKN
 ISQEKVSYNLNKLYKGSFEELESEL SHFLDTKYLFHEKKS VNELQ TILNTSNNECAKLNF
 MKSDNNNNNNNSNIINLLKTEL SHLLSLKENI IKKLLNHIEQNIQNSSNKYTITTYTDINN
 RMEDYKEEIESLEVYKHTIGNIQKEYILHLYENDKNALAVHNTSMQILQYKDAIQNIKNK
 ISDDIKILKKYKEMNQDLLNYYEILDKKLKDNTYIKEMHTASLVQITQYIPYEDKTISEL
 EQEFNNNNNQKLDNQLQDINAMNLNINILQTLNIGINACNTNNKNVEHLLNKKIELKNILN
 DQMKIIKNDDIIQDNEKENFSNVLKKEEEKLEKELDDIKFNNLKM DIHKLLNSYDHTKQN
 IESNLKINLDSFEKEKDSWVHFKSTIDSLYVEYNICNQKTHNTIKQQKNDIIELIYKRIK
 DINQEIIEKVDNYYSLSDKALTKLKS IHFNIDKEKYKNPKSQENIKLLED RVMILEKKIK
 EDKDALIQIKNL SHDHFNADNEKKKQKEKEEDDEQTHYSKKRKVMGDIYKDIKKNLDEL
 NNKNLIDITLNEANKIESEYEKILIDDICEQITNEAKKSDTIKEKIESYKKDIDYVDVDV
 SKTRNDHHLNGDKIHDSFFYEDTLNYKAYFDKLDLYENINKLTNESNGLKSDAHNNNTQ
 VDKLKEINLQVFSNLGNI IKYVEKLENTLHELKDMYEFLETIDINKILKSIHNSMKKSEE
 YSNETKKIFEQSVNITNQFIEDVEILKTSINPNYESLNDDQIDDNIKSLVLKKEEISEKR
 KQVNKYITDIESNKEQSDLHLRYASRSIYVIDLF IKHEI INPSDGKNFDI IKVKEMINKT
 KQVSNEAMEYANKMDEKNKDIIKIENELYNLINNNIRSLKGVKYEKVRKQARNAIDDINN
 IHSNIKTILT KSKERLDEIKKQPNIKREGDV LNNDKTKIAYITIQINNGRIESNLLNIN
 MKHNIDTILNKAMDYMN DVSKSDQIVINIDSLNMNDIYNKDKDLLINILKEKQNM EAEYK
 KMNEMYNYVNETEKEI IKHKKNYEIRIMEHIKKETNEKKKKFMESNNKSLTTLMD SFRSM
 FYNEYINDYNINENFEKHQNILNEIYNGFNESYNIINTKMTEIINDNLDYNEIKEIKEVA
 QTEYDKLNKKVDELKNYLNNI KEQEGHRLIDYIKEKIFNLYIKCSEQQNIIDDSYNYITV

KKQYIKTIEDVKFLLDSLNTIEEKNKSVANLEICTNKEDIKNLLKHVIKLANFSGIIVMS
 DTNTEITPENPLEDNDLLNLQLYFERKHEITSTLENDSDLELDHLGSNSDESIDNLKVYN
 DIIELHTYSTQILKYLDNIQKLKGCNDLVKDCKELRELSTALYDLKIQTTSVINRENDI
 SNNIDIVSNKLNEIDAIQYNFEKYKEIFDNVEEYKTLDDTKNAYIVKKAIEILKNVDINKT
 KEDLDIYFNDLDELEKSLTLSSNEMEIKTIVQNSYNSFSKINKNINDIDKEMKTLPMLD
 ELLNEGHNIDISLYNFIIRNIQIKIGNDIKNIREQENDTNICFEYIQNNYNFIKSDISIF
 NKYDDHIKVDNYISNNIDVVNKHNSLLSEHVINATNIIENIMTSIVEINEDTEMNSLEET
 QDKLLELYENFKKEKNIINNYYKIVHFNKLKEIENSLETYNSISTNFNKINETQNIDILK
 NEFNNIKTKINDKVVELVHVDSTLTLESIQTFNNLYGDLMSNIQDVYKYEDINNVELKKV
 KLYIENITNLLGRINTFIKELDKYQDENNGIDKYIEINKENNSYIIKLKEKANNLKENFS
 KLLQNIKRNETELYNINNIDIMNTGKSVNNIKQKFSSNLPLKEKLFQMEEMLLNINNI
 MNETKRISNTDAYTNITLQDIENNKNKENNNMNIETIDKLIDHIKIHNEKIQAEILIIDD
 AKRKVKEITDNINKAFNEITENYNNEENNGVIKSAKNIVDKATYLNNELDKFLKLNELLS
 HNNNDIKDLGDEKLILKEEEEERKERERLEKAKQEEERKERERIEKEKQEKERLEREKQEQ
 LKKEALKKQEQERQEQQKEEALKRQEQERLQKEEELKRQEQERLEREKQEQQLQKEEELR
 KKEQEKQQQRNIQEELEEQKKPEIINEALVKGDKILEGSDQRMELSKPNVSMNDNTNNSPI
 SNSEITESDDIDNSENIHTSHMSDIESTQTSRHSNTHGQQISDIVEDQITHPSNIGGEKI
 THNDEISITGERNNISDVNDYSESSNIFENGSTINTSTRNTSSTHDESHISPI SNAYDH
 VVSDNKKSM DENIKDKLKIDESITTDEQIRLDDNSNIVRIDSTDQRDASSHGSSNRDDDE
 ISHVGSDIHMSVDIHDSIDTDENADHRHNVNSVDSLSSSDYTDQKDFSSIIKDGGNKE
 GHAENESKEYESQTEQTHEEGIMNPKNKYSISEVDGIKLNEEAKHKITEKLVDIYPSTYRT
 LDEPMETHGPNKPFHMFSGSPYVTEEDYTEKHDDYDKHEDFNERNYSNHNKMDDFVYNAGGV
 VCCVLFFASITFFSMDRSNKDECDFDMCEEVNNNDHLSNYADKEEIIIEIVFDENEEKYF

Mutations of SEQ ID NO:1 are also included in the scope of this invention and include
 embodiments whereby D at amino acid 2471 is replaced with A, K at amino acid 2560 is
 replaced with E, K at amino acid 3090 is replaced with N, N at amino acid 3116 replaced
 with T, N at amino acid 3116 is replaced with Y.

More particularly, the contiguous amino acid sequence may found in the region between
 about 31 amino acids N-terminal of the Prodom PD006364 homology region to about the
 transmembrane domain of Rh2b. The contiguous amino acid sequence may also be
 found in the region from about residue 2027 to 3115 of Rh2b, or more particularly from
 about residue 2027 to about residue 2533 of Rh2b.

In another form of the immunogenic molecule the contiguous amino acid sequence is
 found in the region from about residue 2098 to about residue 2597, or the region from
 about 2616 to 3115 of Rh2b.

In one form of the immunogenic molecule, the Rh molecule is Rh2a, and the contiguous
 amino acid sequence is found in SEQ ID NO: 11:

SEQ ID NO: 11 Amino acid sequence of Rh2a (PlasmoDB Accession No: PF13_0198)

MKTTLFCSISFCNIIFFFLELSHEHFVQGSSNTHGASSVTDNFNFSEEKNLKSFEKGKNNNN
 DNYASINRLYRKPKPYMKRSLINLENDLFRLEPISYIQRYKKNINRSDIFHNKKERGSKV
 YSNVSSFHSFIQEGKEEVEVFSIWGSNSVLDHIDVLRDNGTVVFSVQPYLDIYTCKEAI
 LFTTSFYKDLKSSITKINEDIEKFNEEIIKNEEQCLVGGKTDFDNLLIVLENAEKANVR
 KTLFDNTFNDYKNKKSSFYNCLKNKKNDYDKKIKNIKNEITKLLKNIESTGNMCKTESYV
 MNNNLYLLRVNEVKSTPIDLYLNRAKELLESSSKLVNPIKMKLGDNKNMYSIGYIHDEIK
 DIIKRYNFHLKHIEKGKEYIKRITQANNIADKMKKDELIKKIFESSKHFAFKYSNEMIS
 KLDSLFIKNEEILNNLFNNIFNIFKKKYETYVDMKTIESKYTTVMTLSEHLLEYAMDVLK
 ANPQKPIDPKANLDSEVVKLQIKINEKSNELDNAISQVKTLIIIMKSFYDIIISEKASMD
 EMEKKELSLNNYIEKTDYILQTYNIFKSKSNIINNNSKNISSKYITIEGLKNDIDELNSL
 ISYFKDSQETLIKDDDELKKNMKT DYLN NVKYIEENVTHINEIILLKDSITQRIADIDELN
 SLNININDFINEKNISQEKVSYNLNKLYKGSFEELESEL SHFLDTKYLFHEKKS VNELQ
 TILNTSNNECAKLNFMKSDNNNNNNNSNIINLLKTEL SHLLSLKENI IKKLLNHIEQNIQ
 NSSNKYTITYTDINNRMEDYKEEIESLEVYKHTIGNIQKEYILHLYENDKNALAVHNTSM
 QILQYKDAIQNIKNKISDDIKILKKYKEMNQDLLNYYETLDKKLKDNTYIKEMHTASLVQ
 ITQYIPYEDKTI SELEQEFNNNNQKLDN ILQDINAMNLNINILQTLNIGINACNTNNKNV
 EHLLNKKIELKNILNDQMKIIKNDDIIQDNEKENFSNVLKKEEEKLEKELDDIKFNNLKM
 DIHKLNSYDHTKQNIESNLKINLDSFEKEKDSWVHFKSTIDSLYVEYNICNQKTHNTIK
 QQKNDIIELIYKRIKDIHQEIIIEKVDNYYSLSDKALTKLKSIFHNIDKEKYKNPKSQENI
 KLLIEDRVMILEKKIKEDKDALIQIKNL SHDHFVNADNEKKKQKEKEEDEQTHYSKRRKV
 MGDYIKDIKKNLDELNNKNLIDITLNEANKIESEYEKILIDDICEQITNEAKKSDTIKEK
 IESYKKDIDYVDVDSKTRNDHHLNGDKIHDSFFYEDTLNYKAYFDKLDLYENINKLTN
 ESNGLKSDAHNNNTQVDKLKEINLQVFSNLGNI IKYVEKLENTLHELKDMYEFLETIDIN
 KILKSIHNSMKKSEEYSNETKKIFEQSVNITNQFIEDVEILKTSINPNYESLNDDQIDDN
 IKSLVLKKEEISEKRKQVNKYITDIESNKEQSDLHLRYASRSIYVIDLF IKHEIINPSDG
 KNFDIIKVKEMINKTKQVSNEAMEYANKMDEKNKDIIKIENELYNLINNNIRSLKGVKYE
 KVRKQARNAIDDINNIHSNIKTILTKSKERLDEIKKQPNIKREGDVLNNDKTKIAYITIQ
 INNGRIESNLLNILNMKHNDITILNKAMDYMNVDVSKSDQIVINIDSLNMNDIYNKDKDLL
 INILKEKQNMEEAYKKMNEMYNVNETEKEIKHKKNYEIRIMEHIKKETNEKKKKFMES
 NNSLTTLMDSFRSMFYNEYINDYNINENFEKHQNILNEIYNGFNESYNIINTKMTEIIN
 DNLDYNEIKEIKEVAQTEYDKLNKKVDELKNYLNNIKEQEGHRLIDYIKEKIFNLYIKCS
 EQQNIIDDSYNYITVKKQYIKTIEDVKFLDLSLNTIEKNKSVANLEICTNKEDIKNLLK
 HVIKLANFSGIIVMSDTNTEITPENPLEDNDLLNLQLYFERKHEITSTLENDSDLELDHL
 GSNSDESIDNLKVYNDIIE LH TYSTQILKYLDNIQKLKGDCNDLVKDCKELRELSTALYD
 LKIQITSVINRENDISNNIDIVSNKLNEIDAIQYNFEKYKEIFDNVEEYKTLDDTKNAYI
 VKKAEILKNVDINKTKEDLDIYFNDLDELEKSLTLSSNEMEIKTIVQNSYNSFSDINKNI
 NDIDKEMKTLPMLDELLNEGHNIDISLYNFIIRNIQIKIGNDIKNIREQENDTNICFEY
 IQNNYNFIKSDISIFNKYDDHIKVDNYISNNIDVVKHNSLLSEHVINATNIIENIMTSI
 VEINEDTEMNSLEETQDKLLELYENFKKEKNIINN NYKIVHFNKLKEIENSLETYNSIST
 NFNKINETQNIDILKNEFNNIKTINDKV KELVHVDSTLTLESIQTFNNLYGDLMSNIQD
 VYKYEDINNVELKKVKLYIENITNLLGRINTFIKELDKYQDENNGIDKYIEINKENNSYI
 IKLKEKANLKENFSKLLQNIKRNETELYNINN IKDDIMNTGKSVNNIKQKFSSNLPLKE
 KLFQMEEMLLNINNIMNETKRISNTAAYTNITLQDIENNKNKENNNMNIETIDKLIDHIK
 IHNEKIQAEILIIDDAKRKVKEITDNINKAFNEITENYNNENNGVIKSAKNIVDEATYLN
 NELDKFLLKLNELL SHNNNDIKDLGDEKLILKEEEERKERERLEKAKQEEERKERERIEK
 EKQEKERLEREKQEQLKKEEELRKKEQERQEQQQKEEALKRQEQRERLQKEEELKRQEQR
 LEREKQEQLQKEEELKRQEQRERLQKEEALKRQEQRERLQKEEELKRQEQRERLEREKQEQLQ
 KEEELKRQEQRERLQKEEALKRQEQRERLQKEEELKRQEQRERLERKKIELAEREQHIKSKLE
 SDMVKIIKDELTKEKDEIIKNKD IKLRHSLEQKWLKHLQNILSLKIDSLLKNKNDVIKDN
 ETQLKTNILNSLKNQLYLNLKRELNEI IKYEENQKKILHSNQLVNDLSLEQKTNRLVDIK
 PTKHGDIIYTNKLSNETEMLITSKEKKDETESTKRS GTDHTNSSESTTDDNTNDRNFSRS
 KNLSVAIYTAGSVALCVLIFSSIGLLLIKTN SGDNN SNEINEAFEPNDDVLFKEKDEIIE

ITFNDNDSTI

Mutations of SEQ ID NO:11 are also included in the scope of this invention and include embodiments whereby A at amino acid 2546 is replaced with D, E at amino acid 2613 is replaced with G, R at amino acid 2723 is replaced with K, K at amino acid 2725 replaced with Q.

More particularly, the contiguous amino acid sequence may found in the region between about 31 amino acids N-terminal of the Prodom PD006364 homology region to about the transmembrane domain of Rh2a. The contiguous amino acid sequence may also be found in the region from about residue 2133 to about residue 3065 of Rh2a.

In another form of the immunogenic molecule the contiguous amino acid sequence is found in the region from about residue 2098 to about residue 2597, or the region from about residue 2616 to about residue 3115 of Rh2a.

SEQ ID NO: 13 Amino acid sequence of Rh1 (PlasmoDB Accession No: PFD0110w)

MQRWIFCNIVLHILIYLAEFSSHEQESYSSNEKIRKDYSDDNNYEPTPSYEKRKKEYGKDE
 SYIKNYRGNNFSYDLSKNSSIFLHMGNSSNSKTLKRCNKKKNIKTNFLRPIEEEKTVLNN
 YVYKGVNFLDTIKRNDSSYKFDVYKDTSF LKNREYKELITMQYDYAYLEATKEVLYLIPK
 DKDYHKFYKNELEKILFNLKDSLKLLREGYIQSKLEMIRIHSIDILNEFHQGNIIINDNY
 FNNEIKKKKEDMEKYIREYNLYIYKYENQLKIKIQKLTNEVSINLNKSTCEKNCYNYILK
 LEKYKNI IKDKINKWKDLPEIYIDDKSFSYTF LKDVINNKIDYKTISSFISTQKQLYYF
 EYIYIMNKNTLNLLSYNIQKTDINSSSKYTYTKSHFLKDNHILLSKYTTAKFIDILNKTY
 YYNLYKNKILLFNKYIIKLRNDLKEYAFKSIQFIQDKIKKHKDELSENILQEVNNIYIK
 YDTSINEISKYNNLIINTDLQIVQQKLLLEIKQKKNDITHKVQLINHIYKNIHDEILNKKN
 NEITKII INNIKDHKKDLQDLLLFIQQIKQYNILTDHKITQCNNYYKEI IKMKEDINHIH
 IYIQPILNNLHTLKQVQNNKIKYEEHIKQILQKIYDKKESLKKIILLKDEAQLDITLLDD
 LIQKQTKKQTQTQTQKQTLIQNNETIQLISGQEDKHESNPFNHIQTYIQQKDTQNKNI
 QNLLKSLYNGNINTFIDTISKYILKQKDIELTQHVYTDEKINDYLEEIKNEQNKIDKTID
 DIKIQETLKQITHIVNNIKTIKKDLLKEFIQH LIKYMNERYQNMQQGYNNLTNYINQYEE
 ENNNMKQYITIRNIQKIYYDNIYAKEKEIRSGQYYKDFITSRKNIYNIRENISKNVDMI
 KNEEKKKIQNCVDKYNSIKQYVKMLKNGDTQDENNNNNNDIYDKLIVPLDSIKQNDKYN
 TEHNFITFTNKINTHNKKNQEMMEEFIYAYKRLKILKILNISLKACEKNNKSINTLNDKT
 QELKKIVTHEIDLLQKDILTSQISNKNVLLLNDLLKEIEQYIIDVHKLKKKSNDLFTYYE
 QSKNYFYFKNKKDNFDIQKTINKMNEWLAIKNYINEINKNYQTLYEKKINVLLHNSKSYV
 QYFYDHI INLILQKKNYLENTLTKTIQDNEHSLYALQQNEEYQKVNEKDQNEIKKIKQL
 IEKNKNDILTYENNI EQIEQKNIELKTNAQNKDDQIVNTLNEVKKKIYTYEKVDNQISN
 VLKNYEEGKVEYDKNVVQNVNDADDTNDIDEINDIDEINDIDEINDIDEINDIDEIKDID
 HIKHFDDTKHFDDIYHADDTRDEYHIALSNYIKTELNRNINLQEIKNNI IKIFKEFKSAHK
 EIKKESEQINKEFTKMDVVINQLRDIDRQMLDLYKELDEKYSEFNKTKIEEINNIRENIN
 NVEIWYEKNIIEYFLRHMNDQKDKA AKYMENIDTYKNNIEIISKQINPENYVETLNKSNM

YSYVEKANDLFYKQINNIIINSNQLKNEAFTIDELQNIQKNRKNLLTKKQQIIQYTNEFE
 NIFNEIKNINNILVLTNYKSILQDISQNINHVSIYTEQLHNLYIKLEEEKEQMKTLYHKS
 NVLHNQINFNEDAFINNLLINIEKIKNDITHIKEKTNIYMIDVNKSKNNAQLYFHNTLRG
 NEKIEYLKNLKNSTNQQTITLQELKQVQENVEKVVDIYNQTIKYEEEIKKNYHIITDYENK
 INDILHNSFIKQINMESSNNKKQTKQIIDIINDKTFEEHIKTSKTKINMLKEQSQMKHID
 KTLLEQALKLFVDINSTNNNLDNMLSEINSIQNNIHTYIQEANKSFDKFKIICDQNVND
 LLNKLSLGDLNYMNLKLNQNEIRNMNLEKNFMLDKSKKIDEEKKLDILKVNISNINNS
 LDKLKKYEEALFQKVKEKAEIQKENIEKIKQEINTLSDFVKKPFFFIQLNTDSSQHEKD
 INNNVETYKNNIDEIYNVFIQSYNLIQKYSSEIFSSTLNYIQTKEIKEKSIKEQNQLNQ
 EKEASVLLKNIKINETIKLQKIKNERQNDVHNKEDYNLLQQYLYNMKNEMEQLKKYKN
 DVHMDKNYVENNNGEKEKLLKETISSYDKINNINNKLYIYKNKEDTYFNNMIKVSEILN
 IIKKKQQNEQRIVINAEDSSLINKDEEIKKEINNQIIELNKHNNENISNIFKDIQNIKK
 QSQDIITNMNDMYKSTILLVDIIQKKEEALNKQKNILRNIDNINLNKKENIIDKVIKCNCD
 DYKDILIQNETEYQKLQININHTYEEKKSIDILKIKNIKQKNIQYKNKLEQMNTIINQS
 IEQHVFINADILQNEKIKLEEIKNLDILDEQIMTYHNSIDELYKLGICDNHLITTISV
 VVNKNTTKIMIHKKQKEDIQKINNYIQTNYNIIINEEALQFHRLYGHNLISEDKNLHVH
 IIEQKQNIYTQKEIDISKIHKHVKKGLYSLNEHDMNHDTHMNIINEHINNINILQPYTQLI
 NMIKDIDNVFIKIQNNKFEQIQKYIEIISLEQLNKNINTDNLNKLKDTQNKLINIETEM
 KHKQQLINKMNDIEKDNTDQYMHVDVQNI FEPITLKMNEYNTLLNDNHNNNNINNEHQF
 NHLNLSLHTKIFSHNYNKEQQQEYITNIMQRIDVFINDLDITYQYEEYFYEWNOEYKQIDKN
 KINQHINNINILHVKKQFEHTLENIKNNENIFDNIQLKKKDIDDIIININNTKETYLK
 ELNKKKNVTKKKKVDEKSEINNHTLQHDNQNVEQKNKIKDHNLTTPNNNSSEESHQNE
 QMKEQNKNILEKQTRNIKPHHVHNHNHNHNQNKDSTKLQEQDISTHKLHNTIHEQQSKD
 NHQGNREKKQKNGNHERMYFASGIVVSILFLFSFGFVINSKNNKQEYDKEQEKQQQNDV
 CDNNKMDDKSTQKYGRNQEEVMEIFFDNDYI

The present invention includes a mutated form of SEQ ID NO:13. It is known to the skilled person that there are a large number of single nucleotide polymorphism in Rh1 and these and any other mutations are included within the scope of the invention.

More particularly, the contiguous amino acid sequence may found in the region between about amino acid residue 1 to transmembrane domain of Rh1. The contiguous amino acid sequence may also be found in the region from about residue 1 to about residue 2897 of Rh1.

In one form of the immunogenic molecule, the Rh protein is Rh4, and the contiguous amino acid sequence is found in SEQ ID NO: 3 (PlasmoDB Accession No: PFD1150c), as disclosed below.

MNKNILWITFFYFLFFLLDMYQGNDAIPSEKKNNDPEADSKNSQNQHDINKTHHTNNNYD
 LNIKDKDEKRRKNDNLINNYDYSLLKLSYNKNQDIYKNIQNGQKLKTDIILNSFVQINSS
 NILMDEIENYVKKYTESNRIMYLQFKYIYLQSLNITVSFVPPNSPFRSYDKNLNKDINE
 TCHSIQTLLNNLISSKIIIFKMLETTEQIILLWNNKKISQQNYNQENQEKSKMIDSENEK
 LEKYTNKFEHNIKPHIEDIEKKVNEYINNSDCHLTCSKYKTIINNYIDEIITNTNIYEN
 KYNLPQERIIKNYNHNGINNDNFIEYNILNADPDLRSHFITLLVSRKQLIYIEYIYFIN
 KHIVNKIQENFKLNQNKYIHFINSNNAVNAAKEYEYIIKYYTTFKYLQTLNKSLYDSIYK
 HKINNYSHNIEDLINQLQHKINNLMIIISFDKNKSSDLMLQCTNIKKYTDDICLSIKPKAL

EVEYLRNINKHINKNEFLNKFMONETFKKNIDDKIKEMNNIYDNIYIILKQKFLNKLNEI
 IQNHKNKQETKLNTTTTIQELLQLLKDIKEIQTKQIDTKINTFNMYYNDIQQIKIKINQNE
 KEIKKVLPLQLYIPKNEQEYIQIYKNELKDRIKETQTKINLQKQILELKEKEHYITNKHTY
 LNFTHKTIQQILQQQYKNNTQEKNTLAQFLYNADIKKYIDELIPITQQIQTKMYTTNNIE
 HIKQILINYIQECKPIQNISEHTIYTLYQEIKTNLENIEQKIMQNIQOTTNRLKINIKKI
 FDQINQKYDDLTKNINQMNDKIGLRQMENRLKGKYEEIKKANLQDRDIKYIVQNNNDANN
 NNNNIIIIINGNNQTGDYNHILFDYTHLWDNAQFTRTKENINNLDKNIQININNIKSIRN
 LQNELNNYNTLKSNSIHIYDKIHTLEELKILTQEINDKNVIRKIYDIETIYQNDLHNIEE
 IIKNITSIYYKINILNILIICIKQTYNNNKSIESLKLKINNLTNSTQEYINQIKAIPTNL
 LPEHIKQKSVSELNIYMKQIYDKLNEHVINNLYTKSKDSLQFYINEKNYNNNHDDHNDH
 NDVYNDIKENEIYKNNKLYECIQIKKDVELYNIYDQLFKNISQNYNNHSLSFVHSINNH
 MLSIFQDTKYGKHKNQQILSDIENI IKQNEHTESYKNLDTSNIQLIKEQIKYFLQIFHIL
 QENITTFENQYKDLIIKMNHKINNNDKIDITHIVINDNNTLQEQNRIYNELQNKIKQIKNV
 SDVFTHNINYSQQILNYSQAQNSFFNIFMKFQININDINSKRYNVQKKITEIINSYDIIN
 YNKNNIKDIYQQFKNIQQQLNTTETQLNHIKQININHFKYFYESHQTSIVKNMQNEKLKI
 QEFNKKIQHFKEETQIMINKLIQPSHIHLHKMKLPITQQQLNTILHRNEQTKNATRSYNM
 NEEENEMGYGITNKRKNSETNDMINTTIGDKTNVLKNDDQEKGKRGTSRNNNIHTNENNI
 NNEHTNENNINNEHTNEKNINNEHANENEKNIYNEHTNENNINYEHPNNYQQKNDEKISLOH
 KTINTSQRITIDDSNMDRNNRYNTSSQQKNLHTNNNSNSRYNNNHDKQNEHKYNQKGSSG
 KDNAYYRIFYAGGITAVLLLCSSSTAFFFIKNSNEPHHIFNIFQKEFSEADNAHSEEKEEY
 LPVYFDEVEDEVEDEDEDEDENEENEVENENEDFNDI

The present invention includes a mutated form of SEQ ID NO:3. Mutations that are included within the scope of the invention include those whereby Y at amino acid 12 is replaced with A, L at amino acid 143 is replaced with I, N at amino acid 435 is replaced with K, Q at amino acid 438 is replaced with K, T at amino acid 506 replaced with K, N at amino acid 771 is replaced with S, N at amino acid 844 is replaced with I, K at amino acid 1482 is replaced with R, or N at amino acid 1498 is replaced with I.

More particularly, the contiguous amino acid sequence is found in the region from about the MTH1187/YkoF-like superfamily domain to about the transmembrane domain of Rh4.

In another form of the immunogenic molecule, the contiguous amino acid sequence is found in the region from about residue 1160 to about residue 1370 of Rh4.

Based on results presented herein, Applicant proposes that a broad inhibitory response against functional epitopes of invasion ligands may be needed to convey substantial protective immunity. It is proposed that vaccines should preferably target multiple invasion ligands in order to be fully effective and ameliorate parasite immune evasion strategies. An effective vaccine may include ligands involved in both SA-dependent and

SA-independent invasion. In light of this, it will still be appreciated that immune responses against only single ligands will nonetheless be useful.

Similarly, the skilled person understands that strict compliance with any amino acid sequence disclosed herein is not necessarily required, and he or she could decide by a matter of routine whether any further mutation is deleterious or preferred. Thus, the immunogenic molecules of the present invention include sequences having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to any protein disclosed herein. The immunogenic molecules also include variants (e.g. allelic variants, homologs, orthologs, paralog, mutants, etc.). The molecules may lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus.

Some forms of the composition contain more than one EBA-derived immunogenic molecule, or more than one Rh-derived immunogenic molecule. The composition may contain any combination of two or more immunogenic molecules derived from Rh1, Rh2a, Rh2b and Rh4. The composition may contain any combination of two or more immunogenic molecules derived from EBA175, EBA140 and EBA181. It is further contemplated that any combination of Rh-derived immunogenic molecules with EBA-derived immunogenic molecules may be present in the composition.

As alluded to by the aforementioned disclosure, the invention further provides a composition of the invention for use as a medicament. Accordingly, in a further aspect the present invention provides a method of treating or preventing a condition caused by or associated with infection by *Plasmodium falciparum* comprising administering to a subject in need thereof an effective amount of a composition as described herein. The medicament is a malarial vaccine in one form of the composition.

Vaccines according to the present invention may either be prophylactic (i.e. to prevent infection) or therapeutic (i.e. to treat infection), but will typically be prophylactic. Accordingly, the invention includes a method for the therapeutic or prophylactic treatment of *Plasmodium falciparum* infection in an animal susceptible to *Plasmodium falciparum* infection comprising administering to said animal a therapeutic or prophylactic

amount of the immunogenic compositions of the invention.

The compositions of the invention may elicit both a cell mediated immune response as well as a humoral immune response in order to effectively address a Plasmodium intracellular infection. This immune response will preferably induce long lasting antibodies and a cell mediated immunity that can quickly respond upon exposure to Plasmodium.

Two types of T cells, CD4 and CD8 cells, are generally thought necessary to initiate and/or enhance cell mediated immunity and humoral immunity. CD8 T cells can express a CD8 co-receptor and are commonly referred to as Cytotoxic T lymphocytes (CTLs). CD8 T cells are able to recognized or interact with antigens displayed on MHC Class I molecules.

CD4 T cells can express a CD4 co-receptor and are commonly referred to as T helper cells. CD4 T cells are able to recognize antigenic peptides bound to MHC class II molecules. Upon interaction with a MHC class II molecule, the CD4 cells can secrete factors such as cytokines. These secreted cytokines can activate B cells, cytotoxic T cells, macrophages, and other cells that participate in an immune response. Helper T cells or CD4+ cells can be further divided into two functionally distinct subsets: TH1 phenotype and TH2 phenotypes which differ in their cytokine and effector function.

Activated TH1 cells enhance cellular immunity (including an increase in antigen-specific CTL production) and are therefore of particular value in responding to intracellular infections. Activated TH1 cells may secrete one or more of IL-2, IFN-gamma, and TNF-beta. A TH1 immune response may result in local inflammatory reactions by activating macrophages, NK (natural killer) cells, and CD8 cytotoxic T cells (CTLs). A TH1 immune response may also act to expand the immune response by stimulating growth of B and T cells with IL-12. TH1 stimulated B cells may secrete IgG2a.

Activated TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. Activated TH2 cells may secrete one or more of IL-4, IL-5, IL-6, and IL-10. A TH2 immune response may result in the production of IgG1, IgE, IgA and memory B cells for future protection.

An enhanced immune response may include one or more of an enhanced TH1 immune response and a TH2 immune response.

An enhanced TH1 immune response may include one or more of an increase in CTLs, an increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFN-gamma, and TNF-beta), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. Preferably, the enhanced TH1 immune response will include an increase in IgG2a production.

A TH1 immune response may be elicited using a TH1 adjuvant. A TH1 adjuvant will generally elicit increased levels of IgG2a production relative to immunization of the antigen without adjuvant. TH1 adjuvants suitable for use in the invention may include for example saponin formulations, virosomes and virus like particles, non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), immunostimulatory oligonucleotides. Immunostimulatory oligonucleotides, such as oligonucleotides containing a CpG motif, are preferred TH1 adjuvants for use in the invention.

An enhanced TH2 immune response may include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10), or an increase in the production of IgG1, IgE, IgA and memory B cells. Preferably, the enhanced TH2 immune response will include an increase in IgG1 production.

A TH2 immune response may be elicited using a TH2 adjuvant. A TH2 adjuvant will generally elicit increased levels of IgG1 production relative to immunization of the antigen without adjuvant. TH2 adjuvants suitable for use in the invention include, for example, mineral containing compositions, oil-emulsions, and ADP-ribosylating toxins and detoxified derivatives thereof. Mineral containing compositions, such as aluminium salts are preferred TH2 adjuvants for use in the invention.

Preferably, the invention includes a composition comprising a combination of a TH1 adjuvant and a TH2 adjuvant. Preferably, such a composition elicits an enhanced TH1 and an enhanced TH2 response, i.e., an increase in the production of both IgG1 and

IgG2a production relative to immunization without an adjuvant. Still more preferably, the composition comprising a combination of a TH1 and a TH2 adjuvant elicits an increased TH1 and/or an increased TH2 immune response relative to immunization with a single adjuvant (i.e., relative to immunization with a TH1 adjuvant alone or immunization with a TH2 adjuvant alone).

The immune response may be one or both of a TH1 immune response and a TH2 response. Preferably, immune response provides for one or both of an enhanced TH1 response and an enhanced TH2 response. The TH1/TH2 response in mice may be measured by comparing IgG2a and IgG1 titres, while the TH1/TH2 response in man may be measured by comparing the levels of cytokines specific for the two types of response (e.g. the IFN- γ /IL-4 ratio).

In one form of the method of treatment or prevention the subject is a human. The human may be an infant, a child, an adolescent, or an adult. Use of the vaccine may be especially important in women in child-bearing years. Pregnant women, particularly in the second and third trimesters of pregnancy are more likely to develop severe malaria than other adults, often complicated by pulmonary oedema and hypoglycaemia. Maternal mortality is approximately 50%, which is higher than in non-pregnant adults. Fetal death and premature labor are common.

One way of monitoring vaccine efficacy for therapeutic treatment involves monitoring *Plasmodium falciparum* infection after administration of the compositions of the invention. One way of checking efficacy of prophylactic treatment involves monitoring immune responses systemically (such as monitoring the level of IgG1 and IgG2a production) against the *Plasmodium* antigens in the compositions of the invention after administration of the composition. Serum *Plasmodium* specific antibody responses may be determined post-immunisation and post-challenge.

The uses and methods are for the prevention and/or treatment of a disease caused by *Plasmodium* (e.g. malaria) and/or its clinical manifestations (e.g. prostration, impaired consciousness, respiratory distress (acidotic breathing), multiple convulsions, circulatory collapse, pulmonary oedema (radiological), abnormal bleeding, jaundice, haemoglobinuria, etc.).

The compositions of the present invention can be evaluated in *in vitro* and *in vivo* animal models prior to host, e.g., human, administration. For example, *in vitro* neutralization and/or invasion inhibition is suitable for testing vaccine compositions (such as immunogenic/immunoprotective compositions) directed toward *Plasmodium*.

Reaction to the vaccine may be evaluated *in vitro* and *in vivo* following host e.g. human, administration. For example, response to vaccine compositions may be examined by Enzyme-Linked Immunosorbent Assay (ELISA). For example, ELISA may be conducted as follows: Plates (e.g. flat-bottomed microtiter plates (Maxisorp from Nunc A/S or High Binding from Costar, Cat. No. 3590) may be coated with 50 μ L of peptide solution or crude parasite antigen at 10 μ g/mL in coating buffer. Keep the plate at 4°C overnight. With many proteins or peptides, PBS can be used as a coating solution. Block with 100 μ L of 0.5% BSA in coating buffer for 3 to 4 h at 37°C. Wash 4 times with 0.9% NaCl plus 0.05% Tween. Add 50 μ L of serum samples diluted 1:1000; leave them for 1 h at 37°C. Wash 4 times with 0.9% NaCl plus 0.05% Tween. Add 50 μ L of ALP-conjugated or biotinylated anti-Ig of appropriate specificity at the recommended concentration in Tween-buffer; leave for 1 h at 37°C. Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween. If biotinylated antibody has been used, add 50 μ L of streptavidin-ALP diluted 1:2000 in Tween-buffer; leave the sample for 1 h at 37°C. Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween. Develop the sample with 50 μ L of NPP (1 tablet/5 mL of substrate buffer) and read at OD₄₀₅.

Infection may be established using typical signs and symptoms of malaria. The signs and symptoms of malaria, such as fever, chills, headache and anorexia. Preferably, more specific methods of diagnosis are preferred e.g. using a scoring matrix of clinical symptoms, light microscopy which allows quantification of malaria parasites (e.g. thick or thin film blood smears from patients stained with acridine orange or Giemsa, rapid diagnostic tests (e.g. immunochromatographic tests that detect parasite-specific antigens e.g. HRP2, parasite lactate dehydrogenase (pLDH), aldolase etc) in a finger-prick blood sample, and polymerase-chain reaction.

Vaccine efficacy may be measured e.g. by examining the number and frequency of cases of malaria (e.g. asexual *Plasmodium falciparum* at any level plus a temperature

greater than or equal to 37.5°C and headache, myalgia, arthralgia, malaise, nausea, dizziness, or abdominal pain), time to first infection with *Plasmodium falciparum*, parasitemia, geometric mean parasite density in first clinical episode, adverse events, anaemia (measured by for example packed cell volume less than 25% or less than 15%), absence of parasites at the end of immunization, proportion of individuals with seroconversion to the antigens of the present invention at e.g. day 75 post immunization, proportion with "efficacious seroconversion" to the antigens of the present invention (4-fold elevation in antibody titre) at day 75, number of symptomatic *Plasmodium falciparum* cases after 1, 2, or 3 doses, number of days until *Plasmodium falciparum* positive blood slide, density of *Plasmodium falciparum*, prevalence of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium malariae*, levels of anti-Rh or anti-EBA (e.g. Rh1, Rh2, Rh4, EBA175, EBA181, EBA140 etc.) antibody by ELISA, geometric mean parasite density in first clinical episode, lymphocyte proliferation to Rh or EBA (e.g. Rh1, Rh2, Rh4, EBA175, EBA181, EBA140 etc.) T-cell responses to antigen frequency of fever, malaise, nausea, Malaria requiring hospital admission, cerebral malaria (e.g. Blantyre coma score <2) etc.

The vaccine may be administered using a variety of vaccination regimes familiar to the skiller person. In one form of the invention, the vaccine composition may be administered post antimalarial treatment. Preferred antimalarials for use include the chloroquine phosphate, proguanil, primaquine, doxycycline, mefloquine, clindamycin, halofantrine, quinine sulphate, quinine dihydrochloride, gluconate, primaquine phosphate and sulfadoxine. For example, blood stage parasitaemia may be cleared with Fansidar (25 mg sulfadoxine/0.75 mg pyrimethamine per kg body weight) before each vaccination. In another form of the invention antimalarial (e.g. Fansidar) treatment is given 1 to 2 weeks before the doses (e.g. first and third doses). In another form of the invention antimalarial (e.g. Fansidar) treatment is given before the first dose.

In another form of the invention, 3 doses of vaccine composition (e.g. 0.5 mg adsorbed onto 0.312 g alum in 0.125 mL) is administered in 3 doses, 2 mg per dose to > 5 year olds, 1 mg to under 5 year olds, at weeks 0, 4, and 25. In another form of the invention, 3 doses of vaccine composition (e.g. 1 mg per dose) are given subcutaneously at weeks 0, 4, and 26. In another form of the invention, 3 doses of vaccine composition is administered on days 0, 30, and 180 at different doses (e.g. 1 mg; 0.5 mg). In another

form of the invention, 3 doses of vaccine composition is administered at 3 to 4 month intervals either intramuscularly or subcutaneously. In another form of the invention 3 doses of vaccine composition is administered subcutaneously on days 0, 30, and about day 180. In another form of the invention, the vaccine composition is administered in 2 doses at 4-week intervals (e.g. 0.55 mL per dose containing 4 µg or 15 µg or 13.3 µg of each antigen). In another form of the invention, 3 doses of the vaccine composition is administered (e.g. 25 µg in 250 µL AS02A adjuvant) intramuscularly in deltoid (in alternating arms) at 0, 1, and 2 months. In another form of the invention 4 doses of the vaccine composition is given (e.g. 50 µg per 0.5 mL dose) on days 0, 28, and 150; and dose 4 given in the following year. In another form of the invention, where the vaccine is a DNA vaccine, the vaccine composition is administered in two doses (e.g. 2 mg on days 0 and 21 (2 intramuscular injections each time, 1 into each deltoid muscle)). In another form of the invention, where the vaccine composition comprises an immunogenic molecule covalently linked to another molecule (e.g. *Pseudomonas aeruginosa* toxin A) the composition is administered in 3 doses (e.g. at 1, 8, and 24 weeks).

The present invention may be used to generate invasion-inhibitory antibodies useful as *in vitro* diagnostic reagents, or as therapeutics for passive immunization. The term "antibody" includes intact immunoglobulin molecules, as well as fragments thereof which are capable of binding an antigen. These include hybrid (chimeric) antibody molecules; F(ab')₂ and F(ab) fragments and Fv molecules; non-covalent heterodimers; single-chain Fv molecules (sFv); dimeric and trimeric antibody fragment constructs; minibodies; humanized antibody molecules; and any functional fragments obtained from such molecules, as well as antibodies obtained through non-conventional processes such as phage display. Preferably, the antibodies are monoclonal antibodies. Methods of obtaining monoclonal antibodies are well known in the art.

Various immunoassays (e.g., Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, invasion-inhibition assays, or other immunochemical assays known in the art) can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody which specifically binds to the immunogen. A preparation of antibodies which specifically bind to a particular

antigen typically provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, the antibodies do not detect other proteins in immunochemical assays and can immunoprecipitate the particular antigen from solution.

The surface-exposed antigens of the invention can be used to immunize a mammal, such as a mouse, rat, rabbit, guinea pig, monkey, or human, to produce polyclonal antibodies. If desired, an antigen can be conjugated to a carrier protein, such as bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. Depending on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants include those described above, as well as those not used in humans, for example, Freund's adjuvant.

Monoclonal antibodies which specifically bind to an antigen can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These techniques include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique.

In addition, techniques developed for the production of "chimeric antibodies," the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used. Monoclonal and other antibodies also can be "humanized" to prevent a patient from mounting an immune response against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence differences between rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site directed mutagenesis of individual residues or by grafting of entire complementarity determining regions.

Alternatively, humanized antibodies can be produced using recombinant methods, as described below. Antibodies which specifically bind to a particular antigen can contain antigen binding sites which are either partially or fully humanized.

Alternatively, techniques described for the production of single chain antibodies can be

adapted using methods known in the art to produce single chain antibodies which specifically bind to a particular antigen. Antibodies with related specificity, but of distinct idiotypic composition, can be generated by chain shuffling from random combinatorial immunoglobulin libraries.

Single-chain antibodies also can be constructed using a DNA amplification method, such as PCR, using hybridoma cDNA as a template. Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent.

A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant DNA methods, and introduced into a cell to express the coding sequence, as described below. Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology.

Antibodies which specifically bind to a particular antigen also can be produced by inducing in vivo production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents.

Chimeric antibodies can be constructed. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as "diabodies" can also be prepared.

Antibodies can be purified by methods well known in the art. For example, antibodies can be affinity purified by passage over a column to which the relevant antigen is bound. The bound antibodies can then be eluted from the column using a buffer with a high salt concentration.

In another aspect the present invention provides use of a composition described herein in the manufacture of a medicament for the treatment or prevention of a condition caused by or associated with infection by *Plasmodium falciparum*.

A further aspect of the invention provides a method of screening for the presence of a *Plasmodium falciparum* invasion-inhibitory antibody directed against a reticulocyte-

binding homologue protein (Rh) of a strain of *Plasmodium falciparum* in a subject, comprising obtaining a biological sample from the subject and identifying the presence or absence of an antibody capable of binding to an immunogenic molecule as described herein. The method may further comprise identifying the presence of a *Plasmodium falciparum* invasion-inhibitory antibody directed against an erythrocyte binding antigen (EBA) of a strain of *Plasmodium falciparum* in a subject comprising identifying the presence or absence of an antibody capable of binding to an immunogenic molecule as described herein.

The invention also provides nucleic acid encoding a polypeptide immunogenic molecule of the invention. The nucleotide sequence of Rh2b is given below (SEQ ID NO: 2)

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The nucleotide sequence of Rh4 is given below (SEQ ID NO:4)

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AACATATTAATGGATGAAATAGAAAATTATGTGAAAAAATATACGGAATCGAATCGTATT
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CCTCCGAATTCACCATTTTCGAAGTTATTATGACAAAAATTTAAATAAAGATATAAATGAA
ACTTGTCATTCCATACAAACACTTCTAAACAATCTAATATCTTCCAAAATTATATTTAAA
ATGTTAGAACTACAAAAGAACAATATTACTTTTATGGAATAACAAAAAATTAGTCAA
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CTAGAAAAGTACACAAACAAGTTTGAACATAATATCAAACCTCATATAGAAGATATAGAG
AAAAAAGTAAATGAATATATTAATAATTCCGATTGTCATTTAACATGTTCAAATATAAA
ACAATTATCAATAATTATATAGATGAAATAATAACAATAATACAAACATATACGAAAAC
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ATAACACTTCTTGTTCAGAAAACAATTAATCTATATTGAATATATTTATTTTATTAAC
AAACATATTGTAAATAAAATTCAAGAAAACCTTAAATTAAATCAAAATAAATATATACAT
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CATAAAATAAATAATTATTCTCATAACATTGAAGATCTTATAAACCAACTACAACATAAA
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CAAATATATAAAAAATGAATTAAAGGATAGAATAAAAGAAACACAAACAAAATTAATTTA
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CTAAATTTTACACACAAAACCTATTCAACAAATATTACAACAACAATATAAAAAACAACACA
CAAGAAAAAATACACTAGCACAAATTTTTTATACAATGCAGATATCAAAAAATATATTGAT
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CATATTAAACAAATACTCATAAATTATATACAAGAATGTAAACCTATACAAAATATATCA
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CAAGAAAATATAACCACTTTTCGAAAATCAATATAAAGATTTAATTATCAAAATGAACCAT
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CAAGAATTCAACAAAAAATAACAACCTTCAAGGAAGAAACACAAATTATGATAAACAAAG
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AATGACATGATAAATACCACCATAGGAGACAAGACAAATGTCTTAAAAAATGATGATCAA
GAAAAAGGTAAAAGGGGAACCTCCAGAAATAATAATATTCATACAAATGAAAATAATATA
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AAAACAATTAATACATCACACGTACCATAGATGATTCGAATATGGATCGAAATAATAGA

TATAACACATCATCACAACAAAAAATAATTTGCATACAAATAATAATAGTAATAGTAGA
TACAACAATAACCATGATAAACAAAATGAACATAAATATAATCAAGGAAAATCTTCAGGG
AAAGATAACGCATATTATAGAATTTTTTTATGCTGGAGGAATTACAGCTGTCTTACTTTTA
TGTTCAAGTACTGCATTCTTTTTTATAAAAAACTCTAATGAACCACATCATATTTTTTAAT
ATTTTTTCAAAGGAATTTAGTGAAGCAGATAATGCACATTCAGAAGAAAAAGAAGAATAT
CTACCTGTCTATTTTGATGAAGTTGAAGATGAAGTTGAAGATGAAGTTGAAGATGAAGAT
GAAAATGAAAATGAAGTTGAAAATGAAAATGAAGATTTTAATGACATATGA

The nucleotide sequence of EBA175 is given below (SEQ ID NO: 6)

ATGAAATGTAATATTAGTATATATTTTTTTGCTTCCTTCTTTGTGTTATATTTTGCAAAA
GCTAGGAATGAATATGATATAAAAGAGAATGAAAAATTTTTAGACGTGTATAAAGAAAA
TTTAATGAATTAGATAAAAAGAAATATGGAAATGTTCAAAAACTGATAAGAAAATATTT
ACTTTTATAGAAAATAAATTAGATATTTTAAATAATTCAAAATTTAATAAAAGATGGAAG
AGTTATGGAACCTCCAGATAATATAGATAAAAATATGTCTTTAATAAATAAACATAATAAT
GAAGAAATGTTTAACAACAATTATCAATCATTTTTATCGACAAGTTCATTAATAAAGCAA
AATAAATATGTTCCCTATTAACGCTGTACGTGTGTCTAGGATATTAAGTTTCCTGGATTCT
AGAATTAATAATGGAAGAAATACTTCATCTAATAACGAAGTTTTAAGTAATTGTAGGGAA
AAAAGGAAAGGAATGAAATGGGATTGTAAAAAGAAAAATGATAGAAGCAACTATGTATGT
ATTCCTGATCGTAGAATCCAATTATGCATTGTTAATCTTAGCATTATTAACACATATACA
AAAGAGACCATGAAGGATCATTTTCATTGAAGCCTCTAAAAAAGAATCTCAACTTTTGCTT
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GAAACAAAATTCAGAAGTTTTTAAAGGGGCTCATGGGGAAATAAGTGAACATAAAATT
AAAAATTTTAGAAAAAATGGTGGAATGAATTTAGAGAGAACTTTGGGAAGCTATGTTA
TCTGAGCATAAAAATAATATAAATAATTGTAAAAATATTCCTCAAGAAGAATTACAAATT
ACTCAATGGATAAAAGAATGGCATGGAGAATTTTTGCTTGAAAGAGATAATAGATCAAAA
TTGCCAAAAAGTAAATGTAAAAATAATACATTATATGAAGCATGTGAGAAGGAATGTATT
GATCCATGTATGAAATATAGAGATTGGATTATTAGAAGTAAATTTGAATGGCATAACGTTA
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TATTCAAAATATTGTGATTGTAAACATACTACTCTCGTTAAAAGCGTTTTAAATGGT
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TTATCTGATACACTTCAATTGCATGAAGATACTAAAGAAAATGATAAACTACCCCTAGAA
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ATAAGTGTATCACCAGAAAATTCAAGACCTGAAACTGATGCTAAAGATACTTCTAACTTG
TTAAAATTAAAAGGAGATGTTGATATTAGTATGCCTAAAGCAGTTATTGGGAGCAGTCCT
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TCAGACGATGGTGAATTATATGACGAAAATTCAGACTTATCTACTGTAAATGATGAATCA
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CAACATATTGAGAGTGATCAACAGAAAAACGATATGAAAACCTGTTGGTGATTTGGGAACC
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GAGGAAAACGAAAGACACTTAATAATCAAAACATTAATATTAGTCAAGAAAGGGATTTG
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CAAATTAATCATAGTCATCATGGAAACAGACAAGATCGGGGGGGAATTTCTGGGAATGTT
TTAAATATGAGATCTAATAATAATAATTTTAATAATATTCCAAGTAGATATAATTTATAT
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ATAAAGAAATTAGCAGAAATAAATAAATGTGAGAACGAAATTTCTGTAAAATATTGTGAC
CATATGATTCATGAAGAAATCCCATTA AAAACATGCACTAAAGAAAAACAAGAAATCTG
TGTTGTGCAGTATCAGATTACTGTATGAGCTATTTTACATATGATTCAGAGGAATATTAT
AATTGTACGAAAAGGGAATTTGATGATCCATCTTATACATGTTTCAGAAAGGAGGCTTTT
TCAAGTATGCCATATTATGCAGGAGCAGGTGTGTTATTTATTATATTGGTTATTTTAGGT
GCTTCACAAGCCAAATATCAAAGGTTAGAAAAAATAAATAAAAATAAAATTGAGAAGAAT
GTAAATTAA

The nucleotide sequence of EBA181 is given below (SEQ ID NO:8)

ATGAAAGGGAAAATGAATATGTGTTTGTGTTTTTTCTATTCTATATTATATGTTGTATTA
TGTACCTATGTATTAGGTATAAGTGAAGAGTATTTGAAGGAAAGGCCCAAGGTTTAAAT
GTTGAGACTAATAATAATAATAATAATAATAATAATAATAGTAATAGTAACGATGCG
ATGTCTTTTGTAAATGAAGTAATAAGGTTTATAGAAAACGAGAAGGATGATAAAGAAGAT
AAAAAAGTGAAGATAATATCTAGACCTGTTGAGAATACATTACATAGATATCCAGTTAGT
TCTTTTCTGAATATCAAAAAGTATGGTAGGAAAGGGGAATATTTGAATAGAAATAGTTTT
GTTCAAAGATCATATATAAGGGGTTGTAAAGGAAAAAGAAGCACACATACATGGATATGT
GAAAATAAAGGGAATAATAATATATGTATTCCTGATAGACGTGTACAATTATGTATAACA
GCTCTTCAAGATTTAAAAAATTCAGGATCTGAAACGACTGATAGAAAATTATTAAGAGAT
AAAGTATTTGATTCAGCTATGTATGAAACTGATTTGTTATGGAATAAATATGGTTTTTCGT
GGATTTGATGATTTTTGTGACGATGTAAAAAATAGTTATTTAGATTATAAAGATGTTATA
TTTGGAACCGATTTAGATAAAAATAATATATCAAAGTTAGTAGAGGAATCATTA AACGT
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AGACTATGGAAAACCTATGATACAGCCATATGCTCATTTAGGATGTAGAAAACCTGATGAG
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AAGGAGAAAGAAAAATTGTTAACAGGAGAATGTTCTGTTAATAGAAAAAAATCTGACTGC
TCAACCGGATGTAATAATGAGTGTTATACCTATAGGAGTCTTATTAATAGACAAAGATAT
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TCTATAAAAGTTCAAAATAATTTAAATTTAATTTTTTGAAAGAAATTTTGTTTATAAAGTT
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TGGGCACATTTCTTTTGTAAGGAAAAAGAATATTGGGAATTAAAATTAAATGATAAATGT
ACAGGTAATAATGGAAAATCCTTATGTCAGGATAAAACATGTCAAAATGTGTGTACTAAT
ATGAATTATTGGACATATACTAGAAAATTAGCTTATGAAATACAATCCGTAAAATATGAT
AAAGATAGAAAATTATTTAGTCTTGCTAAAGACAAAAATGTAACACTACATTTTTTAAAGGAA
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CGAACTGACAAAGATTCAGATAGAAACAAAGGAAAAGATACAGCTACTGATACAAAAAAT
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ACTGAACCAGATGCATCTTTAAAAGATGATAAGAAGGAAGTTGATGATGCCAAAAAAGAA
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TCTGAAGAAGTAGGAGAAAAAGAAGCTAAAGAAATTAAAGTAGAACCTGTTGTACCAAGA
GCCATTGGAGAACCAATGGAAAATTCTGTGAGCGTACAGTCCCCTCCTAATGTAGAAGAT
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ATATTAGATGATTCTAGAAGAAATGGAGAAATGACAGAAGGTAGCGAAAGTGATGTTGGA
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GCGAGCGATAGAGAAAAAGAAGAAATTCAAAAATTACTTAATATAGGACATGAAGAGGAT
 GAAGATGTATTAAAAATGGATAGAACAGAGGATAGTATGAGTGATGGAGTTAATAGTCAT
 TTGTATTATAATAATCTATCAAGTGAAGAAAAAATGGAACAATATAATAATAGAGATGCT
 TCTAAAGATAGAGAAGAAATATTGAATAGGTCAAACACAAATACATGTTCTAATGAACAT
 TCATTAAAAATATTGTCAATATATGGAAAGAAATAAGGATTTATTAGAAACATGTTCTGAA
 GACAAAAGGTTACATTTATGTTGTGAAATATCAGATTATTGTTTAAAAATTTTCAATCCT
 AAATCGATAGAATACTTTGATTGTACACAAAAAGAATTTGATGACCCTACATATAATTGT
 TTTAGAAAACAAAGATTTACAAGTATGCATTATATTGCCGGGGGTGGTATAATAGCCCTT
 TTATTGTTTATTTTAGGTTTCAGCCAGCTATAGGAAGAATTTGGATGATGAAAAAGGATTC
 TACGATTCTAATTTAAATGATTCTGCTTTTGAATATAATAATAATAATAATAATAATA
 CCTTATATGTTTGATCAACAAATAAATGTAGTAAATTCTGATTTATATTCGGAGGGTATT
 TATGATGACACAACGACATTTTAA

The nucleotide sequence of EBA140 is given below (SEQ ID NO:10)

ATGAAAGGATATTTTAATATATATTTTTTAATTCCTTTAATTTTTTTTATATAATGTAATA
 AGAATAAATGAATCAATTATAGGTAGAACACTTTATAATAGACAAGATGAATCATCAGAT
 ATTTCAAGGGTAAATTCACCCGAATTAAATAATAATCATAAACTAATATATATGATTCA
 GATTACGAAGATGTAAATAATAAATTAATAAACAGTTTTGTAGAAAATAAAAGTGTGAAA
 AAAAAAAGGTCTTTAAGTTTTTATAAATAATAAAACAAAATCATATGATATAATTCCACCT
 TCATATTCATATAGGAATGATAAATTTAATTCACCTTCCGAAAATGAAGATAATTCTGGA
 AATACAAATAGTAATAATTTTCGCAAATACTTCTGAAATATCTATTGGAAAGGATAATAAA
 CAATATACGTTTATACAGAAACGTACTCATTTGTTTGCTTGTGGAATAAAAAGAAAATCA
 ATAAAATGGATATGTTCGAGAAAACAGTGAGAAAATTACTGTATGTGTTCCCTGATAGAAA
 ATACAACATATGTATTGCAAATTTTTTAACTCACGTTTAGAAACAATGGAAAAGTTTAA
 GAAATATTTTTAATTTCTGTTAATACAGAAGCAAAATTATTATATAACAAAAATGAAGGA
 AAAGATCCCTCAATATTTTGTAAATGAATTAAGAAATAGTTTTTTCAGATTTTAGAAATTCA
 TTTATAGGTGATGATATGGATTTTGGTGGTAATACAGATAGAGTCAAAGGATATATTAAT
 AAGAAGTTCCTCGATTATTATAAGGAAAAAAATGTTGAAAAATTAAATAATATCAAAAA
 GAATGGTGGGAAAAAAATAAAGCAAATTTGTGGAATCACATGATAGTAAATCATAAAGGA
 AACATAAGTAAAGAATGTGCCATAATTCCTCGCGGAAGAACCTCAAATTAATCTATGGATA
 AAAGAATGGAATGAAAACCTTCTTGATGGAAAAGAAGAGATTGTTTTTAAATATAAAAGAT
 AAGTGTGTTGAAAACAAAAAATATGAAGCATGTTTTTGGTGGATGTAGGCTTCCATGTTCT
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 GAACCCCCCAGAAGACAACTTTATGTCTTGACGTACATATCTTTTACATCGTGGTCAT
 GAGGAAGATTATAAGGAACATTTACTTGGAGCTTCAATATATGAGGCGCAATTATTAAAA
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 GATTTGGCAGATATTATCAAGGGATCGGATATAATAAAAGATTATTATGGTAAAAAAATG
 GAAGAAAATTTAAATAAAGTAAACAAAGATAAAAAACGTAATGAAGAATCTTTGAAGATT
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 AGATGGTTTTAAGGAATGGGGAGACGATTTTTGTGAGAAAAGAAAAGAGAAAATATATTCA
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GATAAATACACAAAAGATAAAAAATAAAAAGATGTATGATAATATTGATGAAGTAAAAAAT
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ATAAAATATTTAAATGAAATTAAATATCCTAAAACAAAACACGATATATATGATATAGAT
ACATTTTTCAGATACTTTTGGTGATGGAACGCCAATAAGTATTAATGCAAATATAAATGAA
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GGTCCTTGTTCTAGAGAAGAAAGAAAGAAATTATGTTGTCAGATTTTCAGATTATTGTTTA
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GAAGAATATGATATAGGAGAATCTAATATAGAAGCAACTTTTGAAGAAAATAATTATTTA
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The nucleotide sequence of Rh2a is given below (SEQ ID NO:12)

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 TCTAGTATAGGATTATTACTTATAAGACTAATAGTGGAGATAACAATTCTAATGAAATT
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The nucleotide sequence of Rh1 is given below (SEQ ID NO:14)

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 GTAATGGAGATATTTTTTGATAATGATTATATTAA

As a matter of routine, the skilled person will be able to identify the regions of the above nucleic acid molecules that encode the specific regions described for the Rh and EBA proteins described elsewhere herein. The present invention includes those specific nucleotide subsequences, and any alterations that are available by virtue of the degeneracy of the genetic code. Furthermore, the invention provides nucleic acid which can hybridise to these nucleic acid molecules, preferably under "high stringency" conditions (e.g. 65°C in a 0.1 x SSC, 0.5% SDS solution). Nucleic acid according to the invention can be prepared in many ways (e.g. by chemical synthesis, from genomic or cDNA libraries, from the organism itself, etc.) and can take various forms (e.g. single stranded, double stranded, vectors, probes, etc.). They are preferably prepared in substantially pure form (i.e. substantially free from other Plasmodial or host cell nucleic acids).

The term "nucleic acid" includes DNA and RNA, and also their analogues, such as those containing modified backbones (e.g. phosphorothioates, etc.), and also peptide nucleic acids (PNA), etc. The invention includes nucleic acid comprising sequences complementary to those described above (e.g. for antisense or probing purposes).

The invention also provides a process for producing an immunogenic molecule of the invention, comprising the step of culturing a host cell transformed with nucleic acid of the invention under conditions which induce polypeptide expression.

The present invention will now be more fully described by reference to the following non-limiting Examples.

EXAMPLE 1: MATERIALS AND METHODS

Invasion inhibition assays

Methods for measuring invasion-inhibitory antibodies in serum samples have been described and evaluated in detail elsewhere [Persson, et al. J. Clin. Microbiol. (2006) 44:1665-1673]. Plasmodium falciparum lines 3D7-wt, 3D7ΔEBA175, W2mef-wt,

W2mef Δ EBA175 and W2mefSelNm were cultured in vitro as described [Beeson et al (1999) *J. Infect. Dis.* 180:464-472]. W2mefSelNm was generated from W2mef-wt by selection for invasion into neuraminidase-treated erythrocytes. W2mefSelNm was continuously cultured in neuraminidase-treated erythrocytes to maintain the phenotype. Synchronized (by 5% D-sorbitol) parasites were cultured with human 0+ erythrocytes in RPMI-HEPES medium with hypoxanthine 50 μ g/ml, NaHCO₃ 25mM, gentamicin 20 μ g/ml, 5% v/v heat-inactivated pooled human Australian sera, and 0.25% Albumax II (Gibco, Invitrogen, Mount Waverley, Australia) in 1% O₂, 4% CO₂, and 95% N₂ at 37°C. Invasion inhibition assays were started at late pigmented trophozoite to schizont stage. Inhibitory activity was measured over two cycles of parasite replication. Starting parasitemia was 0.2-0.3%, hematocrit 1%, and cells were resuspended in RPMI-HEPES supplemented as described above. Assays were performed in 96-well U-bottom culture plates (25 μ l of cell suspension + 2.5 μ l of test sample/well). All samples were tested in duplicate. After 48 hours, 5 μ l of fresh culture medium was added. Parasitemia was determined by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) after 80-90 hours using ethidium bromide (10 μ g/ml, Bio-Rad, Hercules, CA, USA) to label parasitised erythrocytes. Incubation time was influenced by the stage and synchronicity of parasite cultures at commencement of the assay, and by the length of the lifecycle of the parasite line used. We confirmed the inhibitory effect of treated samples by testing immunoglobulin purified from the same samples (36. All serum samples tested for inhibitory antibodies were first treated to remove non-specific inhibitors that may be present and to equilibrate pH [Persson, et al. *J. Clin. Microbiol.* (2006) 44:1665-1673]. Serum samples (100 μ l) were dialyzed against phosphate-buffered saline (PBS; pH 7.3) in 50 kDa MWCO microdialysis tubes (2051, Chemicon, Temecula, CA, USA) and subsequently re-concentrated to the original starting volume using centrifugal concentration tubes (100 kDa MWCO; Pall Corp., Ann Arbor, MI, USA). Analysis of flow cytometry data was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Antibodies to MSP1₁₉ (raised against GST fusion protein) used in the assays (at 1:10 final dilution) were generated by vaccination of rabbits and were kindly provided by Brendan Crabb. Samples from non-exposed donors were included as negative controls in all assays, and anti-MSP1 and/or anti-AMA1 antibodies acted as a positive control. Samples were tested for inhibition of the different lines in parallel in the same experiments. A difference between the lines of $\geq$ 25% in invasion was designated as the cut-off for differential inhibition by samples. Preadsorption of treated serum samples

against erythrocytes did not alter their invasion-inhibitory activity. A selection of sera was also tested for antibodies to the surface of uninfected erythrocytes (maintained in culture) by flow cytometry [Beeson et al (1999) J. Infect. Dis. 180:464-472]; there was very little reactivity against normal erythrocytes and there was no relationship between antibody binding to erythrocytes with invasion-inhibitory activity.

Enzyme treatment of erythrocytes

Erythrocytes were first washed with RPMI/HEPES/ 25mM NaHCO₃, pH7.4, and subsequently incubated with neuraminidase (0.067 units/ml; Calbiochem, 45 min) or chymotrypsin (1mg/ml; Worthington Biochemical, 15 min) at 37°C. Control treatment was RPMI-HEPES only. After incubation, chymotrypsin-treated cells were washed once with RPMI-HEPES containing 20% human serum and twice with normal culture medium (containing 5% serum) to inhibit enzyme activity. The neuraminidase-treated cells were washed with parasite culture medium three times. Treated erythrocytes were then used in invasion inhibition assays as described. All results presented are comparisons to control-treated cells.

Antibodies to recombinant proteins by ELISA

96-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with recombinant GST fusion proteins at 0.5 µg/ml in PBS overnight at 4°C. Plates were washed and blocked with 10% skim milk powder (Diploma, Rowville, Australia) in PBS Tween 0.05% for 2 hours. After washing, serum samples (100 µl /well in duplicate), at 1/500 dilution in PBS Tween 0.05% plus 5% skim milk, were incubated for two hours. Plates were washed and incubated for one hour with HRP-conjugated anti-human IgG at 1/5000 (Chemicon, Melbourne, Australia) in PBS Tween 0.05% plus 5% milk. After washing, colour was developed by adding OPhenylenediamine (Sigma, Castle Hill, Australia; stopped with concentrated sulphuric acid) or azino-bis(3-ethylbenthiazoline-6-sulfonic acid) liquid substrate system (Sigma- Aldrich, Sydney; stopped with 1% SDS) and absorbance read by spectrophotometry. All washes were performed with PBS containing 0.05% Tween 20, and all incubations were at room temperature. For each serum, the absorbance from wells containing GST only was deducted from the absorbance from EBA or PfRh GST fusion proteins. Positive and negative controls were included on all plates to enable standardisation. Recombinant proteins used were EBA140 (e.g. amino acids 746-1045), EBA175 W2mef and 3D7 alleles (e.g. amino acids 761-1271), EBA181 (e.g. amino acids

755-1339), Rh4 (e.g. amino acids 1160-1370), and Rh2 (e.g. amino acids 2027-2533). Schizonts were separated on a 60% Percoll gradient, washed three times in serum-free RPMI 1640, pelleted by centrifugation and resuspended. The cells were lysed through freeze-thawing and the supernatant was preserved. Antibody reactivity of a sample was considered positive if the O.D. was $> \text{mean} + 3\text{SD}$ of the nonexposed controls.

Study population and serum samples

Serum samples (50 adults and 100 children aged ≤ 4 years) were randomly selected from a community-based cross-sectional survey of children and adults resident in the Kilifi District, Kenya, in 1998, a year that was preceded with a relatively high incidence of malaria in the region. The area is endemic for *Plasmodium falciparum*. Samples were also obtained from non-exposed adult residents in Melbourne, Australia (n=20) and Oxford, UK (n=20). Ethical approval was obtained from the Ethics Committee of the Kenya Medical Research Institute, Nairobi, Kenya and from the Walter and Eliza Hall Institute Ethics Committee, Melbourne, Australia. All samples were obtained after written informed consent. All serum samples were tested for antibodies by ELISA. A subset of 80 of these samples was randomly selected (26% children < 5 years, 49% children 6-14, 25% adults) for use in invasion inhibition assays. The same 80 samples were used in all comparative inhibition assays.

Papua New Guinea clinical study

206 children aged 5-14, resident in the Madang Province PNG, were enrolled and treated with artesunate to clear any existing parasitemia (Michon et al., AJTMH (2007) 76(6):997-1008). Children were screened every 2 weeks for the presence of blood-stage parasitemia or any signs or symptoms of clinical illness. Malaria episodes were also identified at participant-initiated visit to the local health clinic. Malaria episodes were defined as presence of fever or symptoms of fever together with a parasitemia of *P. falciparum* of greater than 5000 parasites/ul. Antibodies were measured to recombinant PfRh and EBA proteins (as described above). Children were categorized into high, medium, or low responder groups to each antigen on the basis of terciles of rankings, and risk of malaria episodes from time zero to 6 months was calculated for each antibody group and plotted; Figures 9 and 10.

Statistical analysis

Statistical analyses were performed with SPSS and STATA software. The chi squared test or Fischer's exact test was used for comparisons of proportions. For comparisons of continuous variables, Mann-Whitney U test or Kruskal-Wallis tests were used for non-parametric data, and t-tests or ANOVA were used for normally-distributed data, as appropriate. Associations between antibodies to recombinant antigens by ELISA and invasion-inhibitory antibodies were examined by two approaches. We tested for correlations between ELISA OD values and total invasion inhibition by samples, or the extent of differential inhibition of two comparison parasite lines, and we compared the mean or median inhibition by samples grouped as high or low responders according to reactivity by ELISA. For all analyses $p < 0.05$ was classified as statistically significant.

EXAMPLE 2: Invasion phenotypes and properties of defined *Plasmodium falciparum* lines

Variants of the clonal parasite lines W2mef and 3D7 using different invasion pathways were used. Targeted disruption of the gene for EBA175, and selection of W2mef for invasion of neuraminidase-treated erythrocytes, generated parasites that used the SA-independent pathway. We characterised the invasion phenotypes of these parasite lines by evaluating their invasion into chymotrypsin- and neuraminidase-treated erythrocytes compared to normal erythrocytes. Clear differences between the parasite lines in invasion pathway use or phenotype were demonstrated. Invasion of the parental W2mef wild-type (wt) was sensitive to neuraminidase treatment of erythrocytes (SA-dependent invasion) but moderately resistant to chymotrypsin-treatment of erythrocytes. In contrast, invasion of W2mef with EBA175 disrupted (W2mef Δ EBA175) was resistant to neuraminidase treatment (SA-independent invasion) but sensitive to chymotrypsin. Invasion of 3D7-wt and 3D7 with EBA175 disrupted (3D7 Δ EBA175) was resistant to neuraminidase, but the two 3D7 lines differed in their invasion of chymotrypsin-treated erythrocytes. Invasion in the W2mef line, by repeated selection for invasion of neuraminidase-treated erythrocytes, was also associated with a modest reduction in multiplication rate. No substantial differences were found in the proportions of singly or multiply-infected erythrocytes (this is referred to elsewhere as the selectivity index between the different lines (data not shown). This indicates there is no major reduction in the invasion capacity of the transgenic or selected parasites compared to wild-type.

EXAMPLE 3: The use of alternate erythrocyte invasion pathways alters the efficacy of invasioninhibitory antibodies

Differential inhibition by acquired antibodies of isogenic lines that differ only in invasion phenotype indicates that alternate pathways may exist as a mechanism of immune evasion. Inhibitory activity of serum antibodies was compared against W2mef and 3D7 parasite lines with different invasion phenotypes. We tested serum antibodies from a selection of children (n=60) and adults (n=20) resident in a malaria-endemic region of coastal Kenya. Total invasion-inhibitory antibodies were common among this population; 68% inhibited W2mef-wt and 62% inhibited 3D7-wt by >25% compared to non-exposed controls. For these studies, differential inhibition was defined as a $\geq 25\%$ difference in the extent of inhibition between the comparison lines. In all assays we tested inhibition of W2mef-wt and 3D7- wt using untreated erythrocytes, and inhibition of W2mef Δ EBA175, W2mefSelNm, and 3D7 Δ EBA175 was tested with untreated and neuraminidase-treated erythrocytes. We first compared serum antibody inhibition of W2mef-wt to that of W2mef Δ EBA175, which has a stable, but different invasion phenotype to the parental W2mef, and has up-regulated expression of Rh4. W2mef-wt uses SA-dependent invasion mechanisms, whereas invasion of W2mef Δ EBA175 is largely SA-independent. In comparative inhibition assays, we found that 27% of samples differentially inhibited the two lines (e.g. samples 56, 109, and 135 in Figure 1A), indicating that the inhibitory activity of acquired antibodies is influenced by the invasion pathway being used (Figure 1A and 2A). Large differences in inhibitory activity (up to 66%) between the lines were observed for individual samples. Although W2mef Δ EBA175 has switched to use a SA-independent invasion pathway, it remained possible that other ligands involved in SA-dependent invasion (e.g. EBA140, EBA181, Rh1) may still be functional to some extent in W2mef Δ EBA175, despite the switch in phenotype. To inhibit these interactions, and more clearly compare antibodies against SA-dependent versus SA-independent invasion pathways, we also performed antibody inhibition assays using W2mef Δ EBA175 and neuraminidase-treated erythrocytes, in comparison to inhibition of W2mef-wt with normal erythrocytes (Figure 2B). This approach further emphasized differences in antibody activity linked to variation in invasion phenotype. The proportion of samples showing differential inhibition of the two lines was 48% versus 27% when using normal erythrocytes with both lines. The extent of differences in inhibitory activity was strongly increased for some individual samples (e.g. sample 355 in Figure 1A). This indicates that the inhibitory activity of antibodies against ligands of SA-independent invasion was

enhanced once the residual activity of SA-dependent ligands is inhibited by neuraminidase treatment of erythrocytes. Differential inhibition by samples was also observed with W2mef-wt compared to W2mefSelNm (Figure 1B and 2C). The latter isolate is genetically intact and its phenotype was generated by selection for invasion of neuraminidase-treated erythrocytes. Like W2mef Δ EBA175, it uses an alternate SA-independent invasion pathway and has upregulated expression of Rh4. It still expresses EBA175, but does not depend on this ligand for invasion. We found 35% of samples from children and adults differentially inhibited the two lines (e.g. samples 196 and 436, Figure 1B), confirming that a change in invasion phenotype, or pathway, can substantially alter the efficacy of inhibitory antibodies. As expected, the inhibition of W2mefSelNm and W2mef Δ EBA175 by samples was highly correlated ($r=0.61$; $n=80$; $p<0.001$) as these isolates invade via the same pathway and only differ by the presence of EBA175. We also test antibodies for inhibition of W2mefSelNm invasion into neuraminidase-treated erythrocytes (Figure 2D), compared to W2mef-wt in normal erythrocytes, to more clearly evaluate antibodies against SA-independent versus SA-dependent invasion pathways. Overall, 45% of samples differentially inhibited the two lines. Some samples showed greater differences in the inhibition of W2mef-wt and W2mefSelNm than when normal erythrocytes were used (e.g. samples 196 and 436 in Figure 1B). Differential antibody inhibition of 3D7 lines with different invasion phenotypes further confirmed that variation in invasion phenotypes influences the activity of inhibitory antibodies (Figure 1C and Figure 3, A and B). The proportion of samples that differentially inhibited parental 3D7 versus 3D7 Δ EBA175 was 26% when using normal erythrocytes and 37% when using neuraminidase-treated erythrocytes with 3D7 Δ EBA175. These combined results with W2mef and 3D7 lines clearly established that the availability of alternate pathways for erythrocyte invasion is immunologically important and a likely mechanism for evasion of acquired inhibitory antibodies. Antibody inhibition assays used here have been previously validated and described in detail elsewhere [Persson, et al. J. Clin. Microbiol. (2006) 44:1665-1673]. Differences in the inhibitory activity of individual sera against different parasite lines were confirmed by repeat testing. Overall, results from invasion-inhibitory assays were highly reproducible. For example, repeat testing of 33 samples for inhibition of 3D7-wt and 3D7 Δ EBA175 was highly correlated ($r=0.96$ for 3D7-wt and $r=0.94$ for 3D7 Δ EBA175; $p<0.001$). Repeat testing of 40 samples for inhibition using different parasite lines also demonstrated a high correlation between assays ($r=0.83$; $p<0.001$). We confirmed that the differential

inhibitory activity measured in our assays represented inhibition of invasion, and not an effect on parasite intraerythrocytic development. To do this we tested serum samples in inhibition assays and determined parasitemias at different stages of parasite development, over one and two parasite life-cycles (data not shown). We routinely measured antibody inhibitory activity over two cycles of parasite invasion because this substantially increased the sensitivity of antibody inhibition assays and facilitated the detection of differences in inhibition between parasite lines in this study. Differential inhibition of comparison lines by sera was also observed in single-cycle assays

EXAMPLE 4: Antibodies to SA-dependent invasion pathways are common and inhibitory antibodies are acquired against EBA175.

Having established that antibodies can differentially inhibit alternate invasion pathways, we next aimed to further define the acquisition of antibodies to SA-dependent invasion in the population. Of those samples that differentially inhibited W2mef-wt versus W2mef Δ EBA175 (cultured with normal erythrocytes), 26 of 27 had a type-A response, inhibiting the parental W2mef more than W2mef Δ EBA175 ($P < 0.001$; Figure 2). This pattern of inhibition points to inhibitory antibodies targeting EBA175 and other ligands of SA-dependent invasion. Overall, the mean inhibition of W2mef-wt by all samples (39.4%) was significantly greater than W2mef Δ EBA175 (29.4%; $p < 0.01$) (Figure 2). When W2mef Δ EBA175 was cultured with neuraminidase-treated erythrocytes to inhibit any residual SA-dependent interactions, there was an increase in the difference in the mean inhibition of W2mef-wt versus W2mef Δ EBA175 by samples (a difference of 18.9% versus 10% using untreated erythrocytes; $p < 0.01$; Figure 4). Antibodies from 60% of children ≤ 5 years inhibited W2mef-wt to a greater extent than W2mef Δ EBA175 (Figure 2B), whereas among adults, 22% showed this pattern of inhibition ($p = 0.019$). Similar to results from assays using W2mef Δ EBA175, 31% of samples inhibited W2mefwt more than W2mefSelNm (Type A response; Figure 2C), whereas only 4% inhibited W2mefSelNm more than W2mef-wt ($p < 0.001$). Additionally, the mean inhibition of W2mef-wt (39.4%) by all samples was greater than W2mefSelNm (20%; $p < 0.01$) (Figure 4).

The 3D7 parental line invades erythrocytes through largely SA-independent interactions, which limits the usefulness of this parasite line for evaluating antibodies to ligands of SA-dependent invasion. However, some samples inhibited the invasion of 3D7-wt into

normal erythrocytes more than 3D7 Δ EBA175 using neuraminidase-treated erythrocytes (Figure 3B). This indicates the presence of antibodies against the ligands of SA-dependent invasion. In contrast to W2mef, disruption of EBA175 in 3D7 does not lead to a major switch in invasion phenotype. 3D7 Δ EBA175 shows slightly greater resistance to the effect of neuraminidase-treatment of erythrocytes compared to 3D7-wt, and increased sensitivity to inhibition by chymotrypsin-treatment of erythrocytes, consistent with the loss of function of EBA175. Comparing inhibition of 3D7 and 3D7 Δ EBA175 is therefore a useful tool to investigate inhibitory antibodies specifically targeting EBA175. Using this approach, we obtained evidence that EBA175 is a target of inhibitory antibodies, as suggested from studies with W2mef lines. 15% of children and 17% of adults inhibited 3D7-wt more than 3D7 Δ EBA175 (Figure 3A), strongly suggesting that individuals in the population have inhibitory antibodies against EBA175. These antibodies were responsible for up to 47% of the total inhibitory activity measured in some individuals (Figure 3), indicating that EBA175 is an important target of invasion-inhibitory antibodies.

EXAMPLE 6: Inhibition of invasion by antibodies to Rh proteins (e.g. Rh2 and Rh4).

We evaluated the presence of antibodies to ligands of SA-independent invasion by identifying samples that inhibited W2mef Δ EBA175 or 3D7 Δ EBA175 more than the corresponding parental parasites. Invasion of W2mef Δ EBA175 or 3D7 Δ EBA175 into neuraminidase-treated erythrocytes is dependent on ligands of the SA-independent invasion pathway. Using the W2mef line, 5% of samples (Figure 2B) showed a type-B response and inhibited invasion of W2mef Δ EBA175 into neuraminidase-treated erythrocytes more effectively than W2mef-wt. Furthermore, 13% inhibited W2mefselNm more than W2mef-wt (e.g. sample 436, Fig 1B). Type-B responses were more prevalent with the 3D7 parasite lines than W2mef ($p < 0.001$). A substantial number of samples inhibited 3D7 Δ EBA175 more than 3D7-wt (18% of samples when using normal erythrocytes and 16% when using neuraminidase-treated erythrocytes; Figure 3, A and B). No children ≤ 5 years inhibited W2mef Δ EBA175 more than W2mef-wt (Figure 2, A and B). Samples with type-B responses were only seen among older children and adults ($p = \text{not significant}$).

EXAMPLE 7: Acquisition of antibodies to recombinant EBA and Rh proteins.

Differential inhibition of parasite lines that vary in their invasion phenotype, but not genotype, indicates that members of the EBA and Rh proteins are targets of invasion-inhibitory antibodies. We measured antibodies against recombinant EBA and Rh proteins by ELISA to confirm that these proteins are targets of acquired antibodies. Antibody levels to EBA175 (both 3D7 and W2mef alleles), EBA140, EBA181, Rh2 and Rh4 were positively associated with increasing age (Figure 6), being significantly higher among older than younger subjects ($p < 0.001$). There was little or no reactivity of sera from malaria non-exposed subjects. Antibodies to *Plasmodium falciparum* schizont extract were also significantly correlated with age (data not shown), consistent with increasing exposure to blood-stage malaria.

While the foregoing written description of the invention enables one of ordinary skill to make and use what is considered presently to be the best mode thereof, those of ordinary skill will understand and appreciate the existence of variations, combinations, and equivalents of the specific embodiment, method, and examples herein. The invention should therefore not be limited by the above described embodiment, method, and examples, but by all embodiments and methods within the scope and spirit of the invention as broadly described herein.

The invention claimed is:

1. A composition comprising:

(a) at least one immunogenic erythrocyte binding antigen (EBA) molecule of an EBA protein of a strain of *Plasmodium falciparum*, the EBA molecule consisting of an amino acid sequence selected from the group consisting of:

- (i) the amino acid sequence of residue 746 to residue 1045 of SEQ ID NO: 9,
- (ii) the amino acid sequence of residue 761 to residue 1271 of SEQ ID NO: 5, and
- (iii) the amino acid sequence of residue 755 to residue 1339 of SEQ ID NO: 7, and

(b) at least one immunogenic reticulocyte-binding protein homologue (Rh) molecule comprising a contiguous amino acid sequence of a Rh protein of a strain of *Plasmodium falciparum* selected from the group consisting of:

- (i) SEQ ID NO: 1;
- (ii) residue 2027 to residue 3115 of SEQ ID NO: 1;
- (iii) residue 2027 to residue 2533 of SEQ ID NO: 1;
- (iv) residue 2098 to residue 2597 of SEQ ID NO: 1;
- (v) residue 2616 to residue 3115 of SEQ ID NO: 1;
- (vi) SEQ ID NO: 3;
- (vii) residue 1160 to residue 1370 of SEQ ID NO: 3;
- (viii) residue 28 to residue 766 of SEQ ID NO: 3; and
- (ix) SEQ ID NO: 13;

wherein when administered to a subject the composition induces an invasion-inhibitory immune response to the strain, wherein said composition is substantially free from other proteins of *Plasmodium*.

2. The composition according to claim 1 wherein one or more of the strains is a wild type strain.

3. A pharmaceutical composition comprising the immunogenic molecules according to claim 1 and a pharmaceutically acceptable excipient.

4. The composition according to claim 3 wherein the pharmaceutically acceptable excipient comprises a vaccine adjuvant.

5. Use of the composition of claim 3 in treatment or immunization against a condition caused by or associated with infection by *Plasmodium falciparum*.

6. A composition comprising:

(a) at least one immunogenic molecule of a reticulocyte-binding protein homologue (Rh) polypeptide of a strain of *Plasmodium falciparum*, the immunogenic molecule consisting of an amino acid sequence selected from the group consisting of:

- (i) the amino acid sequence of residue 2027 to residue 3115 of SEQ ID NO: 1;
- (ii) the amino acid sequence of residue 2027 to residue 2533 of SEQ ID NO: 1;
- (iii) the amino acid sequence of residue 2098 to residue 2597 of SEQ ID NO: 1;
- (iv) the amino acid sequence of residue 2616 to residue 3115 of SEQ ID NO: 1;
- (v) the amino acid sequence of residue 1160 to residue 1370 of SEQ ID NO: 3;
- (vi) the amino acid sequence of residue 28 to residue 766 of SEQ ID NO: 3; and

(b) at least one immunogenic molecule comprising a contiguous amino acid sequence of an erythrocyte binding antigen (EBA) protein of a strain of *Plasmodium falciparum* having an amino acid sequence selected from the group consisting of:

- (i) SEQ ID NO: 5;
- (ii) SEQ ID NO: 7;
- (iii) SEQ ID NO: 9;
- (iv) residue 746 to residue 1045 of SEQ ID NO: 9;
- (v) residue 761 to residue 1271 of SEQ ID NO: 5; and
- (vi) residue 755 to residue 1339 of the SEQ ID NO: 7,

wherein when administered to a subject the composition induces an invasion-inhibitory immune response to the strain, and wherein said composition is substantially free from other proteins of *Plasmodium*.

Figure 1

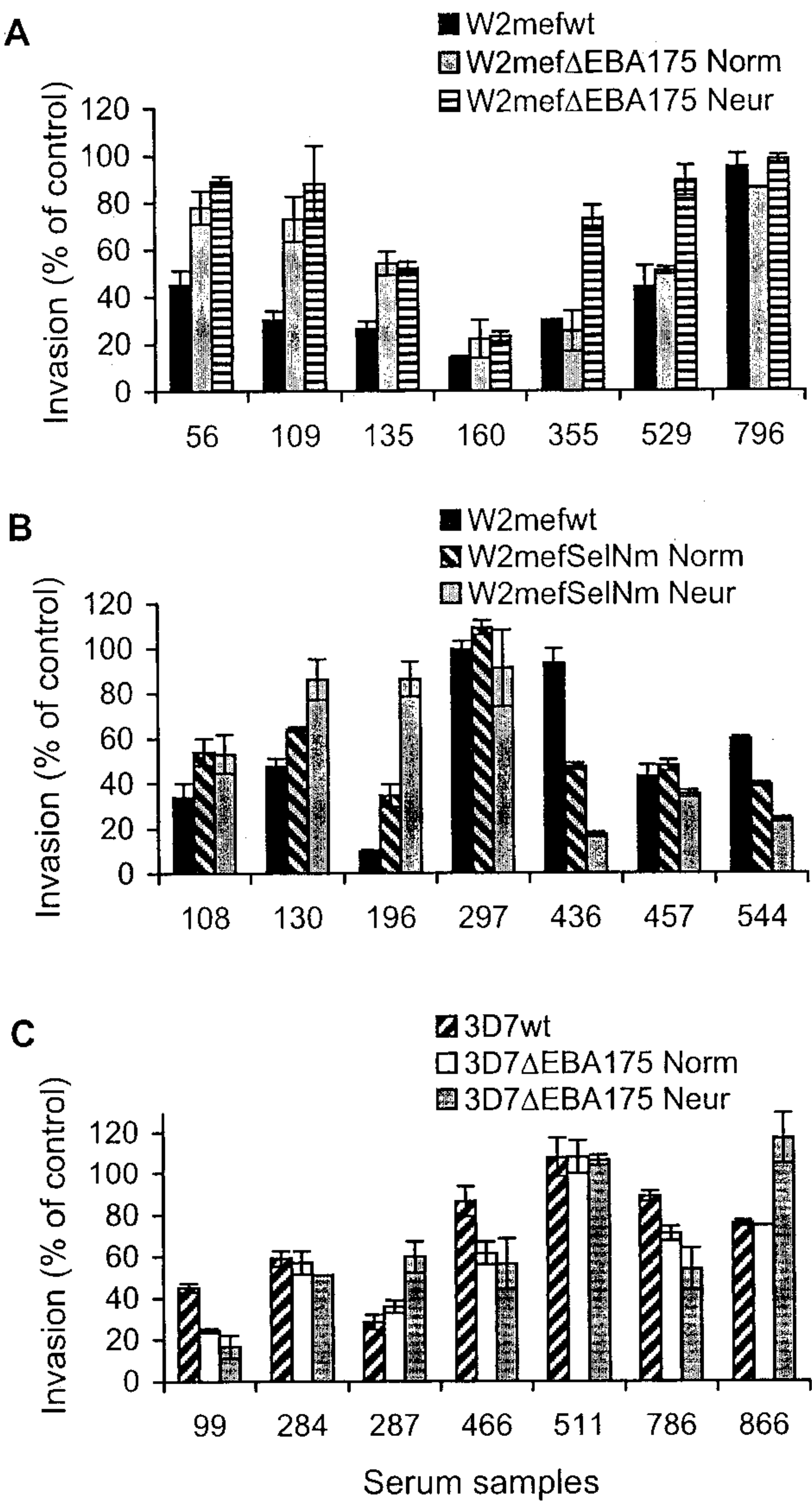


Figure 2

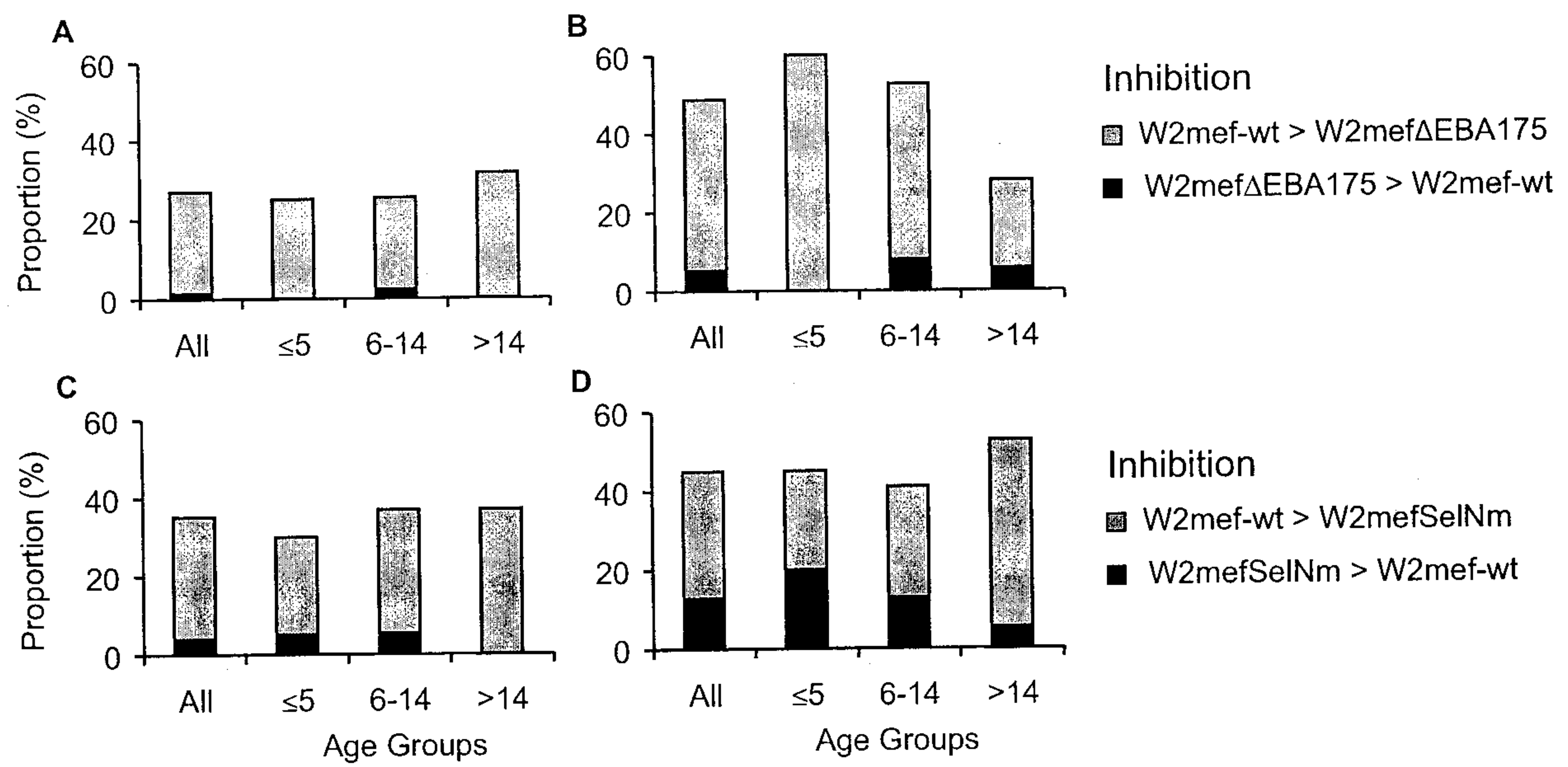


Figure 3

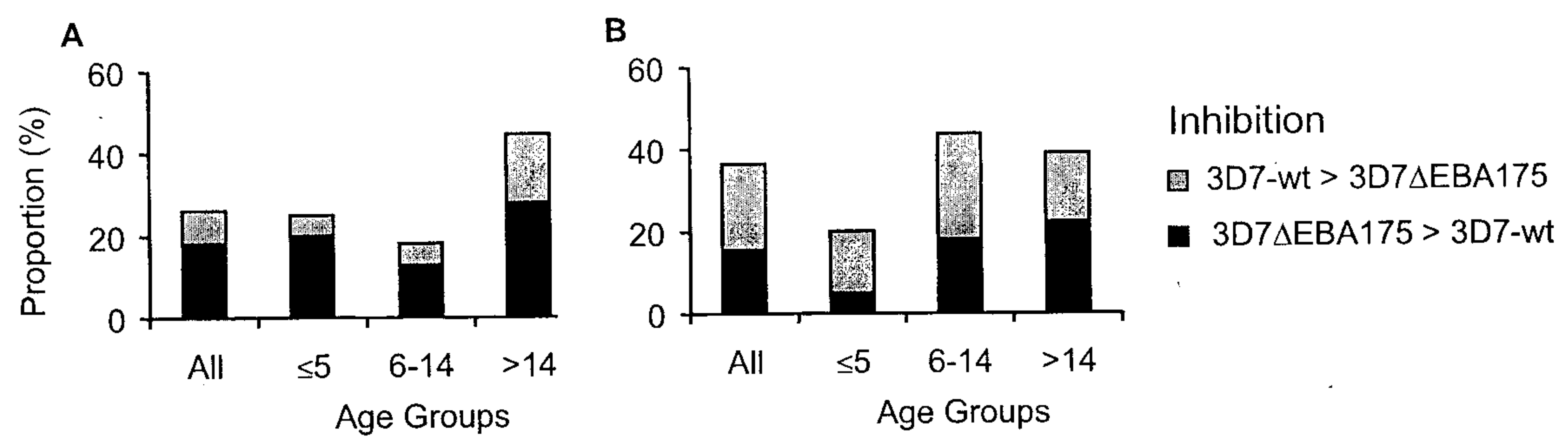


Figure 4

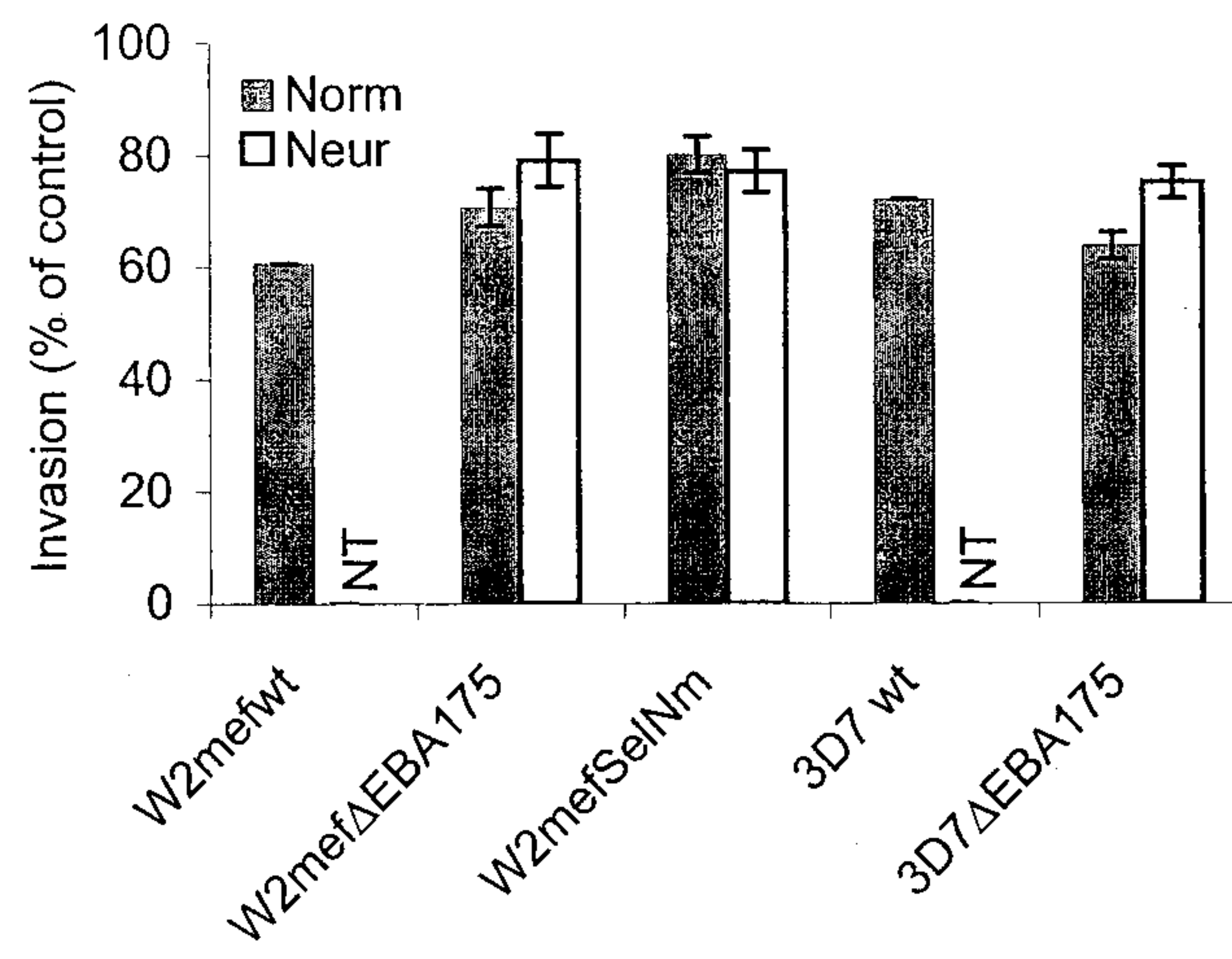


Figure 5

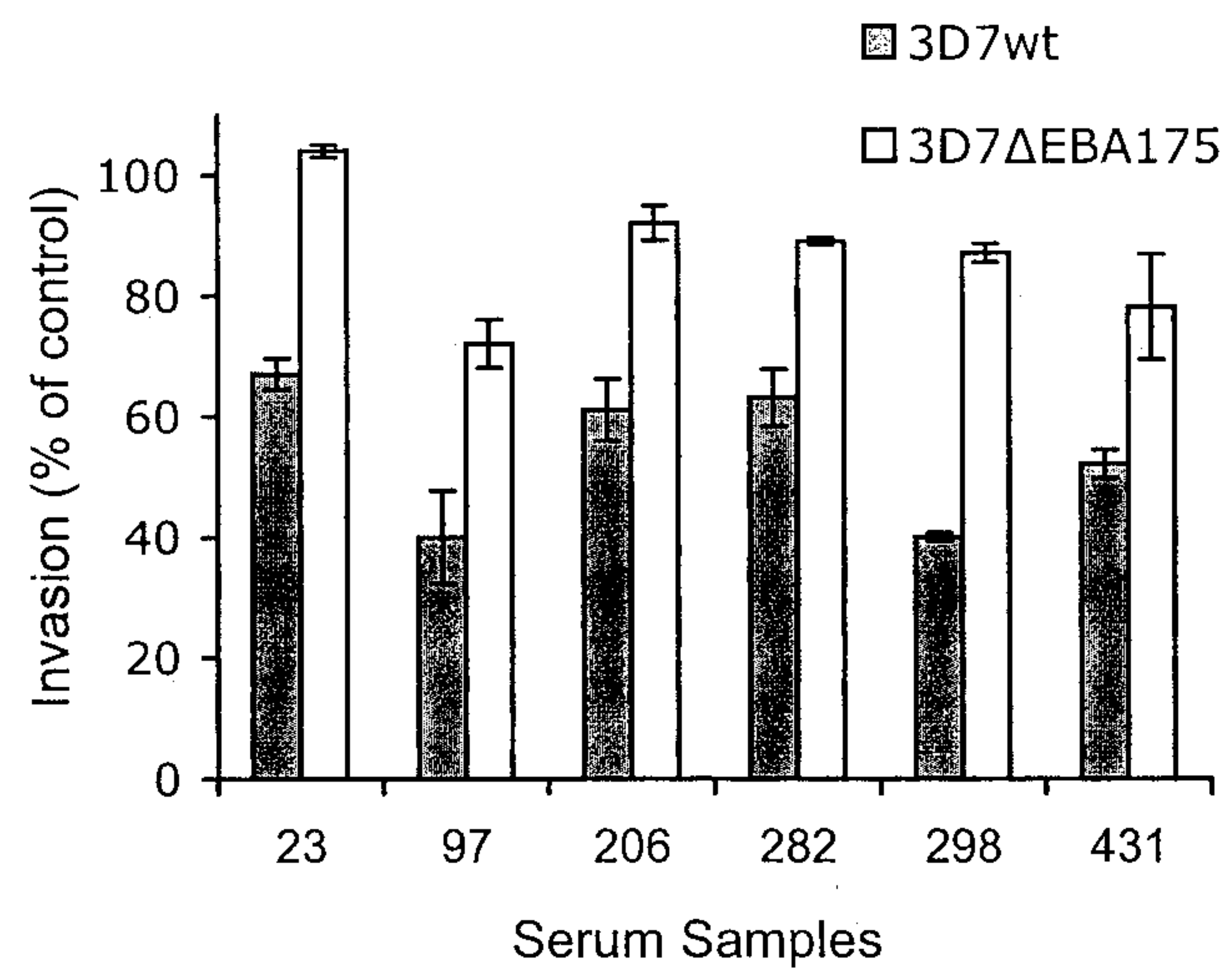


Figure 6

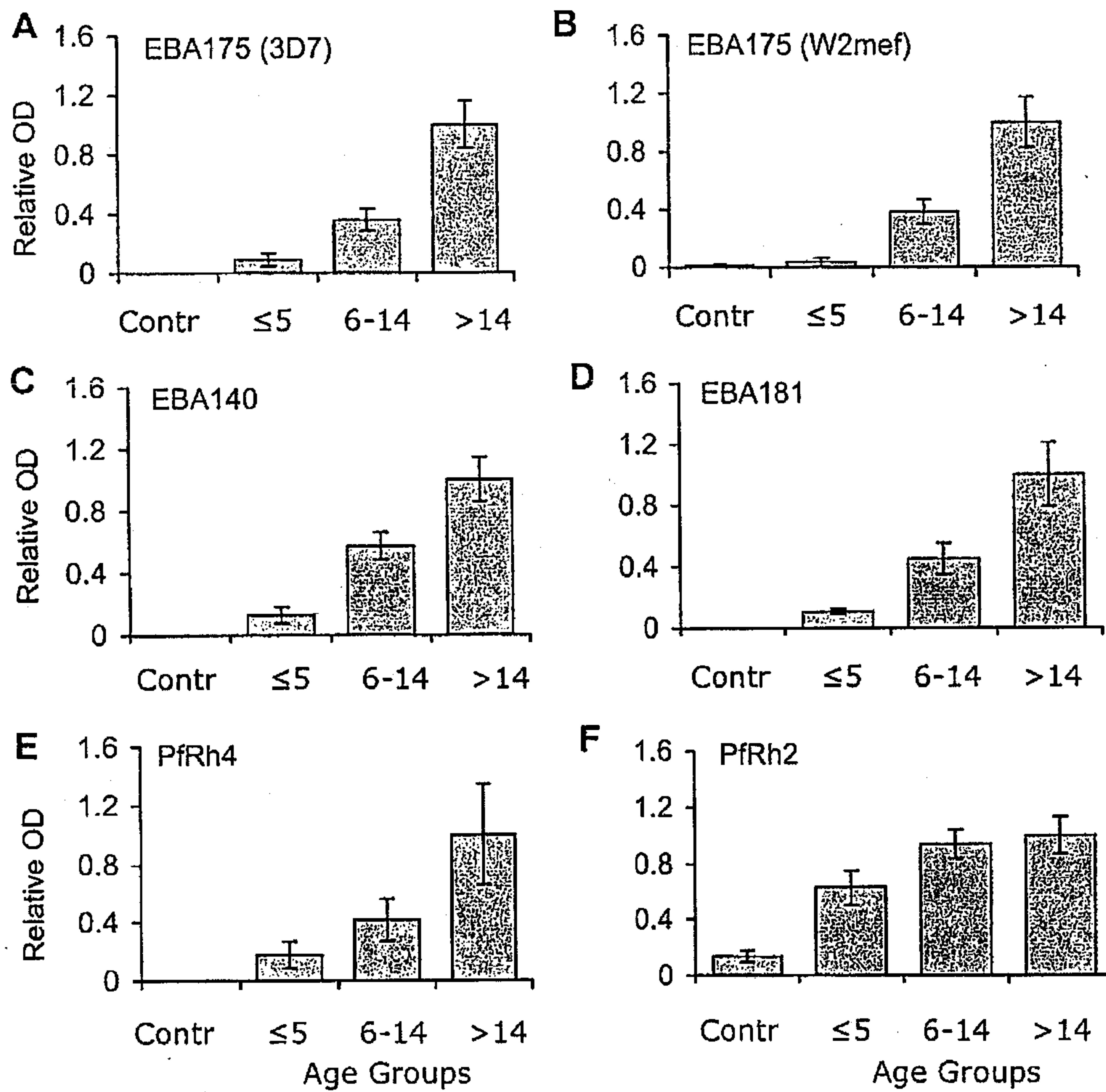


Figure 7

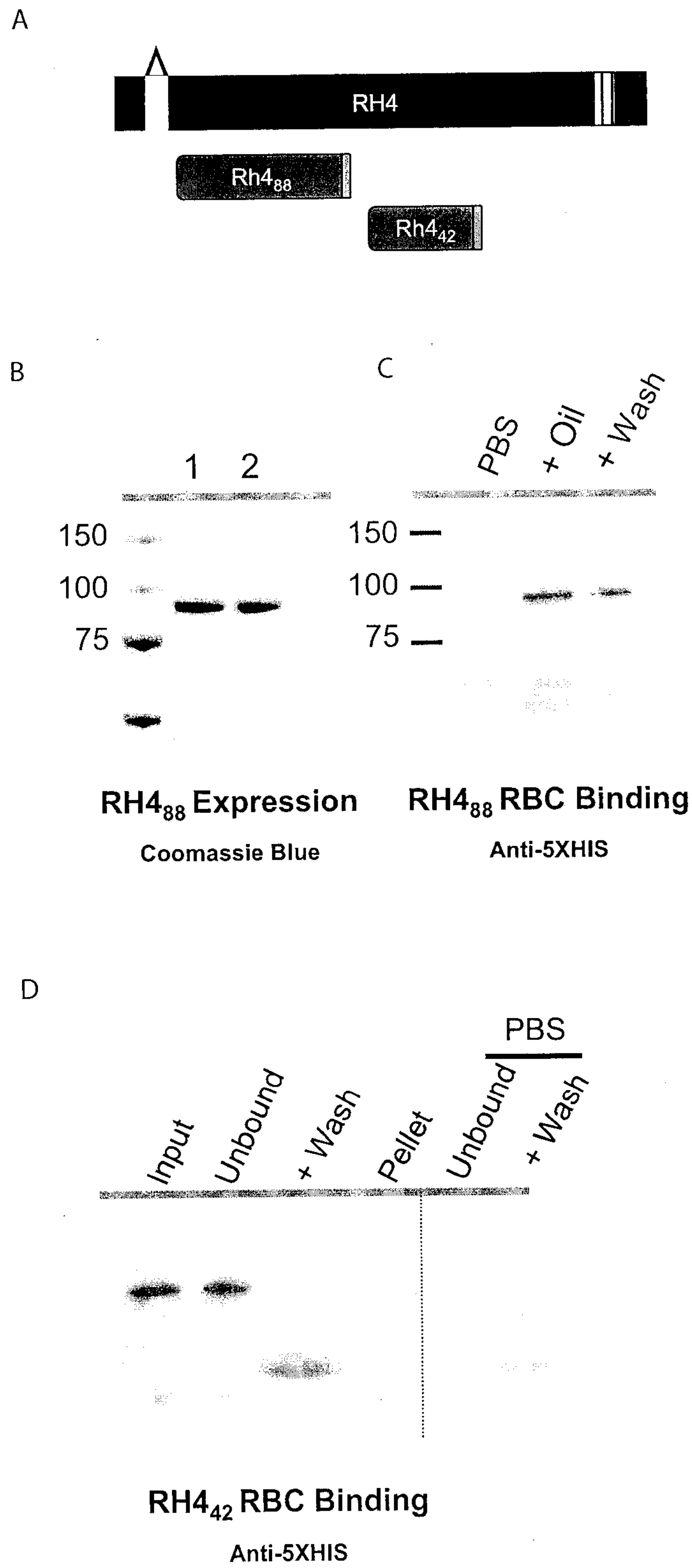
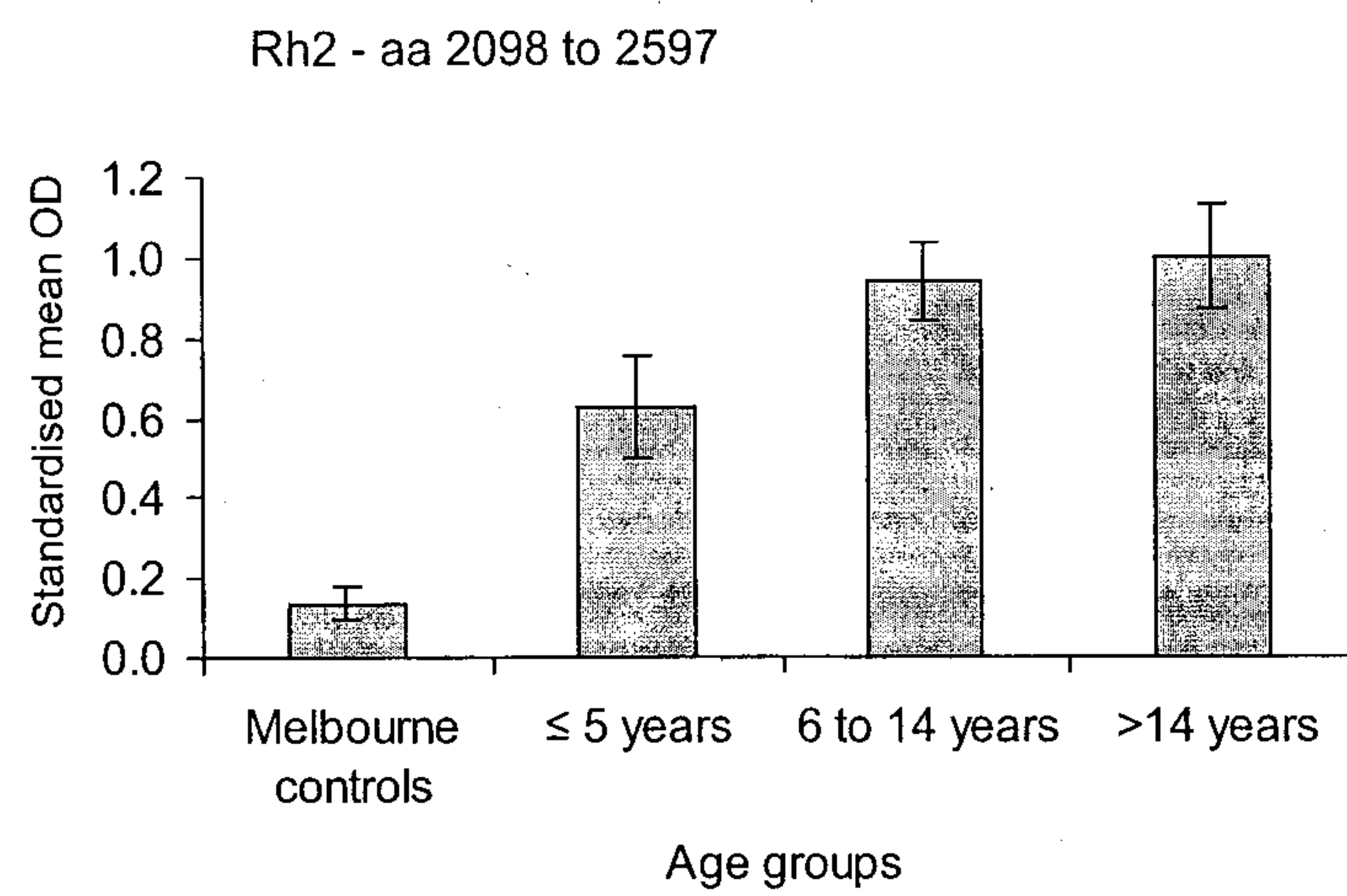


Figure 8

A



B

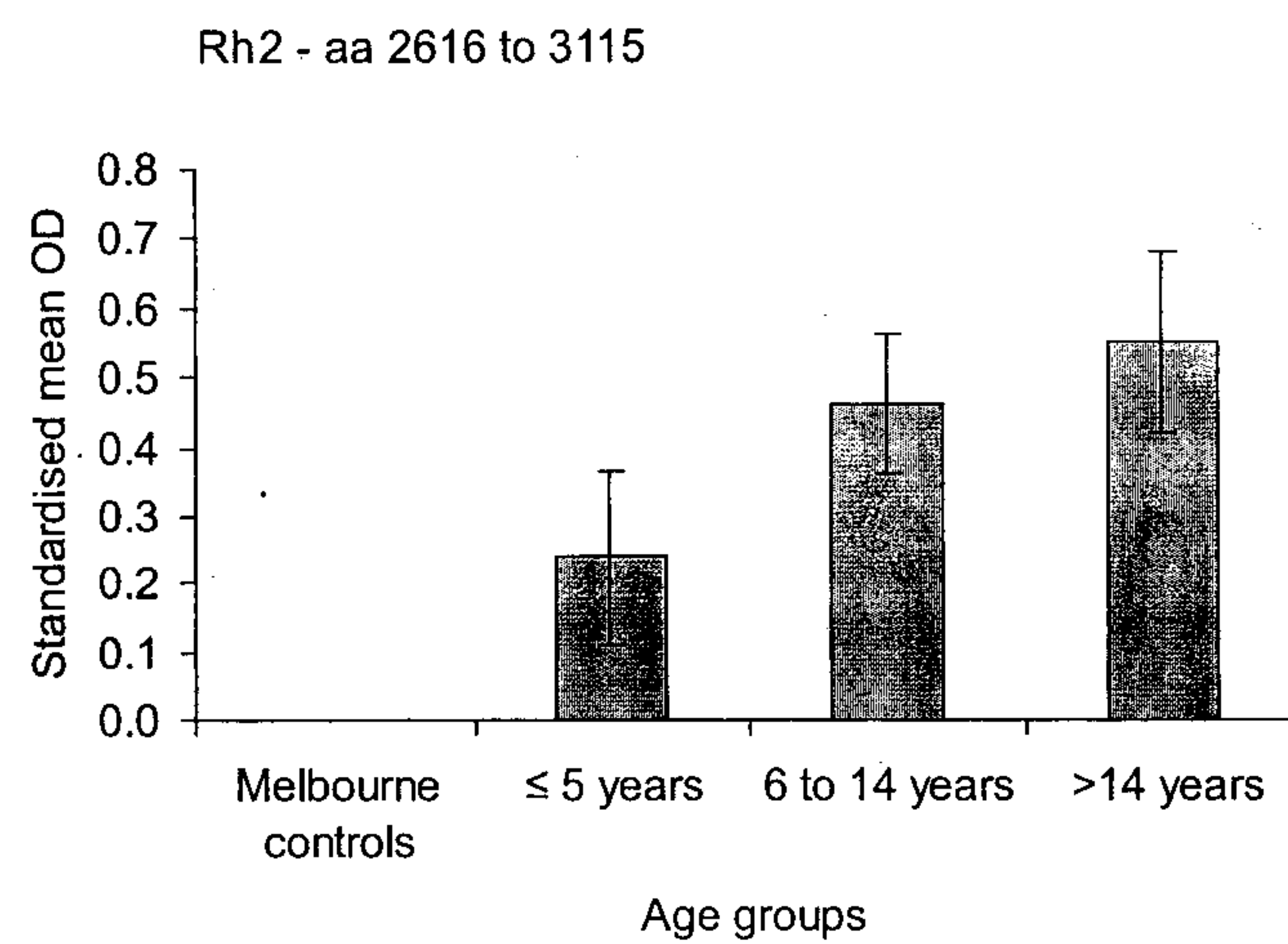


Figure 9

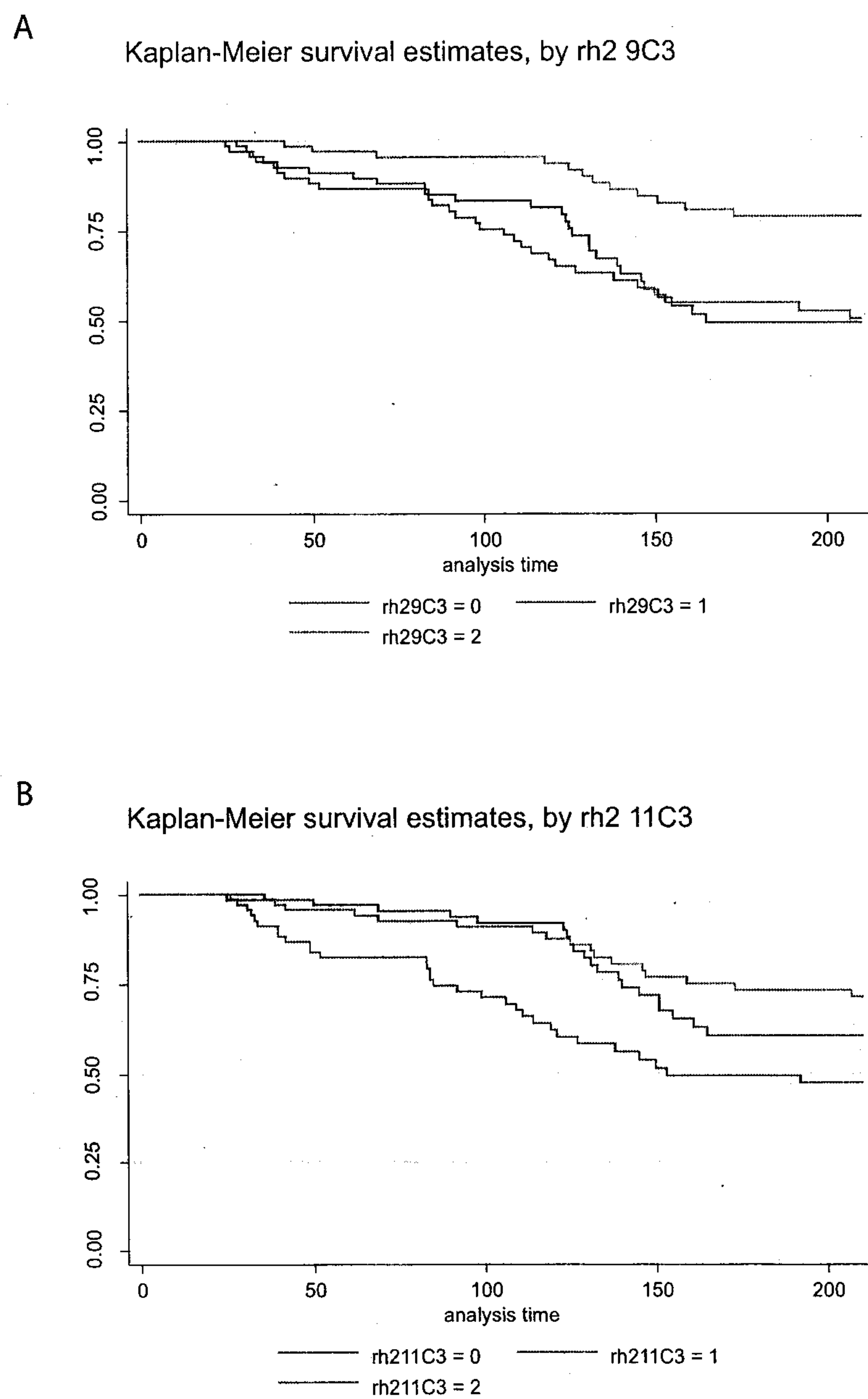


Figure 10

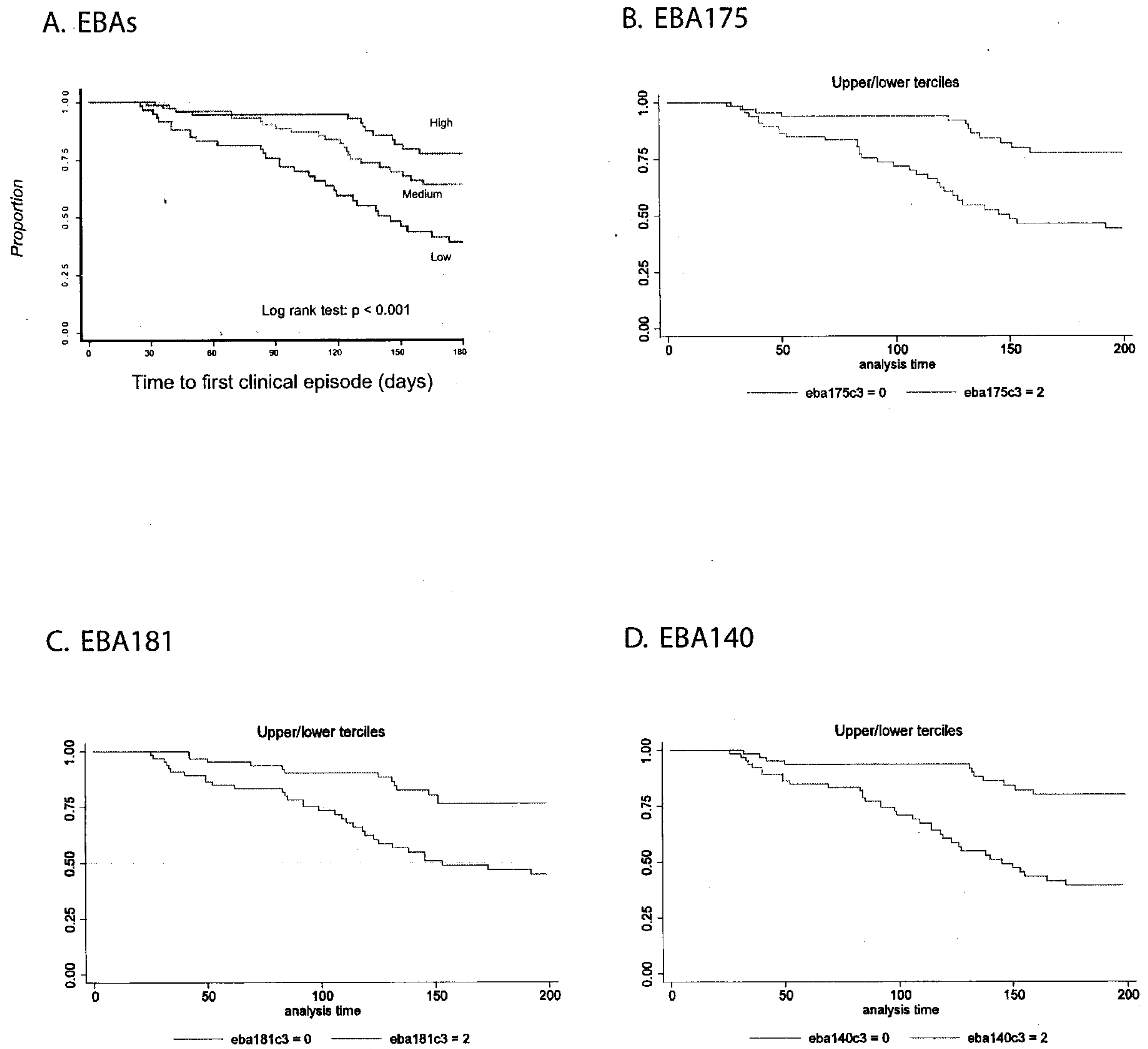


Figure 11

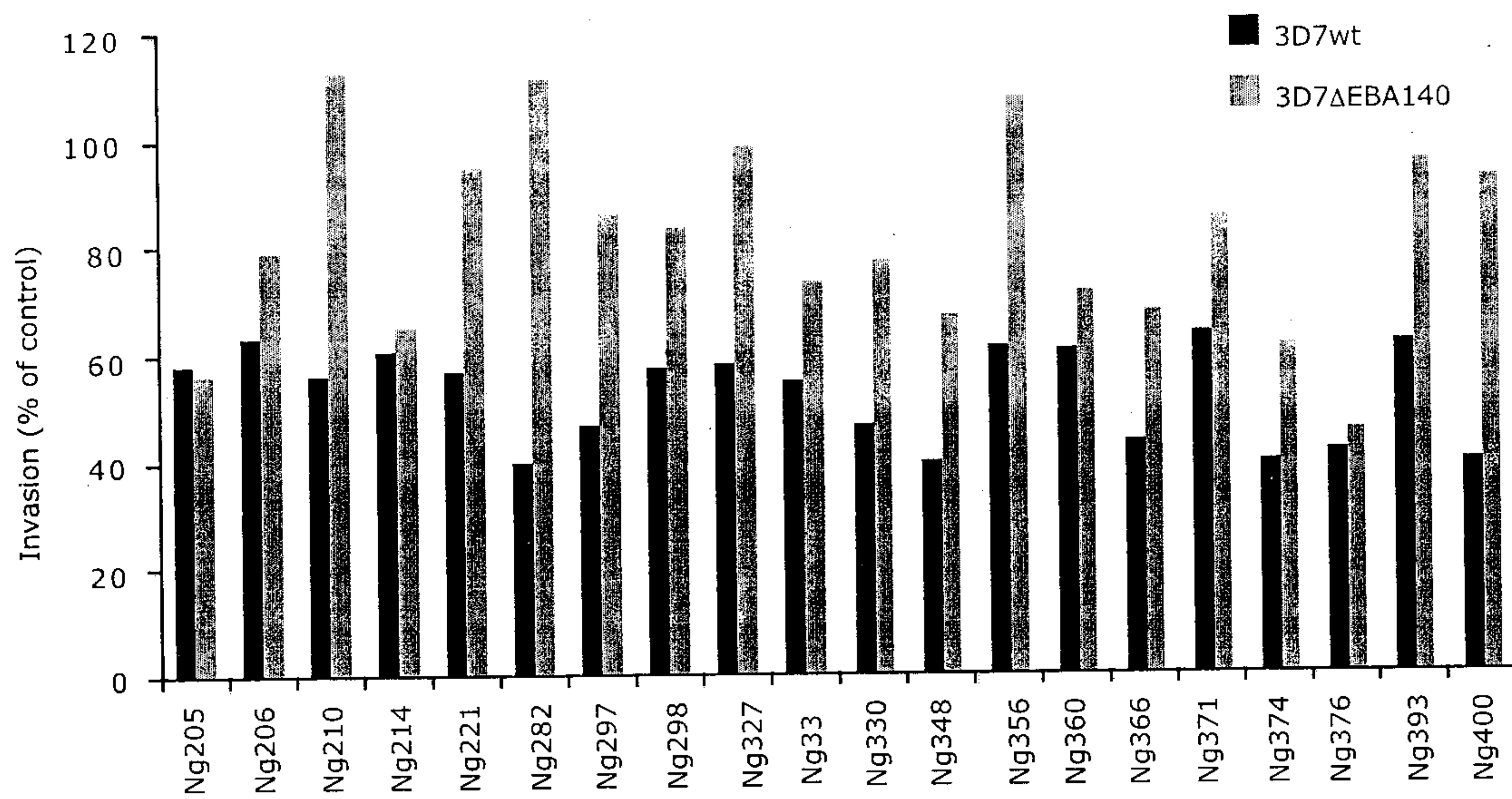


Figure 12

