Phagocytosis-inducing antibodies to *Plasmodium falciparum* upon immunization with a recombinant PfEMP1 NTS-DBL1α domain

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Abstract

**Background:** Individuals living in endemic areas gradually acquire natural immunity to clinical malaria, largely dependent on antibodies against parasite antigens. There are many studies indicating that the variant antigen PfEMP1 at the surface of the parasitized red blood cell (pRBC) is one of the major targets of the immune response. It is believed that antibodies against PfEMP1 confer protection by blocking sequestration (rosetting and cytoadherence), inducing antibody-dependent cellular-inhibitory effect and opsonizing pRBCs for phagocytosis.

**Methods:** A recombinant NTS-DBL1α domain from a rosette-mediating PfEMP1 was expressed in *Escherichia coli*. The resulting protein was purified and used for immunization to generate polyclonal (goat) and monoclonal (mouse) antibodies. The antibodies’ ability to opsonize and induce phagocytosis in vitro was tested and contrasted with the presence of opsonizing antibodies naturally acquired during *Plasmodium falciparum* infection.

**Results:** All antibodies recognized the recombinant antigen and the surface of live pRBCs, however, their capacity to opsonize the pRBCs for phagocytosis varied. The monoclonal antibodies isotyped as IgG2b did not induce phagocytosis, while those isotyped as IgG2a were in general very effective, inducing phagocytosis with similar levels as those naturally acquired during *P. falciparum* infection. These monoclonal antibodies displayed different patterns, some of them showing a concentration-dependent activity while others showed a prozone-like effect. The goat polyclonal antibodies were not able to induce phagocytosis.

**Conclusion:** Immunization with an NTS-DBL1-α domain of PfEMP1 generates antibodies that not only have a biological role in rosette disruption but also effectively induce opsonization for phagocytosis of pRBCs with similar activity to naturally acquired antibodies from immune individuals living in a malaria endemic area. Some of the antibodies with high opsonizing activity were not able to disrupt rosettes, indicating that epitopes of the NTS-DBL1-α other than those involved in rosetting are exposed on the pRBC surface and are able to induce functional antibodies. The ability to induce phagocytosis largely depended on the antibody isotype and on the ability to recognize the surface of the pRBC regardless of the rosette-disrupting capacity.

**Keywords:** Opsonization, Phagocytosis, Rosetting, Antibodies, PfEMP1, NTS-DBL1α

**Background**

Malaria is a devastating parasitic disease endemic in over 97 countries, representing around 3.3 billion people at risk of suffering the disease. There are around 149–303 million malaria cases per year and an estimated of 438,000 deaths, most of which are attributed to *Plasmodium falciparum* infections [1]. The majority of the malaria clinical symptoms are associated with the parasite’s asexual cycle inside the host’s red blood cells (RBCs). This intra-erythrocytic cycle involves extensive modification of the host cell, through the transport of parasite-derived proteins to the RBC cytoplasm and plasma membrane. The major surface antigen *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [2]

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belongs to a large multi-domain protein family (between 200 and 350 kDa), encoded by the hypervariable var gene family [2–4]. var genes are between 6 and 14 kb and have two exons separated by a conserved intron. The first exon encodes a hypervariable extracellular binding region, which includes an N-terminal segment (NTS) and multiple adhesive domains of duffy-binding-like (DBL)-type or cysteine-rich interdomain region (CIDR)-type, sometimes interspersed with C2 inter-domains. The second exon encodes a transmembrane (TM) segment and a more conserved acidic terminal segment (ATS). The DBL and CIDR domains are numbered consecutively from the N-terminus and have been respectively classified into six (α, β, γ, δ, ε and ζ) and five (α, β, γ, δ and pam) different types based on sequence similarities [5, 6]. PfEMP1 is used by the parasite to evade clearance from the human host through two main mechanisms: (1) evasion of the host immune response raised against one PfEMP1 variant, switching to new variants through differential expression of approximately 60 distinct members per genome of the var family [3]; and, (2) evasion of the spleen clearance through sequestration of the pRBCs in the host’s microvasculature [7, 8], which is mediated by the interaction between PfEMP1 and receptors located on the endothelial cell surface (cytoadhesion) or receptors on the uninfected RBC surface (rosetting). Among the different DBL domains, the DBLα is the most conserved [9, 10] and has been identified as a ligand both for rosetting and cytoadhesion [11–13].

After prolonged exposure, individuals living in endemic areas develop immunity to malaria, which manifests as an age-associated decline in the prevalence of severe, then mild clinical episodes [14]. This naturally acquired immunity to malaria is in part due to antibodies since passive transfer of sera from clinically immune adults to children infected with malaria decreases the parasitaemia and reduces the clinical symptoms [15]. There are a large number of studies indicating that the variant antigen PfEMP1 at the surface of the pRBC is the major target of the variant-specific immune response [16–20], and the antibodies are believed to confer protection by blocking sequestration, inducing antibody-dependent cellular-inhibition (ADCI) and opsonizing the pRBCs for phagocytosis. However, the actual variants and epitopes involved as well as the composition, specificity and importance of each possible protective mechanism underlying the naturally acquired anti-PfEMP1 immunity are still unclear. The first antibody protective mechanism has been extensively studied, showing that antibodies against the NTS-DBLα-domain both disrupt rosettes in vitro and protect against the sequestration of pRBC in vivo [21, 22], however it is unclear whether other protective and less studied roles (e.g., pRBC opsonization for phagocytosis) act synergistically with the anti-rosetting/cytoadhesion activity as a possible combined mechanism for parasite clearance. An in vitro study suggests that this is the case [22].

To explore this possibility a recombinant NTS-DBL1α-domain from a rosette-mediating PfEMP1 variant was expressed, in order to investigate if the antibodies elicited upon animal immunization with this domain were able to inhibit rosetting and more importantly to opsonize and induce pRBC phagocytosis in vitro.

**Methods**

**Parasite cultures**

*Plasmodium falciparum* FCR3S1.2 parasites were cultured according to standard methods, and the rosetting phenotype was maintained by enrichment over a Ficoll-gradient [23].

**Production of recombinant protein**

Construct expression of the NTS-DBL1α-domain was performed as previously described [24]. In brief, the NTS-DBL1α-domain spanning amino acids 1–481 (PFIT_bin06900) was codon optimized for expression in bacteria and cloned into the pJ414express vector (DNA2.0). The protein was expressed as a C-terminal 6× histidine-tagged recombinant protein in *Escherichia coli* (Shuffle T7, New England Biolabs); bacteria were grown at 30 °C until they reached an OD$_{600} = 0.6–0.8$ and then induced with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 20 h at 16 °C. Pelleted bacteria were lysed by sonication and the recombinant protein was recovered from the soluble fraction after centrifugation. The protein was purified by immobilized metal affinity chromatography (IMAC) over a TALON Cobalt column (Clontech), followed by size exclusion chromatography on a HiLoad 16/60 Superdex 75 pg column (GE-Healthcare). The purified protein was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using an antibody against the poly-His tag (Qiagen).

**Generation of monoclonal and polyclonal antibodies**

Monoclonal and polyclonal antibodies against the recombinant NTS-DBL1α domain were produced as previously described [24, 25]. Nine mouse monoclonal antibodies were produced in collaboration with the EMBL Monoclonal Antibody Core Facility, Monterotondo, Italy. Briefly, mice were immunized three times with 50 μg of recombinant protein. Antibody levels were measured prior and post fusion by ELISA to select positive cell clones. Monoclonal antibodies were purified over a Protein G agarose column (Pierce ThermoScientific) and subsequently dialyzed and concentrated.
Goat polyclonal antibodies were produced by Agrisera (Vännäs, Sweden). One goat was immunized four times at one-month intervals with 200 μg of protein emulsified in Freund's complete for the first immunization and incomplete adjuvant for the following three immunizations. Final bleeding was carried out 2 weeks after the last immunization and the total IgG was purified on Protein G columns. A set of six human sera from Myanmarese immune adults was used to test for the presence of naturally acquired pRBCs-opsonizing antibodies, able to induce phagocytosis by THP-1 cells in vitro. Sera were collected from asymptomatic adults (18–40 years of age and tested positive for *P. falciparum* infection by microscopy) residents of a malaria endemic area in northern Myanmar (Kachin province).

**Enzyme-linked immunosorbent assay (ELISA)**

For the immune sera set, antibodies against the recombinant NTS-DBL1α were measured by ELISA as previously described [26]. Maxisorp plates (Nunc, Rosklid) were coated overnight at 4 °C with 1 μg of the recombinant antigen dissolved in 15 mM Na₂CO₃ and 35 mM NaHCO₃ (pH 9.6). Plates were washed three times with PBS containing 0.1 % Tween 20 (PBST) and then blocked with 1 % bovine serum albumin (BSA) in PBST for 1 h at room temperature and subsequently washed three times with PBST. All samples were titrated by six serial dilutions in PBS to determine concentration dependency, and they were incubated in triplicate for 1 h at room temperature. Plates were washed three times with PBST, then bound IgG was measured by incubation for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-human IgG (Sigma) diluted 1:1000 in PBS. Plates were washed three times with PBST and developed with SigmaFast p-nitrophenyl phosphate tablets (Sigma) for 15 min. The optical density (OD) was measured at 405 nm in an ELISA reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific). A control pool of six non-malaria exposed Swedish donors was included as well as control wells without serum (background). Seropositivity was determined based on the 1:500 dilutions as the mean OD₄₀₅ plus two standard deviations of the Swedish control pool.

**Flow cytometry assay on infected erythrocytes**

Antibody binding to pRBCs was tested using flow cytometry as previously described [27]. Briefly, the pRBCs were blocked for 1 h with 2 % fetal bovine serum (FBS) in PBS followed by incubation with the human serum samples in a dilution 1:5 for 30 min at room temperature. The pRBCs were washed three times with 2 % FBS in PBS followed by incubation for 30 min at room temperature and protected from light with a goat anti-human IgG antibody coupled to Alexa488 (Molecular Probes®, Life Technologies, dilution 1:200) and ethidium bromide at a final concentration of 2.5 μg/ml to stain the parasite nuclei (added during the last 10 min of the incubation). Finally the pRBCs were washed three times with 2 % FBS in PBS followed by flow cytometry analysis. A control pool of six non-malaria exposed Swedish donors was included. The surface reactivity was expressed as geometric mean fluorescent intensity (MFI) and adjusted according to non-malaria exposed control reactivity and the non-parasitized RBCs reactivity according to the following formula: (pRBCimmune/pRBCnon-malaria)−(RBCimmune/RBCnon-malaria). Samples were considered positive when the adjusted MFI was higher than zero as reported previously [28].

**Rosette disruption assay**

The ability of human serum to disrupt rosettes was tested as described before [29]. A control pool of six non-malaria exposed Swedish donors was included as well as a negative control where no serum was added. Each serum sample was tested in a 1:5 dilution in 50 μl of parasite suspension, at 5 % haematocrit and at least 5 % parasitaemia. The samples were incubated in duplicates at room temperature for 1 h, followed by parasite staining with acridine orange and counting of rosettes under the microscope. For each sample at least 200 pRBCs were counted and the rosetting rate in the presence of serum was calculated relative to the rosetting rate in the negative control (no serum added). Samples were considered positive if disruption was >15 % compared to the control.

The presence of antibodies against the recombinant NTS-DBL1α, the recognition of the native homologous protein expressed on the surface of pRBCs and the rosette disruption activity for the monoclonal and the polyclonal antibodies were measured using similar methodologies and the results were published elsewhere [25].

**Phagocytosis assay**

The phagocytosis assay was performed as described before [22] with modifications. The human monocytic line THP-1 was maintained in RPMI 1640 supplemented with 2 mM glutamine, 0.85 g/L NaHCO₃ (Thermo Scientific) 10 % inactivated fetal bovine serum (FBS), 1000 U/ml of penicillin, 1 mg/ml of streptomycin (Sigma) and 20 mM HEPES at a maximum density of 1 × 10⁶ cells/ml. The cells were checked periodically for surface expression of the Fcy-receptors I (CD64) and II (CD32) on the surface, which is necessary for the antibody-dependent phagocytic activity [30, 31]. In brief THP-1 cells were separately stained with anti-CD32, anti-CD64 or anti-CD16 antibodies (FITC mouse anti-human, 552,883,
Highly synchronized FCR3S1.2 pRBCs (30–32 hpi, 10 % parasitaemia and >70 % rosetting) were used for the phagocytosis assays. They were washed in malaria culture medium (MCM: RPMI 1640, 20 mM HEPES, 0.85 g/L NaHCO₃, 2 mM glutamine, 25 μg/ml Gentamicin); the rosettes were disrupted mechanically (using a 23G syringe) and the pRBCs purified using a VarioMACS magnet. The final parasitaemia and the total cell number were determined and only samples with >80 % purity were used. The pRBCs were resuspended in 2.5 μg/ml ethidium bromide solution in MCM, adjusting the pRBCs’ density to 3.3 x 10⁷ cells/ml. From this suspension, 30 μl per well were distributed in a rounded-bottomed, 96-well plate, the cells were posteriorly pelleted down and re-suspended in the appropriate antibody concentration solution in MCM. A positive (rabbit anti human red blood cells, ab34858, ABCAM, 1:100 dilution) and a negative control (unopsonized control) were always included. Other controls were included: a monoclonal antibody against a non-malaria related antigen (the bacterial protein SlyD), non-immune goat IgG and non-immune Swedish sera controls. The opsonization was performed at 37 °C for 45 min, followed by three washes with MCM and re-suspension in THP-1 cells culture medium. THP-1 cells were washed once and re-suspended at an adjusted density of 5 x 10⁵ cells/ml; 100 μl of this suspension were dispensed in a separate rounded-bottomed, 96-well plate, 50 μl of the opsonized pRBCs were dispensed onto the THP-1 cells mixed gently and incubated for 40 min at 37 °C, 5 % CO₂. The phagocytosis was stopped by centrifugation at 4 °C followed by re-suspension in room temperature ammonium chloride lysing solution (15 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA) for 3 min to lyse non-ingested pRBCs. The lysis was stopped by addition of PBS supplemented with 2 % FBS, followed by three washes. After the final wash the cells were analysed by flow cytometry (FACSCalibur, BD Biosciences, 1:10 dilution) and checked by flow cytometry. The mouse monoclonal antibodies showed variable abilities to opsonize and induce phagocytosis. In general, all antibodies isotypes as IgG2b were not able to induce phagocytosis (Fig. 3a), generating values similar to those of the negative control (unopsonized) and of the non-malaria-related mouse monoclonal antibody (mAbSlyD). This can be explained by the poor binding of this particular isotype to human Fcγ-RI (CD64) [33]. Conversely, antibodies isotypes as IgG2a were able to induce various magnitudes and patterns of phagocytosis (Fig. 3a). The different patterns of phagocytosis were categorized as follows: (1) a prozone-like behaviour, with phagocytosis increasing with initial increases in antibody concentration (up to 40 μg/ml) followed by a decrease when higher antibody concentrations were used (mAbV2–4, mAbV2–7 and mAbV2–16, Fig. 3b); (2) a gradual increase in phagocytosis with increasing antibody concentrations (mAbV2–17.1, Fig. 3c); and, (3) a decrease when higher antibody concentrations were used (mAbV2–17.1, Fig. 3c).
a high and stable phagocytosis induction at all antibody concentrations (mAbV2–13, Fig. 3d). When comparing the monoclonal antibodies in rosette disruption and opsonization for phagocytosis (Table 1), three groups of surface-reacting antibodies emerged: (1) a group effective in both assays (mAbV2–7, mAbV2–13 and mAbV2–17.1); (2) a group effective in rosette disruption but not in opsonization for phagocytosis (mAbV2–3, mAbV2–6, mAbV2–14.1 and mAbV2–14.2), where each group’s ability to induce phagocytosis is linked to their isotype (IgG2a vs IgG2b); and, (3) a group effective in opsonization for phagocytosis but not in rosette disruption (mAbV2–4 and mAbV2–16), indicating that other regions of the NTS-DBL1-α besides those involved in rosetting are exposed on the pRBC surface and are able to induce antibodies with effector functions. A previous study has described a surface-exposed epitope in the sub-domain 2 (SD2) of the DBL-1α that is able to induce strain-transcending antibodies that do not disrupt rosettes [28]. The data presented here contrast with a previous study that describes a clear positive correlation between rosette disruption and opsonization.
for phagocytosis [22]. Instead, the data showed that the most important factors defining the level of phagocytosis are antibody surface reactivity and isotype, independently of the ability to disrupt rosettes.

Polyclonal antibodies generated in goat were able to recognize the recombinant antigen (measured by ELISA) as well as the native protein expressed on the surface of pRBCs (measured by flow cytometry); furthermore, they disrupted the rosettes (Table 1). However, they were not able to induce phagocytosis by the THP-1 cells in vitro. High antibody concentrations (900 μg/ml) generated phagocytosis values similar to total IgG purified from a control goat (Fig. 4a) and attempts to use lower concentrations (<10 μg/ml) to rule out a possible prozone-like effect were unsuccessful. In goats, two IgG isotypes are present: IgG1 and IgG2; only IgG1 is peaking in concentration during immune responses while IgG2, to which cytophilic activity is attributed, remains stable. However, human Fc receptors seem to have a very low affinity for goat IgG [34, 35] explaining the absence of phagocytosis induction by the polyclonal IgG used in this study.

The set of six human sera from Myanmarese immune adults (individually or pooled together) showed the presence of potent, naturally acquired, pRBCs-opsonizing antibodies able to induce phagocytosis (Fig. 4b) as has been shown in other studies [36, 37]. However, even though antibodies against the particular NTS-DBL1-α variant expressed by the parasite (measured by ELISA Additional file 1: Figure S2) were detected in all samples, the observed reactivity against the pRBCs surface (Additional file 2: Figure S1) and the induction of phagocytosis may also originate from other surface-directed antibodies. It is likely that antibodies recognizing other surface-exposed antigens, e.g., other PfEMP1 domains, RIFINs, SURFINs, STEVORs, and possibly others, are present in the samples. Previous studies have suggested that anti-RIFIN antibodies are a dominant component of the overall response against surface-exposed antigens [38, 39], and the variability confined in the predicted extracellular segment of SURFIN 4.2 (the member of the protein family transported to the surface) suggests that it is also likely to be exposed to the immune system and to be a target of positive selection [40]. Therefore, it is still important to convincingly identify if there are additional targets, besides PfEMP1, among the host’s naturally acquired, opsonizing antibodies, particularly in African children who are at the greatest risk of malaria. A recent study has shown correlation between protection against clinical malaria and naturally
acquired, opsonizing antibodies against *P. falciparum* merozoites [41]; it would be interesting to investigate the same protective correlation with opsonizing antibodies against pRBCs.

### Table 1 Properties of the antibodies used in the study

| Antibody type | ELISA reactivity (ITvar60 NTS-DBL1α) | Surface reactivity (FCR3S1.2) | Rosette disruption (FCR3S1.2) | Opsonization for phagocytosis (FCR3S1.2) |
|---------------|--------------------------------------|-------------------------------|-------------------------------|----------------------------------------|
| mAbV2–3 IgG2b | +                                    | +                             | +                             | −                                      |
| mAbV2–6 IgG2b | +                                    | +                             | +                             | −                                      |
| mAbV2–14 IgG2b | +                                    | +                             | +                             | −                                      |
| mAbV2–14.2 IgG2b | +                                    | +                             | +                             | −                                      |
| mAbV2–4 IgG2a | +                                    | +                             | −                             | +                                      |
| mAbV2–7 IgG2a | +                                    | +                             | +                             | +                                      |
| mAbV2–13 IgG2a | +                                    | +                             | +                             | +                                      |
| mAbV2–16 IgG2a | +                                    | +                             | −                             | +                                      |
| mAbV2–17 IgG2a | +                                    | +                             | +                             | +                                      |
| mAbSly Da IgG2b | −                                    | −                             | −                             | −                                      |
| ITvar60 goat IgG | +                                    | +                             | +                             | +                                      |
| Control goat IgG | −                                    | −                             | −                             | −                                      |
| IM1 Serum | +                                    | +                             | −                             | +                                      |
| IM2 Serum | +                                    | +                             | −                             | +                                      |
| IM3 Serum | +                                    | +                             | −                             | +                                      |
| IM4 Serum | +                                    | +                             | −                             | +                                      |
| IM5 Serum | +                                    | +                             | −                             | +                                      |
| IM6 Serum | +                                    | +                             | +                             | +                                      |
| IMP Serum | +                                    | +                             | −                             | +                                      |
| SCP Serum | −                                    | −                             | −                             | −                                      |

Table summarizes the sample name (mAb monoclonal antibody, IM immune malaria, IMP immune malaria pool, SCP Swedish control pool) and antibody type with their corresponding activity (+) in different assays. Positivity was determined based on the cut-off criteria specified in the “Methods” section.

*ELISA reactivity, surface reactivity and rosette disruption activity for these antibodies have been published elsewhere [25] and are accordingly reported here.*

### Conclusion

In summary, the results showed that immunization with a PfEMP1 NTS-DBL1-α domain elicits antibodies that not only have a biological role in rosette disruption, and therefore in sequestration blocking, but also effectively induce opsonization for phagocytosis of pRBCs, a role that could be of great importance during pRBCs clearance in vivo. Moreover, some of the antibodies with high opsonizing activity were not able to disrupt rosettes, indicating that epitopes of the NTS-DBL1-α other than those involved in rosetting are exposed on the pRBC surface and are able to induce functional antibodies that could provide protection.
Overall malaria infection is kept in population. Immune samples were depicted in red and named from 1 to 6. The pooled immune sample (IMP) is also depicted. Swedish control pool (SCP) is depicted in blue.

Additional files

Additional file 1: Figure S2. IgG levels in the human samples against the recombinant NTS-DBL1a (ITfVar60) measured by ELISA. The original OD values were fitted to a 4 Parameter Logistic (4PL) curve. The antibody concentration is expressed as log of the dilution factor (DF). Immune samples are depicted in red and named from 1 to 6. The pooled immune sample (IMP) is also depicted. Swedish control pool (SCP) is depicted in blue.

Additional file 2: Figure S1. Surface reactivity of the human samples measured by FACS. Immune samples are depicted in red and named from 1 to 6. The pooled immune sample (IMP) is also depicted. Swedish control pool (SCP) is depicted in blue.

Abbreviations

NTS-DBL1-a: N-terminal segment-Duffy-binding like alpha; PfEMP1: Plasmodium falciparum erythrocyte membrane protein 1; pRBC: parasitized red blood cell; ADCI: antibody-dependent cellular-inhibitory effect; CIDR: cysteine-rich interdomain region; TM: transmembrane; ATS: acidic terminal segment.

Authors' contributions

MdPQ and MW conceived and designed the experiments. MdPQ and DA performed the experiments. MdPQ analysed the data. QC provided the set of immune adult sera. MdPQ, KM and MW wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declared that they have no competing interests.

Ethics approval

Collection of human samples was conducted according to the principles expressed in the declaration of Helsinki. Ethical permission for human sample collection was obtained in China (The Committee for Laboratory Safety and Ethics, Institute of Zoonosis, Jilin University, permit number 2008-12-20). Animal immunizations were carried out commercially by EMBL (Monterotondo, Italy) according to European Guidelines 86/609/EEC and by Agrisera (Vänna, Sweden) approved by Umé Ethical Review Board (permission A37-10).

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