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A multi-stage malaria vaccine candidate targeting both transmission and asexual parasite life-cycle stages

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ABSTRACT

Effective control and eventual eradication of malaria drives the imperative need for clinical development of a malaria vaccine. Asexual parasite forms are responsible for clinical disease and death while apathogenic gametocytes are responsible for transmission from man to mosquito. Vaccines that combine antigens from both stages may provide direct protection and indirect benefit by reducing the force of infection. We constructed a chimeric antigen composed of a fragment of the *Plasmodium falciparum* (*Pf*) glutamate-rich protein fused in frame to a correctly folded fragment of *Pfs*48/45. The chimera was produced in *Lactococcus lactis* and induced robust antibody responses in rodents to the individual components. Specific antibodies showed strong transmission blocking activity against multiple *Pf*-strains in the standard membrane feeding assay and functional activity against asexual stages in the antibody dependent cellular inhibition assay. The combined data provide a strong rationale for entering the next phase of clinical grade production and testing.

1. Introduction

Considerable reductions in the burden of malaria have been observed in the last decade in association with up-scaling of malaria control efforts. It is, however, acknowledged that further up-scaling with currently available tools are unlikely to result in malaria elimination in the majority of African settings [1]. Novel tools that specifically reduce the transmission of malaria from man to mosquito are needed for this purpose [2]. A vaccine that targets sexual and/or sporogonic development would be of great value for malaria elimination strategies. Classical transmission-blocking malaria vaccines (TBMV), however, do not provide direct protection against clinical disease caused by pathogenic asexual stages. Clinical development of a multi-stage chimeric vaccine incorporating both sexual and asexual blood-stage components inducing functional antibody responses, can address this limitation providing both direct personal protection and delayed benefit by reducing transmission and risk for new infections.

The sexual stage *Pfs*48/45 antigen is a well-established lead TBMV candidate because of its critical role in parasite fertilisation [3,4]. Series of monoclonal antibodies (mAbs) have been generated [5] against distinct B-cell epitopes with the capacity to completely block the generation of infected mosquitoes when tested in the standard membrane feeding assay (SMFA). *Pfs*48/45 is a cysteine-rich protein with multiple disulfide bonds and most transmission blocking B-cell epitopes depend on tertiary structures [6]. Since *Pfs*48/45 is expressed in gametocytes once the parasite undergoes sexual differentiation in the human host [7,8], naturally acquired anti-*Pfs*48/45 antibodies are found in sera from endemic populations, and presence of these antibodies is associated with transmission blocking activity [6,9–11]. Although attractive from this perspective for clinical vaccine development, *Pfs*48/45 has

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proven to be a difficult target for production in most heterologous expression systems [3,4,12–17].

The asexual blood-stage antigen Glutamate-rich Protein (GLURP) has been identified as a target of naturally acquired immunity against malaria [18–20]. Results from epidemiological studies show strong correlation between the presence of cytophilic anti-GLURP antibodies and protection against clinical malaria [20]. IgG preparations derived from endemic sera and from volunteers participating in a phase-1 GLURP vaccine trial have been shown to functionally inhibit parasite growth *in vitro* in co-operation with monocytes [19,21–23]. Finally, GLURP is part of the GMZ2 vaccine which is an asexual hybrid protein currently tested in a multicentre phase IIb trial in Africa. It has been relatively easy to produce GLURP in recombinant form [24,25] and especially food grade *Lactococcus lactis* has been an ideal expression system [24], most likely because *L. lactis* exhibits similar codon bias as *Plasmodia* and therefore does not require codon optimisation or harmonisation prior to heterologous expression [26].

Here, we have produced a correctly-folded functional fragment of *Pfs*48/45 (10C) as a chimeric antigen fused in frame with a section of GLURP (GLURP.RO) in *L. lactis*. Purified R0.10C induced functional antibodies in rats showing both TB activity and asexual growth inhibition against a panel of *Pf* strains of different geographical origin.

2. Materials and Methods

2.1. Construction of plasmid and growth conditions

pLEA5: $glurp_{79\text{-}1500}$ fragment (Genbank M59706) was amplified from F32 gDNA with the primers 5′-CCAGATCTACAAGTGAGAATAGAAATAAACGAATC (GA52) and 5′-CTATACTTGATATAACCTTTTCAGTATTATCTGCTTCATGCTCGCTTTTT-TCCGATTC (GA4), and the $pfs48/45_{475\text{-}1284}$ fragment (corresponding to bp 475–1282 of Genbank: NC_004331.2) was amplified from 3D7 gDNA using the primers 5′-GAATCGGAAAAAAGCGAGCATGAAGCAGATAATACTGAAAAGGTTA-TATCAAGTATAG (GA12) and 5′-CCAGATCTCTAATGGTGATGAT-GGTGATGTGCTGAATCTATAGTAACTGTCATATAAG (GA53). The GA53 primer contains the hexahistidine coding sequence, while GA4 and GA12 are mutually overlapping. The $glurp_{79\text{–}1500}$ and $pfs48/45_{475\text{–}1284}$ fragments were joined by overlapping PCR using equimolar amounts of the two amplicons and the primers GA52 and GA53. The resulting amplicon was purified, digested with *Bgl*II and ligated into *Bgl*II digested pKBR11 [24]. The plasmid was constructed in *Escherichia coli* TOP10 (Invitrogen, Denmark), transformed into *L. lactis* MG1363 by electroporation [27] and verified by DNA sequencing.

Fermentation of *L. lactis* MG1363 containing pLEA5 was performed in 2% yeast extract, 0.05 mM $FeSO_4$, 0.0025 mM $CaCl_2$, 0.00265 mM $MgCl_2$, 0.5 mM citric acid, 1.38 mM ammonium sulphate, 14.7 mM sodium-acetate, 20 mM potassium phosphate buffer pH7.4, 5% glucose supplemented with erythromycin (10 μg/ml) at 30° C. After approximately 3 h of growth, pH was reduced to 6.4 ± - 0.1 and maintained by a pH-controlled intake of 2 M KOH for another 8 h until the cell density was approximately OD_{600} = 11. Clarified culture-filtrates were stored at 4°C.

2.2. Purification of recombinant R0.10C

Culture-filtrates were concentrated 10-fold and buffer exchanged to phosphate buffered saline (PBS), pH 7.4 containing 10 mM Imidazole on a QuixStand Benchtop system (GE Healthcare, Sweden). The recombinant protein was first applied to a 5 ml HisTrap™ HP column (GE Healthcare, Sweden). Bound protein was eluted with 500 mM Imidazole in PBS and further purified by size exclusion chromatography (SEC) on a Superdex S 200 column (GE Healthcare, Sweden). The monomeric fraction was finally purified on a 5 ml HiTrap NHS-activated HP column containing rat mAb 45.1 (epitope I) as described by the manufacturer (GE Healthcare, Sweden). Protein concentrations were measured using a NanoDrop 2000 (Thermo Scientific, USA).

2.3. Immunization and purification of IgG

Wistar Hanover rats (Taconic, Denmark) were immunised with immune-purified R0.10C in a volume of 200 μl by subcutaneous injections. R0.10C was emulsified in either Freund's adjuvant or $Al(OH)_3$. Each rat received three injections at 2-week intervals and was bled at days 0, 14, 28 and 42. IgG was purified as described previously [24].

2.4. ELISA and indirect immunofluorescent antibody (IFA) test

Schizonts and mature gametocytes (stage V) were produced in an automated static culture system in red blood cells of blood group O^+ and non-immune AB serum [28], harvested, purified as described [29] and stored at–70 °C. Asexual and sexual-stage antigens were solubilised by re-suspension in 1% sodium desoxycholate, 10 mM Tris pH 8.0, 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride. After 10 min incubation at room temperature cell debris was removed by centrifuging at 15800 g for 10 min and the supernatants containing schizont or gametocyte extracts were stored at–70 °C. Microtitre plates (Sterilin® ELISA plates, Netherlands) were coated overnight with 100 μl of schizont or gametocyte extract (respectively 85000 or 125000 parasite equivalent per well), blocked with 5% milk in PBS and reacted with serial dilutions of sera in PBS-0.05% Tween 20 (PBST) for 4 h at room temperature. The secondary antibody was rabbit-anti rat IgG-HRP (H + L) (DAKO, Denmark) diluted 1:3000. After 2 h incubation bound secondary antibody was quantitated with tetramethyl benzidine (TMB) substrate solution for 20 min. The colour reaction was stopped with 0.2 N H_2SO_4 and the optical density was read at 450 nm in a Microplate Reader (Labtec BV, Germany). The plates were washed extensively with PBST – 0.5 M NaCl between each incubation step. Serum from a GMZ2 immunised rat was used as positive control in the schizont-ELISA and negative control in the gametocyte-ELISA. Midpoint (EC50) values were calculated using GraphPad Prism, (GraphPad Software, USA). Indirect IFA was performed with cultured sexual and asexual stages of *Pf* parasites air dried onto a multisport slide as previously described [30].

2.5. Standard Membrane Feeding Assay (SMFA)

Antisera obtained from rats immunised with R0.10C were assessed in SMFA as described [31,32]. Briefly, 30 μl of rat serum was mixed with 90 μl of naïve human serum and 150 μl of *in vitro* gametocyte cultures of the *Pf* (NF54, NF166 or NF135) [33] laboratory lines. The mixture was fed to *Anopheles stephensi* mosquitoes through a membrane feeding apparatus. Pre-immune sera served as the controls. Fully engorged mosquitoes were separated and held at 26 °C. Seven days later, midguts of 20 mosquitoes were examined for oocysts. The observed (transmission-blocking) TB activity was determined as the percentage reduction in the arithmetic mean oocyst number in test samples compared to that in controls. The experiment was considered valid when at least 85% of the mosquitoes feeding on control sera were infected. Data were analysed by generalised linear mixed effect models and used to estimate the efficacy of the vaccine by comparing individual rat serum (and pools of sera) against a single rat serum that did not receive a vaccine [34]. A binomial error structure was used for the

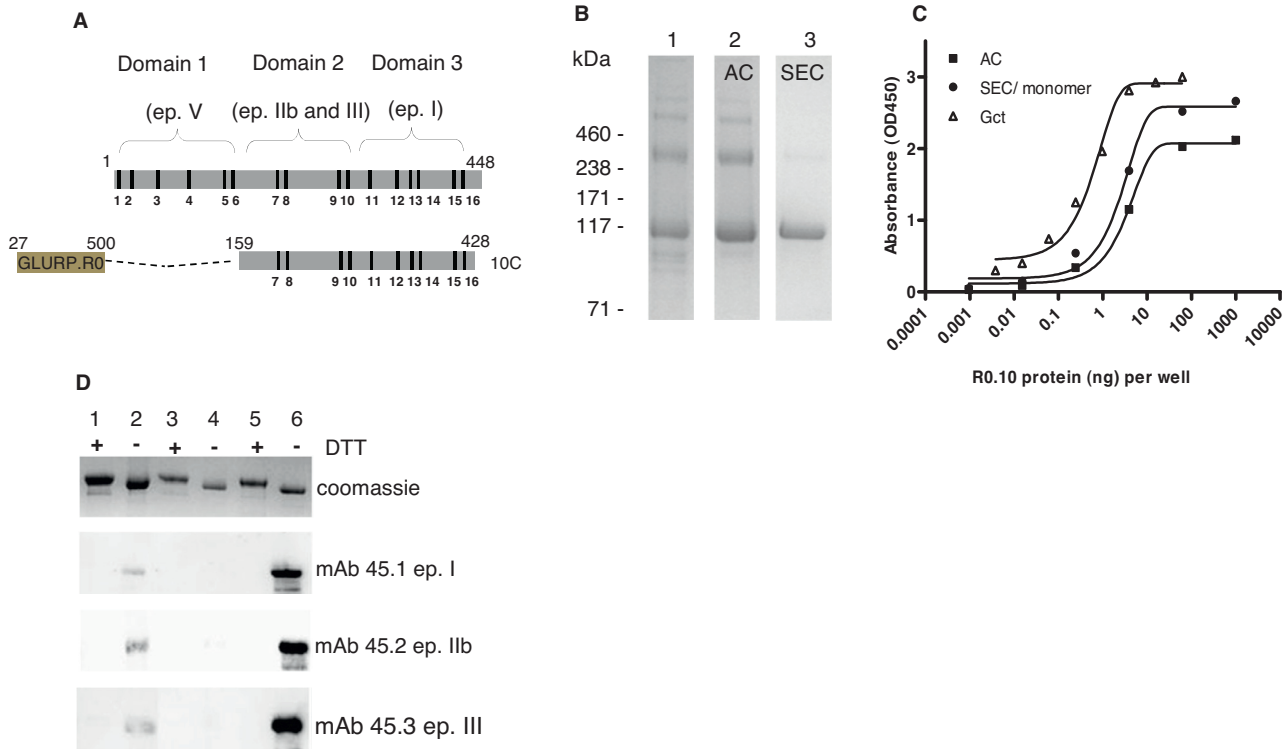

Fig. 1. Expression and purification of monomeric R0.10C. (A) Schematic representation of *Pfs*48/45 and the 10C region cloned into pLEA5. Please note that the GLURP.R0 region does not contain cysteine residues. (B) Coomassie blue-stained 4–12.5% polyacrylamide gel of R0.10C purification steps. Lane 1, Culture supernatant; Lane 2, eluate from AC-column; Lane 3, monomer fraction from the Superdex S200-column (SEC). The sizes (kDa) of the molecular mass markers are indicated. (C) Sandwich ELISA of fractions from purification. ■, eluate from AC column (Lane 2 in panel B); ●, Monomer fraction from the SEC column (Lane 3 in panel B); Δ, Gametocyte extract. The different antigens were captured with mAb 45.1 [37] and detected with anti-His-HRP. X-axis is shown on a logarithmic scale. (D) Immune purification of R0.10C. Upper panel, coomassie blue-stained 4–12.5% polyacrylamide gel of protein fractions from mAb 45.1 immune-purification of R0.10C. Lanes 1 and 2, input; lanes 3 and 4 runthrough; lanes 5 and 6, eluate 2 μg of protein was loaded with or without DTT as indicated. Lower panel: an immune blot analysis of the same gel shown in the upper panel using mAbs 45.1, 45.2 and 45.3 as primary antibody. 1 μg of protein was loaded in each lane.

parasite presence/absence data while a zero-inflated negative binomial distribution was used to describe mosquito oocyst intensity. Models were compared using the likelihood ratio. Treatment (i.e. whether the mosquito received blood mixed with vaccinated rat serum or not) was included as the fixed effect. Oocyst development was allowed to vary at random between individual rats to enable an overall estimate of efficacy to be generated.

2.6. Antibody-dependent cellular inhibition (ADCI)

ADCI assays were performed as described [23], using three different *Pf* (NF54, 7G8 and M7201) laboratory strains (generously donated by Morten A. Nielsen). Each plate included the following controls: (i) normal rat IgG (NIG) from non-immunised rats, (ii) human monocytes without rat IgG, (iii) immunised rat IgG without human monocytes, (iv) purified IgG from hyperimmune African adults as a positive control and (v) pooled IgG from pre-bleeds as a negative control. Effector monocytes were purified from buffy coats from whole blood donated by Danish blood donors. Donors allowed by informed consent that donated blood could be used as normal material for research.

2.7. Sequencing of pfs48/45 and GLURP

DNA was extracted from cultured laboratory lines using QIAamp DNA minikit (Qiagen). The *pfs*48/45 and *glurp* gene fragments were amplified and sequenced as described [35,36]. Nucleotide sequence data are available under accession numbers KF561820, KF561821, KF561822 and KF631400 (*glurp*) and KF561823, KF561824, KF631401, KF631402 and KF631403 (*pfs48/45*).

3. Results

3.1. Expression, purification and characterisation of monomeric R0.10C

Development of a *Pfs*48/45-based subunit vaccine has been a formidable task because correct folding of TB epitopes is essential for its capacity to elicit functional antibodies [4]. Here, we have used a set of TB mAbs to guide the expression of correctly folded *Pfs*48/45. A Chimeric protein (R0.10C) was generated between the N-terminal part of GLURP (R0) and the C-terminal part of *Pfs*48/45 (10C) (Fig. 1A). The corresponding plasmid, pLEA5, was transformed into *L. lactis* MG1363 resulting in clonal secretion of his-tagged R0.10C hybrid protein (Fig. 1B, lane 1). In the absence of the GLURP.R0 fusion partner there was no expression of recombinant *Pfs*48/45-10C domain (data not shown). Purification by affinity chromatography (AC) on a HisTrap HP column resulted in a mixture of monomeric and multimeric R0.10C (Fig. 1B, lane 2) which were separated by size exclusion chromatography (SEC) on a Superdex S-200 column with a yield of 6 mg/L of monomeric R0.10C (Fig. 1B, lane 3). Transmission blocking mAb 45.1 against the conformational *Pfs*48/45 epitope I [30,37] reacted with monomeric R0.10C (Fig. 1C); however, the binding of mAb 45.1 to R0.10C was less efficient compared to native *Pfs*48/45 derived from a gametocyte extract (Fig. 1C), suggesting that monomeric R0.10C contained a mixture of correctly and incorrectly folded 10C. To obtain a pure preparation of correctly folded conformer, monomeric R0.10C was further immune purified on mAb 45.1. Whereas all fractions contained a predominant band corresponding to R0.10C (Fig. 1D), only affinity-purified R0.10C showed strong reactivity with mAb 45.1

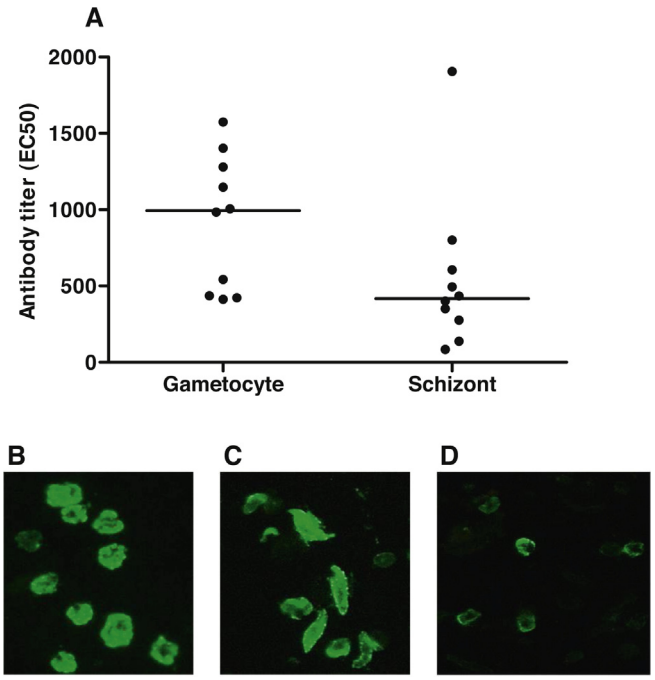

Fig. 2. Antibody responses in rats. Ten rats were immunised with immune-purified R0.10C in Freund's adjuvant. Day 43 serum was tested for antibody reactivity on ELISA plates coated with a gametocyte or schizont extract (A). Antibody titres are expressed as EC50 values. Shown are immunofluorescence microscopy on air-dried *Pf* Schizonts (B), mature gametocytes (C) and macrogametes/zygotes (D).

by western blotting (Fig. 1D). Affinity-purified R0.10C also reacted strongly with the mAbs 45.2 and 45.3 suggesting that both domains 3 (epitope I) and 2 (epitopes IIb and III) adopt a correct fold (Fig. 1D, lane 6). The mAbs did not recognise the run-through suggesting that this form of monomeric R0.10C contains scrambled disulfide bridges (Fig. 1D, lanes 4). There was no antibody reactivity with reduced R0.10C confirming that cysteine connectivity is essential for antigenicity (Fig. 1D, lanes 1, 3 & 5).

3.2. Correctly folded R0.10C elicit antibodies against sexual and asexual stages

In an initial experiment, ten rats were immunised with 20 μg immune-purified R0.10C in Freund's adjuvant (FA). Sera taken 2 weeks after the third immunisation were analysed by ELISA using a gametocyte-extract for the detection of antibodies against native *Pfs*48/45 and a schizont-extract for detection of antibodies against native GLURP (R0 part of R0.10C). Immunised animals showed clear responses against both gametocyte- and schizont-extracts (Fig. 2A). The median midpoint titre in the gametocyte-ELISA was 1/994 (range: 1/413 to 1/1574), and 1/417 (range: 1/84 to 1/1905) in the schizont-ELISA while pre-bleed median midpoint titres were 1/42 and 1/51, respectively. There was a clear reactivity by immune fluorescence assays with air-dried asexual- (Fig. 2B) and sexual/sporogonic-stage parasites (Fig. 2C & D). The immunogenicity of R0.10C was also investigated by immunoblotting of parasite-derived proteins with sera from immunised rats (Supplementary Fig. 1). Rats immunised with R0.10C (lane 1) recognised polypeptides of approximately 48,000 Da and 45,000 Da by SDS-PAGE. These polypeptides were also recognised by mAb 45.1 (lane 2) demonstrating that they correspond to *Pfs*48/45.

3.3. Activity of R0.10C antibodies in functional Pf assays

TB capacity of these antibodies was tested in the SMFA. Two immunisations of R0.10C were sufficient to elicit antibodies which efficiently inhibited oocyst development (>90%; pooled serum samples of 5 rats), whereas pre-bleeds were inactive (Fig. 3A, Supplementary Table 1). After a second booster, sera from 9 out of 10 immunised rats showed strong TB activity (Fig. 3A, Supplementary Table 1). The vast genetic diversity of *Pf* with possible limited functional activity of induced antibodies is a major concern in malaria vaccine development. Sera from five individual rats immunised with R0.10C were therefore tested in SMFA with three geographically distinct *Pf* strains; NF54, NF166 and NF135 [38]. All five samples showed significant TB activity against all parasites isolated (Fig. 3B, Supplementary Table 2) irrespective of polymorphism at amino acid positions 254, 304 and 322 in the *Pfs*48/45 portion of R0.10C (Supplementary Fig. 2A).

Functional activity against asexual blood-stage parasites was tested in the ADCI assay [23]. IgG preparations from all immunised rats showed strong inhibitory effects in the presence of normal human monocytes (Fig. 4A) whereas they had no effect in the absence of monocytes. Pre-bleeds did not promote growth inhibition indicating that the observed ADCI-effects were due to the presence of specific antibodies to GLURP. Comparable inhibition in the ADCI assay was obtained against three genetically diverse laboratory lines; NF54, 7G8 and M7201 (Fig. 5B) despite polymorphisms in the GLURP region of R0.10C (Supplementary Fig. 2B).

3.4. Immunogenicity of R0.10C in aluminium hydroxide

Immunogenicity of R0.10C was also assessed in $Al(OH)_3$ since FA is incompatible with human use. Groups of five rats were immunised 3 times at 2-week intervals with increasing doses of R0.10C/$Al(OH)_3$ ranging from 0.4 to 44.9 μg. Two weeks after the last injection, rats were bled and the functional activity of anti-R0.10C antisera was assessed by SMFA. The strongest immune response was induced by vaccination with 5.6 μg, where 4 out of 5 animals inhibit oocyst development > 90% (Fig. 5A). Increasing the dose to 44.9 μg decreased the number of animals sero-converting to functional antibody titres. This effect is particularly prominent at the 44.9 μg dose. The same dose-dependency was observed when analysing parasite-specific IgG in a gametocyte-ELISA (Fig. 5B) providing a possible explanation for the decreasing functional activity at high doses. Indeed, there was a strong correlation between SMFA activity and gametocyte-specific IgG levels (Fig. 5C, r: 0.7195). In contrast, antibody responses against the GLURP.R0 region showed an increase with increasing dose (Fig. 5B). The relatively low immunogenicity of the *Pfs*48/45-10C domain of R0.10C at high doses may be due to antigenic competition [39].

4. Discussion

The multi-stage life cycle of *Pf* with distinct antigenic composition and function provides the opportunity to simultaneously target both sexual and asexual blood stages for vaccine development purposes. In this study a multi-stage chimera was generated containing the blood-stage vaccine candidate GLURP.R0 fused in frame to C-terminal 10-cysteine (10C) fragment of sexual-stage protein *Pfs*48/45. For the latter we focussed on conformational epitope I, a major target for TB antibodies [30]. Anti-epitope I mAb 45.1 was used to monitor expression and for affinity purification of correctly folded 10C. Interestingly, GLURP.R0 fusion partner greatly enhanced expression of correctly folded *Pfs*48/45 proteins in *L. lactis* possibly through protein stabilisation in the soluble fraction.

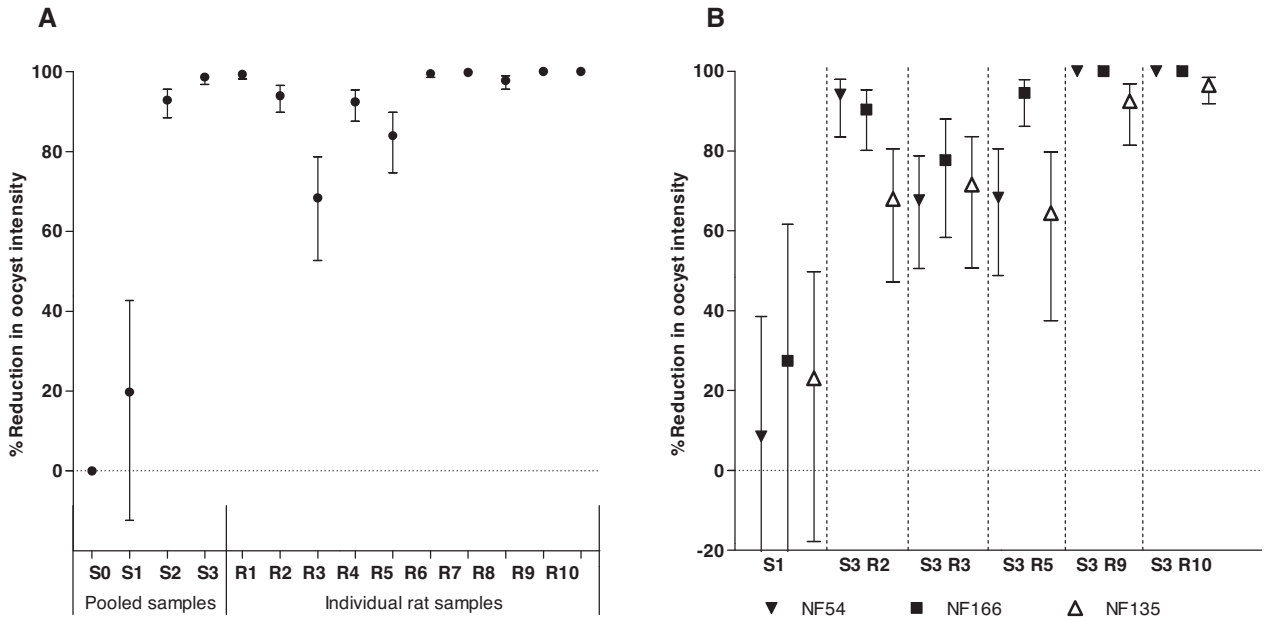

Fig. 3. Functional activity of rat anti-R0.10C antisera in SMFA assay. 10 rats were immunised 3 times with R0.10C adjuvanted in Freund's adjuvant. (A) Specific oocysts' reducing activity compared to control feeds in the SMFA of pooled serum samples S0 (pre bleed), S1 (bleed after 1e immunisation), S2 (bleed after 2e immunisation), S3 (final bleed) and ten individual S3 samples. (B) Selected S3 rat antisera were assessed for functional activity in SMFA using laboratory lines NF54, NF166 and NF135 [38].

Purified, correctly folded R0.10C efficiently elicits TB antibodies in rats. Levels of anti-R0.10C TB antibodies are similar to those previously obtained with a protein fusion between 10C and the maltose-binding protein (MBP) [4] suggesting that the 10C subfragment is appropriate as *Pfs*48/45 target for induction of TB antibodies provided expression of the correct conformer is assured.

The R0 part of R0.10C aims to elicit antibodies with the capacity to inhibit pathogenic asexual parasite proliferation [22], thereby reducing parasite density [40] and clinical disease [41]. GLURP.R0 is also present in GMZ2 which is a combination of GLURP and MSP3 and a leading asexual vaccine candidate currently tested in a multicentre phase IIb trial in Africa. The high ADCI-activity of R0.10C-induced IgG suggests that 10C does not modify the presentation of B-cell epitopes in the R0 segment. It has been previously shown that both human [22] and mouse [24] anti-GLURP antibodies can interact with human monocytes to inhibit parasite growth *in vitro*. Here we show that human monocytes can corporate with rat antibodies to promote ADCI-activity suggesting that rat IgG

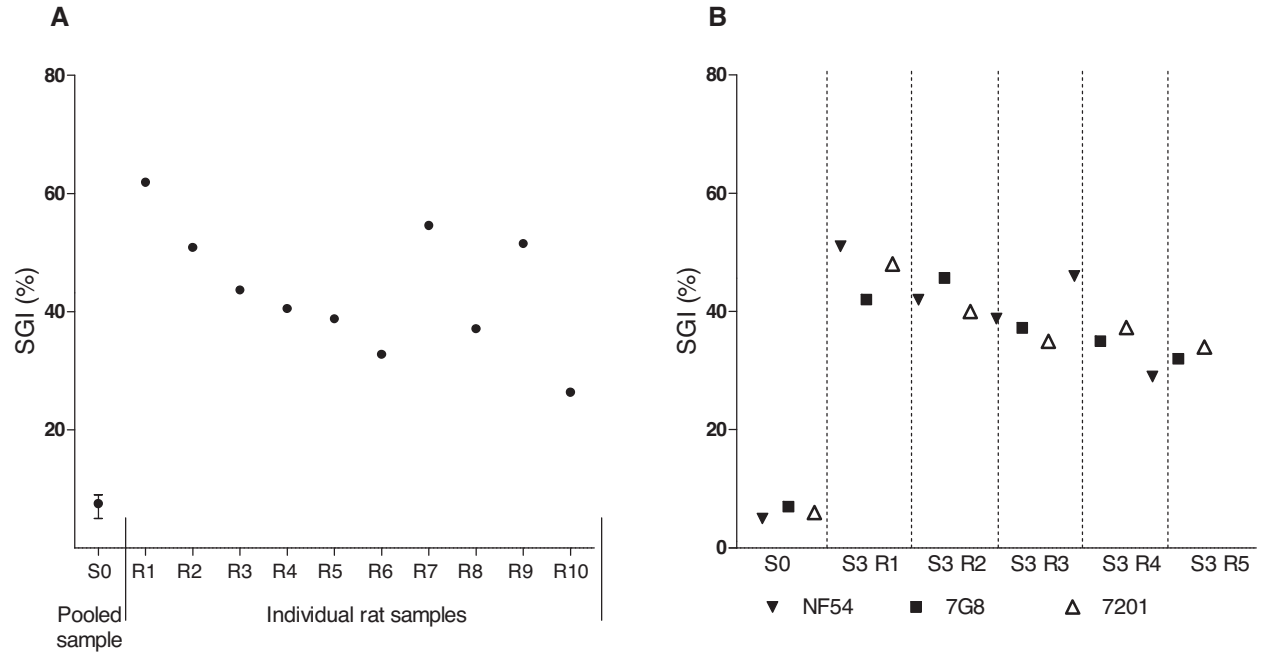

Fig. 4. Functional activity of rat anti-R0.10C antisera in ADCI assay. (A) Specific growth inhibition (SGI) index of ADCI of parasite growth by total IgG purified from pooled S0 samples (pre-bleed), and 10 individual S3 samples (final bleed) from rats immunised with R0.10C. Parasite at the schizont stage were co-cultured with monocytes in the presence of purified antibodies (500 μg/ml of culture in the assay), and parasite growth was monitor after 96 h. (B) Selected IgG samples were assessed for functional activity in ADCI using laboratory lines NF54, 7G8, and M7201. Data shown are representative of at least 2 independent experiments.

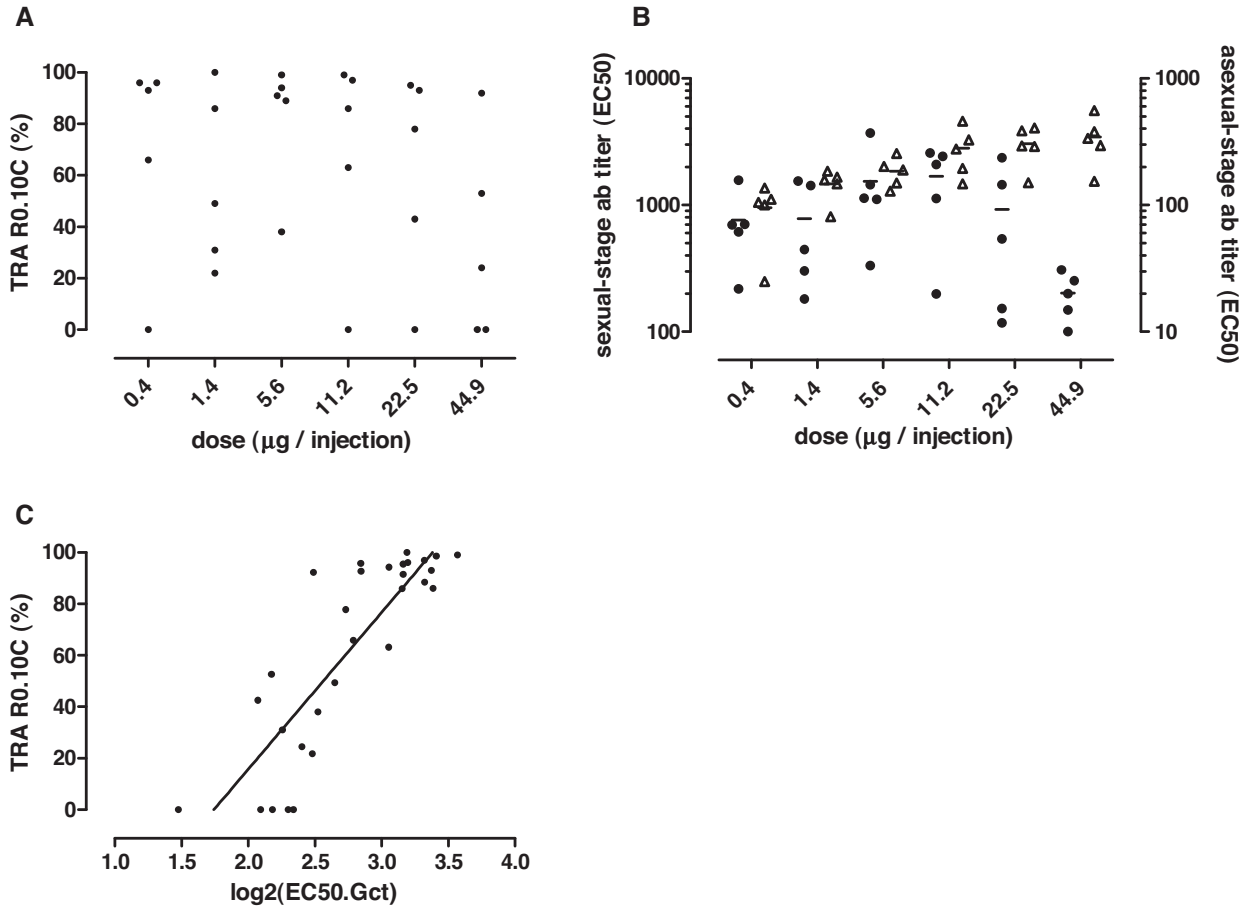

Fig. 5. Functional activity and immune recognition of vaccination antigens. Individual sera from groups of rats (n = 5) immunised with different doses of R0.10C in $Al(OH)_3$ were assessed for functional activity in SMFA (A) and recognition of gametocytes (●) and schizonts (Δ) by ELISA (B). Horizontal lines represent median values. The relationship between functional activity and antibody level for individual rats is shown (C). The coefficient of correlation and P-value is provided in the panel.

engage human Fcγ receptors. This is in agreement with the observation that cytophilic rat IgG2b binds with high affinity to the FcγRI and FcγRIIa-H131 allotype receptors on human monocytes [42]. These findings suggest that specific antibodies generated by immunising small rodent may mimic the ADCI-effect of naturally occurring human antibodies against GLURP [22]. Human clinical trials have further demonstrated that individuals immunised with GLURP.R0 based vaccines elicit specific antibodies which inhibit parasite growth in the ADCI assay [19,43].

A major strength of the R0.10C vaccine candidate with potentially synergistic benefits is the combination of a blood-stage with a sexual-stage component. Since gametocytes originate from asexual forms, reduction of asexual parasitaemia by anti-GLURP antibodies will plausibly reduce the prevalence and density of gametocytes in vaccinated individuals [5]. This may increase the efficiency of 10C induced antibodies to prevent transmission by the remaining gametocytes and potentiate transmission reduction. These combined effects will also decrease the turnover of parasites in the population thereby reducing selection and emergence of escape mutants [33]. Mathematical simulations further indicate that combining blood-stage vaccines with TBMV will lead to higher efficacy in preventing uncomplicated malaria episodes compared to the individual vaccine components and this is evident in all transmission settings [44].

Our results suggest a limited role for strain specificity of vaccine-induced immune responses. The limited antigenic variation observed in the included GLURP and *Pf*s48/45 regions facilitates the generation of broadly inhibiting antibodies as demonstrated by SMFA and ADCI assay using geographically and genetically diverse *Pf* isolates. Whether anti-R0.10C antibodies also inhibit parasite development in naturally infected mosquitoes and in children from geographically different regions of Africa obviously remains to be investigated.

Long-lived antibody responses are thought to be instrumental for acquisition of immunity against clinical malaria [45]. Likewise, effective interruption of the spread of the parasite in the population most likely requires high levels of TB antibodies lasting for at least one transmission season. Both *Pf*s48/45 and GLURP are abundantly expressed in the human host and acquired antibodies against both antigens are present in naturally exposed populations [10,46,47]. Thus, R0.10C induced responses will likely be boosted by natural infections resulting in sustained B-cell memory and effector responses.

In conclusion, we have in a proof-of-concept study, produced a recombinant fusion protein which elicits a bifunctional antibody profile against both asexual and transmission stages of *Pf*. This two-target strategy is conceptually attractive and will constitute a novel means towards control and eventually eradication of malaria once clinical efficacy has been demonstrated.

Conflicts of Interests

The authors report no conflicts of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.03.020.

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