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A *Plasmodium falciparum* GLURP–MSP3 chimeric protein; expression in *Lactococcus lactis*, immunogenicity and induction of biologically active antibodies

Michael Theisen[a,b,*], Soe Soe[c], Katja Brunstedt[a], Frank Follmann[b], Lars Bredmose[d], Hans Israelsen[d], Søren M. Madsen[d], Pierre Druilhe[c]

[a] *Department of Clinical Biochemistry, Statens Serum Institut, Copenhagen, Denmark*
[b] *Department of Infectious Disease Immunology, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen, Denmark*
[c] *Laboratoire de Parasitologie Médicale, Institut Pasteur, Paris, France*
[d] *Department of Lactic Acid Bacteria, Biotechnological Institute, Hørsholm, Denmark*

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Abstract

Plasmodium falciparum malaria is a major cause of morbidity and mortality worldwide. To evaluate the efficacy of a possible vaccine antigen against *P. falciparum* infection, a fusion protein, derived from *P. falciparum* Glutamate-rich protein (GLURP) genetically coupled to *P. falciparum* Merozoite surface protein 3 (MSP3) was produced in *Lactococcus lactis* as a secreted recombinant GLURP–MSP3 fusion protein. The hybrid protein was purified to homogeneity by ion exchange and hydrophobic-interaction chromatography and its composition was verified by MALDI MS, SDS/PAGE and Western blotting with antibodies against antigenic components of GLURP and MSP3. Mice immunized with the hybrid protein produced higher levels of both GLURP- and MSP3-specific antibodies than mice immunized with either GLURP, MSP3 or a mix of both. The protective potential of the hybrid protein was also demonstrated by in vitro parasite-growth inhibition of mouse anti-GLURP–MSP3 IgG antibodies in a monocyte-dependent manner. These results indicate that the GLURP–MSP3 hybrid could be a valuable strategy for future *P. falciparum* vaccine development.

Keywords: *Plasmodium falciparum*; Immunogenicity; Antibodies; *Lactococcus* expression

1. Introduction

The *Plasmodium falciparum* Glutamate-rich protein (GLURP) and the Merozoite surface protein 3 (MSP3) are both targeted by human IgG antibodies, which can inhibit parasite growth either in vitro in a monocyte-dependent manner [1,2] as well as in vivo by passive transfer in *P. falciparum*-humanized SCID mice [3]. The association of human antibodies against these antigens with clinical protection is also indicated by a number of immuno-epidemiological studies, which demonstrate that the levels of GLURP- and MSP3-specific cytophilic antibodies (IgG1 and IgG3) are significantly associated with a reduced risk of malaria attacks [4–6]. The major B-cell epitopes recognized by these human IgG antibodies have been localized to conserved sequences in the $GLURP_{27–489}$ and $MSP3_{212–257}$ regions, respectively [1,6–9].

Since vaccines based on GLURP and MSP3 aim at inducing the same type of immune responses, i.e. high levels of cytophilic antibodies, we decided to produce the respective $GLURP_{25–500}$ and $MSP3_{212–382}$ regions together as a recombinant hybrid-protein in *Lactococcus lactis*. This hybrid offers the possibility to investigate the vaccine potential of both antigens in single immunizations and can potentially increase the immunogenicity by combining a wider range of B and T helper cell epitopes. Different regions of these antigens have previously been produced in *Escherichia coli* fused to various affinity-tags [10–12]. Whereas such additional sequences are advantageous for purification they also pose a potential problem because host immune responses may be biased by foreign sequences. It is, therefore, desirable to explore expression systems, which aim to produce the recombinant protein without a vector-encoded affinity-tag. *L. lactis* was chosen as expression host because (i) it is a well characterized industrial microorganism, generally recognized as safe (GRAS), best known for its use in the production of fermented dairy products, (ii) it can be

* Corresponding author. Tel.: +45-32683734; fax: +45-32683035.
E-mail address: mth@ssi.dk (M. Theisen).

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grown in a defined synthetic medium, (iii) recombinant proteins are secreted into the culture-supernatant, from where they can be easily purified, and (iv) it does not produce toxic substances. In this study, we have used a novel gene expression system, which is based on the pH and growth phase regulated promoter, P170, from *L. lactis* [13–16]. This gene expression system offers a simple fermentation procedure, which has been developed specifically for the P170 promoter. Results obtained by expressing the hybrid GLURP–MSP3 in this system tend to support both the value of the vector for vaccine development and the potential of the combination of the two parasite proteins.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

E. coli DH10B (K-12, F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ* Δm15 Δ*lacX74 deoRrecA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ$^-$ *rpsL nupG*) (Life Technologies) containing the indicated plasmids was grown in Luria broth (LB) supplemented with erythromycin (200 μg/ml). *L. lactis* MG1363 [17] containing the indicated plasmids was grown in either M17 broth (Difco Ltd.) with 0.5% (w/v) glucose or an enhanced synthetic amino acid (SA) medium [18] supplemented with 1 μg/ml of erythromycin. Solidified LB or M17 media was supplemented with 200 or 1 μg/ml of erythromycin, respectively. The vector, pPSM1013 (Fig. 1), is a high-copy number expression plasmid based on the pAMβ1 replicon [19] containing unique restriction sites allowing the construction of in-frame fusions with an optimized secretion signal-peptide sequence, SP310mut2 [16]. The mRNA for the peptide is translated from a plasmid-encoded translational start site and transcribed from the pH and growth phase inducible *L. lactis* promoter, P170 [13–15]. There is essentially no transcription from the P170 promoter at pH values of 7 or more. However, the transcription is induced in the transition to stationary phase at pH values below 6.5. Plasmid pAMJ328 is derived from

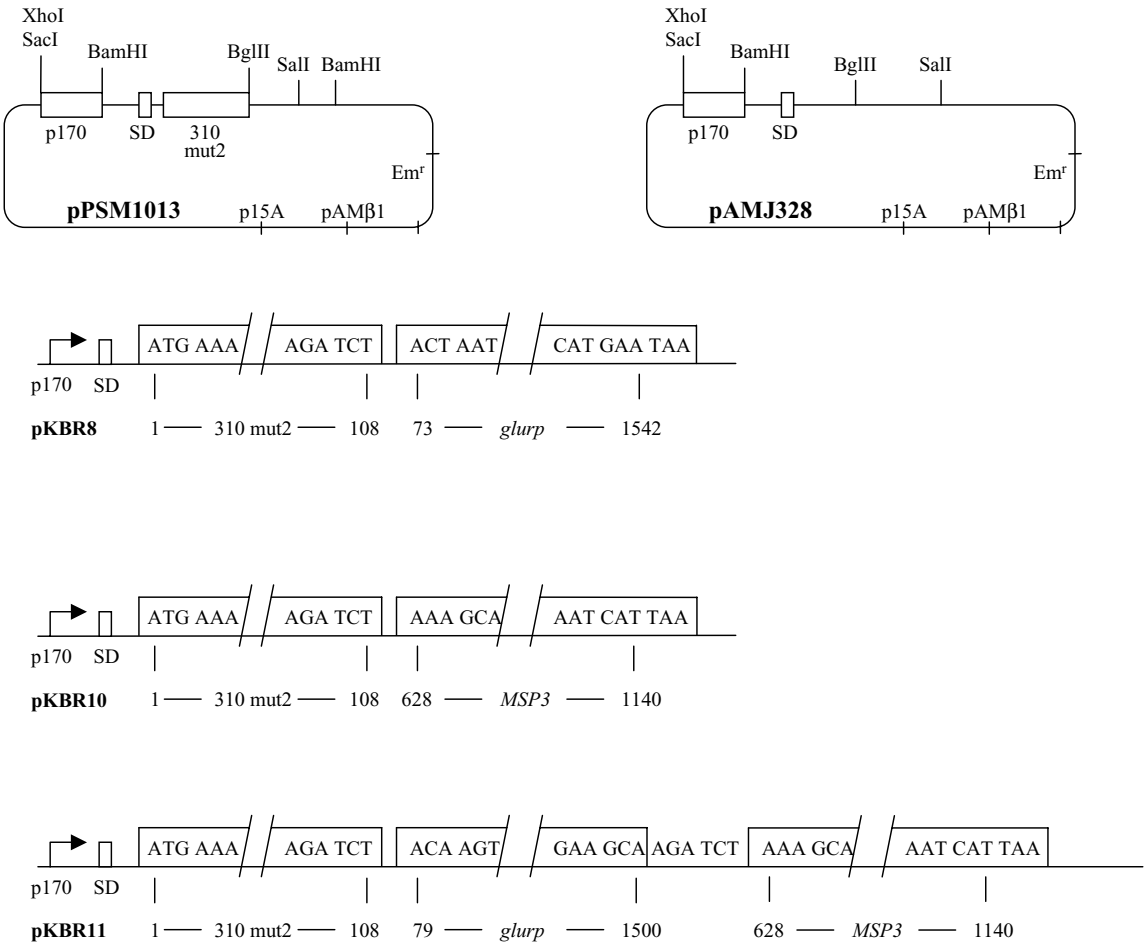

Fig. 1. Schematic representation pf pPSM1013 and pAMJ328 and the expression constructs used in *L. lactis*. The position of vector-encoded restriction sites mentioned in the text, promoter P170, Shine–Dalgarno sequence (SD), and 310mut2 signal peptide are indicated. The signal peptidase is predicted to cleave between amino acid nos. 32 and 33, thus leaving Ala-Glu residues in the N-terminal end of the mature recombinant proteins. The nucleotide numbering of *glurp* and *MSP3* was relative to A in the ATG codon of M59706 and L07944, respectively.

pPSM1013 by deleting all *lacZ* regulatory sequences to avoid transcription from the *lac* promoter and by creating a new cloning region devoid of the signal peptide [20].

2.2. Construction of plasmids expressing GLURP and MSP3 in L. lactis

All plasmids were constructed in *E. coli* DH10B and transformed into *L. lactis* MG1363 by electroporation as described [21]. All plasmid constructions were verified by DNA sequencing.

2.2.1. pMST73

The non-repeat region of FVO *glurp* was amplified with the primers 5′-CCC AGA TCT ACA AGT GAG AAT AGA AAT AAA C (nucleotides 79–100) (counting from A in the ATG start codon of M59706) and 5′-CCC AGA TCT TGC TTC ATG CTC GCT TTT TT CCG AT (nucleotides 1475–1500); digested with *Bgl*II, and the resulting DNA fragment was cloned into *Bgl*II digested pPSM1013.

2.2.2. pKBR5

pMST73 plasmid was digested with *Bam*HI and *Sal*I, and the resulting DNA fragment containing the *glurp* insert was cloned into *Bam*HI-*Sal*I digested pAMJ328.

2.2.3. pKBR7

The non-repeat region of F32 *glurp* was amplified with the primers 5′-AAG TAG ATC TAC TAA TAC AAG TGA GAA TAG AAA TAA AC (nucleotides 73–100), and 5′-GTT CAG ATC TTT ATT CAT GAT GGC CTT CTA GC (nucleotides 1519–1542); the resulting DNA fragment digested with *Bgl*II and cloned into *Bgl*II digested pPSM1013.

2.2.4. pKBR8

Plasmid pKBR7 was digested with *Bam*HI and *Sal*I, and the *glurp* insert was cloned into *Bam*HI-*Sal*I digested pAMJ328.

2.2.5. pKBR9

The C-terminal region of F32 *MSP3* was amplified with the primers 5′-CCC AGA TCT AAA GCA AAA GAA GCT TCT AGT TAT (nucleotides 628–651) and 5′-ATT AGA TCT CAT TTA ATG ATT TTT AAA ATA TTT GGA TA, (nucleotides 1118–1140) (counting from A in the ATG start codon of L07944); the resulting DNA fragment was digested with *Bgl*II and cloned into *Bgl*II digested pPSM1013. This MSP3 region is identical to that of the FC27 allele (accession number L07944) except for the following residues at variable positions in *MSP3*: 735 (T → C) and 948 (A → G).

2.2.6. pKBR10

Plasmid pKBR9 was digested with *Bam*HI and *Sal*I, and the *MSP3* insert was cloned into *Bam*HI-*Sal*I digested pAMJ328.

2.2.7. pKBR11

The *Bgl*II-fragment of pKBR9 was cloned into pKBR5 digested partially with *Bgl*II yielding an in-frame fusion between $glurp_{79–1500}$ and $MSP3_{628–1140}$. This hybrid molecule corresponds to the F32 allele except for the following residues at variable positions in GLURP: Leu-50, Asn-53, Glu-65, Asp-129, Glu-224, Pro-500.

2.3. Fermentation

Fermentation of *L. lactis* MG1363, containing plasmid pKBR8 (GLURP), pKBR10 (MSP3) or pKBR11 (GLURP–MSP3 hybrid), was carried out in 1 l enhanced synthetic medium supplemented with erythromycin (1 μg/ml) and yeast-extract (0.5%, w/v) in 2 l fermentors at 30 °C. The enhanced synthetic medium was based on the SA medium [18] and had the following composition: carbon sources: glucose 83.5 mM (1.5%, w/v), Na-acetate 14.7 mM. Salt concentrations: $CaCl_2$ 1.5 μM, $FeSO_4$ 30 μM, $MgCl_2$ 1.6 mM, $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ 9 nM, H_3BO_3 1.2 μM, $CoCl_2$ 90 nM, $CuSO_4$ 30 nM, $MnCl_2$ 240 nM, $ZnSO_4$ 30 nM, K_2SO_4 0.83 mM, KH_2PO_4/K_2HPO_4 10 mM. 0.3 mM citric acid was used as a complexing agent.

Vitamin concentrations (mg/l): Biotin, 0.3; folic acid, 3; riboflavin, 3; niacinamide, 3; thiamine·HCl, 3; Ca-pantothenate, 3; pyridoxal·HCl, 6. Amino acid concentrations (g/l): L-alanine, 0.9; L-arginine, 0.6; L-asparagine, 0.3; L-cysteine, 0.3; L-glutamic acid, 0.9; L-glutamine, 0.3; glycine, 0.6; L-histidine, 0.15; L-isoleucine, 0.3; L-leucine, 0.3; L-lysine, 0.6; L-methionine, 0.3; L-phenylalanine, 0.6; L-proline, 0.9; L-serine, 0.9; L-threonine, 0.6; L-tryptophan, 0.3; L-valine, 0.3.

The starting pH of the culture medium was adjusted to 7.4. Since *L. lactis* MG1363 produces lactic acid during the growth, pH is declining as cell density increases. After approximately 3 h of growth, pH was reduced to 6 and this level was maintained by a pH-controlled intake of 2 M KOH for another 8 h until the cell density was approximately $OD_{600} = 8$. A 50% glucose solution was added in parallel with the base since this tends to increase the bacterial yield. Bacterial cells were removed from the culture-medium (containing exported protein) by ultrafiltration with a Pellicon 2 Durapore filter (PVDF, 0.22 μm, 0.1 m^2) (Millipore). Culture-filtrates were either used immediately or stored at −20 °C.

2.4. Purification of recombinant proteins

Cell-free culture-supernatants were concentrated on a Millipore Labscale™ TFF System installed with a Pellicon XL Biomax 8 filter (Polypropylene-membrane, 50,000 Da, 50 cm^2) and concentrates were buffer exchanged to 20 mM bis–tris (pH 6.4) on a Sephadex G-25 column (C26/40, 170 ml). Recombinant proteins were first purified on a 5 ml HiTrap Q Sepharose High Performance (Phamacia Biotech) column by applying a gradient of 0–1 M NaCl in column

buffer at a flow-rate of 1 ml/min. Fractions (2 ml) containing the desired recombinant protein were pooled and dialyzed against 20 mM bis–tris (pH 6.4) and applied to a 5 ml HiTrap SP Sepharose High Performance (Phamacia Biotech) column. The recombinant protein was eluted by a gradient of 0–1 M NaCl in column buffer. Fractions (2 ml) containing the desired recombinant protein was pooled and adjusted to 1 M $(NH_4)_2SO_4$ and further purified on a 5 ml Phenyl Sepharose High Performance (Phamacia Biotech) by applying a gradient of 1–0 M $(NH_4)_2SO_4$ in 20 mM bis–tris (pH 6.4) at a flow-rate of 1 ml/min. GLURP and MSP3 were eluted in single peaks whereas the hybrid was eluted in two peaks. Analysis of all fractions was performed by SDS-PAGE. Protein concentrations were measured by the BCA™ protein assay (Pierce, Rockford, Illinois, USA).

2.5. Immunization and purification of mouse IgG

Thirty BALBc/CF1 [22] female mice (7–10 weeks of age) were randomly assigned to three groups. Two groups were immunized with 20 μg of $GLURP_{27–500}$-$MSP3_{212–380}$ hybrid (gr7), or with a mixture of 15 μg $GLURP_{25–512}$ and 5 μg $MSP3_{212–380}$ (gr8) by subcutaneous injections at the base of the tail, respectively; and the third group (gr9) received 15 μg $GLURP_{25–512}$ injected at the base of the tail and 5 μg $MSP3_{212–380}$ injected in the shoulder. All immunogens were emulsified in Montanide ISA720 and each mouse received three injections at 2-week intervals and was bleed on days 0, 14, 28 and 35. Total IgG was purified by $(NH_4)_2SO_4$ precipitation and subsequent purification on DEAE-columns from pooled serum samples taken on day 35 from animals in group gr7 and from pooled day 0 samples. Similarly, IgG was also purified from BALBc/CF1 mice immunized with the individual GLURP and MSP3 recombinant proteins.

2.6. ELISA and serum samples

Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described in detail [10]. The coating concentrations of $GLURP_{25–512}$, $MSP3_{212–380}$, and $GLURP_{27–500}$-$MSP3_{212–380}$ were 0.5, 1.0 and 0.5 μg/ml, respectively. Serial dilutions of plasma from Liberian adults clinically immune to malaria, Danish donors never exposed to malaria [7], and mice were tested on ELISA plates coated with either antigen and the absorbance values were plotted as a function of the reciprocal dilution of plasma samples. The antibody titer was defined as the plasma dilution, which gives an absorbance value of $A_{492} = 1.00$ in the parallel portion of the curves. Heavy and light chains of mouse IgG were determined using Mouse MonoAb ID/SP kit (Zymed) according to the manufacturer's instructions. Sera were diluted 1:200, and plates were washed extensively with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) between each incubation step.

2.7. Competitive ELISA assays

Recombinant $GLURP_{25–518}$ and $MSP3_{212–380}$ and a mixture of these two antigens were added at various concentrations (3.2×10^{-5} to 100 μg/ml) to a pool of plasma from mice immunized with the GLURP–MSP3 hybrid diluted in 1.25% (w/v) milk powder in PBS. The plasma dilution used was adjusted to give an absorbance (A_{492}) of approximately 2.50. The antigen–antibody mixtures were incubated overnight at 4 °C and subsequently the reactivity to GLURP–MSP3 hybrid coated ELISA plates was determined.

2.8. Indirect immunofluorescent antibody (IFA) test

IFA was performed as reported earlier [23]. Briefly, a thin film of RBCs containing predominantly schizonts stages of *P. falciparum* NF54 were incubated with serial dilutions of purified mouse IgG in phosphate buffered saline (PBS pH 7.4) for 30 min at 37 °C in a humid chamber. After washing with PBS, mouse antibodies were revealed with Alexa Fluor conjugated goat anti-mouse IgG (Molecular probe, USA) diluted 1:300 in PBS. After washing the slide was examined under UV light. The endpoint titre was the highest dilution of the antibodies, which produce visible specific immunofluorescence.

2.9. Direct inhibition (DI) and antibody-dependent cellular inhibition (ADCI) assays

Peripheral blood mononuclear cells were isolated from healthy European blood donors without previous exposure to malaria, by density gradient separation on J PREP (Tech-Gen) and subsequently added to 96 wells flat-bottom culture plates (TPP, Switzerland). Each well was washed three times with RPMI, thereby separating non-adherent mononuclear cells from the attached monocytes (2×10^5 monocytes per well). Mature schizonts from fast-growing synchronized in vitro culture of *P. falciparum* NF54 were separated by floatation over 50% Plasmagel, diluted with fresh erythrocytes to a final haematocrit of 2% and a parasitemia of 0.5%, and then added to each well. Purified mouse IgG was dialyzed for 48 h against RPMI and added to the wells at three different concentrations. The final volume in each well was adjusted to 100 μl with RPMI supplemented with 0.5% Albumax. Similar cultures without monocytes were performed in parallel to assess the DI. In all experiments the following controls were run simultaneously on each plate: (i) normal mouse IgG (NIG) from non-immunized mice to assess the non-specific inhibitory components introduced during IgG purification, (ii) human monocytes without mouse IgG to assess the non-specific inhibitory effect of the monocytes, (iii) immunized mouse IgG without human monocytes to assess the direct inhibitory effect of antibodies, (iv) purified IgG from hyperimmune African adults [23] as a positive control, and (v) purified IgG from a pool of serum samples

taken from the 30 animals before the first immunization as a negative control. After 48 and 72 h of growth, respectively, 50 μl of RPMI supplemented with 0.5% albumax (GIBCO), 100 U of penicillin and streptomycin per ml was added per well. After 96 h of growth, parasitemia was determined by microscopic counting of more than 50,000 RBCs on Giemsa stained film. The specific growth inhibitory (SGI) index was calculated as follows: 1−((percent parasitemia with monocytes and test antibodies)/(percent parasitemia with test antibodies/percent parasitemia with monocytes and NIG/percent parasitemia with NIG)) × 100.

The direct inhibition of purified IgG (DI%) on parasite growth, i.e. in the absence of monocytes, was calculated as ((parasitemia of culture with neither antibody nor monocyte) − (parasitemia of test IgG without monocyte))/(parasitemia of culture with neither antibody nor monocyte) × 100.

2.10. RP-HPLC analysis of GLURP and GLURP–MSP3

Samples were analyzed on a HPLC system (Phamacia, Sweden), using a Protein C4 column (VYDAC®, 214TP54, USA) in a acetonitrile:H_2O:TFA buffer system. Purified samples were diluted 1:2 in A-buffer (H_2O + 0.1% (w/v) TFA), applied to the column, and bound material was eluted with a linear gradient (0–80%) of B-buffer (80% acetonitrile + 0.1% (w/v) TFA) over 20 column volumes. Elution was monitored by absorption at 214 nm. Peaks were collected, vacuum dried on a HetoVac (Heto, Denmark) and kept at 4 °C until use.

2.11. MALDI-TOF MS and electrospray mass spectrometry

Samples for peptide mass mapping were cut out of a coomassie stained SDS-PAGE gel. Half a band (approx. 1 μg protein) was washed, dried, reduced and alkylated with iodoacetamide before being digested overnight by modified trypsin (Promega, USA), essentially as described [24]. The supernatant of the digest was applied to GELoader tips (Eppendorff, Germany) packed with Poros 20 R2 reversed phase material (PerSeptive, USA) and eluted with 0.8 μl of α-cyanohydroxycinnamic acid (20 μg/μl in 70% acetonitrile/30% water) directly onto the MALDI target [25]. Analysis was carried out on a PerSeptive Voyager STR (PerSeptive, USA) operated in the reflector mode and the results were analyzed in GPMAW ver. 5.02 (Lighthouse data, Denmark). Electrospray mass spectrometry (ES-MS) of the intact protein was carried out on a Micromass QTOF (Micromass, UK) using a nanospray source.

3. Results

3.1. Expression of glurp and MSP3 in L. lactis

PCR fragments encoding the *glurp*$_{79–1500}$ and *MSP3*$_{628–1140}$ regions were cloned side by side thereby creating an in-frame fusion between a vector-encoded signal-peptide and a GLURP$_{27–500}$-MSP3$_{212–380}$ fusion protein (pKBR11, Fig. 1). This hybrid protein contains two additional amino acid residues created by joining these *glurp* and *MSP3* fragments. The plasmid was transformed into *L. lactis* MG1363 and the resulting strain secreted the GLURP–MSP3 hybrid protein into the culture-supernatant from where it was purified by sequential ion exchange on HiTrap Q and SP Sepharose columns followed by hydrophobic-interaction chromatography on Phenyl Sepharose. Although these purification steps allowed us to remove most of the unwanted *L. lactis* proteins, it also became evident that the GLURP–MSP3 hybrid protein eluted as two distinct peaks from the Phenyl Sepharose column. Subsequent SDS-PAGE showed that these peaks contain protein band of approximately 140 and 143 kDa, respectively (Fig. 2A). When analyzed by immunoblotting both products were specifically recognized by antibodies to GLURP and MSP3, respectively, suggesting that peak 1 may result from incomplete translation of the mRNA and/or from protease cleavage of the primary protein product in peak 2. Both bands were excised for sequence identification using mass spectrometry. The sequence of a total of 16 tryptic fragments derived from peak 2 matched the GLURP and MSP3 sequences listed in Fig. 2B, showing that the recombinant protein corresponds to full-length GLURP$_{27–500}$-MSP3$_{212–380}$ fusion protein, whereas the sequences of 28 tryptic fragments from peak 1 suggests that this recombinant protein is derived from the full-length hybrid protein by proteolytic cleavage in the C-terminal region. The total mass of peak 1 was 67,688 ± 70 Da as determined by MALDI MS suggesting that cleavage occur around residue 586 in the MSP3 region of the hybrid protein (Fig. 2B). The molecular mass of full-length GLURP$_{27–500}$-MSP3$_{212–380}$ was 74,950 ± 20 Da as determined by ES-MS. Assuming that the recombinant protein contain the vector-encoded amino acid residues A-E-R-S at the N-terminal end (Fig. 1), this molecular weight corresponds well to the predicted value of 74939. Thus, the GLURP–MSP3 hybrid protein in peak 2 is intact and contains the amino acid residues predicted from the nucleotide sequence. Subsequent reverse-phase chromatography further showed that the full-length (peak 2) and truncated (peak 1) hybrid proteins eluted with different retention-time from a C4 column (Fig. 2C).

For comparison, the individual *glurp*$_{73–1542}$ and *MSP3*$_{628–1140}$ fragments were also cloned (Fig. 1, pKBR8 and pKBR10) and the resulting recombinant proteins were purified to homogeneity by ion exchange (Fig. 2D, lanes 2 and 3). In accordance with previous observations [10], recombinant GLURP fragments migrate with relative molecular masses of approximately twice the calculated molecular masses. The estimated yields of secreted recombinant proteins are around 30 mg/l for GLURP and GLURP–MSP3 and around 2 mg/l for MSP3.

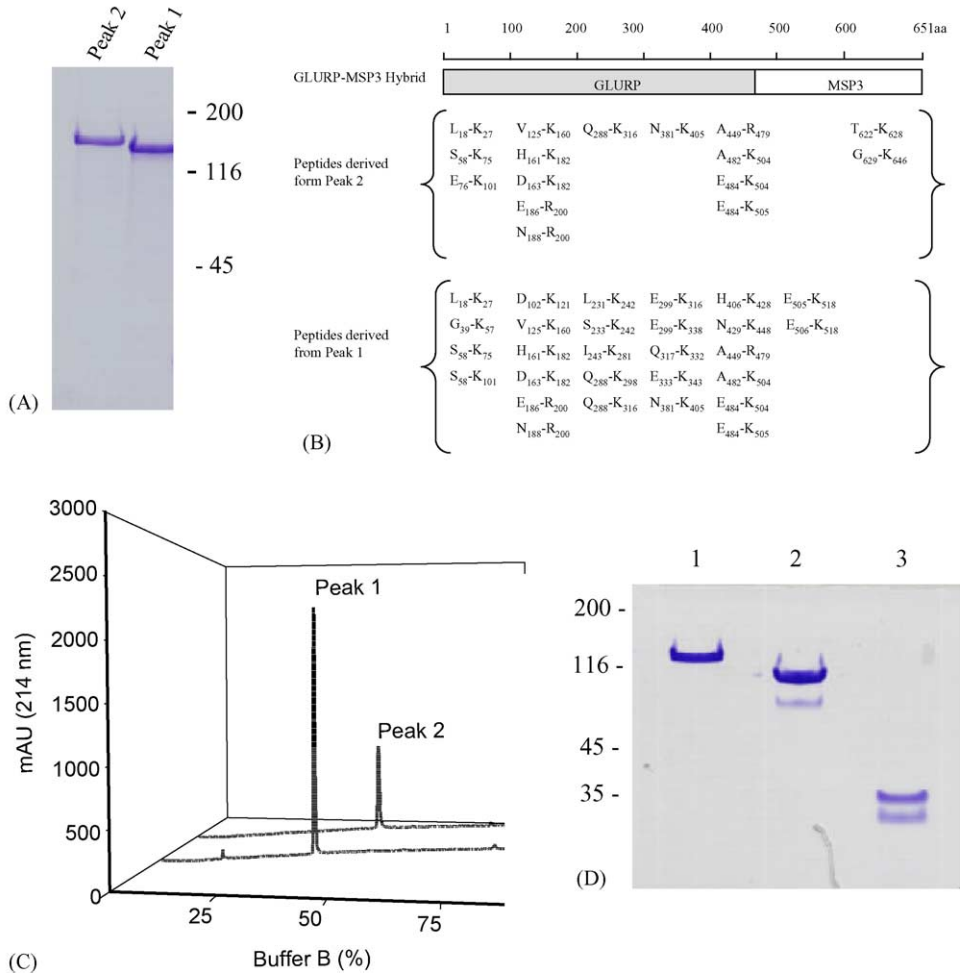

Fig. 2. Purification and sequence verification of the GLURP–MSP3 hybrid protein. (A) Coomassie blue-stained 12.5% polyacrylamide gel of purified GLURP–MSP3 fusion protein in peak 2 and peak 1 from the final Phenyl Sepharose High Performance column. The sizes (kDa) of the molecular mass markers are indicated. (B) List of tryptic peptides that match the GLURP–MSP3 hybrid protein. Amino acid residues are represented using the single letter codes and numbered according their positions in the deduced hybrid protein sequence. The molecular mass of peak 1 was 67,688 ± 70 Da as determined by MALDI MS. (C) HPLC analysis on a C4 column of the GLURP–MSP3 hybrid protein in peaks 1 and 2, respectively. (D) Coomassie blue-stained 12.5% polyacrylamide gel of purified GLURP–MSP3 fusion protein (lane 1), $GLURP_{25–514}$ (lane 2), and $MSP3_{212–380}$ (lane 3) produced in *L. lactis* MG1363. The sizes (kDa) of the molecular mass markers are indicated.

3.2. Antigenicity of recombinant GLURP and MSP3 products

The antigenicity of the recombinant proteins was evaluated by ELISA against IgG antibodies from 71 adult Liberians clinically immune to malaria (Fig. 3). Serial dilutions of all plasma samples were tested on separate plates coated with each recombinant protein and the antigen-specific titer was determined as the dilution giving an absorbance of 1.00. As expected, different plasma contained different amounts of GLURP and MSP3-specific IgG antibodies (Fig. 3A). In general, hybrid-specific antibody titers exceeded those recorded with the individual GLURP and MSP3 antigens (Fig. 3B and C) suggesting that the hybrid molecule provides an adequate presentation of GLURP and MSP3 antigenic determinants, respectively.

3.3. Immunogenicity of recombinant GLURP and MSP3 products

To determine whether the GLURP–MSP3 hybrid molecule is a superior immunogen compared to a mixture of the individual $GLURP_{25–514}$ and $MSP3_{212–380}$ molecules, groups of BALBc/CF1 mice were each immunized subcutaneously with the hybrid molecule in Montanide or with the individual $GLURP_{25–514}$ and $MSP3_{212–380}$ proteins combined in either one syringe or injected separately at two different sites. Sera collected 35 days after the first injection, were tested for IgG antibody reactivity against GLURP and MSP3, respectively. While the mean GLURP–ELISA titer is only marginally higher in the hybrid group than in the other two groups, mean MSP3–ELISA titer is 4.3-fold higher (Kruskal Wallis test, $P < 0.004$) in the group re-

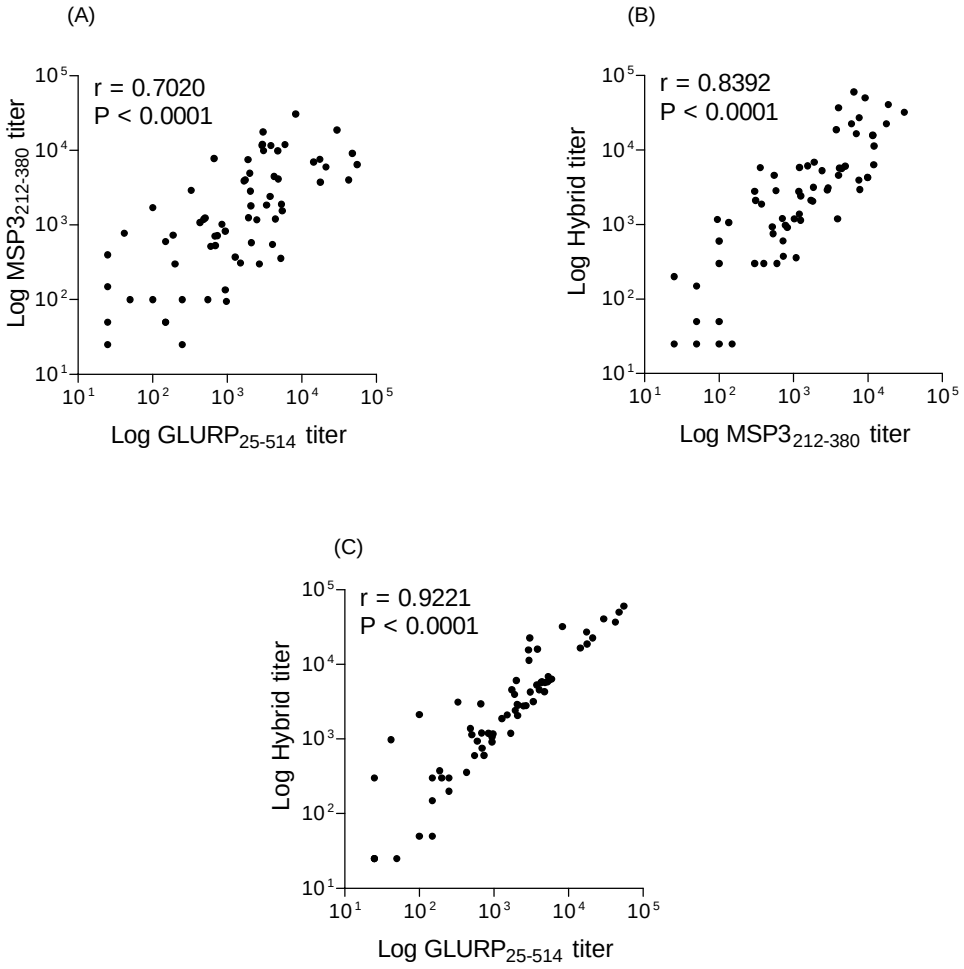

Fig. 3. Patterns of IgG antibody responses to pairs of GLURP and MSP3 derived antigens in 71 plasma samples from adult Liberians clinically immune to malaria. The coefficient of correlation and *P*-value are provided in each panel.

ceiving the hybrid compared to the group receiving both $MSP3_{212–380}$ and $GLURP_{25–514}$ at two different sites (compare gr7 and gr9 in Fig. 4A). At the individual level, mice immunized with the hybrid reacted strongly with both GLURP and MSP3 domains whereas mice immunized with a combination of two molecules tended to mount a predominant antibody response against either GLURP or MSP3. The anti-hybrid IgG antibodies are mainly directed against the GLURP-derived P3, P4, P11, and S3 peptides containing known epitopes for human antibodies [7]; however peptides P5 and P9 which do not contain such epitopes were also recognized (Fig. 4B). Whereas the GLURP and MSP3-specific IgG subclass profiles are similar for all vaccine formulations (Fig. 4C), GLURP-specific IgG antibodies use preferentially the Kappa light chain and MSP3-specific IgG antibodies preferentially the Lambda light chain (data not shown). This difference in light chain was found for all GLURP or MSP3-specific antibodies whether raised against the hybrid or the mixtures of the individual molecules.

The specificity of mouse antibodies to the hybrid was also analyzed by competition-ELISA (Fig. 5). It appears that antibodies to the hybrid are purely GLURP and MSP3-specific, since a mixture of soluble $GLURP_{25–514}$ and $MSP3_{212–380}$ could completely inhibit the binding of anti-hybrid antibodies to immobilized $GLURP_{27–500}$-$MSP3_{212–380}$. Thus, the construction of a GLURP–MSP3 hybrid molecule has not led to the creation of new B-cell epitopes in the overlapping area.

3.4. Reactivity of mouse anti-GLURP and anti-MSP3 sera with native GLURP and MSP3

The immunogenicity of the recombinant GLURP and MSP3 was also investigated by immunoblotting of parasite-derived proteins with sera from mice immunized with each of the three recombinant proteins, hybrid, $GLURP_{25–514}$ and $MSP3_{212–380}$, respectively. As demonstrated in Fig. 6, plasma from mice immunized with $GLURP_{25–514}$, $MSP3_{212–380}$, and the hybrid recognized

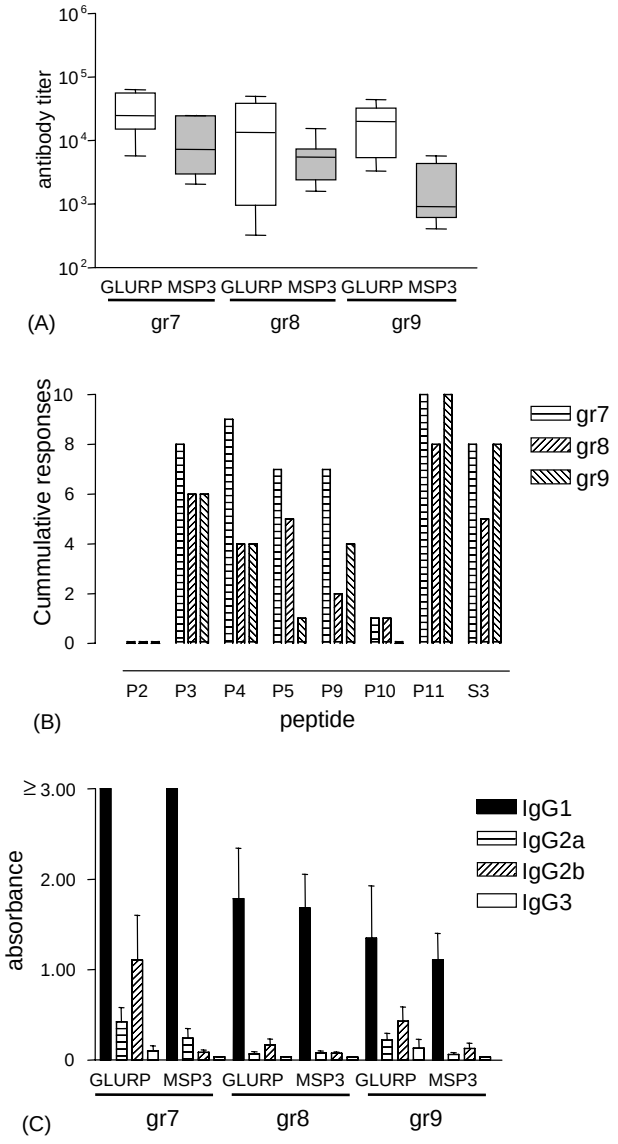

Fig. 4. Antibody responses in mice. Groups of 10 mice were immunized with the hybrid (gr7), a mixture of GLURP and MSP3 in one syringe (gr8), or with GLURP and MSP3 in separate syringes at different sites (gr9). (A) Day 35 plasma samples were tested for antibody reactivity on ELISA plates coated with $GLURP_{25–514}$ or $MSP3_{212–3}$. Box plots show medians, 25th, and 75th percentiles and whiskers show the range of the data. (B) Cumulative responses of mouse sera with eight peptides representing GLURP B-cell epitopes [7] and (C) isotype response of mice for which results are presented in panel (A). Each vertical bar represents the mean absorbance (±S.E.M.) in GLURP- and MSP3-specific ELISAs.

polypeptides of approximately 220,000 Da (lane 1), 48,000 Da (lane 2), and both (lane 3), the apparent molecular masses previously found for GLURP [10] and MSP3 [1] by SDS-PAGE, respectively.

3.5. Biological effect of the hybrid-induced antibodies

Since affinity-purified human antibodies to GLURP and MSP3 can inhibit parasite growth in vitro in cooperation with human monocytes [1,2,7] it was investigated whether anti-hybrid mouse antibodies had a similar effect. Total IgG was isolated from groups of mice, which had received the hybrid (gr7), or the individual $GLURP_{25–514}$ and $MSP3_{212–380}$ molecules, respectively. The three IgG solutions were adjusted to the same IFA end-point, i.e. the same degree of reactivity to parasite proteins to provide comparative data, and their effect on parasite growth was determined. The three IgG suspensions all exerted a strong inhibitory effect in the presence of normal human monocytes whereas they only had a non-significant effect in the absence of monocytes (Table 1). The pre-bleed did not promote inhibition of parasite growth indicating that the observed ADCI-effects are due to the presence of specific antibodies to GLURP and MSP3 (Table 1). There was no correlation between inhibitory effect and the IgG concentration (Table 1).

4. Discussion

Lactic acid bacteria have a long history of use in the production of fermented foods. However, their use in the pharmaceutical field as production hosts for recombinant proteins or as live delivery vehicles for administration of vaccines has only been initiated recently. The testing of new vaccines and therapeutics continuously poses a challenge to the development of suitable gene expression systems for heterologous protein production. The *L. lactis* P170-based gene expression systems employed here has proved ideal for small-scale production of malaria antigens because: (i) the products are biological active without undesired modifications, (ii) the fermentation process is well established, (iii) the recombinant products are secreted, (iv) it offers a simple purification process with little protein degradation, and (v) production yields are acceptable, as compared to other vectors. The GLURP and MSP3 molecules have previously been expressed separately in *E. coli* with and without various affinity-tags [10–12,26], however, these products were considered far less suitable for testing in human beings because host immune responses against the affinity-tag could occur and impede repeated immunizations. In addition, at least GLURP-R0 was highly unstable when produced without a fusion partner in *E. coli* (unpublished data). In our experience the His-tag can also change the physical properties of the recombinant protein, making it less soluble in aqueous solution than the untagged version.

In the present study, we constructed a chimeric malaria protein containing the 5′-end of *P. falciparum glurp* fused in-frame to the 3′-end of *P. falciparum MSP3* as well as the individual GLURP and MSP3 domains in order to identify the main antigenic determinants on the hybrid protein. Each of these constructs gave rise to a major dominant full-length product and a lower-molecular mass band, which corresponds to degradation products. *L. lactis* contains a surface associated housekeeping protease, HtrA, which might be responsible for these smaller molecular-mass products.

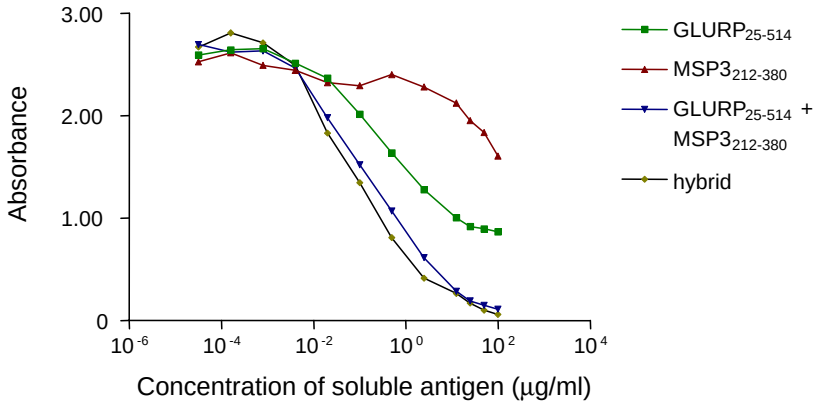

Fig. 5. The hybrid contains only GLURP and MSP3 derived B-cell epitopes. A pool of plasma from mice immunized with the hybrid was pre-incubated with GLURP, MSP3, a mixture of GLURP and MSP3 or the hybrid at the indicated concentrations before being added to ELISA coated with the hybrid. Prior incubation with a mixture of GLURP and MSP3 or the hybrid completely inhibited Ig antibody binding to the hybrid.

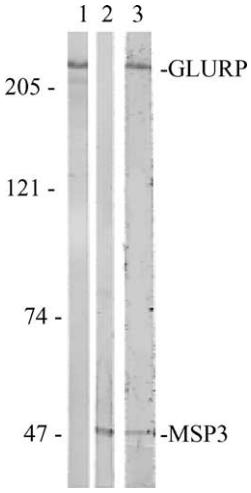

Fig. 6. Immunoblot analysis of *P. falciparum* NF54. A whole cell extract was separated on a 7.5% polyacrylamide gel and subjected to immunoblotting with plasma from mice immunized with $GLURP_{25–514}$ (lane 1), $MSP3_{212–380}$ (lane 2) and GLURP–MSP3 hybrid (lane 3). The sizes (kDa) of the molecular mass markers are indicated.

Inactivation of the *htrA* gene leads to stabilization of several recombinant proteins produced and secreted by *L. lactis* [27,28]. This might also be the case in this study and further investigations should include expression studies of the malaria antigens in an *htrA* mutant strain.

In *L. lactis* the nascent recombinant proteins should be cleaved by leader peptidase I since they contain the predicted cleavage sequence Gln-Ala_Ala-Glu (http://www.cbs.dtu.dk/services/SignalP/) thereby leaving the Ala-Glu amino acid residues attached to the N-terminal end of the mature protein. This prediction was confirmed by the excellent agreement between the theoretical and experimental molecular weights of the hybrid (74,939 Da versus 74,950 ± 20 Da). In addition to the vector-encoded amino acids at the N-terminal end, the hybrid also contains two residues (Arg-Ser) in the fusion junction between GLURP and MSP3. These amino acids, however, did not form part of a novel B-cell epitope in the overlapping area since anti-hybrid antibodies generated in outbreed mice were exclusively directed against antigenic determinants in the respective GLURP and MSP3 regions.

Table 1
Specific growth inhibition (SGI) index and direct growth inhibition (DI) of total IgG purified from serum pools of mice immunized with $GLURP_{27–500}$-$MSP3_{212–380}$ or the individual $GLURP_{25–514}$ and $MSP3_{212–380}$ molecules, respectively

IgG from mice immunized with	IFA titer in assay	IgG concentration in assay (μg/ml)	SGI (%) ± S.D.	DI (%) ± S.D.
Hybrid	30	87	58 ± 3	0
	20	58	64 ± 5	0
	10	29	63 ± 10	0
GLURP	20	55	63 ± 5	0
	10	28	37 ± 3	0
MSP3	20	103	57 ± 4	0
	10	51	58 ± 5	0
Pre-bleed	<5	110	20 ± 10	10 ± 5
	0	55	8 ± 3	10 ± 5

The non-specific growth inhibitory effect of mouse IgG was measured with total IgG purified from the pooled pre-bleeds of all animals for comparison. Each IgG was tested in three different concentrations adjusted according to their IFA titer (anti-hybrid (gr7), 24,300; anti-GLURP, 1300; anti-MSP3, 700). The corresponding IgG concentration is indicated for each assay. SGI and DI values are the results of three independent assays.

The immunogenicity of the hybrid was studied in mice with Montanide used as the adjuvant since Montanide was used in recent clinical trials with long synthetic peptides derived from GLURP and MSP3, respectively. Immunizations with the protein–hybrid consistently generated a stronger antibody response against the individual GLURP and MSP3 domains than any other combination of the two molecules, thereby validating the value of the approach. The difference was most pronounced for the MSP3-specific antibody response suggesting that T cell epitopes located in the GLURP region provide help for B-cell epitopes in the MSP3 region. When the animals were injected with a mixture of the two molecules in one syringe, individual mice tended to mount a predominant antibody response against either GLURP or MSP3. In some animals GLURP was immuno-dominant whereas in other animals it was MSP3. This phenomenon was not observed, when GLURP was injected in one part and MSP3 in another part of the body suggesting that the concomitant presentation of a mixture of individual GLURP and MSP3 proteins to the same antigen-presenting cells may lead to competition. Whichever the underlying mechanism, our data supports the conclusion that the hybrid molecule provides a superior presentation of GLURP and MSP3 antigenic determinants compared to the individual molecules. This conclusion is also in agreement with the observation that the hybrid was more effectively recognized by naturally occurring IgG antibodies in clinically immune African adults than the individual antigens. In fact mice immunized with GLURP alone or fused to MSP3 induced a GLURP-specific antibody response of the same magnitude as that found in naturally exposed individuals (compare Figs. 3A and 4A), suggesting that it is possible to induce the same level of anti-GLURP antibodies by immunization as that found in clinically immune individuals.

Antibodies raised against the hybrid reacted strongly with native parasite proteins by IFA and recognized parasite-expressed GLURP and MSP3 by immunoblotting analysis. The hybrid-specific antibodies did not inhibit parasite growth alone, however, when allowed to collaborate with human monocytes they proved strongly inhibitory in several independent ADCI assays. While there are many examples of mouse antibodies which can interfere with Merozoite invasion of red blood cells [29–34] this study is the first report of a mouse IgG preparation, which act synergistically with human monocytes to inhibit *P. falciparum* parasite growth in vitro. The human FcγIIa receptor is believed to be the primary trigger molecule involved in ADCI [35]. This receptor exists in two different alloforms which are known to be polymorphic with respect to the interaction with mouse IgG1 [36]. The GLURP- and MSP3-specific mouse antibodies were mainly of the IgG1 and IgG2b isotypes, two antibodies which bind well to the most prevalent FcγIIa receptor with arginine at position 131 [37]. This provides pre-clinical indications that hybrid-specific antibodies generated by immunization mimic the ADCI-effect of naturally occurring human antibodies against GLURP [2] and MSP3 [1], respectively.

The value of the hybrid strategy in humans was recently strengthened by immunoepidemiological studies in an Asian setting. Besides confirming the strong statistical association between protection and both anti-GLURP and anti-MSP3 antibody responses, it further showed that in those rare individuals not responding to either antigen, a response to the other antigen was always present and associated with protection (Soe et al., unpublished data). Hence, when presented by the parasite, the antigenicity of the two molecules constituting the hybrid is complementary.

In conclusion, we report a GLURP–MSP3 hybrid molecule, which (i) is produced in a novel expression system as a secreted protein, (ii) is more immunogenic in experimental models than the individual GLURP and MSP3 domains, and (iii) can induce specific antibodies in mice which inhibit parasite growth in collaboration with human monocytes. This new hybrid protein, therefore, offers the possibility to investigate the vaccine potential of GLURP and MSP3 in a single clinical trial.

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