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Genetic linkage of autologous T cell epitopes in a chimeric recombinant construct improves anti-parasite and anti-disease protective effect of a malaria vaccine candidate

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Abstract

We have reported the design of polyvalent synthetic and recombinant chimeras that include promiscuous T cell epitopes as a viable delivery system for pre-erythrocytic subunit malaria vaccines. To further assess the ability of several *Plasmodium* T cell epitopes to enhance vaccine potency, we designed a synthetic gene encoding four *P. yoelii* merozoite surface protein 1 (PyMSP1) CD4⁺ promiscuous T cell epitopes fused in tandem to the homologous carboxyl terminal PyMSP1₁₉ fragment. This Recombinant Modular Chimera (PyRMC-MSP1₁₉) was tested for immunogenicity and protective efficacy in comparative experiments with a recombinant protein expressing only the PyMSP1₁₉ fragment. Both proteins induced comparable antibody responses. However PyRMC-MSP1₁₉ elicited higher anti-parasite antibody titers and more robust protection against both hyper-parasitemia and malarial anemia. Most importantly, passive transfer of anti-PyRMC-MSP1₁₉, but not anti-PyMSP1₁₉ antibodies protected against heterologous challenge. These studies show that protective efficacy can be significantly improved by inclusion of an array of autologous promiscuous T cell epitopes in vaccine constructs.

Keywords

Malaria; chimeric vaccine; T cell epitopes

1. Introduction

Malaria is endemic in more than a hundred countries and territories around the world and it remains a leading cause of mortality in children under five years of age. The implementation of new tools, including long-lasting insecticide-treated nets and artemisinin-based combination therapies, can have a significant impact on malaria transmission. However, the estimated number of clinical cases of malaria worldwide is over 200 million annually [1]. Effective

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vaccines would be a great asset to help control this disease [2]. Ongoing efforts in the search of an effective vaccine have faced several biological constraints typified by the complexity of the parasite antigenic profile and the common occurrence of parasite evasion mechanisms. Nevertheless, immuno-epidemiological data suggest that induction of protective immunity against malaria is technically feasible [3]. Recent reports of the safety and efficacy of the RTS,S vaccine in extended clinical trials [4,5] and its potential for administration using the Expanded Program on Immunization (EPI) schedule [6] have revitalized enthusiasm for developing a second generation of multistage malaria vaccines.

The intricate and diverse antigenic repertoire of malaria parasites is a biological feature that supports the rationale for developing multi-stage vaccines. In proof of principle studies, we have reported the efficacy of synthetic linear peptide chimeras and recombinant subunit chimeras to deliver protective *Plasmodium* immunogens [7–9]. A key component of our vaccine strategy is the inclusion of *Plasmodium* CD4⁺ promiscuous T cell epitopes [7]. The inclusion of homologous promiscuous CD4⁺ T cell epitopes in chimeric vaccines is intended to take advantage of the boosting effect provided by natural parasite exposure in malaria endemic areas. Our first generation of polypeptide chimeras was designed to include linear sequences representing well characterized pre-erythrocytic B cell epitopes [7,9]. Yet structural analyses of several erythrocytic stage vaccine candidates have revealed that protective antibodies predominantly recognize functional domains that exhibit complex tertiary structure [10–15]. To confirm that our strategy to improve the immunogenicity of malaria vaccine candidates can also be used for non-linear structured domains, we designed a *P. yoelii* chimeric recombinant protein comprising autologous promiscuous T cell epitopes assembled in tandem and linked to the carboxyl terminal domain of the merozoite surface protein 1 (PyMSP1).

Merozoite surface protein 1 (MSP1) is considered a leading vaccine candidate [16,17]. It is synthesized as a 190–250-kDa precursor during schizogony and processed during schizogonic development into smaller proteins by two different proteolytic cleavage processes [18–22]. At the time of the erythrocyte invasion, most of the protein complex is shed from the parasite leaving only a 19 kDa carboxyl terminal fragment (MSP1₁₉) anchored to the parasite surface through a glycosyl-phosphatidyl-inositol moiety [20]. The proteolytic cleavage of the large precursor is essential for parasite invasion of erythrocytes [21,23]. Furthermore, antibodies elicited against *Plasmodium falciparum* MSP1₁₉ (PfMSP1₁₉) are able to inhibit both erythrocyte invasion and secondary proteolysis [23]. Importantly, protection against malaria can be induced by immunization with the PyMSP1₁₉ fragment expressed as a fusion protein with glutathione-S-transferase (GST) but not with a DNA vaccine encoding such fusion protein [24–29]. Extensive experimental evidence indicates that protection induced by immunization with PyMSP1 is correlated with the ability to induce a robust antibody response that targets MSP1₁₉ [28,30–32].

T cell reactivity to MSP1, elicited by natural exposure to malaria parasites, is directed toward epitopes located in the amino terminal portion of the protein [33–35]. The compact tertiary structure of the MSP1₁₉, which is maintained by several disulfide bridges [22,36,37], restricts antigen processing and impairs T cell responses to this fragment [38]. Reduction and alkylation of disulfide bonds within recombinant MSP1₁₉ immunogens improve antigen processing, increase generation of T helper cells *in vivo*, and induce high antibody titers in comparison with a non-reduced and alkylated recombinant antigen [39].

Inclusion of exogenous T cell epitopes has been previously tested to improve the immunogenicity of vaccine candidates based on the MSP1₁₉ fragment but is highly dependent on the adjuvant used in the formulation [40–43]. In the present study, we have designed and characterized a second generation of polypeptide chimeras called Recombinant Modular Chimera (PyRMC-MSP1₁₉) that contain four autologous CD4⁺ promiscuous T cell epitopes

fused to the *P. yoelii* MSP1₁₉ fragment. The immune responses induced by immunization with PyRMC-MSP1₁₉ in mice and the functional activity of antibodies elicited in rabbits were compared to those induced by immunization with the PyMSP1₁₉ protein expressed without T cell epitopes. Our data shows that the PyRMC-MSP1₁₉ induces a more robust protective immunity against hyper-parasitemia and severe anemia after homologous experimental challenge in comparison to PyMSP1₁₉. Importantly, although both antibody preparations transfer immunity to C57BL/6 mice to the homologous challenge only anti-PyRMC-MSP1₁₉ IgG is capable of protecting mice against challenge with a heterologous strain.

2. Materials and Methods

2.1. Selection and optimization of *P. yoelii* promiscuous CD4⁺ T cell epitopes

We have reported several putative promiscuous T cell epitopes in the *P. vivax* MSP1 protein using peptide competition assays [8]. Based on these data, we selected the two sequences that were also present in the orthologous *P. yoelii* MSP1. *In silico* analyses were used to evaluate if such orthologous epitopes are predicted to bind promiscuously to multiple MHC class II alleles. We also used prediction algorithms to identify new MHC class II-restricted T cell epitopes in the extended PyMSP1 protein (see below). HLA Class II binding prediction was conducted using the ProPred algorithm [44]. This algorithm uses virtual matrices designed for 51 HLA alleles to calculate binding strength of nonameric segments of the protein and/or peptide [45]. To maximize prediction accuracy, we used a threshold of 3% and peptides predicted to bind 50% of HLA-DR alleles were considered promiscuous for binding. Validation analyses of putative binding motifs were done using the database of MHC ligands and peptide motifs for epitope prediction, SYFPEITHI [46].

2.2. Design and biochemical characterization of the *P. yoelii* PyRMC-MSP1₁₉

A 661 bp *pyrmc-msp1*₁₉ gene was codon optimized and synthesized by Genart (Regensburg, Germany) (Figure 1A and 1B). This synthetic gene includes: 1) Met-Ala on the N-terminus to provide the start signal and decrease degradation in *E. coli* (Ala), 2) two putative *P. yoelii* promiscuous T cell epitopes: Py8 (T₁₅₆-D₁₇₅): TEMPLKKVILGYRKPIENIQD orthologous to the sequence originally described in *P. vivax*, Belem strain (L₁₅₈-D₁₇₇, GenBank accession M60807) [8] and PyT53 (T₁₁₅₄-Q₁₁₇₃): TNRQIRDLSILKARLLKRRKQ orthologous to the sequence also described in *P. vivax* (D₁₁₀₁-N₁₁₁₈) [8], 3) GPGPG spacers, inserted between the amino terminal promiscuous T cell epitopes to enhance stability and antigen processing [47], 4) the extended *P. yoelii* yoelii 17XNL MSP1₁₉ (amino acid H₁₆₁₉ to S₁₇₅₁; GenBank accession XP_726257) that include a 42 amino acid long polypeptide upstream from the first MSP1₁₉ EGF domain, 5) two copies of a *P. berghei* sequence (PbB), derived from the repeat region of the circumsporozoite protein (PPPPNPND)₂, included at the carboxyl terminal end for biochemical characterization of antigenic integrity and to provide an optional affinity purification tag, 6) a carboxyl terminal His-tag for protein purification and 7) restriction sites for subcloning in an expression vector. The *pyrmc-msp1*₁₉ was excised with *NcoI* and *SacI* restriction enzymes and ligated into linearized pET24d(+) vector (Novagen).

A control recombinant protein (PyMSP1₁₉) containing only the PyMSP1₁₉ fragment (H₁₆₆₁ to S₁₇₅₁) and the two carboxy terminal tags, described above, was generated by PCR amplification using the *pyrmc-msp1*₁₉ as template and the following primers: T7 terminator 5' GCTAGTTATTGCTCAGCGG 3' and a reverse primer 5' ATCCCATGGCGCATGTGTGCGTGGAT 3' which contained the *NcoI* restriction site. The KOD amplification system was utilized following the manufacturer's instructions (Novagen, WI). The insert was confirmed by end nucleotide sequencing. Expression of the PyRMC-MSP1₁₉ and PyMSP1₁₉ was performed by re-transforming the positive clones into *E. coli* BL21 (DE3) cells (Novagen, Madison, WI) with kanamycin selection. Protein expression was

induced with 1 mM IPTG for 3 hours, following standard procedures. The recombinant chimeric proteins were purified with a Ni-NTA affinity column according to the manufacturer's instructions (Qiagen, Valencia, CA).

2.3. Western blots of recombinant and native parasite proteins

The integrity of the recombinant proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot. Electrophoresis was performed under reducing conditions on 15% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred into nitrocellulose membranes. After blocking with 3% non-fat dry milk in TBS (0.1M Tris, 0.15M NaCl), the membranes were incubated with anti-PbB species-specific monoclonal antibody (3D11) or anti-His tag monoclonal antibody at 0.2 µg/ml final concentration, or the anti-peptide PyT53 or anti-protein polyclonal antibodies elicited in mice at 1:5,000 sera dilution. Pre-immune sera samples were also used to confirm antigen specificity. After washing with TTBS (0.05% Tween 20, 0.1M Tris, 0.15M NaCl), the membranes were incubated with goat anti mouse IgG (H+L) conjugated to alkaline phosphatase (Promega, Madison, WI). After a final wash, the membranes were incubated with 5-bromo-4-chloro-3-indolylphosphate (BCIP, Promega) and nitroblue tetrazolium (NBT, Promega) to visualize bound antibodies.

For western blot of native antigens, *P. yoelii* 17X or *P. yoelii nigeriensis* N67 parasitized erythrocytes were extracted in 1X PBS containing 1% NP-40 and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Following SDS-PAGE electrophoresis, the proteins were transferred to pure nitrocellulose membranes (BioRad, Laboratories) as described above and then incubated with anti-PyRMC-MSP1₁₉ or anti-PyMSP1₁₉ purified rabbit IgG. Purified rabbit IgG obtained from naïve animals were used as control.

2.4. Parasites and animals

The parental *P. yoelii* 17X and the lethal *P. yoelii nigeriensis* N67/MR4-427 parasites were obtained from the Malaria Research and Reference Reagent Resource Center (MR4/ATCC, Manassas, VA). Six to eight-week old female BALB/c and C57BL6 mice (Jackson Laboratory, Bar Harbor, ME) were immunized on day 0, 20 and 40 subcutaneously in the base of the tail and in the inter-scapular area, using 10 µg of the PyRMC-MSP1₁₉ or PyMSP1₁₉ proteins emulsified in Montanide ISA 51 (Seppic, Fairfield, NJ). Control groups of mice received PBS alone emulsified in the same adjuvant. Twenty days after the last immunization, the mice were challenged by intraperitoneal inoculation with 1×10⁶ *P. yoelii* 17X infected erythrocytes obtained from a donor mouse. For passive transfer experiments, the mice were challenged by intravenous inoculation with 1×10⁴ *P. yoelii* 17X or *P. yoelii nigeriensis* N67 infected erythrocytes per mouse obtained from a donor mouse. Giemsa-stained thin smears were made daily and used to quantify parasitemia by counting the percentage of infected erythrocytes using light microscopy. Additional blood samples from tail snips were used to determine hemoglobin concentration using a HemoCue photometer (HemoCue Inc, Lake Forest, CA).

2.5. Antibody reactivity and functional assays

2.5.1. Enzyme linked immunosorbent assays (ELISA)—The fine specificity of the antibodies elicited by immunization with the PyRMC-MSP1₁₉ or PyMSP1₁₉ was determined by ELISA using Immulon 4HB plates (Thermo Labs Systems, Franklin, MA) coated with 1 µg/ml of the PyRMC-MSP1₁₉ or PyMSP1₁₉, diluted in carbonate buffer (0.2M sodium carbonate and 0.2M sodium bicarbonate, pH: 9.6) or PyT8, PyT53 or PbB synthetic peptides diluted in PBS. The wells were blocked with 5% bovine serum albumin (BSA) in PBS and 0.05% Tween 20 for 2 h at 37°C and then duplicates of serum diluted in PBS with 2.5% BSA and 0.05% Tween 20 were added. The plates were then incubated for 1 h at 37°C and the bound antibodies were then detected using peroxidase-labeled goat anti-mouse total IgG and

H₂O₂/2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate (KPL, Gaithersburg, MD). Optical densities were determined using a VERSAmax ELISA reader (Molecular Device Corporation, Sunnyvale, CA) with a 405 nm filter. The endpoint was measured as the highest dilution of sera having a delta O.D. greater than the mean plus two standard deviations (S.D.) obtained using pre-immune sera. Isotype profiles of the IgG were also determined by ELISA. After sera incubation, plates were washed and incubated with biotinylated rat anti-mouse mAbs IgG1, IgG2a, IgG2b or IgG3 (BD PharMingen, Franklin Lakes, NJ) for 90 minutes. After washing, the bound antibodies were detected using horseradish peroxidase (HRP)-Streptavidin (BD PharMingen) and H₂O₂/2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate (KPL).

2.5.2. Relative avidity of antibodies elicited by immunization with recombinant proteins—The avidity of anti-PyRMC-MSP1₁₉ and PyMSP1₁₉ antibodies was assessed by a thiocyanate elution-based ELISA using sera samples obtained 20 days after the third immunization (Day 60) [8]. The assay was conducted in the same manner as described above with a slight modification. In brief, 0 to 8M ammonium thiocyanate in PBS was added to each well after incubation with the sera dilutions. The plates were incubated for 15 minutes at room temperature before washing and proceeding with the assay as described above.

2.5.3. Passive transfer of anti-PyRMC-MSP1₁₉ or anti-PyMSP1₁₉ IgG—Total IgG were purified from sera obtained from rabbits immunized with either PyRMC- MSP1₁₉ or PyMSP1₁₉ by protein A affinity chromatography using the Affi-Gel protein A MAPS II kit (Bio-Rad, Richmond, CA) and following the manufacturer's instructions. The immunoglobulin G solution was dialyzed extensively and sterilizing by gamma irradiation. Passive transfer studies were conducted with groups of ten female C57BL/6 mice, 6 to 8 weeks of age, by intravenous inoculation of purified IgG on days -1, 0, +1, +3 and +5 relative to the day of challenge. The animals were randomized in three groups that received 500 µg/dose of anti-PyRMC-MSP1₁₉ IgG, anti-PyMSP1₁₉ IgG or IgG derived from pre-immune rabbits as control.

2.5.4. Indirect immunofluorescence assays (IFA)—Sera obtained from BALB/c mice after the third immunization with 50 µg of the PyRMC-MSP1₁₉ or PyMSP1₁₉ control protein were pooled and the antibody reactivity against native protein was evaluated by indirect immunofluorescence. Purified rabbit IgG derived from animals immunized with RMC-MSP1₁₉ or PyMSP1₁₉ were also tested. Blood was drawn from *P. yoelii* 17X or *P. yoelii nigeriensis* N67 infected mice into CPD tubes. The blood samples were washed twice using RPMI 1640 medium and the cells adjusted to 1% hematocrit. Ten microliters of cell suspension was added to wells of 12-well slides (ICN Biomedicals, Inc., Aurora, OH) and air-dried before storage at -20°C. Several dilutions were tested, and the reactivity was evaluated using goat anti-mouse IgG AlexaFluor 488 conjugate antibodies (Invitrogen Corporation, Carlsbad, CA). Parasite nuclei were visualized using 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) included in the anti-fade mounting medium Prolong (Invitrogen Corporation).

2.6. T cell assays

The frequency of peptide-specific T lymphocytes was determined by IFN-γ specific ELISPOT assays conducted *ex vivo* using spleen cells from four BALB/c or C57BL/6 mice 20 days after priming. Assays were performed in nitrocellulose microplates (Millipore, Bedford, MA) coated with rat anti-mouse IFN-γ capture antibody (BD Biosciences Pharmingen, San Diego, CA) following instructions from the manufacturer. Freshly isolated spleen cells from four mice were tested individually and triplicate aliquots were plated in RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids (Invitrogen Corporation), 1 mM sodium pyruvate, 25 U/ml penicillin, 50 µg/ml streptomycin, 5×10⁻⁵ M 2-ME and 10% fetal calf serum. T cells

were activated by addition of 10 µg/ml PyRMC-MSP1₁₉, PyMSP1₁₉ control protein or synthetic peptides representing PyT53 or PyT8 epitopes [7]. After 24 h the plates were washed and incubated with biotinylated rat anti-mouse IFN-γ (BD Biosciences Pharmingen) followed by incubation with Streptavidin-HRP. The reaction was developed using 3-amino-9-ethylcarbazole (AEC) (BD Biosciences Pharmingen) and evaluated in an Immunospot Analyzer (Cellular Technology-Becton Dickinson, San Diego, CA). Concanavalin A (Sigma Aldrich Corp., St Louis, MO) was used as positive control for activation and cells incubated with medium alone served as a negative control for background activation. Duplicate plates were maintained undisturbed for 48 h and the supernatants were collected for cytokine multiplex analysis using the Pierce SearchLight technology (Pierce Boston Technology, Woburn, MA).

2.7. Statistics

Antibody levels were log-transformed and multiple comparisons were conducted using Student's *t*-test. For experimental challenges, differences between groups were evaluated by comparing the integrated numbers in percentages of parasitemia or hemoglobin levels seen in peripheral blood over time, determined as the area under the parasitemia change versus time curves and the area under the hemoglobin change versus time curves calculated by the trapezoid method [48,49]. For parasitemia values, the total areas under the parasitemia change versus time curves were calculated and the mean change values expressed as percentage of parasitemia. For hemoglobin values, the total areas under the hemoglobin change versus time curves were calculated. Subsequently the mean change values in hemoglobin concentration per day were determined by comparison with baseline concentrations and expressed as percentage of reduction in hemoglobin levels. The change from baseline hemoglobin values and the differences among mean parasitemia change curves throughout the follow up period after experimental infection were analyzed by paired Student's *t*-test. To evaluate the correlation between antibody titers and protective efficacy Spearman's rank correlation coefficient was calculated. *P*-values of 0.05 or less were considered significant.

3. Results

3.1. PyRMC-MSP1₁₉ design, expression and biochemical characterization

Two *P. yoelii* putative promiscuous CD4⁺ T cell epitopes were included in tandem at the amino terminal end of the chimeric recombinant protein interspaced by the sequence GPGPG (Figure 1A and 1B). These epitopes are orthologous to two promiscuous CD4⁺ T cell epitopes, referred as PvT8 and PvT53 that we have reported in the *P. vivax* MSP1 protein using peptide competition assays [8]. *In silico* analysis were used to determine whether or not the *P. yoelii* PyT8 and PyT53 sequences are predicted to bind to several class II alleles using the ProPred algorithm [44]. The PyT8 sequence (L₁₅₆-D₁₇₅) is predicted to bind 25 alleles and the PyT53 sequence (T₁₁₅₄-Q₁₁₇₃) 32 alleles out of the 51 HLA-DR alleles available. The binding peptide prediction algorithm was also used to determine putative MHC class II binding domains in the 33 kDa fragment of the native *P. yoelii* MSP1 sequence. Using this approach we defined two regions (I₁₆₂₀-S₁₆₃₁ and S₁₆₄₂-L₁₆₅₅) upstream from the first PyMSP1 epidermal growth factor module that contain putative promiscuous binding sequences. These sequences were predicted to bind 9 and 27 HLA-DR alleles, respectively. The peptide sequences predicted to bind ≥ 25 HLA-DR alleles also exhibited high scores using the SYFPEITHI algorithm (data not shown). The synthetic gene encoding the PyRMC-MSP1₁₉ was codon optimized for expression in *E. coli*. The gene encoding the PyMSP1₁₉ control protein (H₁₆₆₁-S₁₇₅₁) was produced by PCR amplification of the *pyrmcmsp1*₁₉ synthetic gene and contained the two EGF motifs and the C-terminal tags (Figure 1A and 1C).

PyRMC-MSP1₁₉ and PyMSP1₁₉ recombinant proteins were purified from *E. coli* lysates by metal chelate chromatography using a Ni-NTA resin. After initial metal chelate purification, each recombinant construct was further purified using analytical gel filtration chromatography on Superdex 75, purified proteins migrated as a single peak. Analyses by SDS-PAGE showed single bands of the apparent mobility of 26 kDa for PyRMC-MSP1₁₉ and 18 kDa for PyMSP1₁₉. Biochemical identity of the recombinant proteins was established by western blot analysis using a panel of antibodies recognizing different components of the chimeras (Figure 2A). Consistent with the topology of the chimeric proteins, anti-PyT53 antibodies recognize the 26 kDa chimeric protein (PyRMC-MSP1₁₉) but not the 18 kDa control protein (PyMSP1₁₉). The antigenicity of the chimeric proteins was also tested by ELISA (Figure 2B). The same monoclonal or polyclonal antibodies that recognize different domains of the chimeric proteins bound efficiently revealing similar dose-response patterns (Figure 2B). The monoclonal antibody Mab5.2 MRA-94, which recognizes a conformational dependent epitope on the *P. falciparum* MSP1₁₉, was used as a control to test the specie-specificity [50]. This reagent failed to cross-react with the PyRMC-MSP1₁₉ (Figure 2B).

3.2. Humoral immune response in mice immunized with *P. yoelii* PyRMC-MSP1₁₉ or PyMSP1₁₉

The PyRMC-MSP1₁₉ and the PyMSP1₁₉ recombinant chimeric proteins were formulated with Montanide ISA 51 and used to immunize BALB/c and C57BL/6 mice three times twenty days apart by subcutaneous inoculation. Each experimental group and the placebo group, which received adjuvant alone, included ten animals. Sera were collected prior to the first immunization and twenty days after each immunization by tail snips. Sera collected at different time points were tested for recognition using the homologous or heterologous *P. yoelii* proteins used for immunization and a synthetic peptide representing the PbB tag sequence by ELISA. Following immunization with PyRMC-MSP1₁₉ or PyMSP1₁₉ recombinant protein, the antibody responses reached a plateau after the third immunization (Day 60) (Figure 3). After the first immunization, C57BL/6 mice had higher anti-PyRMC-MSP1₁₉ or PyMSP1₁₉-specific antibody titers than BALB/c mice ranging between three to seven folds. However, the overall antibody titers showed no significant differences between strains whether the mice received PyRMC-MSP1₁₉ or PyMSP1₁₉. No significant differences between strains of mice were observed when evaluating the kinetics of the total anti-PyRMC-MSP1₁₉ or anti-PyMSP1₁₉ protein IgG antibody titers when homologous proteins were used as antigens in ELISA assays. Interestingly, the total antibody response against PyRMC-MSP1₁₉ elicited in both strains of mice immunized with the PyMSP1₁₉ control protein were significantly higher (BALB/c, $P=0.01$; C57BL/6, $P=0.04$) than the anti-PyMSP1₁₉ antibody titers elicited in mice immunized with PyRMC-MSP1₁₉ (Figure 3, right panel). Sera obtained from control mice immunized with adjuvant alone were non-reactive with either recombinant protein (data not shown).

Antibody titers elicited against the tag were also determined by ELISA using a synthetic peptide representing the carboxyl terminal *P. berghei* tag (PbB). Antibody titers were significantly higher in BALB/c mice immunized with the PyMSP1₁₉ control protein in comparison with those immunized with PyRMC-MSP1₁₉ (geometric mean antibody titer after the third immunization 1:583,858 versus 1:91,952; $P=0.0001$); no antibody titers differences were seen between the experimental groups in the C57BL/6 mice (geometric mean antibody titer after the third immunization 1:327,680 versus 1:583,859). The quality of antibody responses elicited by immunization with the recombinant proteins was evaluated using sodium thiocyanate avidity assays. To test antibody avidity, various concentrations (1M to 8 M) of sodium thiocyanate were used. Sera obtained from BALB/c or C57BL/6 mice had comparable avidity for PyRMC-MSP1₁₉ or PyMSP1₁₉. The molar concentration of ammonium thiocyanate required to dissociate 50% of bound anti-protein antibodies ranged from 1.9 M to 2.4 M. Measurement of antigen-specific IgG isotypes was used as a surrogate for evaluating Th1 and

Th2 biased responses using sera samples collected three weeks after the third immunization. Anti-PyRMC-MSP1₁₉ and anti-PyMSP1₁₉ specific IgG1, IgG2a, IgG2b, and IgG3 subclass antibodies were determined by ELISA. No significant differences were observed in the IgG subclasses induced by either immunogen or between mouse strains (data not shown).

To compare the reactivity to the native protein expressed by *P. yoelii* infected erythrocytes, sera samples obtained 20 days after the last immunization were tested by IFA. Antibodies elicited in mice by immunization with both recombinant proteins reported here recognized the intact PyMSP1 protein. However, the antibody titers obtained with sera from mice immunized with the PyRMC-MSP1₁₉ were eight to sixteen fold higher than the IFA titers obtained with sera from mice immunized with the MSP1₁₉ control protein when the sera were tested using the homologous strain (BALB/c or C57BL/6 PyRMC-MSP1₁₉ = 1:160,000; BALB/c PyMSP1₁₉ = 1:20,000; C57BL/6 PyMSP1₁₉ = 1:8,000; $P < 0.0001$). Interestingly, sera from mice immunized with the PyRMC-MSP1₁₉, but not sera from mice immunized with the MSP1₁₉ control protein also recognized the heterologous *P. yoelii nigeriensis* parasites by IFA (BALB/c = 1:320 and C57BL/6 = 1:1280).

To characterize the extent of cross-reactivity of anti-PyRMC-MSP1₁₉ or anti-PyMSP1₁₉ antibodies, purified IgG preparations were tested by western blot, ELISA and IFA against homologous (*P. yoelii* 17X) or heterologous parasites (*P. yoelii nigeriensis*). Purified rabbit anti-PyRMC-MSP1₁₉ IgG but not purified pre-immune IgG, reacted with the intact *P. yoelii* MSP1 precursor (~230 kDa) and schizont polypeptides of ~83 ~42, and ~19 kDa estimated molecular mass in an extract of homologous parasites (Figure 4A). Interestingly, schizont polypeptides of ~83, ~42, and ~19 kDa were also detected by purified rabbit anti-PyRMC-MSP1₁₉ IgG in parasite extracts from *P. yoelii nigeriensis*. In sharp contrast, antibodies specific for PyMSP1₁₉ recognized proteins of ~42kDa and 19KDa in parasite extracts from the homologous but not the heterologous strain (Figure 4A). Consistent with the results obtained with western blot analysis, purified anti-PyRMC-MSP1₁₉ antibodies recognized both homologous and heterologous parasite protein extracts by ELISA (Figure 4B). That is in contrast with the poor recognition of the homologous parasite extract using PyMSP1₁₉ antibodies ($P = 0.0006$). Most relevantly, anti-PyMSP1₁₉ antibodies were unable to recognize the heterologous parasite extract ($P = 0.02$) (Figure 4B). Similar reactivity to *P. yoelii* 17X or *P. yoelii nigeriensis* native proteins were observed with anti-PyRMC-MSP1₁₉ antibodies by IFA (Figure 4C).

3.3. Cellular immune response in mice immunized with *P. yoelii* PyRMC-MSP1₁₉ or PyMSP1₁₉

Four out of ten animals included in each group were euthanized twenty days after the first immunization to evaluate T cell reactivity induced by priming with PyRMC-MSP1₁₉ or PyMSP1₁₉ proteins by measuring the frequency of IFN- γ secreting cells by ELISPOT assays and IL-2, IL-5 and IL-10 cytokine levels in 48- hour cell culture supernatants obtained from antigen-stimulated splenocytes by using multiplex analysis. Table 1 shows a balanced cytokine response characterized by production of IFN- γ , IL-2, IL-5 and IL-10 after *ex vivo* stimulation with the recombinant constructs. The frequency of IFN- γ secreting cells and the levels of IL-10 from mice immunized with PyRMC-MSP1₁₉ were significantly higher than the response produced by splenocytes derived from mice immunized with the PyMSP1₁₉ recombinant protein ($P < 0.05$). High prevalence of PyT8 and PyT53 IFN- γ secreting cells were observed in BALB/c mice immunized with PyRMC-MSP1₁₉ in comparison to mice immunized with PyMSP1₁₉. Interestingly, splenocytes from C57BL/6 mice immunized with the PyMSP1₁₉ control protein have high frequency of PyT8 specific IFN- γ secreting cells (Table 1).

3.4. Protection against *P. yoelii* homologous challenge

Groups of six BALB/c and C57BL/6 mice were immunized three times with the PyRMC-MSP1₁₉ or the PyMSP1₁₉ proteins. Control animals received PBS emulsified in Montanide ISA 51 as a placebo. Twenty days after completion of the immunization schedule, the animals were challenged with the parental *P. yoelii* 17X isolate. Age-matched naïve mice were also included as infection controls. Parasitemia was determined daily by light microscopy using Giemsa-stained thin smears and expressed as percentage of parasitemia. The impact of the immunization on the control of malarial anemia was also evaluated by daily determination of the hemoglobin levels. In both strains of mice, parasitemia became detectable in blood smears three days after the experimental challenge (Figure 5). Parasitemia in placebo-immunized mice steadily increased to reach maximum levels between day 9 and 12 after challenge. All naïve BALB/c and C57BL/6 mice were euthanized on day 15 after experimental infection due to hemoglobin levels lower than 6 g/dL (data not shown). Consistent with previous reports of mice immunized with *P. yoelii* MSP1 vaccine constructs, significant reductions in the number of infected erythrocytes in animals that received either PyRMC-MSP1₁₉ or PyMSP1₁₉ were observed in this study (Figure 5). The protective effect of the immunization with PyRMC-MSP1₁₉ or PyMSP1₁₉ in BALB/c was reflected in lower peaks of parasitemias (8.8% and 13.75%, respectively, Figure 5A) when compared to the placebo group (28.5%; PyRMC-MSP1₁₉ $P=0.00022$, PyMSP1₁₉ $P<0.0001$). However, the total area under the parasitemia change versus time curve was lower in mice immunized with PyRMC-MSP1₁₉ than in mice immunized with PyMSP1₁₉ ($P=0.049$). Importantly, two out of six animals immunized with PyRMC-MSP1₁₉ were able to control parasite growth without significant changes in hemoglobin concentration relative to baseline levels (data not shown). One additional mouse had a longer prepatent period (5 days) compared to any of the control groups with a moderate drop in hemoglobin concentration. Consistent with protection against severe anemia, the mean reduction in hemoglobin concentration was lower in mice immunized with PyRMC-MSP1₁₉ in comparison to the reduction in hemoglobin concentration in mice immunized with PyMSP1₁₉ ($P<0.0001$) or placebo ($P=0.0006$) (Figure 5A).

C57BL/6 mice immunized with PyRMC-MSP1₁₉ exhibited a higher degree of protection than the group immunized with PyMSP1₁₉: peaks of parasitemias were 5.97% for PyRMC-MSP1₁₉ and 9.3% for PyMSP1₁₉ with significantly lower parasitemia change versus time curve in mice immunized with PyRMC-MSP1₁₉ ($P=0.0017$) (Figure 5B). Meanwhile, the peak parasitemia for placebo immunized mice were recorded as 19.45% (PyRMC-MSP1₁₉ $P=0.0005$, PyMSP1₁₉ $P=0.0009$). The mean reduction in hemoglobin concentration was lower in mice immunized with PyRMC-MSP1₁₉ in comparison to the reduction in hemoglobin concentration in mice immunized with PyMSP1₁₉ ($P=0.0053$) or placebo ($P<0.0001$) (Figure 5B). Changes in hemoglobin concentration did not correlate with the parasite load (Spearman's rank correlation = 0.18, $P=0.18$).

3.5. Determination of functional activity of antibodies using *P. yoelii* heterologous challenge

Although we were unable to confirm differences in the profile of IgG isotypes or in antibody avidity of antigen-specific total IgG in mice immunized with PyRMC-MSP1₁₉ or PyMSP1₁₉, we observed a significant correlation between total IgG antibody titers and parasite load after experimental challenge, expressed as percentage of parasitemia (Spearman's rank correlation 0.37, $P=0.03$). To assess the functional effect of antibodies raised against PyRMC-MSP1₁₉ or PyMSP1₁₉ on parasite replication, we compared the effect of passively transferred rabbit IgG on the course of infection in C57BL/6 naïve mice. It has been shown that MSP-1 protects against challenge with homologous but not heterologous *P. yoelii* strains [25,51]. We therefore decided to compare the effect of the passively transferred antibodies on the infection with the homologous *P. yoelii* 17X or the heterologous *P. yoelii nigeriensis* N67 isolates. The MSP1 protein in the *P. yoelii nigeriensis* N67 isolate contains a set of 22 polymorphic residues

within the 19 kDa protein fragment in comparison to the *P. yoelii yoelii* isolate used for the design of PyRMC-MSP1₁₉ [52]. Groups of twenty C57BL/6 naïve mice were passively immunized with purified IgG derived from rabbits immunized either with PyRMC-MSP1₁₉ or PyMSP1₁₉. A third group of twenty mice received purified IgG derived from pre-immune rabbits. Ten animals per group were then experimentally challenged either with *P. yoelii* 17X or *P. yoelii nigeriensis*.

Following challenge with the homologous strain, the parasitemias and the reduction in hemoglobin concentration were comparable to those described above for actively immunized mice (Figure 6A). Significantly lower parasitemia change versus time curve was obtained in mice that received purified IgG from PyRMC-MSP1₁₉ immunized rabbits in comparison to mice that received purified IgG from PyMSP1₁₉ immunized rabbits ($P<0.0001$) or normal IgG ($P=0.00063$). Similarly, the reduction in hemoglobin level was lower in mice that received purified IgG from PyRMC-MSP1₁₉ immunized rabbits in comparison to the reduction in hemoglobin level in mice that received purified IgG from PyMSP1₁₉ immunized rabbits ($P<0.0001$) or normal IgG ($P<0.0001$).

All mice in the group that received purified IgG from PyRMC-MSP1₁₉ immunized rabbits were completely protected against experimental challenge with *P. yoelii nigeriensis* (Figure 6B). Eight out of ten mice treated showed sterile immunity, and only two developed very low parasitemia that self-cured between day 6 and 9 after challenge. This is in sharp contrast with mice that received purified IgG from PyMSP1₁₉ immunized rabbits ($P<0.0001$) or normal IgG ($P=0.0005$). Mice with these passive transfers schemes developed high parasitemias and severe anemia and were euthanized between day 7 and 11 after challenge. Two out of ten animals in the group that received purified IgG from PyMSP1₁₉ immunized rabbits also developed high parasitemias that were partially controlled but succumb to the effects of anemia on day 15 post-challenge. Consistent with complete control in parasite growth, changes in the hemoglobin levels were not detectable in mice that received purified IgG from PyRMC-MSP1₁₉ immunized rabbits (Figure 6). In contrast, an accelerated reduction of hemoglobin levels was detected in mice that received either purified IgG from anti-PyMSP1₁₉ immunized rabbits ($P=0.002$) or normal IgG ($P=0.0009$). All mice in these groups had hemoglobin levels lower than 5 g/dL between day 7 and 15 after experimental challenge.

4. Discussion

We have previously reported that polymeric linear peptide chimeras (LPCs) and recombinant protein subunit chimeras, tailored to include several pre-erythrocytic stage linear epitopes, can efficiently induce protective immunity to malaria using the stringent murine model of protection with *P. yoelii* [7,9]. Topological features of these vaccine constructs include amino terminal *Plasmodium* promiscuous CD4⁺ T cell epitopes and the multimeric nature of the resulting chimeric polypeptides [7,9]. It is well known that protective immunity to erythrocytic stage antigens are mediated by epitopes highly dependent on structured domains [11,13,53–55]. Such domains require the establishment of disulfide bonds and the conformation of precisely folded structures that are hard to reproduce using synthetic peptides or multimeric recombinant polypeptides. In the present study, we describe the design of a synthetic gene encoding a *P. yoelii* chimeric protein (PyRMC-MSP1₁₉) that contain a set of promiscuous CD4⁺ T cell epitopes inserted in tandem and fused to the 19-kDa fragment of the *P. yoelii* Merozoite Surface Protein-1 (PyMSP1₁₉). The PyRMC-MSP1₁₉ retained the T-B topology used for the production of pre-erythrocytic vaccine constructs [7,8]. This synthetic gene was codon-optimized for expression in *E. coli* and the soluble recombinant chimera was purified using metal-ion affinity chromatography. Proof of principle studies to evaluate the protective effect of the PyRMC-MSP1₁₉ were done in BALB/c and C57BL/6 mice in comparative studies with a control recombinant protein that express only the MSP1₁₉ fragment (PyMSP1₁₉).

Protective efficacy was evaluated using two different criteria: control of parasitemia and control of malarial anemia. The present study clearly shows that PyRMC-MSP1₁₉ induced anti-parasite immunity capable of protecting the host against the development of hyper-parasitemia and severe anemia. The protective response induced by PyRMC-MSP1₁₉ was more robust than the response induced by PyMSP1₁₉ purportedly due to the presence of T cell epitopes. Such protective effect against challenge with the homologous *P. yoelii* strain can be reproduced by passive transfer of immune IgG, suggesting that antibodies have a crucial role. More importantly, this is the first report showing passive protection against a heterologous strain using anti-MSP1 antibodies, suggesting that the inclusion of several promiscuous T cell epitopes derived from different regions of the protein enhance functional characteristics of the antibodies.

Preclinical and clinical vaccine trials have indicated that the MSP1₁₉ protein fragment is an important target for antibody inhibitory activity but that it is poorly immunogenic [24,26,30, 56–58]. MSP1₁₉ includes two epidermal growth factor (EGF)-like domains responsible for a compact and rigid structure maintained by multiple disulfide bonds [11,37]. The conformational epitopes created by the correct folding of the MSP1₁₉ fragment are essential for the induction of functionally relevant inhibitory antibodies [59], yet these epitopes are poorly immunogenic [33], most likely because disulfide bond rich moieties inhibit the protease activity essential for antigen processing [39]. The cellular response to MSP1₁₉ seems to improve when a denatured recombinant protein is used as immunogen [35]. Several approaches have been used to enhance the immunogenicity of the MSP1₁₉ fragment that include the conjugation of exogenous T cell epitopes to a MSP1₁₉ fusion protein [60], the fusion of MSP1₁₉ to novel protein adjuvants [61], the genetic linkage to hepatitis B virus surface protein [62,63] or the genetic linkage to promiscuous T cell epitopes [40,42,64]. The PyRMC-MSP1₁₉ described here includes two endogenous putative promiscuous T cell epitopes genetically linked to an extended PyMSP1₁₉ protein fragment, which includes a region predicted to contain additional putative promiscuous T cell epitopes. The levels of anti-PyMSP1 specific antibodies determined by ELISA were comparable in both BALB/c and C57BL6 mice immunized with the chimeric or the control protein. However, inclusion of the endogenous promiscuous T cell epitopes improved the recognition of native proteins and the quality of the antibodies determined by *in vivo* neutralization of parasite infectivity. Antibodies with PyMSP1 specificity have been reported to have distinct functional activity that includes neutralization, inhibitory antibodies or antibodies that block protein processing [23,59]. Our data show that PyRMC-MSP1₁₉ and PyMSP1₁₉ induce comparable levels of high avidity antibodies and a similar profile of IgG isotypes. However, significant differences were observed after passive transfer of antigen-specific IgG into naïve hosts suggesting that the inclusion of endogenous T cell epitopes may play a role in the induction of neutralizing, protective antibodies. Comparative experiments using MSP1-based proteins expressed in different systems have confirmed the broad heterogeneity of the functional antibodies elicited by immunization [65]. The proteins reported here were expressed in the same system using a single codon optimized synthetic gene, differences in the functional activity reported can be attributed to the genetic linkage of promiscuous T cell epitopes.

The kinetics of hemoglobin levels and parasitemia after experimental challenge of naïve mice followed a profile consistent with fast-growing or lethal strains for both parasite isolates used here. We confirmed that both recombinant proteins induce protective immunity illustrated by control of hyper-parasitemia and malarial anemia in comparison to placebo-immunized or naïve mice. However, significant differences in the control of parasitemia and malarial anemia were also significant when both the area under parasitemia time curve and the area under hemoglobin time curve in mice immunized with PyRMC-MSP1₁₉ were compared to the areas under time curves in mice immunized with the PyMSP1₁₉ protein. Importantly, this protective effect was induced using a route of immunization (subcutaneous), immunization regime (three

doses) and formulation (Montanide) that are approved for human use. This is in contrast with previous publications whereby *P. yoelii* MSP1 recombinant products required the use of intraperitoneal inoculation, a formulation not permitted for human use (Freund's adjuvant), and complex immunization protocols to induce a protective immune response [30,60,66–69].

Given the reported genetic and functional heterogeneity of the *P. yoelii* 17X isolate [70], and the failure in the past to induce protection against heterologous challenge [25,51], we questioned whether the PyRMC-MSP1₁₉ would render protective immunity against a heterologous *P. yoelii* strain. *P. yoelii* MSP1₁₉ is highly diverse across strains [51,71,72]. *P. yoelii nigeriensis* N67 differs in a total of 22 amino acid residues in comparison to the *P. yoelii* 17XNL reported sequence included in the PyRMC-MSP1₁₉, 9 in the first EGF domain, 3 in the joining region, and 10 in the second EGF domain [72]. Consistent with the extensive variability in this region, immunization with a GST fusion protein expressing PyMSP1₁₉ induced protection against the homologous but not heterologous strain [51]. Moreover, *P. falciparum* MSP1₁₉ contain only four dimorphic amino acids [73,74] and protection to experimental challenge in a non-human primate model of *P. falciparum* using vaccine preparations based on PfMSP1 have confirmed that the protective effect is mediated by antibodies and it is strain-specific [75]. Our results, strongly indicate that the extensive variation in the sequence of *P. yoelii* isolates tested here for protection are not a surmountable factor to induce protective immunity using MSP1 protein chimeras. Thus the development and testing of new vaccine candidates including *P. falciparum* or *P. vivax* MSP1 as recombinant chimeras using the RMC topology described here would be advisable and cost-effective.

The PyRMC-MSP1₁₉ was also able to induce a robust cellular immune response to the homologous recombinant construct as well as to the synthetic peptides representing two individual T cell epitopes. Similarly, antibodies elicited by immunization with the PyRMC-MSP1₁₉ recognize the PyMSP1₁₉ recombinant and the native PyMSP1 proteins. These results suggested that the homologous T cell epitopes can be efficiently processed and presented in mice by immunization with a chimeric protein and that the linkage of several T cell epitopes to the conformationally restricted *P. yoelii* MSP1₁₉ module do not interfere with the folding of such protein fragment into the correct three-dimensional conformation to elicit functional antibodies. Consistent with our data, a chimeric *P. vivax* vaccine construct that contained two T cell epitopes was recently reported [42]. Comparative experiments indicated that a chimeric vaccine including a carboxyl terminal MSP1 promiscuous T cell epitope linked to an exogenous universal T cell epitope was a better immunogen in mice than its counterpart without T cell epitopes. However, the immune response of the *P. vivax* chimeric protein in a non-human primate model seemed to be highly dependent on the adjuvant used, a feature attributed to the low potency of the *Plasmodium* T cell epitopes included in the preparation [42]. It is also possible that the inclusion of amino terminal T cell epitopes interspaced with GPGPG spacers in the PyRMC-MSP1₁₉ design created a more suitable antigen in comparison to the chimeric construct with carboxyl terminal T cell epitopes [42]. In fact, the critical role of the topology in the immunogenicity of chimeric recombinant proteins that include T helper cell epitopes and spacers has been reported [76].

Functional CD4⁺ T cells are required for the induction of a robust antibody response after immunization with protein antigens [77]. Our work using chimeric synthetic peptides containing B cell epitopes fused to promiscuous CD4⁺ T cell epitopes has indicated that anti-peptide and anti-parasite antibody responses are improved by inclusion of *Plasmodium* CD4⁺ T cell epitopes [7,8]. The promiscuous CD4⁺ T cell epitopes included in the PyRMC-MSP1₁₉ described here represent orthologous sequences that we have previously described in *P. vivax* [8] and are known to be immunogenic in mice [7]. These T cell epitopes were originally described for their ability to bind several human MHC class II alleles [8]. *In silico* analysis of the region immediately upstream of the PyMSP1₁₉ fragment indicated that two putative

promiscuous T cell epitopes are present (I₁₆₂₀-S₁₆₃₁ and S₁₆₄₂-L₁₆₅₅). Therefore, we included these two epitopes in the synthetic gene as an extended 5' region that encodes for a 40-amino acid long polypeptide upstream from the first EGF domain (H₁₆₁₉ to K₁₆₆₀). The mice immunized with PyRMC-MSP1₁₉ exhibited protein-specific cytokine production consistent with Th1 and Th2 responses (production of IL-2, IFN- γ , IL-5 and IL-10 after *ex vivo* stimulation with the corresponding recombinant protein). Differences in the pattern of cytokine production in mice immunized with PyRMC-MSP1₁₉ or PyMSP1₉ suggested differences in the kinetics of the cellular immune response [78].

In summary we present here the design and immunological characterization of a malaria recombinant chimeric protein (PyRMC-MSP1₁₉) using the *P. yoelii* rodent malaria model. From amino to carboxy terminal, the PyRMC-MSP1₁₉ was designed to encode two promiscuous CD4⁺ T cell epitopes interspaced with GlyProGlyProGly spacers and linked to an extended *P. yoelii* MSP1₁₉ protein. Codon optimization of the synthetic gene allowed for successful expression in *E. coli*. Further, the inclusion of autologous T cell epitopes did not alter the integrity of the recombinant product, and both T cell epitopes were expressed and induced epitope-specific T cell responses. Most importantly, we found that the PyRMC-MSP1₁₉ is highly immunogenic and induce functional antibodies that protected against heterologous challenge. Finally, the PyRMC-MSP1₁₉ induced protection to both hyperparasitemia and severe anemia. Future studies will evaluate the flexibility of the modular chimeric design to incorporate multi-antigen, multi-stage and multi-species vaccine candidates acceptable for human use.

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Figure 1.

Topology of the synthetic codon optimized *pyrmcmsp1₁₉* and *pymsp1₁₉* genes. (A) Schematic representation of the chimeric proteins reported here. *pyrmcmsp1₁₉* encoded 207 amino acids with the T cell epitopes, schematically represented by double lined boxes interspaced by five amino acid residues (striped boxes), upstream of the 19 kDa fragment of PyMSP1 fragment (GenBank accession XP_726257). PyMSP1₁₉ includes the complete amino acid sequence of the MSP1₁₉ and two carboxyl terminal tags. (B) Sequence of the *PyRMC-MSP1₁₉* protein. The amino acid sequence is shown in single letter code. The sequences enclosed in boxes are the two promiscuous CD4⁺ T cell epitopes originally reported in the *P. vivax* MSP1 protein [7,8]. The amino acid residues GPGPG were included as spacers between individual CD4⁺ T

cell epitopes and the extended *P. yoelii* MSP1₁₉ protein (H₁₆₁₉-S₁₇₅₁). The underlined 42 amino acid long polypeptide, upstream from the first MSP1₁₉ EGF domain, contains two *in silico* predicted T cell epitopes. The carboxyl terminal *P. berghei* CSP repeat tag sequence PPPNPNDPPPPNPND, was included for biochemical characterization of the antigenic integrity and to provide optional affinity purification tag is underlined with a dotted line. A carboxyl terminal (H)₆ tag was included for protein purification. (C) Sequence of the MSP1₁₉ control protein includes amino acid H₁₆₆₁ to S₁₇₅₁, it also included the PbCSP repeat tag.

Figure 2.

Biochemical and immunological characterization of the chimeric proteins described here. (A) SDS -PAGE (left panel) and western blot (center and right panels) of the purified *P. yoelii* PyRMC-MSP1₁₉ and PyMSP1₁₉ proteins expressed in *E. coli* BL21 (DE3). Left panel shows Coomassie stain after SDS-PAGE separation, total bacterial lysate and column purified proteins are shown for PyRMC-MSP1₁₉ (lanes 1 and 2) and PyMSP1₁₉ (lanes 3 and 4). Western blot analysis of the purified PyRMC-MSP1₁₉ (central panel) and PyMSP1₁₉ (right panel) incubated with sera samples from mice immunized with a synthetic peptide representing the promiscuous T cell epitope PyT53 (T₁₁₅₄-Q₁₁₇₃) (lane A and E), mice immunized with the corresponding recombinant protein (lane B and F), the monoclonal antibody 3D11 that recognize the *P. berghei* tag sequence [79] (lane C and G), or an anti-6X His tag monoclonal antibody (lane D and H). The molecular weight markers (BioRad) are indicated to the left. (B) Antigenicity of the *P. yoelii* chimeric proteins determined by ELISA, PyRMC-MSP1₁₉ closed

symbols, PyMSP1₁₉, open symbols. Recombinant chimeras were tested using anti-PyT53 (●○), anti-PyRMC-MSP1₁₉ (▼;), anti- PyMSP1₁₉ (△), 3D11 (◆◇) or anti-His tag (■□). The monoclonal antibody MRA-94 that recognized the *P. falciparum* MSP1 was included as control of antigen specificity (▲▽). Data are presented as the geometric mean O.D. obtained at different concentrations of the corresponding monoclonal antibodies (1 µg to 0.06 ng/ml) or the reciprocal of the sera dilution obtained from mice immunized with *P. yoelii* PyRMC-MSP1₁₉ (dilutions 1:1000 to 1:16384000) or mice immunized with the synthetic peptide PyT53 (dilutions 1:100 to 1: 4096000). Antigen specificity was confirmed using pre-immune sera samples as a control (data not shown).

Figure 3.

Kinetics of the anti- PyRMC-MSP1₁₉ or anti-PyMSP1₁₉ antibody responses in BALB/c and C57BL6 mice immunized with the homologous or heterologous antigen determined by ELISA. Antibody titers were measured 20 days after each immunization of BALB/c (A) or C57BL6 mice (B) with the PyRMC-MSP1₁₉ or anti-PyMSP1₁₉ and expressed as reciprocal geometric mean antibody titers $\pm$ standard deviation (n=6). Sera from mice immunized with the corresponding protein were tested with the homologous (left panel) or heterologous (right panel) antigen on day 20 (black bars), day 40 (stripped bars) and day 60 (white bars).

Figure 4.

Reactivity of the antibodies elicited in rabbits with native *P. yoelii* proteins. (A) Western blot analysis of late stage parasite extracts. The samples were subjected to SDS-PAGE on a 5 to 15% polyacrylamide gradient gel, blotted onto nitrocellulose and then probed with purified IgG from rabbits immunized with PyRMC-MSP1₁₉ (lanes 1 and 2), purified IgG from rabbits immunized with PyMSP1₁₉ (lanes 3 and 4) or IgG from normal rabbits (lanes 5 and 6) at 0.6 μg/ml. The antibodies react with protein extract derived from *P. yoelii* 17X merozoites (lanes 1, 3 and 5) or *P. yoelii nigeriensis* N67 (lanes 2, 4 and 6). The mobility of molecular mass markers is indicated. (B) Recognition of soluble protein extract determined by ELISA using *P. yoelii* 17X, closed symbols, or *P. yoelii nigeriensis* N67 parasite extracts, open symbols. The protein extract were tested using purified IgG from rabbits immunized with PyRMC-MSP1₁₉ (●○), purified IgG from rabbits immunized with PyMSP1₁₉ (▼;△) or purified IgG from normal rabbits (■□). Data are presented as O.D. values at 405 nm using different concentrations of purified IgG preparations. (C) Characteristic immunofluorescence pattern of purified IgG from rabbits immunized with PyRMC-MSP1₁₉ on erythrocytes infected with *P. yoelii* 17X (upper panel) or *P. yoelii nigeriensis* N67 (lower panel). Left panels show parasite nuclei stained with DAPI, central panels show the anti-PyRMC-MSP1₁₉ antibody reactivity using AlexaFluor 488 goat anti-rabbit IgG-specific. The right panels are a merge of the blue and green fluorescence channels. A weak specific fluorescence was detected on erythrocytes infected with *P. yoelii* 17X using PyMSP1₁₉ (data not shown). No specific fluorescence was detected on erythrocytes infected with *P. yoelii nigeriensis* N67 with purified IgG rabbits immunized with PyMSP1₁₉ (data not shown). No specific fluorescence was found with purified IgG derived from normal rabbits using either homologous or heterologous strains.

Figure 5

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Figure 5.

Effects of immunization with either PyRMC-MSP1₁₉ or PyMSP1₁₉ on the time course curves after experimental challenge with *P. yoelii* 17X. BALB/c (A) or C57BL6 (B) mice were immunized three times subcutaneously using Montanide ISA 51 as adjuvant. Placebo control mice received adjuvant alone. The top panels summarize the area under the parasitemia time curves and the bottom panels the area under the hemoglobin time curves, obtained using the trapezoidal method [48,49], in mice immunized with PyRMC-MSP1₁₉ (●), PyMSP1₁₉ (▽) or placebo (■). For parasitemia values, the total areas under the parasitemia change versus time curves were calculated and the mean change values expressed as percentage of parasitemia (n=6). For hemoglobin values, the total areas under the hemoglobin change versus time curves were calculated. Subsequently the mean change values in hemoglobin concentration per day were determined by comparison with baseline concentrations and expressed as percentage of reduction in hemoglobin levels. Data are representative of two independent experiments. 50% reduction in hemoglobin concentration relative to baseline is indicated with broken lines.

Figure 6.

Effects of passive immunization on the time course curves after experimental challenge with *P. yoelii* 17X (A) or *P. yoelii nigeriensis* N67 (B). The top panels summarize the area under the parasitemia time curves and the bottom panels the area under the hemoglobin time curves, obtained using the trapezoidal method [48,49], in mice immunized with PyRMC-MSP1₁₉ (●), PyMSP1₁₉ (▽) or placebo (■). For parasitemia values, the total areas under the parasitemia change versus time curves were calculated and the mean change values expressed as percentage of parasitemia (n=10). For hemoglobin values, the total areas under the hemoglobin change versus time curves were calculated. Subsequently the mean change values in hemoglobin concentration per day were determined by comparison with baseline concentrations and expressed as percentage of reduction in hemoglobin levels. Data are representative of two independent experiments. 50% reduction in hemoglobin concentration relative to baseline is indicated with broken lines. (+) Indicate animals that were euthanized due to severe anemia.

Table 1

Cytokine responses in BALB/c and C57BL/6 mice after priming with PyRMC-MSP1₁₉ or PyMSP1₁₉.

Mouse strain	Group	Autologous protein						PyT8				PyT53			
		INF- γ^a	IL-2 ^b	IL-5 ^b	IL-10 ^b	INF- γ^a	IL-2 ^b	IL-5 ^b	IL-10 ^b	INF- γ^a	IL-2 ^b	IL-5 ^b	IL-10 ^b	INF- γ^a	IL-2 ^b
BALB/c	PyRMC-MSP1 ₁₉	1310±422*	145.4±9.6*	2.6±2.8	174.3±1.2*	112±53	0	6.6±5.4	1.2±1.6	372±155*	1.9±6.6	0.9±1.3	0.1±0.1		
	PyMSP1 ₁₉	34±21	5.3±7.8	64±89	4.6±3.3	15±14	0.8±1.2	0.3±0.2	0.3±1.9	25±22	0	0.5±0.7	0.8±0		
C57BL/6	PyRMC-MSP1 ₁₉	603±38*	130±19.7	1.6±2.2	316.7±13.9*	0	8.5±11.2	4.0±1.7*	0	1	0	0.9±1.2	0		
	PyMSP1 ₁₉	118±80	108±6	0	17±7	176±77*	13±10	0	0	0	0	0	0		

^aNumber of IFN- γ secreting cells determined by ELISPOT after priming using *ex vivo* stimulation of spleen cells with the autologous protein or synthetic peptides representing the sequences PyT8 or PyT53. Data are presented as number of SFC per 10⁶ spleen cells derived from four mice tested individually $\pm$ SD. The frequency of protein or peptide IFN- γ secreting cells were subtracted from the frequency obtained with cells incubated with medium alone.

* $P < 0.05$.

^bCytokine concentration in 48 hours supernatants after *ex vivo* stimulation of pooled spleen cells with the autologous protein or synthetic peptides representing the sequences PyT8 or PyT53. Data are presented in pg/ml determined by multiplex proteomic array. The data represents means $\pm$ SD of cytokine levels expressed in pg/ml. Protein or peptide-stimulated cells were subtracted from the cytokine levels obtained with cells incubated with medium alone.

* $P < 0.05$.