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Protective immune responses elicited by immunization with a chimeric blood-stage malaria vaccine persist but are not boosted by *Plasmodium yoelii* challenge infection

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Abstract

An efficacious malaria vaccine remains elusive despite concerted efforts. Using the *Plasmodium yoelii* murine model, we previously reported that immunization with the C-terminal 19 kDa domain of merozoite surface protein 1 (MSP1₁₉) fused to full-length MSP8 protected against lethal *P. yoelii* 17XL, well beyond that achieved by single or combined immunizations with the component antigens. Here, we continue the evaluation of the chimeric PyMSP1/8 vaccine. We show that immunization with rPyMSP1/8 vaccine elicited an MSP8-restricted T cell response that was sufficient to provide help for both PyMSP1₁₉ and PyMSP8 specific B cells to produce high and sustained levels of protective antibodies. The enhanced efficacy of immunization with rPyMSP1/8, in comparison to a combined formulation of rPyMSP1₄₂ and rPyMSP8, was not due to improved conformation of protective B cell epitopes in the chimeric molecule. Unexpectedly, rPyMSP1/8 vaccine-induced antibody responses were not boosted by exposure to *P. yoelii* 17XL infected RBCs. However, rPyMSP1/8 immunized and infected mice mounted robust responses to a diverse set of blood-stage antigens. The data support the further development of an MSP1/8 chimeric vaccine but also suggest that vaccines that prime for responses to a diverse set of parasite proteins will be required to maximize vaccine efficacy.

1. INTRODUCTION

Plasmodium falciparum has taken a great toll on human health with nearly half the population of the world at risk of infection [1]. The burden of malaria is borne by the poor who, in most cases, cannot afford or access the otherwise available treatments. With the development and spread of drug resistant parasites and less than optimal vector control strategies, an effective malaria vaccine remains the most feasible strategy to control this disease [1]. Merozoite surface proteins are a focus for subunit vaccines designed to block merozoites from invading and replicating within red blood cells (RBCs) and thus preventing malaria associated pathology [2-5]. Merozoite surface protein 1 (MSP1), arguably the lead candidate, is an essential and abundant surface protein synthesized as a ~200 kDa precursor protein during schizont stages [2-6]. Upon synthesis, MSP1 is proteolytically processed into four fragments of 83, 30, 38 and 42 kDa [7-9], which remain non-covalently associated with

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MSP6 and MSP7 [10-11]. This multimeric complex is tethered to the merozoite surface by the C-terminal GPI-anchored 42 kDa fragment (MSP1₄₂) [9]. During invasion, MSP1₄₂ is further cleaved into MSP1₃₃ and MSP1₁₉ releasing the whole complex except for MSP1₁₉ which remains GPI-anchored and is carried into the newly invaded RBC [12].

MSP1₁₉, comprised of two compact and highly conserved epidermal growth factor (EGF)-like domains [6,13-15], is a prime target of protective antibodies [12,16-23] informing its inclusion in all MSP1-based vaccine formulations. However, MSP1₁₉ elicits very poor CD4 T cell responses [24-26] limiting the help needed for B cells to produce protective antibodies of sufficient quantity and quality. In fact, successful efficacy studies in experimental models have required fusion of MSP1₁₉ to heterologous T cell epitopes and/or formulation with Freund's adjuvant; a potent adjuvant but not suitable for use in human subjects. The choice of the larger *Pf*MSP1₄₂ processed fragment for further clinical development was based mainly on the need to incorporate MSP1₃₃ sequences in order to provide parasite-specific T cell epitopes. While *Pf*MSP1₃₃ induces good T cell responses [25,27-28], it is polymorphic [14] which may limit the scope of *rPf*MSP1₄₂ vaccine-induced protection. Furthermore, immunization with MSP1₄₂ induces good antibody responses to MSP1₃₃ but these appear to only contribute modestly to protection [29-30]. Thus far, clinical trials with *Pf*MSP1₄₂ have been disappointing partly due to antigen polymorphism and/or poor potency of the adjuvants tested [31-36]. Even though MSP1-based vaccines still hold promise, the likelihood that a subunit vaccine will be successful in controlling this complex parasite is being questioned.

Previously, we identified MSP8 in *Plasmodium yoelii* 17XL, a rodent malaria parasite, to complement MSP1 and improve the efficacy of MSP-based vaccines [37]. Like MSP1, MSP8 possesses two C-terminal EGF-like domains that share significant homology and possibly similar function [37-39]. Importantly, immunization with *rPy*MSP8 also confers antibody-dependent protection against challenge infection with *P. yoelii* 17XL [37,40]. Unlike MSP1₄₂, MSP8 is well-conserved among *P. falciparum* strains [38] and can therefore provide non-polymorphic, parasite-specific B cell and CD4 T cell epitopes. In our previous studies, we generated a chimeric *rPy*MSP1/8 vaccine by fusing *Py*MSP1₁₉ onto the N-terminus of *Py*MSP8. We showed that immunization with *rPy*MSP1/8 afforded superior protection against *P. yoelii* 17XL malaria compared to immunization with single or admixture of *rPy*MSP1₄₂ and *rPy*MSP8 [41]. In the present study, we set out to further characterize the protective immune responses elicited by this chimeric *rPy*MSP1/8 model vaccine to better understand the basis for the enhanced efficacy. Here, we report on 1) the specificity of *rPy*MSP1/8 immunization-induced T cell and B cell responses; 2) the durability of *rPy*MSP1/8 immunization-induced protective responses; and 3) the variability in boosting *P. yoelii* antigen-specific antibody responses in immunized animals upon challenge infection with blood-stage parasites.

2. MATERIALS AND METHODS

2.1. Experimental animals and parasites

Male BALB/cByJ mice, 5 to 6 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in the Animal Care Facility at Drexel University College of Medicine under specific pathogen-free conditions. Lethal *Plasmodium yoelii* 17XL and non-lethal *P. yoelii* 17X parasites were originally obtained from William P. Weidanz (University of Wisconsin, Madison, WI) and maintained as cryopreserved stabilates. All animal studies were reviewed, approved and conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) of Drexel University College of Medicine.

2.2. Immunization and challenge protocols

Production, purification and refolding of His₆-tagged recombinant proteins rPyMSP1₄₂, rPyMSP8 and rPyMSP1₁₉ as well as glutathione S-transferase (GST) and the fusion protein, GST-PyMSP1₁₉ have previously been described [41]. Groups of BALB/cByJ mice (5-10/group) were immunized subcutaneously with 14 µg of purified rPyMSP1/8 formulated with 25 µg of Quil A as adjuvant (Accurate Chemical and Scientific Corporation, Westbury, NY). Control mice were immunized with 25 µg of Quil A alone. In all experiments, mice were boosted twice at three week intervals with the same regimen used in the priming immunization. This protocol was previously shown to induce protection against a lethal challenge with *P. yoelii* 17XL [41]. Cells and serum were harvested as indicated below. Additional groups of immunized and control mice were challenged by intraperitoneal injection of 1×10^5 *P. yoelii* 17XL parasitized RBCs (pRBCs) obtained from donor mice. Blood parasitemia was monitored through the course of infection by microscopic examination of Giemsa-stained thin blood smears of tail blood. In compliance with the IACUC policy, infected mice were euthanized when parasitemia exceeded 50% and the infection in such animals was recorded as lethal.

For the analysis of antibody response induced by infection only, naïve BALB/cByJ mice (n=5) were infected i.p. with 1×10^5 *P. yoelii* 17X pRBCs and parasitemia monitored. One week following clearance of parasites from circulation, primary infection sera were collected. A second group of naïve BALB/cByJ mice (n=5) were similarly infected. Following resolution of the primary infection, these mice were re-challenged twice with 1×10^7 *P. yoelii* 17X pRBCs. One week after the final rechallenge, tertiary infection sera were collected.

2.3. Antigen-specific T cell proliferation assay

BALB/cByJ mice (5 mice/group) were immunized as above with rPyMSP1/8 formulated with Quil A as adjuvant or with Quil A alone. Approximately 10 weeks after the last immunization, spleens were harvested and single cell suspensions prepared. RBCs were lysed with AKC lysis buffer (0.15 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA, pH 7.0). Membrane debris was removed by filtering the cell suspensions through sterile gauze and the viability of recovered splenocytes determined by trypan blue exclusion. Splenocytes were plated in 96-well round-bottomed Falcon plates (BD Biosciences, San Jose, CA) at a concentration of 1×10^5 cells/well in complete medium consisting of RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM L-glutamine, 0.5 mM sodium pyruvate, 50 µM 2-mercaptoethanol, and 1X streptomycin/penicillin (Invitrogen Corporation, Carlsbad, CA), plus 10% heat-inactivated fetal bovine serum (Atlanta Biologicals Inc., Lawrenceville, GA). Cells were stimulated in triplicate sets with rPyMSP1/8 (1.2 µg/ml), rPyMSP8 (1 µg/ml), GST-PyMSP1₁₉ (1 µg/ml), GST alone (1 µg/ml) or Concanavalin A (1 µg/ml, Sigma-Aldrich). Polymixin B (10 µg/ml, Sigma-Aldrich) was also added to eliminate any effect of LPS. An additional set of wells was left unstimulated and served as the negative control. The plates were cultured at 37°C in 5% CO₂ for 4 days and were pulsed with 1 µCi per well of methyl [³H] thymidine (40-60 Ci/mmol, GE Healthcare, Piscataway, NJ) for the last 18 hours of incubation. Cells were harvested on fiber filters with an automatic cell harvester and incorporation of ³H-thymidine was measured by liquid scintillation counting (Perkin Elmer Life and Analytical Sciences, Shelton, CT). The stimulation index was calculated as the mean counts per minute of stimulated wells divided by the mean counts per minute of unstimulated wells.

2.4. Immunoaffinity chromatography

Serum was collected from rPyMSP1/8 immunized mice two weeks after the final immunization and depleted of antibodies specific to PyMSP1 and PyMSP8 using

recombinant antigens immobilized on a solid matrix. To immobilize rPyMSP8 or rPyMSP1₄₂, Ni-NTA beads (Ni-NTA Superflow matrix, Qiagen Inc, Valencia, CA.) were washed twice in distilled water and charge buffer (0.4 M NiSO₄) and then equilibrated in binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl). After equilibration, 100 µg of either rPyMSP8 or rPyMSP1₄₂ diluted in the binding buffer were added to 100 µl of packed Ni-NTA beads and rocked overnight at 4°C. To immobilize GST-PyMSP1₁₉, glutathione agarose beads (GE Healthcare) were washed three times in TBS (25 mM Tris-HCl pH 8.0, 150 mM NaCl). After washing, 100 µg of GST-PyMSP1₁₉ diluted in TBS were added to 100 µl of packed beads and rocked overnight at 4°C. To remove the unbound antigen, the beads were gently pelleted and washed twice in TBS and twice in TBS containing 0.1% Tween 20 and 1% bovine serum albumin (BSA, Sigma-Aldrich). Equal volumes of anti-rPyMSP1/8 serum from individual mice were pooled and diluted 1:500 in TBS-0.1% Tween 20 + 1% BSA. To deplete antibodies specific to PyMSP8, diluted serum (1 ml per 100 µl of antigen-coupled beads) was applied onto immobilized rPyMSP8 and incubated for 2 hours at 4°C with mixing. The recovered supernatant was then applied to columns containing immobilized GST-PyMSP1₁₉ or immobilized rPyMSP1₄₂ (as indicated in the specific experiment) for an additional 2 hours to remove antibodies specific to PyMSP1. In control experiments, serum was absorbed with either Ni-NTA or glutathione agarose beads devoid of bound recombinant proteins. Following antibody depletion, the reactivity of the control absorbed and antigen-absorbed serum against rPyMSP1/8, rPyMSP8, rPyMSP1₄₂ or GST-PyMSP1₁₉ was analyzed by ELISA.

2.5. Antigen-specific ELISA

Serum was collected from immunized and control mice approximately two weeks following the third immunization, just prior to *P. yoelii* 17XL challenge infection and at later time points following immunization and challenge as indicated in specific experiments. The titers of antigen-specific antibodies were measured by ELISA as previously described [41]. Briefly, high-binding ELISA plates (Easy-Wash; Corning Costar Corporation, Cambridge, MA) were coated with 0.25 µg per well of rPyMSP1/8, rPyMSP8, rPyMSP1₄₂ or GST-PyMSP1₁₉ diluted in 100 mM Na₂CO₃-NaHCO₃ pH 9.6, and incubated overnight at 4°C. Antigen coated plates were washed and blocked for 1 hour in TBS containing 5% nonfat dry milk. Two-fold serial dilutions of absorbed sera in TBS-0.1% Tween-20 + 1% BSA were added to antigen coated wells and incubated for 1 hour at room temperature. Bound antibodies were detected using horseradish peroxidase-conjugated rabbit antibody specific for mouse immunoglobulin G (Zymed Laboratories, South San Francisco, CA) and ABTS (2, 2'-azinobis(3-ethylbenzthiazolinesulfonic acid)) as substrate (KPL, Inc, Gaithersburg, MD). For each dilution, the mean absorbance values at 405 nm (A_{405}) of the pooled sera from adjuvant control mice ($n = 5$) were subtracted as background. For each serum sample, A_{405} values of between 1.0 and 0.1 were plotted and titer calculated as the reciprocal of the dilution of serum that yielded an A_{405} of 0.5. A high titer pool of serum obtained from rPyMSP1/8 immunized mice ($n=5$) was included in each assay as an internal reference to normalize the data between assays.

2.6. Metabolic labeling and immunoprecipitation of parasite proteins

When parasitemia in *P. yoelii* 17XL infected BALB/cByJ mice was approximately 20-30%, blood was collected into RPMI 1640 containing 20 mM MOPS, pH 7.4 and heparin (10 units/ml). RBCs were washed twice and resuspended at 20% hematocrit in methionine-free RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 20 mM HEPES, pH 7.4 and 10 µg/ml hypoxanthine. EasyTag express mix [³⁵S] methionine/cysteine (>1000 Ci/mmol, Perkin-Elmer) was added at a concentration of 100 µCi/ml and cells cultured at 37°C in 5% CO₂ for 3 hours. An equal volume of RPMI 1640 with methionine (supplemented with 2 mM L-glutamine, 20 mM HEPES, pH 7.4 and 10 µg/ml hypoxanthine) was then added and

incubation continued for an additional hour. RBCs were pelleted and washed twice in PBS, pH 7.4. The cell pellet was resuspended in 10 volumes of solubilization buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate) and rocked for 2 hours at 4°C. Any remaining insoluble material was removed by centrifugation at 13,000 rpm for 20 minutes at 4 °C. The amount of incorporated label was determined by liquid scintillation counting of proteins precipitated by 5% trichloroacetic acid. For each sample, approximately 1×10^5 cpm of parasite proteins were pre-precipitated with normal BALB/cByJ sera diluted 1:50 to reduce non-specific binding. Labeled parasite proteins were then immunoprecipitated with mouse sera (1:50) collected following rPyMSP1/8 immunization and *P. yoelii* 17XL challenge infection. Protein G agarose beads (Invitrogen) were used to collect antigen-antibody complexes. Precipitated proteins were separated by SDS-PAGE on 10% polyacrylamide gels. Gels were fixed and treated with a fluorographic reagent (Amplify, GE Healthcare). Immunoprecipitated proteins were visualized by autoradiography.

2.7. Statistical analysis

The statistical significance of the differences in mean peak parasitemia and in stimulation indices in T cell proliferation assays was determined by two tailed, unpaired Student's t-test. The significance of differences in lethality between groups was determined by Mantel-Haenszel log rank test (GraphPad Prism 4.0; GraphPad Software Inc., San Diego CA). Probability (*p*) values < 0.05 were considered significant.

3. RESULTS

3.1. PyMSP1/8 vaccine induces robust T cell responses confined to epitopes within MSP8

MSP1₁₉ has been reported to elicit poor T cell responses [24-27]. In developing the chimeric rPyMSP1/8 vaccine, it was expected that T cell recognition of PyMSP8 associated epitopes would be adequate to provide help for the production of antibodies to both PyMSP8 and PyMSP1₁₉ components. Alternatively, the enhanced antibody response to PyMSP1₁₉ observed could have been due to direct T cell recognition of PyMSP1₁₉ as a result of increased accessibility of epitopes in the chimeric recombinant molecule relative to the native protein. To test this, BALB/cByJ mice were immunized three times at three-week intervals with rPyMSP1/8 formulated with Quil A as adjuvant or with Quil A alone. Two weeks after the last immunization, one set of mice from each group (n=5) was challenged with a lethal dose of *P. yoelii* 17XL and the infection monitored. As previously reported [41], mice immunized with rPyMSP1/8 developed a low grade parasitemia with all animals clearing the infection while control mice succumbed to the infection by day 12 (data not shown). The second set of mice was not challenged but rested to allow vaccine-induced immune responses to subside. Approximately 10 weeks after the third rPyMSP1/8 immunization, splenocytes were harvested and stimulated in vitro with equimolar amounts of rPyMSP1/8, rPyMSP8, GST-PyMSP1₁₉ or GST. Antigen-specific T cell proliferation was quantified by [³H] thymidine incorporation after 4 days of culture. As shown in Figure 1, stimulation of splenocytes from rPyMSP1/8 immunized mice with either rPyMSP1/8 or rPyMSP8 induced high T cell proliferative responses relative to the adjuvant control mice (*p*≤0.001). In contrast, T cell proliferation upon stimulation with GST-PyMSP1₁₉ was minimal and similar in magnitude to that induced by GST alone (*p*=0.23). These results indicate that rPyMSP1/8 elicited robust T cell responses that were restricted to the epitopes within the PyMSP8 fragment of the chimeric protein.

3.2. The enhanced efficacy of the PyMSP1/8 vaccine cannot be explained by improved conformation of protective epitopes

The enhanced efficacy of immunization with rPyMSP1/8 in comparison to an admixture of rPyMSP1₄₂ and rPyMSP8 correlated with a marked increase in the titer of PyMSP1₁₉-specific antibodies [41]. In addition to quantity, it is also possible that differences in the conformation of the relevant epitopes on these recombinant MSP-based vaccines affected the quality of the antibody responses elicited upon immunization. To test this possibility, antibodies elicited by immunization with rPyMSP1/8 that could bind to rPyMSP8 or GST-PyMSP1₁₉ were removed from immune sera by adsorption on immobilized recombinant antigens as outlined in Figure 2A. The reactivities of unabsorbed and absorbed sera were then compared by ELISA on wells coated with GST-PyMSP1₁₉, rPyMSP8 or rPyMSP1/8. As shown in Figure 2B, removal of antibodies that reacted with rPyMSP8 and GST-PyMSP1₁₉ reduced the reactivity of the anti-rPyMSP1/8 sera on rPyMSP1/8 coated wells by >95%. Alternatively, antibodies elicited by immunization with rPyMSP1/8 that could bind to rPyMSP8 or rPyMSP1₄₂ were removed from sera by immunoadsorption on matrices containing the respective antigens. The reactivities of unabsorbed and absorbed sera were compared by ELISA as above. Removal of antibodies that reacted with rPyMSP8 and rPyMSP1₄₂ also reduced the reactivity of the anti-rPyMSP1/8 sera on rPyMSP1/8 coated wells by >95% (Figure 2C). These data indicate that the PyMSP1 EGF-like domains of GST-PyMSP1₁₉, rPyMSP1₄₂ and rPyMSP1/8 bear a similar, if not identical, conformation. Likewise, the overall conformation of PyMSP8 is similar when expressed alone or as part of the chimeric rPyMSP1/8 antigen. It appears unlikely that enhanced efficacy of immunization with rPyMSP1/8, in comparison to a combined formulation of rPyMSP1₄₂ and rPyMSP8, was due to an improved conformation of protective B cell epitopes or the generation of novel epitopes in the chimeric molecule.

3.3. PyMSP1/8 vaccine induces long-lasting protective responses

The ability of rPyMSP1/8 vaccine-induced protection to persist over time was tested as outlined in Figure 3A. BALB/cByJ mice were immunized three times with rPyMSP1/8 formulated with Quil A as adjuvant or with Quil A alone. Following the third immunization, one set of rPyMSP1/8 immunized and control mice (group #1) was rested for approximately 15 weeks and then challenged with *P. yoelii* 17XL pRBCs and the course of infection monitored. In parallel, a second set of rPyMSP1/8 immunized and control mice (group #2) was challenged two weeks after the last immunization with *P. yoelii* 17XL pRBCs and the course of infection monitored. Despite the long period between immunization and challenge infection in group #1 mice, the protective efficacy of the rPyMSP1/8 vaccine remained high with 8/9 mice surviving an otherwise lethal challenge infection (Figure 3B). Parasitemia in adjuvant control mice was fulminant and resulted in 100% mortality by day 12 post-infection. Group #1 mice (Figure 3B) cleared blood-stage parasites from circulation by day 30 with a slightly higher mean peak parasitemia compared to group #2 mice (Figure 3C) that were challenged two weeks following the last immunization. This difference however, was not statistically significant ($p=0.42$). Following clearance of the primary infection, group #2 mice were then rested for approximately 2.5 months during which no parasite recrudescence was detected. On day 106 following the primary challenge infection, group #2 mice were rechallenged with *P. yoelii* 17XL pRBCs and course of infection monitored. As shown in Figure 3D, all rPyMSP1/8 immunized mice in group #2 were solidly protected against the secondary infection with no patent parasitemia detected. As expected, all age-matched, naïve control mice infected at the same time succumbed to *P. yoelii* 17XL malaria by day 11. Combined, these data indicate that rPyMSP1/8 vaccine-induced immune responses are sustained for a significant period of time in the absence of parasite exposure and that immunized mice are protected against repeated infections.

3.4. *P. yoelii* challenge infection does not boost vaccine-induced antibody responses

The prolonged period of protection following rPyMSP1/8 immunization could potentially be explained by high and sustained levels of PyMSP8 and/or PyMSP1₁₉ specific antibodies in serum. Alternatively, exposure to *P. yoelii* pRBCs upon challenge infection could further boost rPyMSP1/8 vaccine-induced responses to protective levels. The latter possibility was of particular interest considering that the T cell response elicited by rPyMSP1/8 immunization was largely restricted to PyMSP8 epitopes. In the experimental design outlined in Figure 3A, serum was collected from group #2 mice just prior to the first *P. yoelii* 17XL challenge infection (day 0) and then again post-infection on day 45. A third set of serum samples was collected on day 102 just prior to secondary *P. yoelii* 17XL challenge infection and then again post-clearance, on day 143. For group #1 mice, serum samples were collected at the same time points as group #2. The only major difference was that group #1 mice were challenged only once and this primary challenge was delayed until day 106 (according to the group #2 timeline).

As shown in Figure 4A and 4B, analysis of prechallenge day 0 sera showed high titers of antibodies specific for the chimeric rPyMSP1/8 as well as the individual PyMSP1₁₉ and PyMSP8 components. By day 45, antibody titers to each antigen dropped approximately 3-4 fold ($p < 0.001$). Surprisingly, the drop in group #1 and group #2 mice was comparable and did not appear to be influenced by *P. yoelii* 17XL challenge of group #2 mice. Between day 45 and day 102, the level of antibodies specific for both PyMSP1₁₉ and PyMSP8 remained essentially unchanged in both groups of mice. Analysis of serum collected on day 143 following *P. yoelii* 17XL challenge infection on day 106 also showed no significant change in PyMSP1₁₉ or PyMSP8 specific antibodies. While rPyMSP1/8 elicited antibodies do persist in circulation over time, the vaccine-induced antibody responses to PyMSP1₁₉ and PyMSP8 were not boosted by repeated exposure to *P. yoelii* 17XL infected RBCs. These data raised the possibility that PyMSP1₁₉ and/or PyMSP8 are poorly immunogenic during the normal course of *P. yoelii* malaria. To address this, antigen-specific antibodies induced upon challenge of naïve mice with non-lethal *P. yoelii* 17X were measured. As shown in Figure 5, PyMSP1₁₉-specific and PyMSP8-specific IgG antibodies were readily detected in convalescent serum of mice that suppressed single or multiple *P. yoelii* 17X challenge infections. Both PyMSP1₁₉ and PyMSP8 are immunogenic in unimmunized mice during the normal course of *P. yoelii* malaria.

3.5. *P. yoelii* challenge infection of rPyMSP1/8 immunized mice elicits significant responses to a diverse set of parasite proteins

The lack of boosting of vaccine-induced responses upon challenge infection raised questions regarding the ability of rPyMSP1/8 immunized mice to mount any response to the parasite upon challenge infection. To determine if rPyMSP1/8 immunized mice mounted significant antibody responses to antigens other than PyMSP1 and PyMSP8 upon challenge infection, day 143 sera from mice challenged once (group #1) or twice (group #2) were used to immunoprecipitate metabolically labeled *P. yoelii* 17XL blood-stage antigens. As shown in Figure 6A, group #1 mice mounted significant antibody responses to several other parasite antigens upon primary exposure during infection. The extent of seroreactivity generally corresponded to the level of parasitemia that developed in individual mice with higher parasitemia associated with stronger antibody responses against a diverse set of antigens. Of significant interest, these infection-induced antibody responses to *P. yoelii* 17XL blood-stage antigens were markedly boosted by a secondary *P. yoelii* 17XL challenge as shown in Figure 6B with sera from group #2 mice. Of note, group #2 mice never developed patent parasitemia upon secondary infection. While the vaccine-induced response to PyMSP1₁₉ and PyMSP8 was not boosted by a primary or secondary *P. yoelii* 17XL challenge, mice

mounted robust immune responses to a diverse set of blood-stage antigens upon primary infection and such responses could be boosted by a subsequent infection.

4. DISCUSSION

It is generally accepted that an effective *Pf*MSP1-based vaccine will incorporate the C-terminal EGF-like domains of MSP1₁₉, the critical targets of protective merozoite neutralizing antibodies [12,16-23]. We also know from studies in mice and non-human primates that quite high titers of MSP1₁₉-specific antibodies are required for protection [19-20,22,29,41-45]. In these experimental models, protective levels of MSP1₁₉-specific antibodies can be elicited by MSP1-based vaccines formulated with potent adjuvants such as Freund's adjuvant and/or by fusion of MSP1₁₉ to strong, heterologous T cell epitopes. Unfortunately, immunization of human subjects with r*Pf*MSP1₄₂ has not induced sufficiently high titers of MSP1 specific antibodies [31-36], which explains in part, the lack of efficacy in a recently concluded study in Kenyan children [36]. The potency of human-compatible adjuvants, the polymorphism of protective B cell epitopes of MSP1 and the relative strength of MSP1 associated T cell epitopes have all been considered in the discussion of the poor immunogenicity and efficacy. In an attempt to address some of these obstacles, we focused on improving the design of the vaccine construct by coupling MSP1₁₉ with the conserved and relatively immunogenic epitopes of MSP8 to generate a chimeric MSP1/8 vaccine. We previously reported that a chimeric rPyMSP1/8 vaccine, formulated with Quil A as adjuvant, markedly improved immunogenicity of PyMSP1₁₉ and afforded superior protection against lethal *P. yoelii* 17XL infection compared to rPyMSP1₄₂ and rPyMSP8, alone or in combination [41].

In the current study, we set out to further characterize the chimeric MSP1/8 as a vaccine candidate to determine if its further development was likely to overcome some of the difficulties encountered with r*Pf*MSP1₄₂ vaccines. Our first question was straightforward. Upon protective immunization with rPyMSP1/8, do T cells recognize MSP1 and/or MSP8 associated epitopes? Ex vivo antigen-specific splenocyte proliferation assays showed that immunization with rPyMSP1/8 + Quil A induced strong T cell responses that were exclusively restricted to the epitopes within the PyMSP8 component of the chimeric vaccine. Failure of PyMSP1₁₉ to induce any significant T cell response was expected and consistent with previous reports [24-27]. We infer from these data that MSP8 specific T cells provided sufficient help to both MSP1 and MSP8 specific B cells leading to the production of high levels of antigen-specific IgG. The significance of this finding is three-fold. First, the immunogenicity of MSP1₁₉ is improved when coupled to MSP8 instead of MSP1₃₃. MSP1₃₃ is a target of T cell responses [25,27-28], but we have not been able to induce as high of a PyMSP1₁₉ specific antibody response upon immunization with rPyMSP1₄₂ [41]. Second, the induction of strong, protective T and B cell responses upon immunization with rPyMSP1/8 did not depend on the use of Freund's adjuvant. Quil A is the predecessor of QS21 which has been employed as an adjuvant component in vaccine clinical trials involving RTS,S [46-47], *Pf*AMA1 [48-49] and *Pf*MSP1 [31-33,35-36]. Third, the use of MSP8 as a fusion partner for MSP1₁₉ instead of heterologous GST or tetanus toxoid T cell epitopes provides an opportunity to boost vaccine-induced responses by natural infection. This will be very important for immunization of human subjects where subunit vaccine-induced responses have been suboptimal relative to those observed in animal model systems. As important, MSP8 is highly conserved among *P. falciparum* isolates [38], significantly reducing potential problems related to epitope polymorphism.

Maintaining proper conformation of both rPyMSP1 and rPyMSP8 is critical for the generation of protective antibodies [18,40]. The second question of interest to us was largely technical. Was it possible that the conformation of PyMSP1₁₉ and/or PyMSP8 portions of

rPyMSP1/8 more accurately mimicked the native antigens in comparison to individual rPyMSP1 or rPyMSP8 antigens? Could it be that an interaction between PyMSP1₁₉ and PyMSP8 within the chimeric rPyMSP1/8 resulted in the formation of novel protective epitopes? Based on our series of absorption studies, the data convincingly show that the overall conformation of PyMSP8 in the chimeric rPyMSP1/8 was similar to that of non-fused PyMSP8. We also show that the conformation of the protective EGF-like domains of PyMSP1₁₉ in rPyMSP1/8, GST-PyMSP1₁₉ and rPyMSP1₄₂ was essentially identical since each MSP1 recombinant antigen effectively depleted PyMSP1₁₉-specific antibodies from anti-rPyMSP1/8 serum. Finally, we found no evidence for the creation of novel protective epitopes in the chimeric vaccine as all antibodies induced by immunization with rPyMSP1/8 bound to either MSP1 or MSP8 component antigens. Thus, the improved efficacy of the rPyMSP1/8 vaccine could not be explained by the differences in conformation of critical B cell epitopes. These data are very encouraging for the further development of a chimeric *P. falciparum* MSP1/8 vaccine as they demonstrate our ability to produce a recombinant product in a heterologous expression system that contains multiple, correctly formed disulfide bonds to yield native-like conformational domains.

The restriction of the T cell response elicited by rPyMSP1/8 immunization to PyMSP8 associated epitopes raised the third question. Would *P. yoelii* challenge infection boost PyMSP8 as well as PyMSP1₁₉ specific antibody responses? Following the third PyMSP1/8 + Quil A immunization, we rested animals for almost 4 months to allow immunization-induced immune responses to peak and contract to steady-state levels in order to increase the probability of detecting a boost upon infection. We learned that both PyMSP1₁₉ and PyMSP8 specific antibody levels dropped approximately 3-4 fold from peak values within 45 days, but then remained relatively constant. The lack of an antigen depot effect with the use of Quil A only as adjuvant may have contributed to this initial drop in titer. Fortunately, the persisting antibody level was still high and sufficient to confer significant protection against *P. yoelii* 17XL challenge infection. Jeamwattanaalert et al [50] have reported that sustained, protective levels of antibodies can be elicited by multiple immunizations with PyMSP1₁₉ emulsified in Freund's adjuvant or Montanide ISA51 + CpG ODN. However, with Quil A as adjuvant, 8 of 9 rPyMSP1/8 immunized animals survived the delayed challenge infection, but the mean peak parasitemia was somewhat higher than in mice challenged shortly after their third immunization, when antibody titers were at maximal levels. These data suggest that antibody levels in this rested group of immunized animals were near a threshold level for protection at the time of challenge. Nevertheless, we feel that this result is encouraging for the development of a *Pf*MSP1/8 chimeric vaccine as the model may indicate that a significant level of protection can be expected even for infrequently exposed individuals in endemic areas. Unexpected and somewhat disappointing was the finding that after immunization-induced responses declined to a steady state level, exposure to blood-stage parasites failed to boost rPyMSP1/8 primed antibody responses.

There are several factors that could explain the failure of infection to boost rPyMSP1/8 vaccine-induced responses. We considered the possibility that PyMSP8 itself may be poorly immunogenic in the context of natural infection. This does not appear to be the case as naïve animals infected with non-lethal *P. yoelii* 17X mount reasonable antibody responses to both PyMSP1₁₉ and PyMSP8. *Pf*MSP8 also appears to be immunogenic in *P. falciparum* infected individuals [38]. Alternatively, it may be possible that prechallenge PyMSP1₁₉ and PyMSP8 specific antibody levels interfered with infection-induced responses. Earlier reports have indicated that high titers of antibodies induced by immunization of non-human primates by repeated immunization with *Pf*MSP1-based vaccines formulated with Freund's adjuvant are not readily boosted upon *P. falciparum* challenge [51-52]. Although we allowed immunization induced responses to decline to a steady state level prior to challenge, antibody concentrations were still quite high. In fact, the level of antibodies specific for

PyMSP1₁₉ and *PyMSP8* were 8-30 fold higher in *PyMSP1/8* immunized mice on day 102 compared to levels induced by a primary *P. yoelii* 17X infection in naïve mice. In effect, such antibodies could facilitate clearance of specific antigen before T and B cell responses could be adequately boosted. If this is so, we would predict that in situations where vaccine induced antibody responses are suboptimal, boosting upon natural infection may be more likely. Such boosting of suboptimal *PyMSP1₁₉* vaccine induced responses upon challenge infection has been demonstrated [53]. Finally, in a recent study, Yoshida et al [54] showed that a baculovirus-based *PyMSP1₁₉* vaccine administered intranasally and a GST-*PyMSP1₁₉*/Alum vaccine administered intraperitoneally could both induce high titers of protective antibodies. However, natural boosting of *PyMSP1₁₉*-specific antibody responses by infection only occurred in mice immunized with the baculovirus-based *PyMSP1₁₉* vaccine. In studies involving human subjects, it will be important to determine if different vaccine platforms, adjuvants and/or routes of infection are more or less likely to promote boosting upon systemic exposure to malaria parasites.

It has also been reported that malaria specific memory T and B cells can be targeted for deletion during blood-stage malaria [55-57] and that pre-existing vaccine-induced humoral responses can impede the ability of mice to respond to other parasite antigens during infection [53]. Considering the possibility of a more global suppression or impairment of infection-induced immune responses in r*PyMSP1/8* immunized mice, we addressed our final question. Upon exposure to parasites following challenge infection, do r*PyMSP1/8* immunized mice mount a primary immune response to other blood-stage antigen(s)? Our data clearly showed that r*PyMSP1/8* immunized mice mounted significant antibody responses to a diverse set of parasite antigens when challenged with *P. yoelii* 17XL blood-stage parasites. Perhaps as important, these responses were boosted upon a second challenge of r*PyMSP1/8* immunized mice, which led to essentially complete protection, with no detectable blood parasitemia. This is consistent with the previous data showing that *PyMSP1₁₉*-specific antibodies alone are not sufficient to completely protect and that an active, infection-induced immune response is still needed [19,58]. Our present data and these previously reported data continue to inform the effort to develop a blood-stage malaria vaccine. Clearly, high levels of merozoite specific antibodies are necessary to influence the course of blood-stage malaria. Ongoing efforts to improve the immunogenicity of MSP-based vaccines and to induce sustained and boostable antibody responses are on the critical path. However, such antibodies may only provide an initial period of protection during which time the host needs to mount an immune response to a significantly larger set of parasite proteins. In this regard, inactive or attenuated whole-blood-stage vaccines that could prime for such a heterogeneous response are appealing. In reality however, these will be limited by practical issues related to production, mass distribution and/or safety. Novel approaches still need to be developed that attempt to diversify vaccine-induced immune responses toward a significantly large set of parasite antigens. Since it is likely that the magnitude of the response to individual antigens elicited by such a multicomponent vaccine may not be optimal, boosting upon natural infection will also be a requirement for efficacy.

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Figure 1. rPyMSP1/8 vaccine induces robust T cell responses confined to epitopes within MSP8 BALB/cByJ mice (5 mice/group) were immunized three times with rPyMSP1/8 with Quil A as adjuvant (open bars) or with Quil A alone (closed bars). Approximately 10 weeks after the third immunization, splenocytes were harvested and stimulated with rPyMSP1/8, rPyMSP8, GST-PyMSP1₁₉ or GST as indicated on the x-axis. After four days of culture, proliferation was quantitated by ³H-thymidine incorporation. The stimulation index was calculated as mean counts per minute in stimulated cultures/mean counts per minute in unstimulated cultures. Mean values $\pm$ SD are shown.

Figure 2. The conformation of protective B cell epitopes on rPyMSP1/8, rPyMSP8 and rPyMSP1 based vaccines is comparable

Antibodies specific to PyMSP8 and PyMSP1₁₉ were depleted from anti-rPyMSP1/8 sera by sequential absorption as outlined in panel A over matrices of immobilized rPyMSP8 and GST-PyMSP1₁₉ (panel B) or rPyMSP8 and rPyMSP1₄₂ (panel C). For controls, pooled anti-rPyMSP1/8 serum was passed over Ni-NTA or glutathione agarose beads devoid of bound recombinant antigens. Reactivity of the antigen-adsorbed sera (broken lines) and control-adsorbed sera (solid lines) against rPyMSP1/8 (●), rPyMSP8 (▲), GST-PyMSP1₁₉ (■) or rPyMSP1₄₂ (◆) was determined by ELISA.

Figure 3. Immunization with rPyMSP1/8 vaccine induces long lasting protective responses

A. Schematic summarizing immunization and challenge protocol. **B.** Group #1 BALB/cByJ mice were immunized with rPyMSP1/8 with Quil A as adjuvant (▽, n=9) or with Quil A alone (◇, n=8). The mice were rested for approximately 15 weeks following the last immunization and then challenged with 1×10^5 *P. yoelii* 17XL pRBCs (delayed primary). Course of infection was monitored by Giemsa stained thin smears of tail blood. **C.** In parallel, group #2 BALB/cByJ mice were immunized as above with rPyMSP1/8 (▼, n=10) or with Quil A alone (◇, n=8). Two weeks following the last immunization, mice were challenged with 1×10^5 *P. yoelii* 17XL pRBCs (*P. yoelii* 17XL - primary) and the course of infection monitored. **D.** Approximately 13 weeks following the primary challenge infection, rPyMSP1/8-immunized group #2 mice (▼) were re-challenged with 1×10^5 *P. yoelii* 17XL pRBCs (*P. yoelii* 17XL - secondary) and course of infection monitored. At this time, age-matched mice immunized with Quil A alone (◇, n=5) were used as controls. For all challenge studies, mean % parasitemia ($\pm$ SD) versus days post-infection are shown. 'D' refers to the number of deceased animals at each time point.

Figure 4. *P. yoelii* challenge infection does not boost rPyMSP1/8 vaccine-induced antibody responses

Total IgG antibody titers (mean $\pm$ SD) against GST-*PyMSP1*₁₉, (□), rPyMSP8 (▒) and rPyMSP1/8 (■) in sera collected from rPyMSP1/8 immunized mice were determined by ELISA. **A.** Sera from group #1 mice were collected two weeks following the third immunization with rPyMSP1/8 + Quil A (day 0), and subsequently on days 45, 102 and 143. Mice in group #1 were challenged with *P. yoelii* 17XL on day 106 as indicated. **B.** Sera from group #2 mice were collected two weeks following the third immunization with rPyMSP1/8 + Quil A just prior to *P. yoelii* 17XL challenge infection (day 0) and again on days 45, 102 and 143. Mice in group #2 received a second challenge infection with *P. yoelii* 17XL on day 106 as indicated.

Figure 5. *PyMSP1₁₉* and *PyMSP8* are immunogenic in unimmunized mice during the normal course of *P. yoelii* malaria

Naïve BALB/cByJ mice (5 mice per group) were infected once (primary infection sera) or three times (tertiary infection sera) with non-lethal *P. yoelii* 17X pRBCs. Sera were collected from both groups approximately one week following clearance of blood-stage parasites. Total IgG antibody titers (mean $\pm$ SD) against GST-*PyMSP1₁₉*, ($\square$) and r*PyMSP8* ($\blacksquare$) were determined by ELISA. A pool of sera from uninfected mice was non-reactive with both antigens at the serum dilutions assayed.

Figure 6. *P. yoelii* challenge infection of rPyMSP1/8 immunized mice elicits significant responses to a diverse set of parasite proteins

Final bleed serum (day 143) from rPyMSP1/8 immunized animals challenged once (**panel A, group #1**) or twice (**panel B, group #2**) with *P. yoelii* 17XL were used to immunoprecipitate metabolically labeled *P. yoelii* 17XL proteins from mixed blood-stages. Pooled hyperimmune sera (HIS) or sera from Quil A immunized mice (C) were used as positive and negative controls respectively. Immunoprecipitated proteins were resolved by 10% SDS-PAGE and visualized by autoradiography. Molecular weight markers are indicated. Below each lane are peak parasitemia levels in individual mice measured following primary infection of group #1 mice (A) or secondary infection of group #2 mice (B).