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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 ($MSP1_{19}$) 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 *Py*MSP1/8 vaccine. We show that immunization with r*Py*MSP1/8 vaccine elicited an MSP8-restricted T cell response that was sufficient to provide help for both $PyMSP1_{19}$ and *Py*MSP8-specific B cells to produce high and sustained levels of protective antibodies. The enhanced efficacy of immunization with r*Py*MSP1/8, in comparison to a combined formulation of r$PyMSP1_{42}$ and r*Py*MSP8, was not due to improved conformation of protective B cell epitopes in the chimeric molecule. Unexpectedly, r*Py*MSP1/8 vaccine-induced antibody responses were not boosted by exposure to *P. yoelii* 17XL infected RBCs. However, r*Py*MSP1/8 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 MSP6 and MSP7 [10,11]. This multimeric complex is tethered to the merozoite surface by the C-terminal GPI-anchored 42 kDa fragment ($MSP1_{42}$) [9]. During invasion, $MSP1_{42}$ is further cleaved into $MSP1_{33}$ and $MSP1_{19}$ releasing the whole complex except for $MSP1_{19}$ which remains GPI-anchored and is carried into the newly invaded RBC [12].

$MSP1_{19}$, 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, $MSP1_{19}$ 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 $MSP1_{19}$ 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*$MSP1_{42}$ processed fragment for further clinical development was based mainly on the need to incorporate $MSP1_{33}$ sequences in order to provide parasite-specific T cell epitopes. While *Pf*$MSP1_{33}$ induces good T cell responses [25,27,28], it is polymorphic [14] which may limit the scope of r*Pf*$MSP1_{42}$ vaccine-induced protection. Furthermore, immunization with $MSP1_{42}$ induces good antibody responses to $MSP1_{33}$ but these appear to only contribute modestly to protection [29,30]. Thus far, clinical trials with *Pf*$MSP1_{42}$ 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

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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 r*Py*MSP8 also confers antibody-dependent protection against challenge infection with *P. yoelii* 17XL [37,40]. Unlike $MSP1_{42}$, 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 r*Py*MSP1/8 vaccine by fusing $PyMSP1_{19}$ onto the N-terminus of *Py*MSP8. We showed that immunization with r*Py*MSP1/8 afforded superior protection against *P. yoelii* 17XL malaria compared to immunization with single or admixture of r*Py*$MSP1_{42}$ and r*Py*MSP8 [41]. In the present study, we set out to further characterize the protective immune responses elicited by this chimeric r*Py*MSP1/8 model vaccine to better understand the basis for the enhanced efficacy. Here, we report on (1) the specificity of r*Py*MSP1/8 immunization-induced T cell and B cell responses; (2) the durability of r*Py*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, 5–6 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 *P. 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 His_6-tagged recombinant proteins r*Py*$MSP1_{42}$, r*Py*MSP8 and r*Py*MSP1/8 as well as glutathione S-transferase (GST) and the fusion protein, GST-*Py*$MSP1_{19}$ have previously been described [41]. Groups of BALB/cByJ mice (5–10/group) were immunized subcutaneously with 14 μg of purified r*Py*MSP1/8 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 3-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 r*Py*MSP1/8 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 NH_4Cl, 10 mM $KHCO_3$ 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 1× 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 r*Py*MSP1/8 (1.2 μg/ml), r*Py*MSP8 (1 μg/ml), GST-*Py*$MSP1_{19}$ (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% CO_2 for 4 days and were pulsed with 1 μCi per well of methyl [^{3}H] thymidine (40–60 Ci/mmol, GE Healthcare, Piscataway, NJ) for the last 18 h of incubation. Cells were harvested on fiber filters with an automatic cell harvester and incorporation of ^{3}H-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 r*Py*MSP1/8 immunized mice 2 weeks after the final immunization and depleted of antibodies specific to *Py*MSP1 and *Py*MSP8 using recombinant antigens immobilized on a solid matrix. To immobilize r*Py*MSP8 or r*Py*$MSP1_{42}$, Ni-NTA beads (Ni-NTA Superflow matrix, Qiagen Inc., Valencia, CA.) were washed twice in distilled water and charge buffer (0.4 M $NiS0_4$) 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 r*Py*MSP8 or r*Py*$MSP1_{42}$ diluted in the binding buffer were added to 100 μl of packed Ni-NTA beads and rocked overnight at 4 °C. To immobilize GST-*Py*$MSP1_{19}$, 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-*Py*$MSP1_{19}$ 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-r*Py*MSP1/8 serum from individual mice were pooled and diluted 1:500 in TBS–0.1% Tween 20 + 1% BSA. To deplete antibodies specific to *Py*MSP8, diluted serum (1 ml per 100 μl of antigen-coupled beads) was applied onto immobilized r*Py*MSP8 and incubated for 2 h at 4 °C with mixing. The recovered supernatant was then applied to columns containing immobilized