



Protective immune mechanisms against pre-erythrocytic forms of *Plasmodium berghei* depend on the target antigen



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ABSTRACT

Pre-erythrocytic malaria vaccines are believed to either stop the injected sporozoites from reaching the liver or to direct cellular immune responses towards eliminating infected hepatocytes. The present study reveals for the first time the anatomical sites at which these immune mechanisms act against the malaria parasites. To determine the mechanisms leading to protection mediated by two previously characterized vaccines against either the circumsporozoite protein (CSP) or the cell traversal protein for ookinetes and sporozoites (CelTOS), mice were immunized and subsequently challenged by subcutaneous injection of salivary gland sporozoites of luciferase-transgenic *Plasmodium berghei* parasites. The *In Vivo* Imaging System (IVIS) was used to identify the anatomical site where the vaccine-induced immune response eliminates sporozoites after injection. The data demonstrate that CSP-based immunity acts at the site of infection (skin) whereas CelTOS-based immunity is only partially efficient in the skin and allows reduced levels of liver infection that can be subsequently cleared. The results of this study challenge assumptions regarding CSP-mediated immune mechanisms and call into question the validity of some commonly used assays to evaluate anti-CSP immune responses. The knowledge of the mechanism and events leading to infection or immune defense will guide supportive treatment with drugs or combination therapies and thus accelerate the development of effective antimalarial strategies.

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Introduction

Malaria vaccines that target the pre-erythrocytic stage of the parasite life cycle have the greatest potential for inducing sterile protection by targeting to a developmental bottleneck. The circumsporozoite protein (CSP) is the best characterized sporozoite antigen and many different vaccine platforms have been employed to study its prophylactic potential. Our laboratory has been successful in inducing complete protection in inbred and outbred mouse strains using a *Plasmodium berghei* CSP-based DNA vaccine delivered by gene gun. This protection is mediated solely by CSP-specific antibodies directed to the C-terminus of the antigen without the requirement for cytolytic T cells [1]. The clinically

most promising vaccine candidate based on *Plasmodium falciparum* CSP, RTS,S, appears to primarily mediate protection through CSP-specific antibodies since high titers reportedly correlate with protection [2]. However, other vaccine platforms targeting CSP such as heterologous prime/boost approaches using DNA and adenoviral vectors predominantly lead to the induction of strong cellular responses against the infected hepatocytes (reviewed in [3]). CelTOS, on the other hand, is a relatively novel antigen identified based on its essential role during cell traversal of parasites in the host [4]. We have previously reported on a recombinant PfCelTOS protein-based vaccine and shown its protective potential as a vaccine candidate in a murine model [8]. Immunity to PfCelTOS appears unique due to its ability to mediate cross-species protection. In the homologous *P. berghei* challenge model, the recombinant PbCelTOS ortholog induced protection that was mediated by both CelTOS-specific antibodies and CD4⁺ and CD8⁺ T cells [5]. It is likely that these antibodies interfere with the motility and the traversal of host cells by the sporozoites and that T cells act on the intra-hepatic parasites, however, without direct access to events occurring *in vivo* such models are speculative at best.

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Sporozoites embark on a treacherous journey through the mammalian host once they are deposited in the skin by the mosquito. Studies have shown that only a small portion of the sporozoites arrive in the liver and succeed in infecting hepatocytes [5]. A significant proportion of the sporozoites either remain in the skin where they are capable of developing into exo-erythrocytic forms [6], while others migrate to or are transported into draining lymph nodes where they impact the immune response. No matter which path the sporozoites take, they are exposed to immune cells and molecules which result in their neutralization and lead to the inhibition of invasion of hepatocytes. Thus, salivary gland sporozoites administered by direct intravenous injections may bypass the interactions that sporozoites have during a natural infection and instead of taking up to 24 h to reach their target, arrive at their destination within 15 min after injection [6]. Previous studies in our laboratory compared the various challenge routes (intravenous vs. subcutaneous injection of dissected sporozoites and compared these to mosquito bite) and revealed that a non-natural route of infection can introduce artifacts in the evaluation of vaccine efficacy [7]. Such artifacts may minimize efficacy readouts of investigational vaccines assessed in non-natural animal models. Recently, the noninvasive *In Vitro* Imaging System (IVIS[®]) was used to quantify parasite liver stage development and vaccine efficacy of whole parasite approaches that target the induction of liver stage immunity [8–10]. In the current study, using two pre-erythrocytic targets delivered by their optimal vaccination platform, we elucidate the contribution of antibodies at the natural site of infection using *P. berghei* luciferase transgenic sporozoites and the noninvasive *in vivo* imaging system with IVIS[®]. We propose that employing this type of *in vivo* real-time analysis of immune mechanisms may directly guide future vaccine development by revealing effector mechanisms.

Materials and methods

Immunizations

Six-week old C57BL/albino (NCI, Frederick, MD) and C3He mice (Charles River Laboratories, Wilmington, MA) were immunized with either empty plasmid DNA, or plasmid encoding *P. berghei* CSP without the GPI-anchor (designated PbCSP(-A)) or recombinant *P. falciparum* CelTOS (*Pf*CelTOS) protein adjuvanted in Montanide ISA-720 (Seppic Inc., Fairfield, NJ) using previously established regimens [1,5]. Although both mouse strains gave us comparable results with regards to *in vivo* imaging and the protection observed, the data reported here are exclusively from using the C3He mice.

Sporozoite challenge

The luciferase-transgenic *P. berghei* parasite line was generated at WRAIR, Division of Experimental Therapeutics and produces luminescent oocysts and sporozoites. Laboratory-reared female *Anopheles stephensi* mosquitoes (Division of Entomology, WRAIR) were maintained after feeding on infected Swiss ICR mice at 18 °C for 17–22 days. Salivary glands were dissected from malaria-infected mosquitoes and sporozoites isolated as previously described [11]. Each mouse was inoculated subcutaneously in both inguinal regions with approximately 25,000 (C57BL/albino) or 5000 (C3He) sporozoites suspended in a total volume of 100 μ l on day 0 (50 μ l suspension was injected into each inguinal area). To ensure that inoculated sporozoites were viable following the isolation procedure, they were stained with a vital dye containing fluorescein diacetate (50 mg/ml in acetone) and ethidium bromide (20 μ g/ml in phosphate-buffered saline; Sigma Chemical Co.,

St. Louis, MO) and counted in a hemocytometer. The viability of sporozoites ranged from 90% to 100%.

In vivo image system (IVIS)[®]

In vivo imaging of bioluminescence activity from luciferase expressing *P. berghei* infected mice was performed using an IVIS Spectrum instrument (Perkin Elmer, Waltham, MA). Mice were evaluated at 24, 48, and 72 h post sporozoite inoculation to determine liver- and blood-stage malaria infection. Mice received 150 mg/kg luciferin (Gold Biotechnology, St. Louis, MO) intraperitoneally in a volume not to exceed 150 μ l. Three minutes post luciferin administration the mice were anesthetized with inhaled isoflurane. The mice were positioned ventral side up in the IVIS on a 37 °C platform. The mice continued to receive isoflurane through the nose cone delivery method while IVIS imaging was performed. The camera exposure time was set for 1 and 5 min for the 24, 48, and 72 h time points with f-stop = 1 and large binning was selected for the image capture. Quantitative analysis (photons) of bioluminescence emitted from the abdominal area or regions of intensity (ROI) was performed by measuring the luminescence signal intensity using the Living Image[®] 3.0 software (Perkin Elmer). The ROI, whose measurements are expressed in total flux of photons, was set to measure the abdominal area at the location of the liver for whole body imaging. Three-D bioluminescent imaging tomography was performed with the software using sequential images taken with filters ranging from 580 to 660 nm [12].

Results

In this study, we sought to identify the anatomical sites at which immune mechanisms exhibit their effect against malaria parasites after inducing CSP- or CelTOS-based immunity through vaccination [11,13]. In pilot studies, we determined the susceptibility of various mouse strains to luciferase-transgenic sporozoite infection and the optimal dose of salivary sporozoites able to provide an IVIS signal at 48 h after subcutaneous challenge (data not shown). Next, mice were immunized with either the PbCSP(-A) plasmid delivered by gene gun [1] or recombinant *Pf*CelTOS protein adjuvanted in Montanide ISA 720 delivered by needle and syringe subcutaneously [5], both model systems previously established in our laboratory and having shown to provide sterile protection. Mice were challenged with live sporozoites by subcutaneous injection and imaged at 24, 48 and 72 h (Fig. 1). The quantitative data for the images in Fig. 1 are plotted as the individual kinetic response for each mouse in Fig. 2. At 24 h post infection, some parasites were detected at the injection site in mice immunized with an empty vector (control plasmid) or *Pf*CelTOS, but not in PbCSP(-A)-vaccinated animals. By 48 h, 9/10 mice in the empty-vector control plasmid group, 6/9 mice in the *Pf*CelTOS vaccine group, and 1/9 mice in the PbCSP(-A)-vaccine group, had an established, detectable liver infection. With regards to parasite burden, the differences in the liver ROI between the empty-vector control group and the PbCSP(-A)-immunized mice was shown to be statistically significant ($p = 0.0009$, Fisher's exact test) (Fig. 3). The difference in liver burden between the two vaccine groups was also significant ($p = 0.017$, Fisher's exact test) indicating that mice immunized with *Pf*CelTOS had a higher parasite burden than mice immunized with PbCSP(-A) plasmid at 48 h. By 72 h, no parasites were detected in any PbCSP(-A)-vaccinated mouse, while 8/9 mice in the *Pf*CelTOS vaccine group had cleared their liver-stage parasite load to levels below the detection threshold (Figs. 1 and 3). At this time point, both vaccine groups were significantly different from the empty-vector control group ($p < 0.003$, Fisher's exact test). Nominal luminescence localized to the mouth and snout area for

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