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Plasmodium falciparum synthetic LbL microparticle vaccine elicits protective neutralizing antibody and parasite-specific cellular immune responses

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ABSTRACT

Epitopes of the circumsporozoite (CS) protein of *Plasmodium falciparum*, the most pathogenic species of the malaria parasite, have been shown to elicit protective immunity in experimental animals and human volunteers. The mechanisms of immunity include parasite-neutralizing antibodies that can inhibit parasite motility in the skin at the site of infection and in the bloodstream during transit to the hepatocyte host cell and also block interaction with host cell receptors on hepatocytes. In addition, specific CD4+ and CD8+ cellular mechanisms target the intracellular hepatic forms, thus preventing release of erythrocytic stage parasites from the infected hepatocyte and the ensuing blood stage cycle responsible for clinical disease. An innovative method for producing particle vaccines, layer-by-layer (LbL) fabrication of polypeptide films on solid $CaCO_3$ cores, was used to produce synthetic malaria vaccines containing a tri-epitope CS peptide T1BT* comprising the antibody epitope of the CS repeat region (B) and two T-cell epitopes, the highly conserved T1 epitope and the universal epitope T*. Mice immunized with microparticles loaded with T1BT* peptide developed parasite-neutralizing antibodies and malaria-specific T-cell responses including cytotoxic effector T-cells. Protection from liver stage infection following challenge with live sporozoites from infected mosquitoes correlated with neutralizing antibody levels. Although some immunized mice with low or undetectable neutralizing antibodies were also protected, depletion of T-cells prior to challenge resulted in the majority of mice remaining resistant to challenge. In addition, mice immunized with microparticles bearing only T-cell epitopes were not protected, demonstrating that cellular immunity alone was not sufficient for protective immunity. Although the microparticles without adjuvant were immunogenic and protective, a simple modification with the lipopeptide TLR2 agonist Pam_3Cys increased the potency and efficacy of the LbL vaccine candidate. This study demonstrates the potential of LbL particles as promising malaria vaccine candidates using the T1BT* epitopes from the *P. falciparum* CS protein.

1. Introduction

Malaria is a leading cause of morbidity and mortality in much of the developing world, affecting 200–500 million people and causing over 1 million deaths each year. There is a general consensus in the malaria community that vaccines will make an important contribution to malaria disease control [1,2]. *Plasmodium* parasites have a complex life cycle within the mammalian host that is initiated by injection of sporozoites by the infected mosquito as it takes a blood meal. The parasites enter the blood system and travel to the liver where they invade hepatocytes and undergo a multiplication cycle, ultimately releasing thousands of merozoites from each infected hepatocyte. The merozoites rapidly invade erythrocytes and undergo multiple cycles of replication and erythrocyte invasion, resulting in clinical disease progressing to morbidity and mortality unless treated. Pre-erythrocytic vaccines aim to prevent development of the blood stage parasites responsible for clinical disease by targeting the extracellular sporozoite and the intracellular hepatic stages, both of which express circumsporozoite (CS) protein [3].

Abbreviations: CFA, complete Freund's adjuvant; CFSE, carboxyfluorescein succinimide ester; CS, circumsporozoite; DP, designed peptide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; IFA, incomplete Freund's adjuvant; LbL, layer-by-layer; MC, microcapsule; MP, microparticle; NP, nanoparticle; OVA, ovalbumin; PGA, poly-l-glutamic acid; PLL, poly-l-lysine; PLL-FITC, PLL conjugated to fluorescein isothiocynate; sulfo-NHS, 3-sulfo-N-hydroxysuccinimide; TSNA, Transgenic Sporozoite Neutralization Assay.

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Two *P. falciparum* vaccine candidates which currently show the most promise, attenuated sporozoites and RTS,S, both elicit immune responses to CS protein epitopes. The *P. falciparum* whole sporozoite vaccine is prepared by dissection of radiation or genetically attenuated parasites from the salivary glands of mosquitoes that have fed on *Plasmodium*-infected human blood. Numerous logistical hurdles must be overcome for this vaccine candidate, including the use of human blood, inability to grow sporozoites *in vitro*, limited capacity for scale-up and requirement for cold chain storage [4–6]. RTS,S is a virus-like particle composed of a recombinant protein fusing hepatitis B surface antigen (HBsAg) to a truncated *P. falciparum* CS protein [7,8]. Clinical efficacy of RTS,S requires a complex adjuvant formulation containing monophosphoryl lipid A and a purified saponin derivative, QS21, in an oil-in-water emulsion or liposome formulation. In Phase III trials of RTS,S in Africa in infants, vaccine-induced immunity is seen in only 33–55% of the patients and immunity is not sterile as the protected children remain infected with *P. falciparum* but experience milder clinical disease [9,10]. Although these two vaccine candidates show promise and validate the CS protein as a viable vaccine antigen, they also demonstrate the need for more efficacious subunit vaccines that are manufactured by a robust and scalable process, elicit immunity comparable to that obtained in sporozoite-immunized hosts, and minimize inflammatory responses related to the use of potent adjuvant formulations. We have constructed synthetic microparticle vaccines made by layer-by-layer (LbL) fabrication [11] and loaded with a designed peptide (DP) containing the T1BT* epitopes of *P. falciparum* CS protein. In the current study we show that the LbL vaccines elicited neutralizing antibodies and effector T-cells specific for the CS epitopes, and protected immunized mice from mosquito challenge with *Plasmodium* sporozoites expressing *P. falciparum* CS repeats [12]. A simple modification of the particles by addition of the TLR2 ligand Pam_3Cys increased the potency and efficacy of the vaccine. This study demonstrates that LbL fabrication can yield efficacious malaria vaccines using a scalable process and non-biologic raw materials.

2. Materials and methods

2.1. LbL particle fabrication

Peptides were synthesized and analyzed by standard techniques [11]. Fig. 1 shows the location and sequence of the T1, B, and T* epitopes in *P. falciparum* CS protein. Table 1 describes the DP used to make the LbL microparticles. $Pam_3Cys.T1_3B_5$ (DP-2167) was prepared by manual coupling of Pam_3Cys-OH (EMD Millipore) to resin-bound DP-2163 ($T1_3B_5$) in 4:1 N-methylpyrrolidinone/dichloromethane using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation. $CaCO_3$ microparticles (2–4 μm diameter) were obtained from PlasmaChem GmbH (Germany, catalog # PL-CA3). Poly-l-lysine hydrobromide salt (PLL, 15 kDa, catalog # P6516), FITC labeled poly-l-lysine (PLL-FITC, 15–30 kDa, catalog # P3543), poly-l-glutamic acid sodium salt (PGA, 14.5 kDa, catalog # P4636), and 1 M HEPES buffer (catalog #H-3662) were obtained from Sigma-Aldrich (USA). All LbL microparticles (MP) were fabricated as previously reported [11] by alternately layering PGA (negative charge) and PLL (positive charge) on $CaCO_3$ cores to build up a 7-layer base film, and capping with an outermost layer of DP (Table 1). To prepare MP-1141, the base film was chemically crosslinked by treatment with 200 mM EDC and 50 mM sulfo-NHS (Sigma–Aldrich) in 0.2 M phosphate buffer, pH 6.5, for 30 min at room temperature prior to layering DP. Following deposition of the DP, the mature LbL microparticles were washed and stored as damp pellets at 4 °C. The microcapsule MC-1142 was fabricated by dissolving the solid $CaCO_3$ core of MP-1141 by treatment with 0.5 M EDTA (pH 8.0) for 30 min. The microcapsules were recovered by centrifugation (2000 *g* for 5 min), washed twice, resuspended, and stored in suspension at 4 °C. The final architecture of all constructs was $CaCO_3$:PGA:PLL-FITC:PGA:PLL:PGA:PLL:PGA:DP. PGA, PLL and DP contents were measured by amino acid analysis, and endotoxin content was determined by the Limulus Amebocyte Lysate assay (#50-647U, Lonza, Walkersville, MD) [11].

2.2. Mice and immunizations

Female C57BL/6J (H-2^b) and BALB/cJ (H-2^d) mice, 6–8 weeks of age, were obtained from Jackson Laboratories. Mice were housed in microisolator cages and given food and water *ad libitum*. Animal studies were approved by the Northeast Life Sciences (New Haven, CT) Institutional Animal Care and Use Committee. LbL constructs were resuspended in PBS and diluted to deliver 10 μg DP per dose *via* the footpad (f.p.). Control mice were immunized with 10 μg DP in CFA (prime) and IFA (boost) or mock-immunized with PBS. Mice were immunized on days 0, 21, and 42 unless otherwise specified in the Figure legends.

Hybridomas secreting anti-CD4 (GK1.5) and anti-CD8 (2.43) monoclonal antibodies were obtained from the American Type Culture Collection and maintained as instructed. Monoclonal antibodies were purified from culture supernatants by ammonium sulfate precipitation, dialyzed into PBS, and stored at −80 °C. For *in vivo* T-cell depletion, mice received a single i.p. injection of anti-CD4 Mab GK1.5 (50 μg), anti-CD8 Mab 2.43 (50 μg), or a cocktail of both antibodies. Flow cytometry analysis of spleen cells confirmed that each antibody depleted >95% of the respective cell phenotype (data not shown).

2.3. Antibody assays

Mice were bled by retro-orbital puncture on the indicated days and sera were tested by ELISA [11] using plates coated with T1B peptide lacking the $K_{20}Y$ tail. Antibody endpoint titers were based on the final serum dilution yielding an OD against T1B peptide greater than twice the OD against unrelated antigen (BSA).

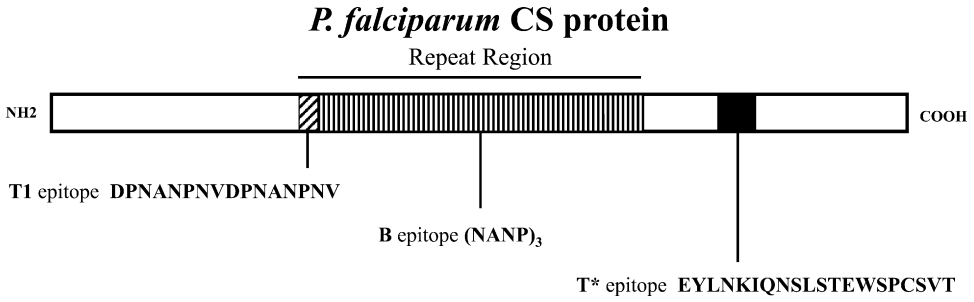

Fig. 1. *P. falciparum* CS protein showing locations and sequences of T1, B, and T* epitopes, and design of $T1BT^*K_{20}Y$ peptide.