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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.

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Keywords

malaria vaccines; microparticle; peptide; sporozoite

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].

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]. Figure 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 minutes 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 minutes. The microcapsules were recovered by centrifugation (2000*g* for 5 minutes), 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). Functional antibody responses were measured by the Transgenic Sporozoite Neutralization Assay (TSNA) [13, 14].

2.4. T-cell assays

Splenic single-cell suspensions were analyzed in IFNγ and IL-5 ELISPOT using commercial reagents (eBioscience) and plates (Millipore Corporation) and following the manufacturers' instructions. Cytotoxic effector cells were detected by an *in vivo* CTL assay as described [11]. Target cells were pulsed with 5 μg/ml of the specified target peptide and labeled with 10 μM of CFSE (Invitrogen, C34554); control target cells were not pulsed with peptide and labeled with 1 μM CFSE.

2.5. Protection from parasite challenge

PfPb is a recombinant *P. berghei* (mouse pathogen) carrying a transgene containing the entire repeat region of *P. falciparum* CS protein (human pathogen); antibodies to *P. falciparum* T1B protect mice from challenge with PfPb sporozoites [12, 14]. Naive and immunized mice were challenged by exposure to bites of PfPb-infected mosquitoes on day 56 (5-15 bites per mouse); this challenge protocol has been shown to reliably elicit patent infection in naive mice [14]. Forty hours post-challenge, the mice were sacrificed and livers were harvested and homogenized in TriReagent (Molecular Research Center, Inc.) using a Polytron PowerGen 500 (Fisher Scientific). RNA was isolated using QIAgen Mini Prep Kit and converted to cDNA using iScript Reverse Transcription Supermix (BioRad), both according to the manufacturers' directions. Parasite burden was monitored by quantifying *P. berghei* 18S rRNA levels in qPCR [13].

2.6. Statistical analyses

Comparisons between multiple treatment groups were analyzed by the non-parametric Kruskal-Wallis test. Pair-wise comparisons were analyzed by the non-parametric Wilcoxon rank sum test with Bonferroni correction; $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed using SAS v. 9.3 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Immunogenicity

C57BL/6J mice were immunized with MP-1140, MP-1141, or MC-1142, each loaded with DP 2062 (T1BT*K_{20}Y). Antibody responses were tested by ELISA and TSNA, while T-cell responses were tested by ELISPOT. MP-1141 and MC-1142 were the most potent LbL constructs, eliciting antibody titers (Figure 2A) and IFNγ+ responses (Figure 2B) comparable to the positive control mice. Figure 2A also shows that the T1B ELISA results correlate with the level of functional antibody activity measured in the TSNA (r^2=0.79, P=0.0004 by Pearson Correlation Coefficient analysis of individual serum titers in both assays), demonstrating the utility of the ELISA as a rapid screening method for measuring functional anti-T1B antibody responses.

3.2. Efficacy

Mice were immunized with MP-1141 or MC-1142 and challenged by exposure to bites of PfPb-infected mosquitoes. Forty hours post-challenge, parasite burden in livers was monitored by quantifying *P. berghei* 18S rRNA levels via qPCR. Protection is defined as ≥90% reduction in parasite burden compared to naïve, challenged mice. Immunization with MP-1141 protected 8 of 10 mice and resulted in a 94% reduction in average parasite burden in the treatment group (P<0.05, Wilcoxon rank sum test), comparable to control mice immunized with DP 2062 in Freund's adjuvant (Figure 3A). Immunization with MC-1142 protected half of the mice but did not result in a significant reduction in the group average parasite burden compared to PBS control.

Sera collected from the mice prior to challenge were tested in the TSNA to measure parasite-neutralizing activity that effectively blocked sporozoite invasion of human hepatoma cells *in vitro*, defined as >90% reduction of parasite rRNA levels in HepG2 cells measured by qPCR. A comparison of TSNA activity with *in vivo* efficacy showed that efficacy was associated with potent neutralizing antibody activity in half of the MP-1141-immunized mice (Figure 3B, red circles). However, there were several mice in both immunized groups that were protected from parasite challenge *in vivo* while mounting only modest neutralizing antibody responses (Figure 3B and 3C, black circles), suggesting that cellular mechanisms may also be involved in protection.

3.3. Role of cellular immunity in efficacy of LbL particles

The detection of IFNγ-secreting cells in ELISPOT (Figure 2B) suggests potential activation of cytotoxic effector T-cells following LbL particle immunization, as found in our previous study [11]. The generation of malaria-specific cytotoxic effector cell responses was examined in an *in vivo* CTL assay using BALB/c mice since C57BL/6J mice fail to develop strong CTL responses to CS protein and there is a known H-2^d restricted CD8+ T-cell epitope contained within the T* epitope [15]. Mice were immunized with PBS or MP-1141, and 7 days later were depleted of CD4+, CD8+, or both T-cell phenotypes by administration of the relevant monoclonal antibodies. The next day, *in vivo* CTL activity was measured [11]. Figure 4A shows that a modest level of killing of T*-loaded target cells was detected in the immunized mice with intact T-cell populations. Depletion of CD8+ cells did not decrease the *in vivo* CTL activity while depletion of CD4+ cells completely prevented effector activity, indicating that immunization with LbL MP bearing the T1BT* antigen elicits CD4+ cytotoxic effector cells, in agreement with published results demonstrating CD4+ effector activity in human volunteers [16, 17].

In light of the T-cell responses detected in ELISPOT (Figure 2B) and *in vivo* CTL assay (Figure 4A), and the apparent discordance between efficacy and TSNA titers in several of the immunized mice (Figure 3B and 3C), we examined the contribution of cellular immunity to efficacy of LbL microparticles. To test the efficacy of cellular responses alone, in the absence of T1B-specific antibody responses, we constructed MP loaded with T-cell epitopes from the CS protein of *P. berghei*, the mouse pathogen (Table 1). BALB/c were used in this study since both the CD4+ and CD8+ T-cell epitopes are recognized in H-2^d mice. Mice were immunized on days 0 and 28 with MP containing *P. berghei* CD4+ T-cell epitopes (MP-1182), CD8+ T-cell epitopes (MP-1183), a fusion peptide containing both T-cell epitopes (MP-1184), or DP fusion peptide in Freund's adjuvant. On day 35, an *in vivo* CTL experiment was performed using target cells loaded with the immunizing epitope(s). Immunization with MP loaded with either *P. berghei* T-cell epitope elicited effector activity against target cells loaded with the immunizing peptide (Figure 4B). However, the CTL activity was not sufficient to protect the mice against challenge with PfPb sporozoites which express the *P. berghei* T-cell epitopes (Figure 4C), suggesting that the efficacy reported in Figure 3A was antibody-mediated.

3.4. Improved vaccine potency by Pam_3Cys modification

Clinical trials of malaria peptide vaccines have demonstrated that adjuvants can significantly increase antibody and cellular responses, but frequently at the cost of increased reactogenicity [18]. The use of TLR agonists that more precisely target innate immunity may help avoid excessive inflammatory responses associated with potent adjuvants. Pam_3Cys, a synthetic lipopeptide TLR2 agonist, is an especially attractive innate immune stimulator for the LbL approach since it can be incorporated directly into DP. We designed a repeat peptide modified by coupling Pam_3Cys to the amino terminal residue. Microparticles were fabricated with $T1_3B_5K_{20}Y$ (MP-1167) or $Pam_3Cys.T1_3B_5K_{20}Y$ (MP-1164) on the

outermost layer. We used multiple copies of the repeat sequences ($T1_3B_5$) in order to increase spatial separation between the lipid moiety and the epitopes, thus reducing the possibility that Pam_3Cys modification of the DP might mask the target epitopes in the MP-1164 construct.

C57BL/6 mice were immunized with MP-1141, MP-1167, or MP-1164; mice immunized with PBS or with DP-2062 (T1BT*) in CFA were included as positive controls. ELISA analysis of sera collected on day 28 shows that MP-1164 containing the Pam_3Cys-modified DP was comparable to the positive control DP-2062 (T1BT*) in Freund's adjuvant and statistically more potent than MP-1167 containing the same DP without Pam_3Cys (P=0.02, Wilcoxon rank sum test) (Figure 5A). MP-1164 also yielded an antibody isotype profile identical to that in the positive control group, including the Th1-associated IgG2c isotype that was minimally induced by MP-1167 or MP-1141 (Figure 5B), each of which lacks Pam_3Cys. The Pam_3Cys-modified MP-1164 was as efficacious as DP 2062 peptide/CFA positive control group, protecting 90% of the mice from liver stage infection (Figure 5C). Protection correlated with neutralizing antibody most strongly in the MP-1164 group (Figure 5F), modestly in the MP-1141 group (Figure 5D), and weakly in the MP-1167 group (Figure 5E). Thus, a simple Pam_3Cys modification of the DP yields an improved LbL vaccine that elicits more potent antibody responses and provides a higher level of protection from parasite challenge.

4. Discussion

The goal of developing a vaccine to eliminate the pre-erythrocytic stages of *P. falciparum*, thus preventing the blood stage infection responsible for clinical disease, was triggered by studies showing that immunization with attenuated sporozoites elicited protective immunity in experimental rodents, monkeys, and human volunteers [19]. The CS protein was the first protective antigen identified using immune sera and cells obtained from these sporozoite-immunized and protected hosts. The central repeat region of CS contains the protective B-cell epitope, namely the $(NANP)_n$ repeat sequence, which elicited sterile immunity in a small clinical trial of patients immunized with $(NANP)_3$-TT tetanus toxoid conjugate [20]. T-cell epitopes of CS include the T1 epitope, which is conserved in all *P. falciparum* strains of diverse geographical origin [21], and T*, a "universal" T-cell epitope recognized by multiple HLA class II molecules [22]. In a small clinical trial, a tri-epitope Pam_3Cys-modified T1BT* branched peptide elicited sporozoite-neutralizing antibodies and polyfunctional CD4+ T-cell clones [23, 24], including clones that directly lysed target cells pulsed with CS peptide, as was found with clones from *P. falciparum* sporozoite-immunized volunteers [16, 17]. These trials provided the first demonstration that CS peptide vaccines can elicit human antibody and CD4+ T-cells with fine specificity and potential effector function comparable to those elicited by attenuated *P. falciparum* sporozoites [23, 25].

The difficult synthesis of the tetrabranched $(T1BT^*)_4$-Pam_3Cys peptide precluded the scale-up necessary to produce quantities needed to prevent the >500,000 deaths caused by *P. falciparum* malaria each year [26, 27]. The value of the CS epitopes as vaccine targets necessitated the search for more efficient ways to manufacture efficacious vaccines. For this purpose, we incorporated the T1BT* epitope into synthetic microparticles made by LbL fabrication of multilayer films on solid supports, using the methodology previously applied to LbL nanoparticle vaccines [11]. Mice immunized with the T1BT* LbL microparticles, in the absence of exogenous adjuvant, generated parasite-neutralizing antibody responses that correlated with protection from parasite invasion of the liver cells following challenge. This is a key finding, since the first immune effector mechanism identified in *Plasmodium* sporozoite-immunized hosts was sporozoite-neutralizing antibodies specific for the CS

central repeat region [28-30], represented by the *P. falciparum* T1B epitopes contained in the current constructs.

T1BT* LbL-immunized mice also developed specific cellular responses including CD4+ T-cell cytotoxic effector activity, in agreement with isolation of cytotoxic CD4+ T-cell clones from human volunteers immunized with either T1BT* peptides or live attenuated *P. falciparum* sporozoites [16]. We found that immunization with LbL microparticles containing only T-cell epitopes failed to provide protection even though robust cytotoxic effector activity was detected. Thus, it appears that parasite-neutralizing antibodies elicited by T1BT*-LbL vaccination were required for efficacy.

Previous studies showed that a T1BT* branched peptide modified with Pam_3Cys elicited high titers of anti-repeat antibodies in mice and humans [24, 31]. LbL microparticles loaded with a Pam_3Cys-modified $T1_3B_5$ DP elicited anti-repeat antibody responses that were quantitatively (titer) and qualitatively (opsonizing isotype) superior to those elicited by microparticles loaded with $T1_3B_5$ DP. Protection from liver stage infection correlated more strongly with neutralizing antibody responses in the mice immunized with the Pam_3Cys-modified particle compared to mice immunized with unmodified particles. These results demonstrate that a simple Pam_3Cys modification of the DP yields an LbL microparticle vaccine candidate that can elicit high titer protective antibody responses.

Incorporation of antigenic epitopes in particulate vaccines elicits improved T-cell and antibody responses to model antigens, tumors, and numerous pathogens [32-43], including malaria [44-51]. Enhanced immunogenicity of particle vaccines is concomitant with efficient phagocytosis of the particles and activation of DC [42, 43, 52], initiating the immunological reactions that culminate in antigen-specific adaptive immunity which can be augmented by inclusion of innate immune stimulators [53, 54]. LbL microparticles offer several advantages for development of particulate vaccines: they are readily fabricated using straightforward methodologies, are stable at room temperature in lyophilized form (unpublished observations), can include multiple target antigen epitopes, and can be modified to include innate immune stimulants that increase vaccine potency. The current study demonstrates the utility of synthetic LbL microparticles as a potent delivery platform for malaria vaccines that elicit protective immune responses including both cellular and humoral components.

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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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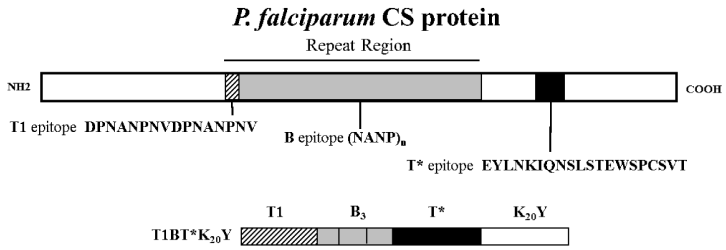


Figure 1.

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

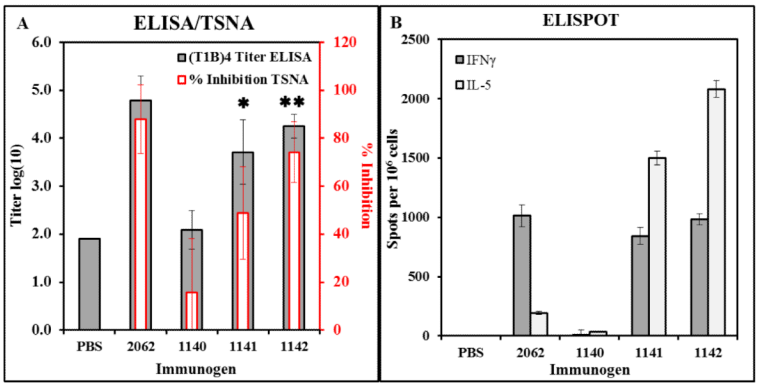


Figure 2.

Immunogenicity and efficacy of LbL constructs containing malaria CS epitopes. Constructs are defined in Table 1. C57BL/6 mice were immunized with doses adjusted to deliver 10 μg of DP on days 0, 21, and 42. (A) Sera were collected on day 28 and tested for T1B-specific IgG titers by ELISA and functional antibodies by TSNA. Results show mean±SD of 5 mice per group for antibody titer (grey bar) and % inhibition (red bar); pooled sera from 5 mock-immunized (PBS) mice were used for both assays. * P<0.05 for ELISA compared to 1140; ** P<0.05 for ELISA and TSNA compared to 1140. (B) Spleen cells were restimulated with T1BT* peptide in IFNγ and IL-5 ELISPOT plates, and the number of spot-forming cells on each plate was counted in an AID ViruSpot Reader. Results show mean±SD of 3 mice per group.

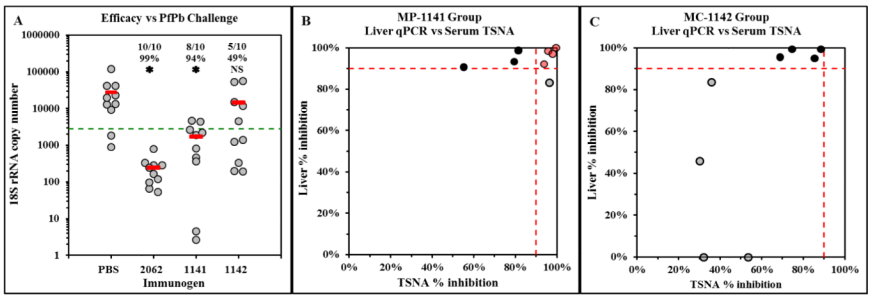


Figure 3.

Protective efficacy of T1BT* microparticles. In a repeat experiment, immunized mice were challenged by exposure to the bites of PfPb-infected mosquitoes on day 56. (A) Parasite burden in the livers was measured by qPCR 2 days post-challenge. Results show parasite rRNA copy number of individual mice (gray circles) and mean value for each group (red bars); insets show number of mice per group that were protected (≥90% reduction of parasite rRNA), group % reduction of parasite rRNA, and ✱ $P<0.05$, all compared to PBS control group; NS = not significant. (B and C) Comparison of *in vivo* protection from parasite challenge (Y-axis) and *in vitro* neutralizing activity of sera (X-axis). Eight randomly-selected individual sera from (A) were tested in each group. Dotted lines indicate ≥ 90% efficacy *in vivo* (Y-axis) or neutralizing activity *in vitro* (X-axis). Red = mice with ≥ 90% activity in both measurements; black = mice with ≥ 90% *in vivo* protection and ≤ 90% *in vitro* neutralizing activity; gray = mice with ≤ 90% *in vivo* protection.

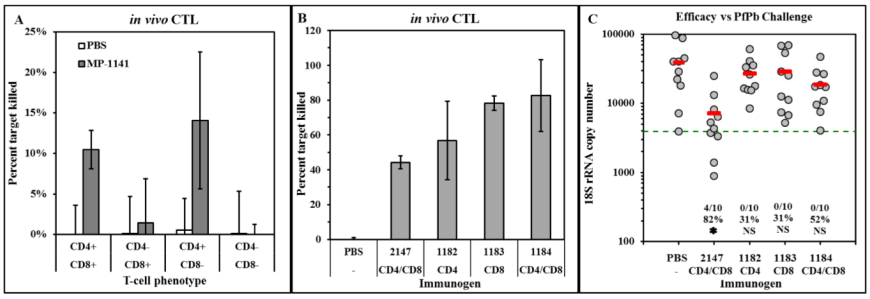
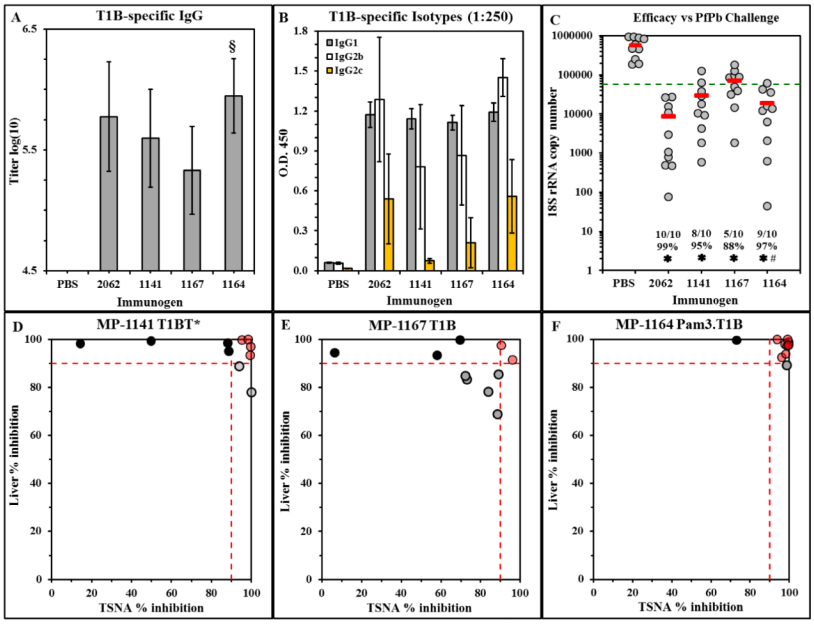


Figure 4.

Cellular immunity and efficacy induced by LbL constructs containing malaria CS epitopes. (A) BALB/c mice were mock-immunized with PBS or immunized with MP-1141, and 7 days later were depleted of CD4+ or CD8+ cells or both. *In vivo* CTL activity was measured the day following depletion. Results show mean±SD percent peptide-specific killing in 3 mice per group. X-axis shows phenotype of T-cells remaining in the mice on day of challenge. (B and C) BALB/c mice were immunized on days 0 and 28 with DP 2147 (*Pb* CD4+:CD8+ fusion peptide) in Freund's adjuvant or MP loaded with *Pb* CD4+ peptide (MP-1182), CD8+ peptide (MP-1183) or CD4+:CD8+ fusion peptide (MP-1184) as indicated (10 μg of DP in each dose). (B) On day 35, *in vivo* CTL activity was measured in three mice per group. Results show mean±SD percent specific killing of cells pulsed with target peptide. (C) The remaining 10 mice per group were challenged by exposure to PfPb-infected mosquitoes, and parasite burden in the liver 40 hours later was measured by qPCR. Results are shown as described in the legend to Figure 3A.



!! A synthetic microparticle vaccine elicits protection from malaria in mouse models.
!! The vaccine is made using the layer-by-layer (LbL) fabrication method.
!! The vaccine includes a peptide subunit of the *Plasmodium* CS protein.
!! All components are chemically synthesized; no biological materials are used.

Figure 5.

Immunogenicity and efficacy of Pam3Cys-modified *Pf* repeat MP. C57BL/6 mice were immunized with the indicated treatments on days 0, 21 and 42. (A) On day 28, sera were tested in ELISA against T1B peptide. Results show the mean±SD anti-T1B IgG antibody titer of 10 mice per group. § $P < 0.05$ compared to the MP-1167 group. (B) The T1B ELISA was repeated with a 1:250 dilution of individual sera, and each serum was probed with isotype-specific detection antibodies. Results show the mean±SD of 10 mice per group. (C) On day 56, mice were challenged by exposure to PfPb-infected mosquitoes, and parasite burden in the liver 40 hours later was measured by qPCR. Results are shown as described in the legend to Figure 3A. # $P = 0.05$ compared to the MP-1167 group. (D, E, F) Comparison of *in vitro* neutralizing activity of sera and *in vivo* protection from parasite challenge. Each circle represents an individual mouse from (C). Results depict *in vitro* neutralizing antibody activity on the X-axis compared to *in vivo* protection on the Y-axis, 10 mice per group. Dotted lines indicate ≥ 90% *in vivo* efficacy (Y-axis) or neutralizing activity *in vitro* (X-axis). Red, black, and gray circles are defined in the legend to Figure 3B and 3C.

Table 1

Designed peptides and LbL particle constructs. MP = microparticle; MC = microcapsule. *Pf* sequences are from *P. falciparum* CS protein, *Pb* sequences are from *P. berghei* CS protein. $K_{20}Y$ ($Lys_{20}Tyr$) is the polyelectrolyte tail which drives the assembly of soluble DP into the LbL film. In DP-2062, the B repeat sequence $(NANP)_3$ is flanked by the T1 repeat (N-terminal) and the T* epitope (C-terminal). In DP-2163 and DP-2167, $(NANPNVDP)_3$ is three copies of the T1 repeat sequence, and $(NANP)_5$ is five copies of the B repeat sequence. The SKKKK linker sequence was included in both DP-2163 and DP-2167. All LbL constructs contained 3.2-7.7 μg DP per mg $CaCO_3$ and < 0.05 endotoxin units per μg DP.

Particle #	DP #	Epitope(s) and source	Sequence
MP-1140 MP-1141 MC-1142	DP-2062	T1BT* *Pf*	DPNANPNVDPNANPNV$(NANP)_3$EYLNKIQNSLSTEWSPCSVTSGNGK_{20}Y
MP-1182	DP-2145	CD4 *Pb*	LEFVKQIRDSITEEWSQCNVK_{20}Y
MP-1183	DP-2146	CD8 *Pb*	KNNNNDDSYIPSAEKILEFVK_{20}Y
MP-1184	DP-2147	CD8:CD4 *Pb*	KNNNNDDSYIPSAEKILEFVKQIRDSITEEWSQCNVK_{20}Y
MP-1167	DP-2163	$T1_3B_5$ *Pf*	SKKKK$(NANPNVDP)_3(NANP)_5K_{20}$Y
MP-1164	DP-2167	Pam3.$T1_3B_5$ *Pf*	Pam_3CSKKKK$(NANPNVDP)_3(NANP)_5K_{20}$Y