Protective Antibody and CD8⁺ T-Cell Responses to the \textit{Plasmodium falciparum} Circumsporozoite Protein Induced by a Nanoparticle Vaccine

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Abstract

\textbf{Background:} The worldwide burden of malaria remains a major public health problem due, in part, to the lack of an effective vaccine against the \textit{Plasmodium falciparum} parasite. An effective vaccine will most likely require the induction of antigen specific CD8⁺ and CD4⁺ T-cells as well as long-lasting antibody responses all working in concert to eliminate the infection. We report here the effective modification of a self-assembling protein nanoparticle (SAPN) vaccine previously proven effective in control of a \textit{P. berghei} infection in a rodent model to now present B- and T-cell epitopes of the human malaria parasite \textit{P. falciparum} in a platform capable of being used in human subjects.

\textbf{Methodology/Principal Findings:} To establish the basis for a SAPN-based vaccine, B- and CD8⁺ T-cell epitopes from the \textit{P. falciparum} circumsporozoite protein (PICSP) and the universal CD4 T-helper epitope PADRE were engineered into a versatile small protein (≈125 amino acids) that self-assembles into a spherical nanoparticle repetitively displaying the selected epitopes. \textit{P. falciparum} epitope specific immune responses were evaluated in mice using a transgenic \textit{P. berghei} malaria parasite of mice expressing the human malaria full-length \textit{P. falciparum} circumsporozoite protein (Tg-Pb/PICSP). We show that SAPN constructs, delivered in saline, can induce high-titer, long-lasting (1 year) protective antibody and poly-functional (IFN-γ, IL-2) long-lived central memory CD8⁺ T-cells. Furthermore, we demonstrated that these Ab or CD8⁺ T-cells can independently provide sterile protection against a lethal challenge of the transgenic parasites.

\textbf{Conclusion:} The SAPN construct induces long-lasting antibody and cellular immune responses to epitope specific sequences of the \textit{P. falciparum} circumsporozoite protein (PICSP) and prevents infection in mice by a transgenic \textit{P. berghei} parasite displaying the full length PICSP.

Introduction

The worldwide burden of malaria remains a major public health problem due to the lack of an effective vaccine against \textit{Plasmodium falciparum}, the causative agent of the deadliest human malaria [1]. There is no recombinant or viral based vaccine that induces long-lasting protective immune responses. Protection studies conducted using RTS,S as well as other available data, suggest that a robust antibody response coupled to a vigorous PICSP epitope specific CD8⁺ T-cell response will likely be needed for a highly effective pre-erythrocytic stage malaria vaccine [2–4]. But the induction of both of these arms of the immune system by a single vaccine against malaria has been difficult to achieve. Nanoparticle based vaccines have been shown to be effective in the induction of immune responses in animal models without the need for an adjuvant [5–10]. Most of these particles are based on polymers that encapsulate antigen or create a solid support to which protein antigens are chemically coupled and therefore do not necessarily have control over an ordered array presentation of epitopes.

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We have previously reported using the basic properties of amino acids and the tenets of structural biology to design a short linear protein monomer that combines with identical monomers to form a self-assembling protein nanoparticle (SAPN) with predictable and controllable conformation and presentation of epitopes. Furthermore, we demonstrated these SAPN could elicit a CD4+ T-cell dependent, high titer, long-lived protective antibody response against a mouse malaria, *P. berghei*, sporozoite challenge [11] in mice. We report here modifications of that original SAPN design to make a vaccine that could be used in humans against the malaria parasite *P. falciparum*. This required change in the core or scaffold to eliminate sequences that might cross react with human proteins. We also included three previously identified CD8+ T-cell epitopes from the *P. falciparum* circumsporozoite protein (PfCSP) as well as copies of the central PfCSP repeat sequence of four amino acids NANP. Since the target of the induced immune responses are directed against the single sporozoite protein, PfCSP, we choose to utilize a transgenic *P. berghei* parasite clone that normally infects rodents [12] to test the efficacy of the vaccines. These transgenic parasites express full length PfCSP on the surface of the sporozoite stage and infect mice similar to wild type *P. berghei* sporozoites thus allowing us to directly test the functionality of immune responses, both antibody and cellular, made against the *P. falciparum* CSP. As control vaccine constructs we designed monomers that when assembled would have scaffolds identical to those of the PfCSP-SAPNs but displayed on their surface the B-cell epitope repeat amino acid sequence of the *P. vivax* sporozoite protein, VK210 CSP [13].

**Results**

**Expression of Monomer Protein and Refolding to Form a Nanoparticle**

The gene for each monomer was cloned into a bacterial expression plasmid and transformed into *E. coli* cells for expression. Purity of the monomer was determined by SDS-PAGE (Figure 1). After purification the denaturant was removed and self-assembly of each of the different monomers (Figure 2A) into nanoparticles was driven by the interaction of the trimeric and pentameric oligomerization domains creating α-helical rod-like coiled-coils [14] (Figure 2B). By both transmission electron microscopy and dynamic light scatter measurements the final SAPNs had a size of about 40 nm and formed uniform, non-aggregating nanoparticles (Figure 2C, D).

**SAPN Vaccines Expressing PfCSP**

Repeating Region B-cell Epitopes Protect Against a Transgenic *P. berghei* Sporozoites Displaying the PfCSP

Following two or three doses of vaccine high titer epitope specific antibodies to the NANN peptide were produced in both C57BL/6 and Balb/c mice (Figure 3A). Following challenge with sporozoites of the Tg-Pf/PfCSP parasite two wks post third dose of vaccine 90% to 100% of mice were protected compared to 0% in the unimmunized infectivity control mice (Figure 3B & Table 1). Because our standard route of SAPN administration, i.e., is not a route commonly used to deliver vaccines to humans we tested i.m. administration in parallel experiments. The route of administration of PfCSP-SAPN made no statistically significant difference in either the NANN-specific antibody titers observed (p = 0.45) (Figure 3A) or the levels of protection achieved (p = 0.99) in either strain of mice tested (Figure 3B).

**Protective Antibody Response is Long-lived**

One of the most desirable qualities of a vaccine is long-term induction of an effective immune response. This would be especially advantageous for a malaria vaccine because in many areas where malaria is endemic there are periods of high or low transmission paralleling rainy and dry sessions, respectively. To investigate how long after immunization with PfCSP-SAPN protective antibody levels would persist, we immunized mice at wk 0, 2 and 4 and challenged them either at wk 6, 8, 12, 16, 28, 40 or 52 of the study with the Tg-Pf/PfCSP sporozoites. After the third dose of vaccine the mice did not receive booster doses of vaccine nor were they exposed to parasites until the day of challenge. Mice maintained high antibody titers out to wk 52 (Figure 3C). Even up to wk 40, 75% to 100% of mice were protected following challenge and 50% of animals were protected even up to wk 52 (Figure 3D). One mouse from the wk 16, wk 28 and wk 52 test groups died before challenge from non-malaria, non-vaccine related causes. Analysis of pre-challenge antibody titers and avidity index of individual mice revealed that while there was no significant difference (p = 0.45) in the group mean pre-challenge antibody titers, those individual mice whose titer dropped below about 2200 (7.3 μg/ml) (Figure 3C) and avidity...
index $\leq 0.32$ were not protected. All mice with anti-$Pf$CSP-SAPN antibody avidity index $\geq 0.46$ were protected (Table 2). Whereas greater than 95% mice immunized with the $Pf$CSP-SAPN were protected from a lethal challenge none were protected if the animals were immunized with SAPN with an identical scaffold to the $Pf$CSP-SAPN but displaying $P.\text{vivax}$ CSP repeat epitopes on its surface, the $Pv$CSP-SAPN (Figure 4A).

Figure 2. Sequences, formation and structural analysis of SAPN. (A) The amino acid sequences of the monomers used to make the SAPN in this study. Black: flanking regions (His-tag, thrombin cleavage site, proteosomal cleavage sites, linkers). Green: coiled-coil pentamer domain (Trp-zipper); Blue: coiled-coil trimer domain; Red: predicted B-cell epitopes of $P.\text{falciparum}$ or $P.\text{vivax}$ CSP repeat region; Yellow: predicted human HLA restricted CD8$^+$ T-cell epitopes $P.\text{falciparum}$ CSP; Magenta: universal CD4 T-helper epitope (PADRE) as a part of the trimer domain. (B) SAPNs are formed by the oligomerization of 3- and 5-stranded coiled-coiled domains within a single polypeptide monomer. Shown is the in silico prediction of the SAPN with icosahedral symmetry. Colors are representative of the sequences as described in (A). (C) Individual nanoparticles are visualized using transmission electron microscopy. The bar represents 100 nm. (D) The size distribution of the nanoparticles in solution is monitored using dynamic light scattering.

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SAPN Platform can Present T-cell Epitopes to Induce Protective CD8+ T-cells

Between 90% and 100% of mice immunized with PjCSP-KMY-SAPN, and 40–60% of PkCSP-KMY-SAPN-immunized mice were protected against lethal challenge (Figure 4A). While the protection in PjCSP-KMY-SAPN-immunized mice could be attributed to both antibody and cell mediated immune responses, the protection in PkCSP-KMY-SAPN-immunized mice could only be attributed to a protective cellular response since antibodies to P. vivax CSP repeat epitopes do not cross-react with epitopes in P. falciparum CSP repeat region or against the Tg-Pb/Pf-CSP parasite. To further determine that cells and not antibodies were responsible for the observed protection in the experiments involving PkCSP-KMY-SAPN we immunized a strain of MHC...
Class I knockout mice [15] with the SAPN containing the CD8+ T-cell epitopes (Figure 4B). Knockout mice immunized with PFCSP-KMY-SAPN were not protected from challenge, whereas wild-type mice receiving PFCSP-KMY-SAPN were protected. Naïve mice that received sera from PFCSP-SAPN or PFCSP-KMY-SAPN immunized mice survived challenge and mice that received splenocytes or enriched CD8+ T-cells from PFCSP-KMY-SAPN immunized mice were protected (Figure 5). But neither splenocytes nor serum from PFCSP-SAPN-immunized mice conferred protection to naïve mice and mice that received only serum from PFCSP-KMY-SAPN-immunized mice were not protected (Figure 5B). However, mice were protected if they received splenocytes from PFCSP-KMY-SAPN-immunized mice (Figure 5B). Finally, the passive transfer of highly enriched CD8+ T-cells from the liver and spleens of mice immunized with PFCSP-KMY-SAPN conferred protection against Tg-Pbs/PFCSP sporozoite challenge of naïve recipient mice (Figure 5e). Together, these experiments strongly indicated that a SAPN vaccine platform carrying P. falciparum CSP CD8+ T-cell epitopes was capable of inducing CD8+ T-cells that were directly involved with the protection against an otherwise lethal challenge of sporozoites.

An additional desirable quality of a malaria vaccine would be one that had the ability to induce multi-functional [16], long-term central memory (CD44hiCD62LhiIFNγ+IL-2+) markers of memory cells. We found that immunization did not disturb the homeostatic balance of the naïve T-cell compartment in the blood, spleen, liver or draining lymph nodes (Figure 6A). But we did observe the induction of about 2–8% increase over baseline of effector memory (CD44hiCD62LhiIFNγ+IL-2+) or long-term central memory (CD44hiCD62LhiIFNγ+IL-2+) markers (Figure 6B–D) in these organs (Figure 6B–D and Figure S1A–E). The long-term central memory (TLCM) cells specifically responding by producing both IFNγ and IL-2 when exposed in vitro to each of the K, M, or Y peptides.

Discussion

Our goal for this study was to determine if we could design and construct a SAPN that could be potentially used in humans to induce strong immune responses to the human malaria P. falciparum CSP epitopes. First, we demonstrated that SAPNs could elicit high-titer, high-avidity, and long-lasting protective antibodies to epitopes of the repeat region of the circumsporozoite surface protein of P. falciparum. Second, we established that SAPNs could induce antigen-specific CD8+ T-cells responses that played an active role in a protective immune response. These data demonstrate that unique, structure-designed nanoparticles, administered without adjuvant, have the ability to induce both antibody and cellular responses in mice that, together or independently, are able to steriley protect mice against sporozoites which display on their surface the CSP of the human malaria parasite P. falciparum.

We had previously reported the use of a SAPN-based vaccine to induce protective immune responses in a mouse malaria model
However, a part of the core scaffold (designated P4c) in our early mouse vaccine construct, P4c-Mal could not be used as basis for a vaccine destined to be used in humans because it contained sequences corresponding to the human cartilage oligomeric matrix protein (COMP). Therefore, to develop a SAPN-based vaccine that could be potentially used in humans, the COMP in P4c was replaced with a de novo designed high tryptophan content sequence (Trp-zipper) that, like COMP, formed a pentameric coiled-coil domain (Figure 7). Surprisingly, this new construct, T81c-Mal, did not induce antibody production in mice and protection against parasite challenge was lost. We reasoned that the removed COMP sequence contained a CD4 helper epitope and therefore we added the pan-allelic DR epitope (PADRE) [20] into the newly designed scaffold to make the construct T81c-8-Mal. This restored antibody production in mice and, subsequently, protection from challenge.

Figure 4. SAPN vaccination induces protective cellular immune responses in mice. SAPN based vaccines present CD8⁺ T-cell epitopes to stimulate a protective cellular immune response. (A) C57BL/6 mice immunized with a SAPN containing only P. falciparum CSP B-cell epitopes (PICSP-SAPN) or a SAPN containing both P. falciparum CSP B- and T-cell epitopes (PICSP-KMY-SAPN). n = 10; Error bars show means ± s.d. of three separate experiments. (B) Either sera or total splenocytes were transferred from immunized mice to naive mice which were challenged 24 h post-transfer. n = 10; data shown is one of two experiments. (C) In order to determine if CD8⁺ T-cells were involved in protection we immunized wild-type C57BL/6 mice (WT) and MHC Class I knockout (MHC1 KO) mice with SAPN containing PICSP specific CD8⁺ epitopes. Mice were challenged with 5,000 Tg-Pb/PICSP sporozoites. n = 10. *P < 0.01; ***P < 0.001. doi:10.1371/journal.pone.0048304.g004

Figure 5. Sera or Cell Transfer Studies. (A) Pooled sera isolated from mice immunized with PICSP-SAPN or PICSP-KMY-SAPN but not sera from PvCSP-SAPN or PvCSP-KMY-SAPN immunized mice transferred to naive mice conferred protection from challenge with Tg-Pb/PICSP sporozoites. (B) In a separate experiment sera or washed splenocytes from mice were transferred. Whereas sera from PICSP-SAPN or PICSP-KMY-SAPN transferred protection sera from PvCSP-SAPN or PvCSP-KMY-SAPN immunized mice did not. On the contrary, total splenocytes from PICSP-KMY-SAPN or PvCSP-KMY-SAPN transferred protection while splenocytes from PICSP-SAPN or PvCSP-SAPN did not. (C) Two wks post final immunization with PICSP-KMY-SAPN 1.33x10⁶ enriched CD8⁺ T-cells were adoptively transferred to naive animals which were then challenged 72 hrs post transfer. *P < 0.05. Mice were challenged with 5,000 Tg-Pb/PICSP sporozoites. n = 10. doi:10.1371/journal.pone.0048304.g005
If sporozoites make their way to the liver they can avoid antibody by entering hepatocytes and undergoing developmental transformation and replication. In the liver stage of development CSP is no longer produced therefore all detectable CSP is a product of the initial invading sporozoite parasite [21]. The CSP is processed and peptide epitopes are presented on the hepatocyte surface in the context of MHC Class I molecules [22,23]. It has been shown that \textit{P. yoelii} CSP epitope specific CD8\(^+\) T-cells can kill hepatocytes containing developing malaria parasites [24]. But the induction of CSP-specific CD8\(^+\) T-cells using recombinant protein or a multiple antigenic peptide array vaccine has been difficult to achieve without the co-administration of potent immuno-stimulators that are not suitable for human vaccine development [25]. Reports have indicated that CD8\(^+\) T-cell responses could also be induced by antigen-containing small particles (20–200 nm) that are trafficked to the lymph nodes where they are taken up by antigen presenting cells, processed and presented to adaptive immune cells [26]. To test if SAPN induced protective CD8\(^+\) T-cells we engineered on to the nanoparticle scaffold three predicted CD8\(^+\) T-cell epitopes (KP KDELDY, MP NDPNPR NV & YLNKQNSL) from the \textit{P. falciparum} 3D7 clone. The epitopes were selected because of the prevalence of the human HLA B35, B7 and A2.1 haplotypes in the world’s population. About 40% of the world is HLA-A2 [27] with between 35–50% in every ethnicity in the USA [28]. In addition, the HLA-A2.1, HLA-B7 and HLA-B35 haplotype associated \textit{PfCSP} CD8\(^+\) T-cell epitopes have all been associated with protection from malaria in Africans and other ethnic groups [29–32] thus a vaccine based on these CD8\(^+\) T-cell epitopes should have a broad coverage throughout the world. If the vaccine is tested with a \textit{P. falci parum} 3D7 clone challenge, all three CD8 epitopes will be present on the CSP of the invading sporozoite. However, of the three 3D7 clone epitopes chosen only one of the sequences, the M peptide, had a 100% homology match to the PfCSP of the Wellcome strain CSP protein (Table 3). This single 100% homology match, and perhaps the lower homology matched epitopes, did however induce protective CD8\(^+\) T-cells as demonstrated by the cell transfer studies presented in Figure 4 and Figure 5. This is encouraging because it suggests that a single vaccine containing several different HLA specific CD8\(^+\) T-cell epitopes could have broad coverage in a malaria endemic area with multiple parasite strains.

\textbf{Figure 6. CD8\(^+\) T-lymphocyte population profiles.} \textit{PICSP-KMY-SAPN} immunized mice show induction of long-lived Ag-specific memory cells and residency in both secondary lymph and non-lymphoid organs (A–D). Two to five wks post final immunization total lymphocytes from designated organs were cultured, stimulated with peptides, stained and characterized by flow cytometry. Data are presented as a percentage of the CD8\(^+\) T-cell population normalized to peptide stimulated saline control minus media alone. Data was segregated based on phenotype and location for (A) naive, (B) effector memory, (C) central memory, and (D) long-term central memory cell populations and characterized as Naïve, TEM, TC or TCM based on expression levels of CD44, CD62L, IL-2 and IFN\(\gamma\). The FACS gating strategy is shown in Figure S1. Error bars represent the mean ± s.d. observed in 4 separate experiments, two mice each, over the course of 5 wks post vaccination. LN = lymph node; SPL = spleen.

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One of the more interesting aspects of SAPN formulation is the ability to trigger immune responses without the need of an adjuvant. In our previous study we have shown that potential traces of LPS were not the reason for the adjuvant effect of SAPN [11]. Furthermore, in vitro studies have shown that SAPN activate dendritic cells in vitro to over-express the co-stimulatory molecules CD40 and CD86 (Figure S2). This is consistent with an adjuvant effect of SAPN. However, the exact mechanisms by which SAPN activate these dendritic cells as well as the receptor(s) involved in SAPN recognition by dendritic cells are still unclear and is currently under investigation.

The promise of vaccination against malaria depends on epitope selection and delivery to the immune system to induce strong, long-lasting immune responses. In this study, we performed the first essential steps to show the ability of a new vaccine platform to deliver PfCSP B- and T-cell epitopes to induce long-lived humoral and cellular responses. Certainly the epitope approach would be of limited value if the selected sequences were highly variable. But, the central repeat region of the PfCSP is highly conserved. While there is significant divergence in the CD8+ T-cell epitopes in the flanking regions we have shown that we can incorporate at least 3 different epitopes, encompassing major HLA haplotypes into the

**Figure 7. Schematic representation of redesigning the scaffold for SAPN-based P. falciparum CSP vaccine.** The cartilage oligomerization matrix protein (COMP) was replaced with a de novo designed sequence (Trp-zipper) that, like COMP, forms a pentameric coiled-coil domain. Sequences coding for the universal CD4 T-cell helper epitope, the pan-allelic DR epitope (PADRE) were incorporated into the trimeric coiled-coil domain without disrupting the stoichiometry needed to oligomerize. The P. berghei circumsporozoite surface protein repeat (PbCSP) epitopes were replaced with PfCSP repeat epitopes to form PfCSP-SAPN. Three PfCSP CD8+ T cell epitopes were then engineered into the pentameric coiled coil domain to form PfCSP-KMY-SAPN.

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### Table 3. Amino acid sequences of the three predicted P. falciparum CSP CD8+ T-cell epitopes (K, M and Y) based on binding to major human HLA haplotypes.

| Peptide Name | K | M | Y |
|--------------|---|---|---|
| HLA Restriction | B35 | B7 | A2.1 |
| Sequence in Pf- or PfCSP-KMY-SAPN polypeptide | KPKDELGY | MPNDPNRNV | YLNKIQNSL |
| Sequence in 3D7 Strain CSP | KPKDELGY | MPNDPNRNV | YLNKIQNSL |
| Sequence in Wellcome Strain CSP | KPKDELGY | MPNDPNRNV | YLNKIQNSL |

Shown are comparisons to the known sequence in the 3D7 strain of P. falciparum CSP (used for human volunteer challenge trials) and to the Wellcome Strain of P. falciparum CSP sequence expressed in the Tg-Pb/PfCSP sporozoites (used in these mouse studies). Underlined residues in Wellcome strain highlight the differences from the 3D7 strain.

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SAPN design which gives the vaccine a wider effective range. Using protein engineering we can change the vaccine to modify epitope presentation. For example, several different T-cell epitopes determined optimal for control of local parasite strains or improved responses from indigenous human MHC haplotype populations can be engineered into the monomer sequence to be assembled into the final vaccine product. The use of rodent transgenic parasites expressing *P. falciparum* molecules represented a powerful and convenient model to dissect and evaluate the effectiveness of the immune responses against a human malaria antigen. In future studies with human volunteers we will aim to demonstrate the safety and immunogenicity of this nanoparticle malaria vaccine and to test its effectiveness against a challenge of *P. falciparum* sporozoites.

**Materials and Methods**

**Ethics Statement**

Research with mice was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All animal work was conducted under protocols approved by the WRAIR/NMRC Institutional Animal Care and Use Committee.

**Redesign of the Scaffold**

The redesign of the core scaffold to be use as the basis for human SAPN vaccine constructs was as schematically presented in **Figure 7**. The DNA sequence encoding the cartilage oligomeric matrix protein (COMP) was replaced with sequence that encodes a *de novo* designed Trp-zipper sequence: WQTWNKWDQWSN&DWQAKWDDWAR-WRALWM, that forms a pentameric coiled-coil domain [33] (Figure 2). Sequences coding for the universal CD4 T-cell helper epitope, the pan-allergic DR epitope (PADRE) was incorporated into the trimeric coiled-coil domain without disrupting the stoichiometry needed to oligomerize followed by a replacement of the *P. berghei* circumsporozoite surface protein repeat (*Pf*CS) epitopes with *Pf*CS repeat epitopes to form *Pf*CS-PAPN. Three *Pf*CS CD8+ T-cell epitopes were then engineered on to the pentamer coiled coil domain to form *Pf*CS-KMY-SAPN.

**P. vivax** CSP Containing SAPN

Using the plasmids that expressed the *Pf*CS-PAPN and *Pf*CS-KMY-SAPN monomer proteins we switched the sequences that encoded the *Pf*CS repeats for sequences that were designed to express monomer proteins containing the *P. vivax* CSP (VK210) repeat sequence DRAAGQPAGDRADGQPA (Figure 2).

**Plasmids, Protein Expression, Purification and Characterization**

Plasmids (pET24 backbone) containing inserted genes to encode the desired monomer proteins were transformed into BL21 (DE3) *E. coli*, Tuner strain, cells and grown in LB Broth to OD_{600}=0.8 and induced with 0.5 mM IPTG for 2 h. Cells were collected by centrifugation and resuspended in 40 mM cold Cracking Buffer (6 M guanidine-HCl, 20 mM Tris-HCl, 100 mM NaH_{2}PO_{4}, pH 8.0) and disrupted by a single pass through a high-pressure microfluidizer (Model 1109; Microfluidic Corp.). The total cell lysate was centrifuged at 12,000 rpm, 30 min. The monomer protein was purified from the supernatant by nickel nitrilotriacetic acid (Ni-NTA)-agarose chelating resin affinity chromatography followed by two polishing ion-exchange chromatography steps utilizing an FPLC system (AKTA Purifier, GE Healthcare). The first, SP-Sepharose, enriched the monomer protein and the second, Q-Sepharose, bound and removed endotoxin. Throughout the purification protocol the monomer was kept in a denatured state by maintaining an 8 M urea concentration in the buffer. Buffer pH and salt concentrations were changed as required to retain or elute the monomer protein. All elutions were monitored by UV absorbance at OD_{280}. The monomers were allowed to combine into a nanoparticle by dialysis of the final eluted protein solution against multiple changes of Refolding Buffer (20 mM Tris-HCl, 5% Glycerol (vol/vol), pH 7.5) to remove the urea. The nanoparticle size was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) as described below. Endotoxin contamination of the final sample was determined by the pyrochrome *Lamalus* amebocyte lysate (LAL) test (Associates of Cape Cod) following the manufacturer’s recommended instructions and was routinely <10 EU mg\(^{-1}\) of protein. Aliquots of material were quickly thawed at RT and an aliquot was checked by DLS for confirmation of particle integrity.

**Transmission Electron Microscopy**

The sample was negatively stained with 1% (wt/vol) uranyl acetate (SPI Supplies) and observed with a FEI Tecnai T12 S/TEM at an accelerating voltage of 80 kV (FEI). The protein concentration of the construct was about 0.05 mg mL\(^{-1}\).

**Dynamic Light Scattering**

DLS experiments were carried out on a Zetasizer Nano S Instrument (Malvern), with a 633 nm He-Ne laser. All measurements were carried out at RT in the final reconstituting buffer containing 20 mM Tris-HCl, pH 7.5 and 5% glycerol (v/v).

**Mice**

Six-to-eight-wk old female C57BL/6 (H-2\(^b\)) and BALB/C (H-2\(^k\)) mice were used for most protection experiments. In addition, an MHC Class I (MHC I) knockout (KO) strain (B6.129P2-B2mtm1Unc/J) was used to determine the role of CD8+ T-cells in the immune response to the SAPN. All mice were obtained from The Jackson Laboratory.

**Immunization**

All mice were immunized either intramuscularly (i.m.) or intraperitoneally (i.p.) with 10 μg of protein in 0.1 mL of Refolding Buffer per immunization, on d 0, 14 and 28 unless otherwise noted.

**Challenge with Parasites**

A clone of the transgenic *P. berghei* parasite [12], expressing the full-length *P. falciparum* CSP was used. In this transgenic parasite (Tg-Pf/PfCSP) the endogenous *csp* gene from *P. berghei* had been replaced with the full-length *csp* gene from *P. falciparum* thus allowing testing of responses to both B- and T-cell *Pf*CSP specific epitopes. The sporozoite of this clone has been previously demonstrated to invade mouse hepatocytes and initiate a lethal blood infection in mice [12,34]. Cryopreserved mouse blood containing infected RBCs of the original clone was injected into C57BL/6 mice and after 5 days whole blood was isolated and cryopreserved for future use. For infectious sporozoites an aliquot of frozen blood stage parasites was thawed and injected into mice. When gametocytes were observed to be above 3% of infected cells by stained slide examination *Anopheles stephensi* mosquitoes were
were analyzed by Luminex goat anti-mouse IgG-PE (Jackson ImmunoResearch). Samples

Briefly, 96-well microplates (Dynax) were coated with 100 ng of the synthetic CSP peptide (Eurogentec North America) or 50 ng of the VMP001, per well, overnight at 4°C and then blocked for 1 h at 37°C with BlockerTM Casein in PBS (Thermo Scientific Inc). Plates were washed three times (PBS, 0.05% Tween20) and incubated for 1 h at 37°C with individual mouse sera in duplicate wells per serum sample. Plates were washed again and incubated for 1 h at RT with 1:5,000 diluted (10× diluted solution of BlockerTM Casein in PBS) secondary anti-mouse immunoglobulin (total IgG, IgM and IgA) labeled with horseradish peroxidase (Southern- Biotechnology Associates). Plates were washed and developed by adding 2, 2′-azino-di (3-ethylbenthiazoline sulfo-nate)-peroxidase (ABTS) substrate (Kirkegaard & Perry Laboratories) for 1 h at RT. The reaction was measured using a BioTek Synergy™ 4 Microplate reader by determining optical density at 405 nm (OD405). Antibody titers were determined as the dilution which gave an OD405 = 1 by the SoftMax Pro v5.2 ELISA Analysis Software (Molecular Devices LLC).

Determination of Infection Following Parasite Challenge

Parasitemia was determined by examining Giemsa-stained thin smears prepared with blood from each mouse from 6–15 d post-challenge. Parasitic animals were euthanized immediately following detection of blood stage parasites and an animal was considered fully protected if no parasites were detected by 15 d post challenge.

Determination of Antibody Titer

Antibody responses against the PfCSP peptide [NANP]6 or PfCSP recombinant protein VMP001 (containing 9 copies of the PfCSP VK210 repeat) [35] were measured by the enzyme-linked immunosorbent assay (ELISA) as previously described [11]. Briefly, 96-well microplates (Dynax) were coated with 100 ng of the synthetic PfCSP peptide (Eurogentec North America) or 50 ng of the VMP001, per well, overnight at 4°C and then blocked for 1 h at 37°C with BlockerTM Casein in PBS (Thermo Scientific Inc). Plates were washed three times (PBS, 0.05% Tween20) and incubated for 1 h at 37°C with individual mouse sera in duplicate wells per serum sample. Plates were washed again and incubated for 1 h at RT with 1:5,000 diluted (10× diluted solution of BlockerTM Casein in PBS) secondary anti-mouse immunoglobulin (total IgG, IgM and IgA) labeled with horseradish peroxidase (Southern- Biotechnology Associates). Plates were washed and developed by adding 2, 2′-azino-di (3-ethylbenthiazoline sulfo-nate)-peroxidase (ABTS) substrate (Kirkegaard & Perry Laboratories) for 1 h at RT. The reaction was measured using a BioTek Synergy™ 4 Microplate reader by determining optical density at 405 nm (OD405). Antibody titers were determined as the dilution which gave an OD405 = 1 by the SoftMax Pro v5.2 ELISA Analysis Software (Molecular Devices LLC).

In vitro Cell Stimulation, Surface and Intracellular Staining

To assess cell profiles following in vitro stimulation with either KPKDELKDY (K), MPNDPNNRV (M), or YLNIKQONSL (Y) peptides, single cell suspensions were made from the spleen, draining lymph nodes, blood and liver of PfCSP-KMY-SAPN immunized or saline control mice. Livers were perfused with saline, and lymphocytes were separated from hepatocytes using Percoll gradient treatment. Red blood cells from the spleen, liver and blood were lysed using an 8.3 g L⁻¹ ammonium chloride solution. Cells were seeded in 96 well plates with 100 μg designated peptide or media alone for 5 h in the presence of GolgStop. Cells were then harvested and stained for anti-mouse APC-conjugated anti-TCRβ (H57–597), Pacific Blue-conjugated anti-CD4 (RM4-5), FITC-conjugated anti-CD8α (53–6.7), PE-Cy5-conjugated anti-CD44 (IM7), PE-conjugated anti-CD62L (MEL-14), Alexa Fluor 700-conjugated anti-IFNγ (XM1G.1.2), and PE-Cy7-conjugated anti-IL-2 (JES6-5H4) all purchased from BD Pharmingen and analyzed by flow cytometry. Prior to intracellular staining with Alexa Fluor 700 anti-IFNγ (XM1G.1.2) and PE-Cy7-conjugated anti-IL-2 (JES6-5H4), cells were treated with Cytofix/Cytoperm reagents (BD Pharmingen) per manufacturer’s recommendations.

Adoptive Transfer of Serum or Cells

Serum or cells (total splenocytes or enriched CD8⁺ T-cells) were harvested two wks after the third immunization from mice for the adoptive transfer experiments. 100 μL of pooled serum (non-diluted) from immunized or non-immunized (injected with saline) mice was transfused to each of five 6–8 wk old naïve C57BL/6 female mice. Single cells suspensions were made from organs of immunized or saline control mice. CD8⁺ T-lymphocytes were enriched from the liver and spleens of immunized mice with a lineage depletion kit (the cocktail of antibodies was against CD4, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC Class II, and Anti-Ter-119 (Miltenyi Biotec)). Using antibodies to CD3e, CD4 and CD8 it was determined by flow cytometry that >98% of the enriched cells were CD8⁺ T-lymphocytes. A total of 20×10⁵ total splenocytes or 1.3×10⁶ enriched CD8⁺ T-cells were intravenously injected into naïve C57BL/6 mice. Mice were challenged with 5,000 sporozoites 24 h after receiving total splenocytes or 72 h after receiving enriched CD8⁺ T-cells.

Statistical Calculations

p-values between groups were calculated using unpaired students t-test. Evaluation of variance in (Table 1) was done by 1-way ANOVA. Comparison of Kaplan-Meier survival curves (Figure 5C) were done by log-rank (Mantel-Cox) test using Prism GraphPad v5.0.

Supporting Information

Figure S1 Representative gating strategy for determination of cell phenotypes. (A) Cells from their respective organs were harvested from PfCSP-KMY-SAPN immunized or PBS sham immunized mice and were selected on a lymphocyte gate and (B) further characterized for expression of TCRβ. (C) TCR β⁺ cells were subdivided into CD8⁺ or CD4⁺ cells. (D) CD8⁺ T-cells were characterized as Naïve, TEM, TCM or TLCM based on expression levels of (E) CD44, CD62L, IL-2 and IFNγ. Shown is determination of TCM.

(TIF)
Figure S2  In vitro re-stimulation of dendritic cells with SAPN induces expression of co-stimulatory molecules, CD40 and CD86. Human dendritic cells were incubated overnight with media (negative control (Red)), 10 ng (Orange) or 1 ng (Green) LPS as positive controls or 5 μg test SAPN (Blue). Cells were then stained with markers of co-stimulatory molecules, CD40 (A) and CD86 (B).

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Author Contributions

Conceived and designed the experiments: SAK MEM PB DEL. Performed the experiments: SAK MEM TAPFD CB QG DD YY CM RS. Analyzed the data: SAK MEM AC PB DEL. Contributed reagents/materials/analysis tools: RS AC PB. Wrote the paper: SAK MEM DEL.

References

1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005) The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 434: 214–217.
2. Doolan DL, Martinez-Allier N (2006) Immune response to pre-erythrocytic stages of malaria parasites. Curr Med Mol 6: 169–185.
3. Douradinha B, Doolan DL (2011) Harnessing immune responses against Plasmodium for rational vaccine design. Trends in Parasitology 27: 274–283.
4. Partnership TRCT (2011) First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med 365.
5. Akagi T, Wang X, Uto T, Baba M, Akashi M (2007) Protein direct delivery to dendritic cells using nanoparticles based on amphotilic poly(aminoc acid) derivatives. Biomaterials 28: 3427–3436.
6. Kalkandem M, Pietrini GA, Xiang SD, Mottram PL, Crimeen-Irwin B, et al. (2006) Methods for nano-particle based vaccine formulation and evaluation of their immunogenicity. Methods 40: 20–29.
7. Minino G, Scholzen A, Tang CK, Hanley JE, Kalkandem M, et al. (2007) Poly-L-lysine-coated nanoparticles: a potent delivery system to enhance DNA vaccine efficacy. Vaccine 25: 1316–1327.
8. Mottram PL, Leong D, Crimeen-Irwin B, Gloster S, Xiang SD, et al. (2007) Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus. Mol Pharm 4: 73–84.
9. Reddy ST, Reher A, Schmelke HG, Hubbell JA, Swartz MA (2006) In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release 112: 26–34.
10. Reddy ST, Swartz MA, Hubbell JA (2006) Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends Immunol 27: 573–579.
11. Kaba SA, Brandel C, Guo Q, Mittleholzer C, Raman S, et al. (2009) A Nonadjuvanted Polyepitope Nanoparticle Vaccine Confers Long-Lasting Protection against Rodent Malaria. Journal of Immunology 183: 7268–7277.
12. Tewari R, Spaccapelo R, Bistoni F, Holder AA, Crisanti A (2002) Function of region I and II adhesive motifs of Plasmodium falciparum circumsporozoite protein in sporozoite motility and infectivity. Journal of Biological Chemistry 277: 47613–47619.
13. Rosenberg R, Wirtz RA, Lanar DE, Sabbagontj J, Hall T, et al. (1989) Circumsporozoite Protein Heterogeneity in the Human Malaria Parasite Plasmodium Vivax. Science 243: 973–976.
14. Raman S, Machaidze G, Lusing A, Asbi U, Burkhard P (2006) Structure-based design of peptides that self-assemble into regular polyhedral nanoparticles. Nanomedicine 2: 95–102.
15. Koller BH, Marrack P, Kappler JW, Smithies O (1990) Normal development of CD4(+) cells is involved in protection against malaria. Faseb Journal 13: A943–A949.
16. Alexander J, Oseroff C, Dahllberg C, Qin MS, Ishioka G, et al. (2002) A decapeptide polypeptide primes for multiple CD8(+) IFN-gamma- and Th1 lymphocyte responses: Evaluation of multiple peptide polypeptides as a mode for vaccine delivery. Journal of Immunology 168: 6198–6199.
17. Florins I, Washburn MP, Raine JD, Anthony RM, Granger M, et al. (2002) A proteomic view of the Plasmodium falciparum life cycle. Nature 419: 520–526.
18. BougnSE T, Torgler R, Romero JF, Renaud L, Corradin G (2007) Plasmodium berghei-infected primary hepatocytes process and present the circumsporozoite protein to specific CD8(+) T cells in vitro. Journal of Immunology 178: 7054–7063.
19. Cockburn IA, Tse SW, Radtke AJ, Vinivasan P, Chen YC, et al. (2011) Dendritic Cells and Hepatitis C. Use Distinct Pathways to Process Protective Antigens from Plasmodium in vivo. Plos Pathogens 7.
20. Rodrigues MM, Cordey AS, Arreaza G, Corradin G, Romero P, et al. (1991) CD8(+) cytotoxic T-cell clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria. International Immunology 3: 579–585.
21. Chai SK, Clavijo P, Tam JP, Zavala F (1992) Immunogenic properties of multiple antigen peptide systems containing defined T-epitope and B-epitope. Journal of Immunology 149: 2305–2309.
22. Manosova V, Place A, Bauer M, Schwarz K, Sangal P, et al. (2008) Nanoparticles target distinct dendritic cell populations according to their size. European Journal of Immunology 38: 1404–1413.
23. Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR (2011) Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. Nucleic Acids Research 39: D913–D919.
24. Ellis JM, Henson V, Slack R, Ng J, Hartzman RJ, et al. (2000) Frequencies of HLA-A2 alleles in five US population groups - Predominance of A*02011 and identification of HLA-A*02011. Human Immunology 61: 334–340.
25. Chinchilla M, Pasenti MF, Medina-Moreno S, Wang JY, Gonzalez-Daarte OG, et al. (2007) Enhanced immunity to Plasmodium falciparum circumsporozoite protein (PfCSP) by using Salmonella enterica serovar Typhi expressing PfCSP and a PICSP-encoding DNA vaccine in a heterologous prime-boost strategy. Infect Immun 75: 3769–3779.
26. Tammenga C, Segheli M, Regis D, Chauang I, Epstein JE, et al. (2011) Adenovirus-5-Vectored P. falciparum Vaccine Expressing CSP and AMA1. Part B: Safety, Immunogenicity and Protective Efficacy of the CSP Component. Plos One 6.
27. Wang RB, Richie TL, Baraceros MF, Rahardjo N, Gay T, et al. (2005) Boosting of DNA vaccine-elicted gamma interferon responses in humans by exposure to malaria parasites. Infect Immun 73: 2603–2607.
28. Yang OO, Lewis MJ, Reed EF, Grejton DW, Kalilani-Phiri L, et al. (2011) Human leukocyte antigen class I haplotypes of human immunodeficiency virus-I-infected persons on Likoma Island, Malawi. Human Immunology 72: 877–889.
29. Liu J, Yong W, Deng YQ, Kallenhach NR, Lu M (2004) Atomic structure of a tryptophan-zipper pentamer. Proceedings of the National Academy of Sciences of the United States of America 101: 16156–16161.
30. Grauer AC, Mauduit M, Turnbull R, Romero JF, Tewari R, et al. (2007) Sterile Protection against Malaria Is Independent of Immune Responses to the Circumsporozoite Protein. Plos Pathogens 3.
31. Wang RB, Richie TL, Baraceros MF, Rahardjo N, Gay T, et al. (2005) Boosting of DNA vaccine-elicted gamma interferon responses in humans by exposure to malaria parasites. Infect Immun 73: 2603–2607.
32. Yang OO, Lewis MJ, Reed EF, Grejton DW, Kalilani-Phiri L, et al. (2011) Human leukocyte antigen class I haplotypes of human immunodeficiency virus-I-infected persons on Likoma Island, Malawi. Human Immunology 72: 877–889.
33. Liu J, Yong W, Deng YQ, Kallenhach NR, Lu M (2004) Atomic structure of a tryptophan-zipper pentamer. Proceedings of the National Academy of Sciences of the United States of America 101: 16156–16161.
34. Grauer AC, Mauduit M, Turnbull R, Romero JF, Tewari R, et al. (2007) Sterile Protection against Malaria Is Independent of Immune Responses to the Circumsporozoite Protein. Plos One 2.
35. Yadava A, Sabbagontj J, Washington MA, Ware LA, Majam V, et al. (2007) A novel chimeric Plasmodium vivax circumsporozoite protein induces biologically functional antibodies that recognize both VK210 and VK247 sporozoites. Infection and Immunity 75: 1117–1185.