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A Nonadjuvanted Polypeptide Nanoparticle Vaccine Confers Long-Lasting Protection against Rodent Malaria

Stephen A. Kaba,* Clara Brando,* Qin Guo,* Christian Mittelholzer, † Senthilkumar Raman, † David Tropel,† Ueli Aebi, † Peter Burkhard, ‡ and David E. Lanar2* 

We have designed and produced a prototypic malaria vaccine based on a highly versatile self-assembling polypeptide nanoparticle (SAPN) platform that can repetitively display antigenic epitopes. We used this platform to display a tandem repeat of the B cell immunodominant repeat epitope (DPPPPNPN)2 of the malaria parasite Plasmodium berghei circumsporozoite protein. Administered in saline, without the need for a heterologous adjuvant, the SAPN construct P4c-Mal conferred a long-lived, protective immune response to mice with a broad range of genetically distinct immune backgrounds including the H-2b, H-2d, and H-2k alleles. Immunized mice produced a CD4+ T cell-dependent, high-titer, long-lasting, high-avidity Ab response against the B cell epitope. Mice were protected against an initial challenge of parasites up to 6 mo after the last immunization or for up to 15 mo against a second challenge after an initial challenge of parasites had successfully been cleared. Furthermore, we demonstrate that the SAPN platform not only functions to deliver an ordered repetitive array of B cell peptide epitopes but operates as a classical immunological carrier to provide cognate help to the P4c-Mal-specific B cells. The Journal of Immunology, 2009, 183: 7268–7277.
peptides coassemble into SAPN (Fig. 1B). The assembly is driven by the multiple coiled-coils formed by the two oligomerization domains. Ideally, such SAPN will have icosahedral symmetry and thus resemble VLPs in their architecture (26). Because both the N- and C-terminal ends of the peptide are exposed on the surface, the SAPN are nicely suited for the presentation of B cell epitopes in a repetitive-Ag display. To test the vaccine efficacy of the platform to display peptides of malaria parasites, we selected the tandem repeat (DPPPPNPND)\(_6\) of the B cell immunodominant repeat epitope of the malaria parasite Plasmodium berghei circumsporozoite protein (PbCSP) (31) to be displayed on the SAPN surface. It has previously been shown that this tandem repeat, when displayed as a multiple antigenic peptide and administered in CFA, can confer protection in mice against a lethal challenge of live P. berghei sporozoites (32). In this paper, we show that P4c-Mal, the malaria epitope displaying SAPN, when administered in PBS, can confer a long-lived protective immune response to mice without the need of a heterologous adjuvant and that the Abs produced in response to the SAPN are of higher avidity than Abs produced against a near full-length recombinant PbCSP (R-PbCSP) delivered in the adjuvant Montanide ISA-720. Mice that received P4c-Mal were protected against an initial challenge of parasites as much as 6 mo after the last immunization and for up to 15 mo against a second challenge after an initial challenge of parasites had successfully been cleared. Furthermore, we show that immunization with P4c-Mal SAPN generate CD4\(^+\) T cell-dependent Abs which account for this protection.

Materials and Methods

Strains of mice

Six-to-eight-week old female C57BL/6 (H-2\(^b\)) and BALB/c (H-2\(^d\)) mice were used for most protection experiments. In addition, C6D8 knockout strain B6/129S2-Cd1d\(^{null}\)/J, the MHC class II (MHC II) knockout (KO) strain B6.129S2-H2\(^{dnull}\)/J, the CD4 knockout strain B6.129S2- Cd4\(^{null}\)/J, or the allogenic nude strain B6.Cg-Foxn1\(^{-/-}\)/J of mice were used to dissect the immune response to the SAPN. Two strains of TLR4-deficient (C57BL/10ScNJ and C3H/He3) mice were used to test for the presence of endotoxin. All mice were obtained from The Jackson Laboratory.

Gene cloning

The sequence encoding the SAPN peptide was ligated into the BamHI/EcoRI restriction sites of mpPEP-T expression vector, which corresponds to pPEP-T lacking the laminin oligomerization domain (33). Oligonucleotides encoding PbCSP B cell epitope (DPPPPNPND)\(_6\) were then cloned into the XmnI/EcoRI restriction sites of the SAPN expression construct to yield the final amino acid sequence MGHHHHHHHHHGGSDKGQSLVQPSGDEKR, or the aliphatic native strain B6.Cg-Foxn1\(^{-/-}\)/J of mice were used to dissect the immune response to the SAPN. Two strains of TLR4-deficient (C57BL/10ScNJ and C3H/He3) mice were used to test for the presence of endotoxin. All mice were obtained from The Jackson Laboratory.

Protein purification, refolding, and analysis of the nanoparticle polypeptide

Recombinant protein was expressed, purified, and refolded essentially as previously described (26). Briefly, the LP was expressed as a recombinant protein in Escherichia coli and purified by metal affinity chromatography followed by two polishing ion-exchange chromatography steps. The first, Q-Sepharose, captured the endotoxin, and the second, SP-Sepharose, concentrated the protein. Throughout the purification protocol the LP was kept in a denatured state in 8 M urea, then the LP was slowly folded by stepwise dialysis to remove the urea. The shape and size of the P4c-Mal nanoparticles were studied by using transmission electron microscopy (TEM) and dynamic light scattering (DLS) (27). TEM analysis was performed as previously described (29) and photographed on a Zeiss EM910 transmission electron microscope (Carl Zeiss). The hydrodynamic diameter of the SAPN was measured using a Zetasizer Nano S (Malvern Instruments) DLS instrument in PBS at 25°C and pH 7.5.

SAPN containing different ratios of C-terminal peptides

To determine the percent of PbCSP peptide in a SAPN that was needed to still be protective, we prepared a series of SAPN containing different ratios of LP containing either the PbCSP repeat peptide epitope or the random peptide sequence RD. After each LP-PbCSP or LP-RD was expressed and purified, they were mixed in the ratio of 0:100, 10:90, 25:75, 50:50, 75:25, or 100:0 (LP-PbCSP:LP-RD). Urea was then removed by dialysis and final SAPN analyzed as described (27).

Endotoxin analysis of the final product

Endotoxin levels in the final product were determined using the Pyrochrome Limulus ameocyte lysate (LAL) test (Associates of Cape Cod) following the manufacturer’s instructions and as reported previously (34).

Immunization of mice

All animal study protocols were reviewed and approved by the Walter Reed Army Institute of Research (WRAIR) Institutional Animal Care and Use Committee, conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals. Mice were randomly divided into groups of 5 or 10 and immunized i.p. three times at 14-day intervals. Where indicated, a positive control group was immunized with irradiated P. berghei sporozoites according to methods described in Ref. 35.

Passive transfer of serum and splenocytes

Ten BALB/c or C57BL/6 mice were immunized three times at 14 days intervals with 10 \(\mu\)g of P4c-Mal per mouse per injection (11) in the presence or absence of Montanide ISA-720. One week after the third immunization, animals were killed and bled by cardiac puncture for processing of serum. Spleens were surgically removed for harvesting of splenocytes. Serum or splenocytes from the 10 animals were pooled. Groups of 10 naive BALB/c or C57BL/6 mice were transfused with 100 \(\mu\)l of pooled serum (nondiluted), 1 \(\times\) 10\(^5\) cells, or both. The infectivity control group received neither cells nor serum. Animals were challenged with live sporozoites 16 h after the transfer.

Challenge of mice with live P. berghei sporozoites

P. berghei sporozoites (ANKA strain), maintained by cyclical transmission in mice and Anopheles stephensi, were dissected from mosquitoes 21–23 days after their infectious blood meal and used within 6 h. Fourteen days after the final immunization or at other specific times on long-term memory experiments, mice were challenged with a lethal dose of live P. berghei sporozoites by i.v. inoculation. C57BL/6, MHC KO, and nude mice were injected with 1000 sporozoites and BALB/c mice were injected with 4000 sporozoites per mouse.

Determination of infection following parasite challenge

Parasitemia was determined by examining Giemsa-stained thin smears from days 6–15 after challenge. Parasitemic animals were euthanized within 24–48 h of detecting blood stage parasites and an animal was considered fully protected if no parasites were detected by 20 days post challenge. In no case did an animal develop low-grade parasitemia and self-cure.

Cellular response (ELISPOT)

Splenocytes from P4c-Mal immunized or naïve animals were resuspended at a concentration of 4 \(\times\) 10\(^5\) cells/dish in complete medium. One hundred microliters of cell suspension was distributed into 96-well ELISPOT plates for IL-2 release determination and stimulated with P4c-Mal protein (5 \(\mu\)g/well) or Con-A (5 \(\mu\)g/ml) or left in medium alone with or without anti-CD4\(^+\) blocking Ab (2 \(\mu\)g/ml). To activate memory T cells, all cultures contained 4 \(\mu\)g/ml each of anti-CD28 and anti-CD49d Abs (36). The number of cells secreting IL-2 was determined.

Ab titer in response to vaccination

Ab responses to the PbCSP peptide (DPPPPNPND)\(_6\) were measured by ELISA as previously described (37). Briefly, 96-well microplates (Dynax) were coated with 100 ng of the synthetic PbCSP peptide (GenScript) per well overnight at 4°C. The Ag was blocked for 1 h at 37°C with PBS (pH 7.4) containing 0.05% Tween 20 and 1% casein (Sigma-Aldrich). Plates were washed three times with PBS-T and incubated for 2 h at room temperature with individual mouse sera in triplicate wells per serum sample. Plates were washed again and incubated for 1 h at room temperature with...
1/5000 diluted (PBS) secondary anti-mouse Ig (total IgG) or the different Ig subcategories (IgG1, IgG2a, IgG2b, and IgG2c) Abs labeled with HRP (Southern Biotechnology Associates). Plates were washed and developed by adding ABTS substrate (Kirkegaard & Perry Laboratories). Color reaction was measured in a Dynatech MR5000 microplate reader by determining OD at 405 nm (OD_{405 nm}). The results were calculated as mean OD_{405 nm} readings of duplicate assays ± SDs.

Ab avidity index (AI) determination

An initial concentration of sodium isothiocyanate (NaSCN) that eluted 50% of the total bound IgG was determined by the method described by Pullen et al. (38). Then two side-by-side 4-fold serial dilutions for a second ELISA were done (in triplicate). After three washes with PBS (pH 7.4) containing 0.05% Tween 20 and one wash with PBS, 100 μl of the concentration of NaSCN (1.5 M dissolved in PBS) measured in the initial avidity determination was added to one set of the serial dilutions on the plate and plain PBS was added to the other dilution series. The same elution concentration of NaSCN determined for total IgG was used for elution of IgG subclasses. After 15-min incubation, all wells were washed and developed as described above. The AI was calculated based on the ratio of the areas derived from under the curves obtained by the plot of OD (OD_{405 nm}) and log of the sera dilution in the ELISA experiment with and without NaSCN treatment as described (39).

Statistics

Student’s t tests were performed using Excel software (Microsoft). The association of Ab titer and protection was determined using logistic regression analysis in the program SAS (SAS Institute).

Results

Preparation and characterization of P4c-Mal SAPN

The LP was expressed, purified, and folded to form nanoparticles. Although the calculated molecular mass of the LP was 14.7 kDa, the protein ran at a relative molecular mass of ~17 kDa on SDS-PAGE (Fig. 1C). TEM showed the particles to be ~25 nm in diameter (Fig. 1D) and DLS analysis indicated a uniform distribution of unclumped, single nanoparticles (data not shown). The endotoxin levels were below the level of detection (0.005 EU/ml) of the LAL chromogenic assay.

The P4c-Mal SAPN induce high Ab titer leading to protection

Groups (n = 10) of C57BL/6 and BALB/c mice were immunized with 10 μg P4c-Mal or P4c (a SAPN without the PbCSP peptide epitope) in either PBS or Montanide ISA-720. Other groups of mice were immunized with 10 μg near full-length R-PbCSP protein (40) in PBS or in Montanide ISA-720 (R-PbCSP/M). Negative controls were age- and sex-matched C57BL/6 and BALB/c mice that had received either saline only or saline and Montanide ISA-720. One day before the first immunization and 14 days after each immunization, mice were bled for determination of Ab titers. As shown in Fig. 2A, Ab titers reached maximum levels after two immunizations with the adjuvanted P4c-Mal, whereas titers of the non-adjuvanted P4c-Mal and adjuvanted R-PbCSP-immunized mice achieved maximum levels after the third immunization.

Fourteen days after the last immunization, animals were challenged i.v. with live sporozoites. More than 95% of mice immunized with P4c-Mal, both with and without Montanide ISA-720, or R-PbCSP in Montanide ISA-720 did not develop any parasitemia and thus showed complete protection against challenge with viable sporozoites (Fig. 2B). This ability to prevent parasitemia and thus prevent malaria following sporozoite challenge is equivalent to what is only achieved with the whole, irradiated sporozoite immunization regime. In contrast, as few as 5% of animals administered saline, saline and Montanide ISA-720, or R-PbCSP in saline did not develop parasites and survived until 11 days post challenge. No animal was observed with blood stage parasites that did not die naturally or was killed according to protocol. These results show that immunization with P4c-Mal had a significant ability to induce a protective immune response in the presence as well as in the absence of adjuvant. The ability to protect against challenge was directly correlated with Ab titer (p < 0.001). This was true in both BALB/c and C57BL/6 mice.

To determine whether the Abs alone were responsible for protection, serum and spleen cells were collected from the protected animals and transferred into strain-matched naïve animals. C57BL/6 or BALB/c mice were divided into four groups of five animals each, and each mouse received 100 μl of serum from immunized animals by i.p. injection, 1 × 10^7 total splenocytes by i.v. injection from immunized donors, or a mixture of both splenocytes and serum. A group of animals for each strain was injected with PBS and served as infectivity controls. Animals from both strains that received serum or splenocytes plus serum showed 100% resistance to lethal challenge whereas all animals that received cells alone or those injected with PBS became parasitemic by day 10 (Table 1).

SAPN need to contain at least 50% of LP carrying a specific epitope

The SAPN are made by self-assembly of LPs that contain a B cell epitope on their C-terminal ends. By mixing different LP-expressing peptide epitopes it is, theoretically, possible to make a SAPN that displays several different peptide epitopes and, therefore, perhaps to create a multiple antigenic SAPN. We wanted to determine the percentage of LP in a SAPN carrying a unique epitope that would be required to achieve an immune response. Mixing LP with the PbCSP peptide and LP with a random peptide in different ratios...
before self-assembly allowed us to form SAPN with different percentages of PbCSP on their surface. Only SAPN that contained 25% or more of the PbCSP peptide induced high Ab titers against PbCSP peptide. SAPN with 50% or more of the PbCSP peptide developed high titers after three immunizations, but those with 75% or more induced the highest titers (Fig. 3).

Protective immune response is long lasting

The ability to induce immune memory is an important component of any vaccine. Therefore, we wanted to determine the extent of the longevity of the protective immune response after immunization. We asked the question in two ways: first, how long after the final immunization would an effective immune response exist and second, analogous to a field situation where subsequent exposure to infected mosquitoes occurs, how long after an initial response to an effective challenge would the mouse remain protected? In the first experiment (Fig. 4A) groups of 10 mice were immunized and then challenged either 2 wk or 1, 3, or 6 mo later. The ability to resist infection slowly dropped over time but a majority of mice were still able to resist infection from lethal challenge up to 6 mo after the third immunization with P4c-Mal in PBS.

In the second set of experiments, five groups (n = 5) of mice received three doses of vaccine. All mice were challenged 2 weeks after the third P4c-Mal immunization with a lethal dose of sporozoites. All animals survived and individual groups were then challenged either 1, 3, 6, 9, or 15 mo later. As shown in Fig. 4B, all mice survived a second challenge up to 9 mo later and after 15 mo 60% of mice were still protected. At the time of challenge in all experiments, five control mice (immunized with PBS and not initially challenged) were given sporozoites and in all instances they became parasitemic.

Ab production is CD4+ T cell dependent

No P. berghei-specific or universal Th epitope was intentionally incorporated into the SAPN design. This raised the question as to whether the observed Ab response was T cell dependent or independent. To answer this question, two different strains of mice deficient in functional CD4+ T cells, MHC II KO, or nude, or their wild-type control strain, C57BL/6, were immunized with P4c-Mal. Two weeks post-third immunization, the MHC II KO and all nude mice became infected. Only C57BL/6 mice immunized with P4c-Mal developed an immune response that prevented the appearance of blood stage parasites in these mice.

Table 1. Protection is transferred by serum

<table>
<thead>
<tr>
<th>Group</th>
<th>Transfer</th>
<th>Number of Balb/c Mice Developing Parasitemia by Day 10</th>
<th>Number of C57BL/6 Mice Developing Parasitemia by Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µl undiluted serum</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>100 µl undiluted serum + 1 × 10^7 splenocytes</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>3</td>
<td>1 × 10^7 splenocytes</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>
parasites (Table 2). Thus, the absence of functional CD4+ T cells impairs the ability of mice to generate protective Abs when immunized with P4c-Mal.

Ag-specific CD4+ cells are generated in response to SAPN immunization

Determining that CD4+ T cells are needed to initiate the humoral immune response raised the question of how these cognate CD4+ helper cells are generated. The SAPN is a complex protein structure and we suspected that a CD4+ stimulatory epitope was likely to be part of the LP that makes up the SAPN. To test this hypothesis, a group of C57BL/6 mice (n = 5) were immunized with P4c-Mal and mice and control groups were euthanized 2 weeks post-third dose of vaccine. All mice survived challenge. One group (n = 5) each of vaccinated and challenged mice or mice receiving saline only were challenged a second time either 1, 3, 6, 9, or 15 mo later with live sporozoites.

Table 2. T cell dependency of protective immune response with P4c-Mal immunization

<table>
<thead>
<tr>
<th>Mice</th>
<th>Immunized with</th>
<th>% Developing Parasitemia After Challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>PBS</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>P4c-Mal</td>
<td>0</td>
</tr>
<tr>
<td>Nude</td>
<td>PBS</td>
<td>100</td>
</tr>
<tr>
<td>Nude</td>
<td>P4c-Mal</td>
<td>100</td>
</tr>
<tr>
<td>MHC II KO</td>
<td>PBS</td>
<td>100</td>
</tr>
<tr>
<td>MHC II KO</td>
<td>P4c-Mal</td>
<td>100</td>
</tr>
</tbody>
</table>

* By day 10 post-challenge with 1000 sporozoites.

FIGURE 5. P4c-Mal induces the production of IL-2 in CD4+ T cells. Splenocytes from P4c-Mal immunized (IM) or naive C57BL/6 mice were cultured with P4c-Mal nanoparticles, Con A, or medium with or without anti-CD4+ T cell-blocking Abs. After 18 h the numbers of cells secreting IL-2 were determined. Values are representative of one of four separate experiments. *, p > 0.001.
bound endotoxin we would not be able to protect TLR4-null mice with a P4c-Mal preparation. Therefore, we immunized two different TLR4-null, endotoxin-resistant mouse strains C57BL/6/10ScNJ (TLR4<sup>−/−</sup>) and C3H/He3c (defective LPS-response allele Tlr4<sup>−<sub>4</sub>−/−</sup>) as well as their wild-type equivalent C57BL/6 and C3H mice, respectively, with P4c-Mal. After immunization with P4c-Mal in PBS and sporozoite challenge, three of five TLR4<sup>−/−</sup> mice and four of five Tlr4<sup>−<sub>4</sub>−/−</sup>-deficient mice and five of five of each of their wild-type counterparts were protected. All mice that survived developed moderate to high Ag-specific Ab titers whereas all mice that died failed to develop any significant Ag-specific Ab. The fact that we were not able to protect 100% of all the TLR4-null mice is not surprising. It has been shown that there is an increased susceptibility to pathogens when TLR4 signaling is impaired (41). However, the ability to protect 70% of TLR4-null mice strongly indicates that LPS is not involved in the stimulation of the immune system in response to SAPN immunization.

**IgG subclass Ab responses**

Anti-PbCSP peptide Ab levels were determined in serum from C57BL/6 and BALB/c mice immunized with P4c-Mal. The geometric mean values of the total IgG anti-PbCSP Abs or IgG subclass Abs in both strains are illustrated in Fig. 6. We measured IgG2a in BALB/c mice and IgG2c in C57BL/6 mice. C57BL/6 mice lack the Igh-1a allele that codes for the IgG2a but instead express IgG2c from the Igh-1b allele (42, 43). Sera were pooled from 10 mice in each group 2-week post-third immunization. Immunization with P4c-Mal and P4c-Mal/M resulted predominantly in the induction of specific IgG1 and IgG2a/c Abs. C57BL/6 mice immunized with R-PbCSP/M resulted in the induction of only IgG1 Abs (Figs. 6B and 7A).

In C57BL/6 mice, P4c-Mal induced a significantly larger amount of IgG1 Ab than P4c-Mal/M (<i>p</i> < 0.001; Fig. 6B). Although over four times the amount of IgG1 was produced in BALB/c mice than in C57BL/6 (<i>p</i> < 0.0002), no significant difference was observed in the amount of IgG1 subclass Abs when BALB/c were immunized with either P4c-Mal or P4c-Mal/M (<i>p</i> = 0.2). The formulation of P4c-Mal with Montanide ISA-720 significantly affected IgG2a responses in BALB/c mice (<i>p</i> < 0.001). IgG2c Ab levels produced in C57BL/6 mice immunized with either P4c-Mal or P4c-Mal/M are statistically the same (<i>p</i> = 0.4).

**Determination of AI of Abs made against PbCSP repeat epitope**

The chaotropic ELISA elution assay was used for determination of the AI of the Abs induced by SAPN-presented or recombinant protein-presented epitopes. In Fig. 7, the OD<sub>405 nm</sub> values of the ELISA from each group of mice were plotted against the respective log of the sera dilutions. Rather than using a single specific point on the curve to determine the AI, the area under the curve was used (39). Table 3 shows the calculated AI for sera from R-PbCSP/M, P4c-Mal, and P4c-Mal/M Ags. Immunization with R-PbCSP/M induced only IgG1 subclass Ab. The AI value (0.4) for total IgG for R-PbCSP/M was significantly lower (<i>p</i> = 0.005) than for any SAPN-PbCSP peptide-induced IgG, regardless of whether the epitope was delivered in adjuvant (<i>p</i> > 0.5) or saline (<i>p</i> > 0.5). AI values for the total IgG induced by P4c-Mal or P4c-Mal/M were statistically similar. For IgG1, there was no significant difference among the AI values for R-PbCSP/M (<i>p</i> > 0.1) and the P4c-Mal (<i>p</i> > 0.5) or P4c-Mal/M (<i>p</i> > 0.5). For IgG2a/c and IgG1, there was no significant difference between the AI values Abs induced by either of the SAPN-PbCSP peptides. Remarkably, the AI for IgG2c was close to 1, indicating that this particular IgG subclass had an extremely high avidity for the circumsporozoite protein peptide epitope.

**Discussion**

Despite the recent limited successes of RTS,S in field trials (8, 44), the development of a vaccine for malaria has been a disappointing one. Neither live attenuated viral vectors nor DNA-based vaccines, alone or in combination, have worked effectively (3–5, 45–48). In addition, the effectiveness of protein based vaccines, even RTS,S, has been limited by their selection of a coadministered adjuvant (49). We present here a new platform technology based on self-assembling polypeptides that form nanoparticles (27) to induce a protective immune response against whole organism challenge in vivo. We have found that the SAPN functions as a vehicle for the presentation of selected antigenic epitopes and acts as a powerful immunostimulatory entity.

To test the feasibility of this new platform to induce an immune response against an otherwise lethal infectious agent, we chose to construct a vaccine that presents a known protective B cell epitope as a test Ag. We reasoned that the platform would have to be substantially better in the induction of an immune response and in demonstrating protective efficacy than many of the other existing
The PbCSP repeat sequence, (DPPPPNPN)D, has been shown, when chemically conjugated to tetanus toxoid (32) or administered in CFA (50), to induce Abs that prevent \( P. berghei \) sporozoites from establishing a blood stage infection in mice. In the former, the Ab titer was directly proportional to the dose of immunogen; however, the dose was limited because of the toxicity of the tetanus toxoid carrier. In the latter, a chemically synthesized multiple antigenic peptide suffered because the synthesis of the vaccine could not be easily controlled and reproduced. A similar approach of immunization with B cell and Th cell epitopes was later tested with a hepatitis B core Ag VLP (51). This VLP, displaying the PbCSP repeat sequence on its immunodominant loop in combination with the Th epitopes T1 and T*, ultimately failed as it too was not immunogenic enough on its own and repeated administration with a strong adjuvant caused serious side effects (52).

Table 3. AI measurements of PbCSP peptide-specific Ab produced in C57BL/6 or Balb/C mice

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG2c</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-PbCSP</td>
<td>0.40 (P = 0.005)</td>
<td>0.62 (P = 0.136)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>P4c-Mal</td>
<td>0.79 (P = 0.614)</td>
<td>0.84 (P = 0.674)</td>
<td>*</td>
<td>0.67 (P = 0.763)</td>
<td>0.97 (P = 0.549)</td>
</tr>
<tr>
<td>P4c-Mal/M</td>
<td>0.78 (P = 0.614)</td>
<td>0.88 (P = 0.674)</td>
<td>*</td>
<td>0.60 (P = 0.763)</td>
<td>0.92 (P = 0.549)</td>
</tr>
<tr>
<td>Balb/c mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4c-Mal</td>
<td>0.06 (P = 0.733)</td>
<td>0.82 (P = 0.470)</td>
<td>0.53 (P = 0.846)</td>
<td>0.54 (P = 0.279)</td>
<td>*</td>
</tr>
<tr>
<td>P4c-Mal/M</td>
<td>0.52 (P = 0.733)</td>
<td>0.71 (P = 0.470)</td>
<td>0.51 (P = 0.846)</td>
<td>0.90 (P = 0.279)</td>
<td>*</td>
</tr>
</tbody>
</table>

*, Insufficient Ab made to make calculations.
Immunization of mice with the P4c-Mal SAPN in saline provided complete protection to a lethal challenge of sporozoites 1 month after the third dose of vaccine. Subsequent challenge of the same mice showed a sustained immune response capable of protective efficacy against an otherwise lethal challenge of live sporozoites as late as 15 mo later. This level of protection has only been demonstrated previously with the whole cell irradiated sporozoite model of immunization (35). We have shown that Abs were the effector of protection as serum was required to transfer protection whereas cells alone could not and that the response was T cell-dependent as MHC II KO animals were incapable of mounting a protective response.

The T cells were demonstrated to be SAPN-specific, providing cognate help to the P4c-Mal peptide-specific B cells. It is well established that to achieve a T cell-dependent B cell response, B cells need to interact with the cognate T cells that provide help for the Ab production. At the same time, only activated cognate T cells can provide help, and T cell activation only occurs upon simultaneous stimulation of the TCR and the ligand for costimulatory molecules. Conventionally, adjuvant induces expression of high levels of costimulatory molecules on the dendritic cells (DC) presenting Ag to T cells, thus enabling them to simultaneously provide signal 1 (Ag) and signal 2 (costimulatory molecule). With this platform, we did not need to use adjuvant, raising the question: What was the initializing DC maturation to provide signal 2? Studies in our laboratory have shown that this T cell help came from a sequence lacking with the COMP pentameric oligomerization domain. When this region was replaced with another pentameric oligomerization domain, T cell help was greatly diminished. However, inclusion of a universal Th epitope (PADRE) restored the activation potential of the SAPN (our manuscript in preparation).

Other reports using nanoparticles to present Ag have used inert substrates such as gold particles coated with azobenzene disulfide dye (53) or polymers of proteinaceous materials as the substrate. Polypeptides containing T or B cell epitopes bound to polymers of glutamic acid or poly-L-lysine-coated polystyrene (10, 54–56) have been used to enhance DNA vaccine efficacy (12). Most of the immunostimulatory effects of those nanoparticles could be attributed to their interaction with DC in the skin or the lymph nodes. Recent studies have presented convincing evidence that small size (20–30 nm) particles are preferentially transported to the lymph nodes where the particles are presented to immature DC (14, 15, 57, 58). Once internalized in the DC, the nanoparticles are processed and antigenic epitopes are released to activate MHC class I and II pathways, leading to induction of both CD4+ and CD8+ T cell activation.

The high levels of IgG1 and IgG2a/c, as well as a considerable IgG2b subclass of Ig, reflect a mixed Th1/Th2 response. In mice, IgG1 responses are usually associated with Th2 responses, whereas high levels of IgG2a, sometimes associated with IgG2b and IgG3, are thought to reflect Th1 responses. Several parameters influence the IgG subclass responses to proteins, including the dose of Ag used, use of adjuvants or routes of immunization, and the intrinsic immunogenicity of the protein itself (59–65). We did not see major differences in IgG subclasses with different routes of immunization (data not shown) and adjuvants added to SAPN did not influence IgG subclass distribution. Therefore, the major effector of the IgG subclass appears to be associated with an intrinsic immunogenic property of the SAPN.

A surprising finding in these studies was the high avidity and subclass specificity of the Abs produced. Of note is the extremely high avidity of the IgG2c subclass Abs. Immunization with R-PbCSP in the adjuvant Montanide ISA-720 did not induce any detectable IgG2c Abs, whereas high titer, high avidity IgG2c Abs were generated against P4c-Mal with or without adjuvant. Clearly the effectiveness of a vaccine that works predominately by induction of neutralizing Abs, as is the mode of action of the immune response induced in these studies, is dependent not only on the amount of Ab induced but also the avidity of those Abs. The complex cellular events that are needed to generate high-affinity Ab secreting cells are not clearly understood. B cells in the germinal centers undergo somatic hypermutation of V(DJ) Ig genes and competition for Ag retained on the germinal center follicular DC results in the selection of B cells secreting the highest affinity Abs (66). Many things influence the combined affinity (avidity) of Abs to antigenic determinants, including the valence of the Ab and the valence of the Ag. The physical Ag properties or adjuvant assets that influence the maturation process, which may be essential for the protective efficacy, have not been characterized following immunization with T cell-dependent protein Ags but is clearly important in vaccine development (67). The coiled-coil nature of the SAPN may influence the presentation of the Ag and maturation of the Ab affinity because linear recombinant protein with the same epitope given without adjuvant (R-PbCSP) or a modified P4c-Mal LP that would not form a SAPN (data not shown) did not induce any significant Ab and failed to provide a protective immune response.

The nanoparticles used in this study appear to display B cell epitopes in a very immunostimulatory array. The particles are small, ~25 nm in size, and contain strong hydrophobic oligomerization domains that dictate their self-assembly. Recently we have shown that they are efficiently taken up by and activate mouse macrophages and bone marrow-derived DC (manuscript in preparation). The platform has recently been used to effectively present an otherwise poor antigenic determinant to make mAbs against a conserved actin domain (28), as well as to induce in vitro neutralizing Abs against a SARS virus epitope (26). We have now extended the use of this platform to induce an in vivo immune response that is protective against challenge with a lethal parasite. When administered in saline alone, the SAPN are capable of inducing maturation of a long-term memory response to a B cell epitope. The induced Abs protect against an otherwise lethal challenge of sporozoites. It should be noted that when the leading malaria vaccine candidate, RTS,S, itself a VLP vaccine formulation, was administered to mice in saline, no significant anti-circumsporozoite protein repeat or HBsAg Ab was detected (68). This clearly shows that nanoparticles or VLP, a priori, are not the key to effective immune responses. We are now determining whether the SAPN platform can be used to make a vaccine against \textit{P. falciparum} and \textit{P. vivax} malaria; whether it can induce a CD8+ mediated immune response, and whether it is effective in higher order mammals such as rabbits and nonhuman primates. Furthermore, to make a vaccine for human use, autoimmune reactions could develop due to the immune responses induced against the COMP sequence in the SAPN scaffold because, although the sequence was derived from mouse COMP, it also has a high homology to human COMP. Therefore, in advanced designs, we have replaced the COMP with a de novo designed sequence without homology to any human sequences in the protein databank.

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