A Role for Immune Responses against Non-CS Components in the Cross-Species Protection Induced by Immunization with Irradiated Malaria Sporozoites

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Introduction

Sporozoites inoculated by the mosquito must invade and develop within hepatocytes in order to generate merozoites that can then initiate the pathogenic erythrocytic phase. Thus, this obligatory transient phase of the life cycle is an attractive target for interventions to inhibit parasite development fully, as this would prevent both disease and transmission. Sterile immunity against pre-erythrocytic (PE) stages is an all-or-none phenomenon, pre-erythrocytic (PE) stages. However, this role for CS was questioned when we recently showed that immunization with irradiated sporozoites (IrrSpz) of a P. berghei line whose endogenous CS was replaced by that of P. falciparum still conferred sterile protection against challenge with wild type P. berghei sporozoites. In order to investigate the involvement of CS in the cross-species protection recently observed between the two rodent parasites P. berghei and P. yoelii, we adopted our gene replacement approach for the P. yoelii CS and exploited the ability to conduct reciprocal challenges. Overall, we found that immunization led to sterile immunity irrespective of the origin of the CS in the immunizing or challenge sporozoites. However, for some combinations, immune responses to CS contributed to the acquisition of protective immunity and were dependent on the immunizing IrrSpz dose. Nonetheless, when data from all the cross-species immunization/challenges were considered, the immune responses directed against non-CS parasite antigens shared by the two parasite species played a major role in the sterile protection induced by immunization with IrrSpz. This opens the perspective to develop a single vaccine formulation that could protect against multiple parasite species.

Abstract

Immunization with irradiated Plasmodium sporozoites induces sterile immunity in rodents, monkeys and humans. The major surface component of the sporozoite the circumsporozoite protein (CS) long considered as the antigen predominantly responsible for this immunity, thus remains the leading candidate antigen for vaccines targeting the parasite’s pre-erythrocytic (PE) stages. However, this role for CS was questioned when we recently showed that immunization with irradiated sporozoites (IrrSpz) of a P. berghei line whose endogenous CS was replaced by that of P. falciparum still conferred sterile protection against challenge with wild type P. berghei sporozoites. In order to investigate the involvement of CS in the cross-species protection recently observed between the two rodent parasites P. berghei and P. yoelii, we adopted our gene replacement approach for the P. yoelii CS and exploited the ability to conduct reciprocal challenges. Overall, we found that immunization led to sterile immunity irrespective of the origin of the CS in the immunizing or challenge sporozoites. However, for some combinations, immune responses to CS contributed to the acquisition of protective immunity and were dependent on the immunizing IrrSpz dose. Nonetheless, when data from all the cross-species immunization/challenges were considered, the immune responses directed against non-CS parasite antigens shared by the two parasite species played a major role in the sterile protection induced by immunization with IrrSpz. This opens the perspective to develop a single vaccine formulation that could protect against multiple parasite species.

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transgenic mice [8,9]. Further indications that sterile protection can be obtained independently of immune responses to the CS were obtained when immunization with \textit{P. berghei} IrrSpz whose endogenous CS was replaced by that of \textit{P. falciparum} fully protected mice from challenge with wild type \textit{P. berghei} [10].

It had been recently suggested that anti-CS responses might be implicated in the cross-species protection that has been observed between \textit{P. yoelii} and \textit{P. berghei} in the context of IrrSpz immunization [11], possibly because of the extensive sequence homology between the N- and C-terminal of their CS because the repeat regions are quite distinct (Figure S1). Indeed, adoptive transfer of a CD8+ T cell clone specific for the \textit{P. yoelii} CS CD8+ immunodominant epitope protected mice from challenge with \textit{P. berghei} sporozoites [12]. In order to investigate the actual role of immune responses induced against the CS in cross-species sterile protection we exploited the gene replacement approach [13] to generate \textit{P. berghei} sporozoites expressing the CS of \textit{P. yoelii} (\textit{P. berghei} [PyCS]), instead of the endogenous CS, for use with those of wild type \textit{P. berghei} and \textit{P. yoelii} in reciprocal immunization/challenge experiments. This also afforded us the opportunity to characterise the role of CS in sterile protection in the two widely used rodent models of IrrSpz immunization.

Results

T Cell Responses

Groups of BALB/c mice were immunized with three doses of \textit{P. berghei}, \textit{P. berghei} [PyCS] or \textit{P. yoelii} IrrSpz. Cross-reactive T cell responses induced by the immunizations were assessed by ELISPOT using long peptides corresponding to N-terminal or C-terminal regions of \textit{P. berghei}, and \textit{P. yoelii} CS, which contain all the potential CD4 and CD8 epitopes (Figure 1). Whereas splenic T cells from mice immunized with \textit{P. berghei} irradiated sporozoites only recognized peptides derived from the \textit{P. berghei} CS, those from mice immunized with \textit{P. berghei} [PyCS] or \textit{P. yoelii} IrrSpz also recognized the C-terminus peptides derived from \textit{P. berghei}, in addition to the peptides derived from \textit{P. yoelii} CS. This cross-species recognition was more substantial for mice immunized with \textit{P. yoelii} IrrSpz, which surprisingly additionally recognized the long peptide derived from the heterologous N-terminus of the \textit{P. berghei} CS but not of the homologous \textit{P. yoelii} CS (Figure 1 middle and left panels). This unexpected observation, confirmed in duplicate experiments, remains as yet unexplained.

To determine if this cross-reactivity observed for the peptide derived from the C-terminus of the CS molecule were due to CD8+ T cells recognizing the immunodominant CD8 epitope located in this region, as previously suggested using CS-specific T cell clones [12], a set of peptides containing this immunodominant CD8 epitope (9-mer and 17-mer for \textit{P. berghei} and 9-mer and 17-mer for \textit{P. yoelii}) were tested by ELISPOT. This was not the case, because the cross-reactivity was found to be minimal (Figure 2a–2d).

Thus, immunization with \textit{P. yoelii} but not \textit{P. berghei} IrrSpz induced cross-reactive anti-CS T cells, most likely CD4+ T cells. The magnitude of the cross-reactivity was different depending on the context in which the \textit{P. yoelii} CS was (i.e. whether it was expressed in a \textit{P. berghei} or in \textit{P. yoelii} sporozoite background).

Antibody Responses

The levels of antibodies induced after immunization with three injections of IrrSpz from the three parasite lines were assessed by ELISA using peptides corresponding to the three domains of CS (N-terminus, repeat region and C-terminus), and by IFA using whole sporozoites. Anti-CS specific IgG and IgM induced by immunization with \textit{P. berghei} IrrSpz were solely directed against the homologous \textit{P. berghei} but not heterologous \textit{P. yoelii} CS peptides (Figure 3 and Figure S4). The antibodies induced by immunization with the two other lines (\textit{P. berghei} [PyCS] or \textit{P. yoelii}) not only recognized their homologous CS peptides but also cross-reacted with the heterologous C-terminal peptides derived from \textit{P. berghei} CS, with higher levels observed for IgG as compared to IgM (Figure 3A and Figure S4).

Immunization with IrrSpz induced high levels of IgG against homologous but none against heterologous sporozoites. Sera from mice immunized IrrSpz had an IFA titre of $\approx 1/200$ 000 on wet homologous sporozoites (for which only surface antigens are accessible) (Figure 4A), and an IFA titre of $\approx 1/400$ 000 on dried methanol-fixed sporozoites (for which both intracellular and surface antigens are accessible) (Figure 5). By contrast, negligible IFA IgG titres (below 1/10) were obtained against the wet or the dried methanol-fixed sporozoites expressing the heterologous CS.

Figure 1. CS-specific T cells induced by immunization with irradiated sporozoites. Mice were immunized 3 times with IrrSpz from three parasite lines. The frequency of epitope-specific CD8+ or CD4+ T cells in spleens was assessed by IFN-$\gamma$ ELISPOT using long CS peptides 10 days after the last immunization. Long peptides PyNt, PbNt and PyCt, PbCt correspond to the \textit{P. yoelii} or \textit{P. berghei} CS N- and C-terminal region of CS, respectively. These peptides encompass potential CD4+ and CD8+ T cells epitopes. Results are expressed as the mean±SEM of epitope–specific T cells from 5 mice per group.

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Since sporozoites from these lines differed only for the CS, this meant that IgG were predominantly directed against the CS. The titres of IgM responses induced against the homologous sporozoites were one to two orders of magnitude lower (corresponding to IFA titres of 1/200–1/1600) against wet sporozoites than those observed for IgG (Figure 4B). Similar results were obtained with sera obtained from animals immunized only once as opposed to three times with IrrSpz, though in this case the antibody levels were much lower (1/50–1/100).

The fact that the CS cross-reactivity of the antibodies induced by IrrSpz immunization was revealed only when peptides but not when whole sporozoites were used suggested that the cross-reactive antibodies induced by immunization with \textit{P. yoelii} [PyCS] IrrSpz recognized epitopes that were not exposed in the CS expressed by \textit{P. berghei} salivary gland sporozoites.

It was possible to gather some qualitative estimate of the contribution that anti-CS humoral reactivities made to the inhibition of parasite invasion and development as compared to those directed against other antigens. This was achieved in an in vitro assay where sporozoites were added in the presence of sera (used at 1/10 dilution) and the numbers of liver stage parasites that reached maturity were subsequently counted. Sera from animals immunized 3 times with IrrSpz were strongly inhibitory (>90%) to invasion and development of the homologous parasites (Figure 4C). When the heterologous combinations were similarly assayed, inhibition was also observed but it varied in intensity (Figure 4C). The sera from \textit{P. berghei} IrrSpz-immunized mice were moderately inhibitory (30%–40%) against the heterologous \textit{P. yoelii} sporozoites (Figure 4C, top line, right panel). Since cross-reactivities induced against \textit{P. yoelii} sporozoites were only due to the IgM fraction and since very little cross-reactive IgM to \textit{P. yoelii} CS peptides were detected in the sera from \textit{P. berghei} IrrSpz-immunized mice (Figure S4), this indicated that anti-CS IgG but not IgM contributed to more than half of the inhibition measured in vitro against homologous parasites while the remaining inhibition was mediated by IgM against other non-CS antigens.

When the sera from mice immunized with \textit{P. berghei} [PyCS] IrrSpz were tested, inhibition of \textit{P. berghei} sporozoites was high (90%). This contrasted with a weak (30%) inhibitory activity of sera raised by \textit{P. yoelii} IrrSpz immunization against \textit{P. berghei} sporozoites (Figure 4C, left panel, from top to bottom). However, we could not draw meaningful conclusions as to the likely role of the anti-CS versus anti-non-CS component of these inhibitory activities. This is evident when one compares the high cross-reactivity observed for IgG in ELISA against CS peptides (Figure 3) with a low cross-reactivity for the same sera when wet or air-dried methanol-fixed sporozoites were used (Figures 4 and S5). Nonetheless, other indications of the differential contribution to sterile immunity can be obtained from \textit{in vivo} challenge studies.
Sterile protection was equally observed in 80 to 100% of the BALB/c mice inoculated once or three times with *P. berghei* IrrSpz and then challenged with *P. berghei* or *P. berghei* [PyCS] sporozoites (Figure 5A). We also obtained identical results using another *P. berghei* [PyCS] clone (data not shown). Protection was not restricted to BALB/c because outbred CD1 mice immunized once or three times with *P. berghei* IrrSpz and then challenged with *P. berghei* or *P. berghei* [PyCS] were also fully protected (Figure S6). These results indicated that when *P. berghei* IrrSpz were used for immunization, they induced sterile immunity independently of the *P. berghei* CS.

Next, experiments were performed to determine if this was equally true for the *P. yoelii* CS. Mice immunized once or three times with *P. berghei* [PyCS] IrrSpz and then challenged with *P. berghei* or *P. berghei* [PyCS] were also fully protected (Figure S6). These results indicated that when *P. berghei* IrrSpz were used for immunization, they induced sterile immunity independently of the *P. berghei* CS. Next, experiments were performed to determine if this was equally true for the *P. yoelii* CS. Mice immunized once or three times with *P. berghei* [PyCS] IrrSpz and then challenged with either *P. berghei* or *P. berghei* [PyCS] sporozoites were also protected (Figure 5B). However, since we detected antibody and T cell cross-reactive responses against long peptides derived from the *P. berghei* CS after immunization with *P. berghei* [PyCS] or *P. yoelii* IrrSpz (Figures 1 and 3), it was not possible to ascertain to what extent the anti-CS cross-reactive responses as opposed to the immune responses to non-CS antigens contributed to the sterile protection observed.

In order to address this point, mice were immunized once with IrrSpz from the 3 parasite lines and challenged with *P. yoelii* sporozoites. Sterile protection was obtained in 60% of the mice immunized with one injection of *P. yoelii* IrrSpz and challenged with homologous *P. yoelii* sporozoites but not in mice challenged with the heterologous *P. berghei* sporozoites (Figure 6A). On the other hand, 1 of the 5 mice immunized with *P. berghei* [PyCS] IrrSpz was completely protected against a *P. yoelii* sporozoite challenge (Figure 6A, left panel). This indicated that the presence of the *P. yoelii* CS in a *P. berghei* background could not account for sterile protection induced. Sterile protection is an all-or-none phenomenon that depends on maximal inhibition of parasite invasion and growth in the liver. Therefore, we quantified parasites in the livers of immunized and challenged mice in order to determine to what extent immunization with the 3 parasite lines inhibited the development of *P. yoelii* sporozoites. A single immunization with *P. yoelii* IrrSpz induced a significant 98.9% reduction of parasite liver load as compared to non-immunized mice (Figure 6A, right panel). Immunization with *P. berghei* IrrSpz reduced by 57% *P. yoelii* liver load as compared to non-immunized mice, a difference that did not reach statistical significance. When immunization was

### Figure 3. IgG antibody responses to *P. yoelii* and *P. berghei* CS domains.

Pooled serum samples from groups of five mice immunized 3 times with IrrSpz from the different parasite lines were analyzed by ELISA using peptides covering domains of the *P. berghei* (A) or *P. yoelii* (B) CS. Data are expressed as differential absorbance where values from pooled normal serum were subtracted from experimental values. The data presented are representative of 2 experiments. doi:10.1371/journal.pone.0007717.g003
performed with *P. berghei* [PyCS] IrrSpz, hepatic development of challenge *P. yoelii* was significantly reduced (83.4%) as compared to non-immunized mice (Figure 6A, right panel). This indicated that PyCS in the context of *P. berghei* sporozoites did induce an immune response that inhibited *P. yoelii* liver stage development significantly. The contribution of the CS to liver stage inhibition was evident, but it was not possible to deduce a quantitative measure of this contribution to the overall inhibition, though simple subtraction indicated that this could be at least 30%.

We then performed experiments where the mice were immunized 3 times with IrrSpz before challenge with *P. yoelii* sporozoites. Complete or near-complete sterile immunity was observed for each combination (Figure 6B). The CS had no role in the cross-species sterile protection induced after 3 injections of *P. berghei* IrrSpz because immunization with *P. berghei* induced no cross-reactive immune response to the *P. yoelii* CS (Figures 1, 2 and 3). When we performed the reverse experiment, immunization with three doses of *P. yoelii* IrrSpz, which induced cross-reactive anti-CS immune responses, 66% of the immunized mice were fully protected from challenge with *P. berghei* sporozoites (Figure S7). However, immunization with a single dose of *P. yoelii* IrrSpz could not protect any of the BALB/c mice from a similar challenge. These observations indicated that in mice immunized with *P. yoelii* IrrSpz, a cross-reactive anti-CS immune response component contributes to cross-species sterile protection in addition to the non-CS cross-reactive one. However, the relative magnitude of these two components could not be deduced from these experiments with confidence.

**Discussion**

More than forty years have passed since the demonstration that immunization with irradiated sporozoites induces sterile protection...
against a sporozoite challenge [2]. The majority of investigations aimed at elucidating these protective mechanisms, and at developing vaccines that reproduce them, has been based on the CS, a protein that was quickly discovered to make up the bulk of the proteins at the sporozoite surface [14] and to be the main target of antibody responses [4]. Two independent studies using distinct approaches have recently put the central role of CS in the acquisition of sterile immunity into question. The first based on mice made tolerant to the CS of *P. yoelii* provided indirect evidence for the role of other parasite antigens [8]. The second based on gene replacement in *P. berghei* provided conclusive evidence that sterile immunity can be induced independently of specific immune responses to CS [10]. Demonstration that immunization with the IrrSpz of one species can induce sterile protection against a sporozoite challenge by another was subsequently made [11,12]. The possibility that non-CS antigens were implicated in cross-protection was raised, but a role for CS was favoured because of the relative sequence similarities between the CS of the two rodent malaria species used, *P. berghei* and *P. yoelii*. Furthermore, previous observations had shown that adoptive transfer of a T cell clone derived from *P. yoelii* IrrSpz-immunized mice and specific to the *P. yoelii* CS immunodominant CD8 epitope protected against a *P. berghei* sporozoite challenge [12]. In the studies presented here, we exploited gene replacement technology to investigate the role of the *P. yoelii* and *P. berghei* CS in the acquisition of sterile protection induced by IrrSpz immunization, and to ascertain to what extent immune responses to CS are implicated in the cross-species protection.

The data presented for immunization with *P. berghei* IrrSpz confirmed our previous conclusions that sterile protection was independent of immune responses specific to the *P. berghei* CS [10], and furthermore demonstrated that cross-species protection against a *P. yoelii* sporozoite challenge was equally independent of these anti-CS immune responses. On the other hand, the conclusions from the reciprocal immunization, i.e. IrrSpz carrying the *P. yoelii* CS were less clear-cut. In this case a role for specific anti-CS immune responses in protection could not be dismissed, because we found evidence for their significant contribution to the inhibition of sporozoite invasion and development in hepatocytes both in vitro and in vivo. Although formal quantitative evaluation of this contribution was precluded, we estimated that it could plausibly account for up to 40% of the sterile protection observed after heterologous sporozoite challenge. This asymmetrical role for the CS in sterile protection was unexpected. This raises the possibility that a similar phenomenon might operate with the different parasites species that infect humans.

A possible explanation for our observations could be that the immune responses against CS and non-CS antigens are induced differentially in the two rodent malaria species. In our hands, the principal difference between the two model species lay in the number of IrrSpz injections that were required to induce sterile protection. Sterile protection in all animals was obtained after a single IrrSpz injection. By contrast, for *P. yoelii*, boosting with multiple IrrSpz doses would be required to achieve the levels of immune responses to non-CS proteins needed to confer sterile protection. Formal demonstration of this hypothesis must await the identification of these non-CS antigens.

The fact that the immune responses induced against CS in some models has little bearing on the acquisition of sterile immunity conferred by immunization with IrrSpz, should not be taken as basis to rule out inclusion of the CS alone or in combination with other antigens in vaccine formulation. First, others and we have...
shown that immune responses induced by various formulations against CS can significantly reduce liver stage development and even confer sterile immunity in immunized animals [15–18]. In humans, this has proven to be more difficult to achieve, but the induction of sterile immunity in half or more of the volunteers immunized by the RTS,S vaccine remains a very promising result [19–21]. Failure to achieve equivalent levels of sterile protection in adults and children living in African endemic areas [22–24] must be offset by the observations of reduced incidence of clinical malaria episodes in trials in Mozambique, Kenya and Tanzania [22,25,26].

It might be that the CS in Nature actually plays a role in immune evasion. The highly biased antibody responses to CS and its dominance on the sporozoite surface could lead to a monopolization of the antibody responses mounted by the host against sporozoites. In this way, the CS would deviate the host defences away from other antigens more apt at being targets of the sterile protective immunity, such as those induced by immunization with IrrSpz. In such as case, the identification of these non-CS antigens should be strongly encouraged, a point of view increasingly adopted by the community [27]. This antigen subset might also be implicated in the protective mechanisms that underlie the potent cross-species protection obtained through immunization with IrrSpz reported here and elsewhere [11]. The task of identifying these protective antigens will be facilitated by the availability of the entire genomic sequences of malaria parasites.

The prospect of inducing cross-species protection against malaria pre-erythrocytic stage, akin to that reported here, in humans is an exciting one. The perception that immunization with the irradiated \textit{P. falciparum} or \textit{P. vivax} sporozoites does not confer sterile protection against challenge with sporozoite of the heterologous species rests on observations made on a single volunteer immunized with sub-optimal doses of \textit{P. falciparum} irradiated sporozoites, and who was not protected from a single subsequent \textit{P. vivax} sporozoite challenge [28]. It would be judicious to undertake further trials of this nature in order to confirm or to refute the possibility that cross-species protective responses against the parasite’s pre-erythrocytic stages can be acquired in humans. Indeed, the armamentarium to fight against malaria would be substantially enhanced, if it could be demonstrated that a single vaccine capable of protecting against the two most prevalent and pathogenic species of malaria could be developed. The recent exciting advances in the development of practical live sporozoite vaccination strategies [29–34] would make it possible to explore this strategy before elucidating the nature of the cross-species protective antigens.

### Materials and Methods

#### Ethics Statement

All experiments and procedures involving mice were approved by the “Direction Departementale des Service Veterinaires de Paris, France (Authorisation No 75–129) and performed in...
compliance with regulations of the French Ministry of Agriculture for animal experimentation (1987).

**Construction of a Transgenic *P. berghei* Whose CS Gene Was Replaced by That of *P. yoelii***

This was done as depicted in Figure S2A. Briefly, plasmid pPyCS (cl9) was digested with *Apo I* + *Xba I* to release the targeting insert (~8.7 kb) from the plasmid backbone. The insert was then purified, from a gel following electrophoresis, by phenol/ chloroform extraction. Purified schizonts of a cloned line of *P. berghei* ANKA strain were transformed with 5–10 μg of targeting DNA using the Amaxa programme U33 and subsequently injected intraperitoneally (i.p.) into phenyl hydrazine-treated mice as described previously [13]. Pyrимethamine resistant parasites were selected in the TO mice as described previously [35], and cloned in mice by limiting dilution. *P. berghei* ANKA expressing the *P. yoelii* CS protein was referred to as *P. berghei* [PyCS]. Replacement was confirmed by DNA hybridisation (Figure S2B) and by immuno-staining experiments (Figure S3). For the former, genomic DNA was isolated from parasites as previously described [13], 5 μg of genomic DNA were digested with *Eco RV*, electrophoresed on 0.8% agarose gel and blotted onto nylon Hybond-N+ membrane (Amersham). The following DNA fragments were used as probes: a) 1.1 kb fragment amplified from the 3′ UTR sequence of the *Plbs* gene with the primers 3′UTR1CS (5′-ATA AAG ATT ACG CAT GAT TAT A) and 3′UTR2CS (5′-GAG TAC TCA CGA ATC CGA AAT AAG); and b) a 1.1 kb fragment of the PyCS gene with primers PyCS1 (5′-ATG AAG AAG TGT ACG ATT TTA GTT GTA GCG) and PyCS2 (5′-TCA ATT AAA GAA TAC TAG AAT). All hybridization experiments were carried out as described previously [13].

**Mice and Plasmodium Sporozoites**

BALB/cJ and CD1 female mice were purchased from Harlan Laboratories (Gannat, France) and were housed in pathogen-free rodent barrier facility. *P. yoelii yoelii* 17XNL, clone 1.1, a *P. berghei* ANKA cloned line transfected with a GFP molecule derived from selected in the TO mice as described previously [36], and cloned in mice by limiting dilution. *P. berghei* ANKA expressing the *P. yoelii* CS protein was referred to as *P. berghei* [PyCS]. Replacement was confirmed by DNA hybridisation (Figure S2B) and by immuno-staining experiments (Figure S3). For the former, genomic DNA was isolated from parasites as previously described [13], 5 μg of genomic DNA were digested with *Eco RV*, electrophoresed on 0.8% agarose gel and blotted onto nylon Hybond-N+ membrane (Amersham). The following DNA fragments were used as probes: a) 1.1 kb fragment amplified from the 3′ UTR sequence of the *Plbs* gene with the primers 3′UTR1CS (5′-ATA AAG ATT ACG CAT GAT TAT A) and 3′UTR2CS (5′-GAG TAC TCA CGA ATC CGA AAT AAG); and b) a 1.1 kb fragment of the PyCS gene with primers PyCS1 (5′-ATG AAG AAG TGT ACG ATT TTA GTT GTA GCG) and PyCS2 (5′-TCA ATT AAA GAA TAC TAG AAT). All hybridization experiments were carried out as described previously [13].

**Peptides**

Peptides Py3 [(QGPGAP)₃] and Ph2 [(DPPPPNPN)₂], corresponding to the repeat regions of the CS protein of *P. yoelii* and *P. berghei*, respectively, were used in ELISA as previously described [18,40]. The following peptides: PyB1 (SYVPSEAQ), PyBIL (SYVPSAEQILEFVKQIS), containing the dominant H2-K₂-restricted CD8⁺ T cell epitopes in the *P. yoelii* CS [6,41], PhB1 (SYVSPAEEKILFVKQISSQ) and PhBIL (SYVSPAEEKILFVKQISSQ), containing the H2-K₄-restricted CD8⁺ T cell epitopes in the *P. berghei* CS [5,6,12] were used in ELISpot assays. Lysophilized material was resuspended in sterile distilled water at 10 mg/ml, aliquotted, and stored at −20°C until use. The following long peptides corresponding to NH₂-terminal and COOH-terminal parts of the two different CS were kindly given by Gianpietro Corradini (Institute of Biochemistry, University of Lausanne): *P. yoelii* CS long peptides (PyLN), PyNt (N-terminal region, amino acid segment 20–138: PYYGQNKSVQ AQRNNLYENN LHL SNGKIN NIVRNLLGD AGKPEEKKDD PKPDKGKKNL PKEKKDLHK EKK DDPPKD PKKDDPPKNED) and PyCt (C-terminal region, amino acid segment 277–345: NEDSVPSAE...
concentration 10^9 cells were incubated overnight with the different peptides (final concentration 37 μg/ml) and with 30 U/ml of recombinant penicillin-streptomycin solution (100X, stock solution, Gibco) and incubated at 37°C in 3.5% CO2 for 24 hours. After removal of medium from the culture chambers, 10,000 sporozoites were added in 100 μl of fresh supplemented medium. Inhibition of sporozoite and liver stage development assay was performed as previously described [15]. Briefly, sera (1:10 dilution) were added to hepatocyte cultures at the time of sporozoite inoculation and removed 3 hours later. Medium was replaced by fresh supplemented medium. Cultures were fixed with cold methanol after 45 hours. Sera from control naive mice were used as control. Schizont numbers were assessed in triplicate cultures by immunofluorescence assay using antibodies against PyHSP70.1 that recognizes P. yoelii liver stages as previously described [45]. Percent inhibition was calculated by comparing the numbers of parasites in the experimental cultures with the numbers in control wells.

Supporting Information

Figure S1 Alignment of protein sequences from the CSP sequences used in this study. CSP was amplified by PCR using primers flanking the 5’ and 3’ ends of the CSP gene (underlined in figure). Sequences of P. yoelii CSP (GenBank accession number: bankit1261217, GQ862302) and of P. berghei GFP CSP (GenBank accession number: bankit1261246, GQ862302) were obtained and compared. The P. yoelii CSP from Pb (PyCS) was identical to the CSP from P. yoelii 1.1 (confirmed by sequencing). Pre-, post and repeat regions are highlighted in green, and differences in non-repetitive regions are highlighted in yellow.

Figure S2 The P. berghei CS (PbCS) locus and the integration of the PyCS coding sequences. A Map of the PyCS construct and schematic representation of the WT and targeted PbCS loci. To direct the 5’ recombination event, a 1.1 kb 5’ UTR sequence (thin grey box) of PbCS (wide black box) was inserted in front of the 1.1 kb PyCS coding region (wide white box). A 302 bp sequence corresponding to the PbCS 3’ UTR (thin white box) was placed downstream of PyCS. A further 848 bp of the PbCS 3’ UTR (thin white box) was inserted downstream of the DHRF-TS transcription unit (hatched box). The relative position of Eco RV (E) cleavage sites is indicated. Thick black lines (a, b) indicate the positions of the probes used in Southern blot experiments. B. Southern blot analyses of the parasites. Genomic DNA from WT and transgenic PyCS-5 parasites was digested with Eco RV and hybridized with the 2 different probes (a, b) to ascertain the correct integration of the constructs. Size markers are in kilobases (kb). The integrity of the inserted DNA fragment was also confirmed by PCR and sequence analysis (data not shown). These analyses demonstrated that the targeting construct (Figure S2A, panel a) had correctly integrated in the transgenic parasite thereby placing the PyCS coding sequence under the control of the P. berghei CS regulatory sequences and directing the downstream insertion of the selectable marker DHFR-TS (Figure S2B, panel b).

Figure S3 Antibodies to different regions of P. yoelii or P. berghei CS recognize homologous but not heterologous CS on sporozoites. Monoclonal antibodies specific to the repeat regions are highlighted in green, and differences in non-repeat regions are highlighted in yellow.

ELISPOT Assay

PVDF microplates (Millipore, Bedford, MA, USA) were coated overnight at 4°C with 15 μg/ml of an anti-mouse IFN-γ rat mAb (clone AN18, Mabtech AB, Sophia Antipolis, France) diluted in PBS. After extensive washes and 2-hour incubation at 37°C with RPMI medium containing 10% foetal calf serum, 3×10^9 spleen cells were incubated overnight with the different peptides (final concentration 10 μg/ml) and with 30 U/ml of recombinant IFN-γ (clone AN18, Mabtech AB, Sophia Antipolis, France) diluted in PBS. After extensive washes and 2-hour incubation at 37°C with RPMI medium containing 10% foetal calf serum, 3×10^9 spleen cells were incubated overnight with the different peptides (final concentration 10 μg/ml) and with 30 U/ml of recombinant human IL-2. The plates were then washed, incubated with 2 μg/ml of biotinylated anti-mouse IFN-γ monoclonal antibody (clone R1-6A2, Mabtech AB) diluted in PBS containing 0.5% bovine serum albumin for 2 h at 37°C, and then overnight at 4°C. Plates were subsequently incubated with extravidin-coupled alkaline phosphatase (Sigma-Aldrich) diluted in PBS. After adding the BCIP/NBT substrate (Sigma-Aldrich), IFN-γ spot forming cells were counted under a stereomicroscope and expressed as the number of spots per million tested cells.

ELISA

The presence and level of antibodies to Py3 and Pb2 peptides and to PyLP and PbLP were detected by ELISA as described previously [18,40]. Briefly, 96-well flat-bottom plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 1 μg/ml of peptide in PBS, pH 7.8, by overnight incubation at 4°C. After extensive washes, and a 1-hour-incubation with 200 μl of PBS containing 0.05% Tween and 1% BSA, wells were incubated for 1 hour at 37°C with 100 μl of mouse sera diluted 1/100 in PBS-Tween-BSA. After two washes, wells were incubated for 45 min at room temperature, either with goat IgG anti-mouse IgM (Invitrogen SAIL, Cergy Pontoise, France) or with a biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, Newmarket, United Kingdom) diluted in PBS-Tween. The wells containing the goat IgG anti-IgM antibody were washed and further incubated with a biotinylated rabbit anti-goat IgG (Sigma-Aldrich, Saint-Quentin Fallavier, France) diluted in PBS-Tween for 45 min at room temperature, then washed and incubated with extravidin-coupled alkaline phosphatase (Sigma-Aldrich) diluted in PBS-Tween for 1 h at room temperature. Phosphatase activity was measured using 4-methylumbelliferyl phosphate (Sigma-Aldrich) as a substrate and the fluorescence at 355/460 nm was measured using a spectrophotometer (Victor 1420, Wallac Oy, Turku, Finland).

Immunofluorescence Assay (IFA)

Sera from mice immunized with irradiated sporozoites were tested by immunofluorescence using wet or air-dried methanol-fixed sporozoites from the different Plasmodium lines, in order to detect surface or total antigen content as described previously [43].

Sporozoite Invasion and Development Inhibition Assay

Human hepatoma cells, HepG2-C8 (8×10^3 cells/well) [44], which are fully susceptible to P. yoelii and P. berghei sporozoites, were cultured in eight-chamber plastic Lab-Teck slides (Nunc, Naperville, IL) in William’s E medium (GIBCO, Edinburgh, Scotland) supplemented with 5% FCS (GIBCO), 1% penicillin-streptomycin solution (100X, stock solution, Gibco) and incubated at 37°C in 3.5% CO2 for 24 hours. After removal of medium from the culture chambers, 10,000 sporozoites were added in 100 μl of fresh supplemented medium. Inhibition of sporozoite and liver stage development assay was performed as previously described [15]. Briefly, sera (1:10 dilution) were added to hepatocyte cultures at the time of sporozoite inoculation and removed 3 hours later. Medium was replaced by fresh supplemented medium. Cultures were fixed with cold methanol after 45 hours. Sera from control naive mice were used as control. Schizont numbers were assessed in triplicate cultures by immunofluorescence assay using antibodies against PyHSP70.1 that recognizes P. yoelii liver stages as previously described [45]. Percent inhibition was calculated by comparing the numbers of parasites in the experimental cultures with the numbers in control wells.

Supporting Information

Figure S1 Alignment of protein sequences from the CSP sequences used in this study. CSP was amplified by PCR using primers flanking the 5’ and 3’ ends of the CSP gene (underlined in figure). Sequences of P. yoelii CSP (GenBank accession number: bankit1261217, GQ862302) and of P. berghei GFP CSP (GenBank accession number: bankit1261246, GQ862302) were obtained and compared. The P. yoelii CSP from Pb (PyCS) was identical to the CSP from P. yoelii 1.1 (confirmed by sequencing). Pre-, post and repeat regions are highlighted in green, and differences in non-repeat regions are highlighted in yellow.

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Figure S2 The P. berghei CS (PbCS) locus and the integration of the PyCS coding sequences. A Map of the PyCS construct and schematic representation of the WT and targeted PbCS loci. To direct the 5’ recombination event, a 1.1 kb 5’ UTR sequence (thin grey box) of PbCS (wide black box) was inserted in front of the 1.1 kb PyCS coding region (wide white box). A 302 bp sequence corresponding to the PbCS 3’ UTR (thin white box) was placed downstream of PyCS. A further 848 bp of the PbCS 3’ UTR (thin white box) was inserted downstream of the DHRF-TS transcription unit (hatched box). The relative position of Eco RV (E) cleavage sites is indicated. Thick black lines (a, b) indicate the positions of the probes used in Southern blot experiments. B. Southern blot analyses of the parasites. Genomic DNA from WT and transgenic PyCS-5 parasites was digested with Eco RV and hybridized with the 2 different probes (a, b) to ascertain the correct integration of the constructs. Size markers are in kilobases (kb). The integrity of the inserted DNA fragment was also confirmed by PCR and sequence analysis (data not shown). These analyses demonstrated that the targeting construct (Figure S2A, panel a) had correctly integrated in the transgenic parasite thereby placing the PyCS coding sequence under the control of the P. berghei CS regulatory sequences and directing the downstream insertion of the selectable marker DHFR-TS (Figure S2B, panel b).

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Figure S3 Antibodies to different regions of P. yoelii or P. berghei CS recognize homologous but not heterologous CS on sporozoites. Monoclonal antibodies specific to the repeat regions of the P. yoelii yoelii 17XNL (NYS1) (3) or the P. berghei ANKA (3.28) (4) CS and polyclonal antibodies (1/100 dilution) against the N-terminal or the C-terminal regions of the P. yoelii yoelii 17XNL...
CS were tested by IFAs on dried methanol-fixed sporozoites. Antibodies directed against the repeats or the flanking regions of the P. yoelii CS recognized only P. yoelii and P. berghei [PyCS] but not P. berghei sporozoites. Antibodies to the repeat regions of P. berghei CS recognized only P. berghei parasites. References: (1)Charoenvit, Y., et al. 1987, Characterization of Plasmodium yoelii monoclonal antibodies directed against stage-specific sporozoite antigens. Infect Immun 55: 604–608, (2)Weber, J. L., et al. 1987. Plasmodium berghei: cloning of the circumsporozoite protein gene. Exp Parasitol 63: 295–300.

**Figure S4** IgM antibody responses to P. yoelii and P. berghei CS domains. Pooled serum samples from groups of mice immunized with the different parasite lines were analyzed by ELISA against domains. Pooled serum samples from groups of mice immunized with the sporozoites from groups of mice immunized with the sporozoites from different parasite lines were analyzed by IFAT against dried and methanol-fixed sporozoites to detect the total CS and other antigens content using secondary antibodies specific to IgG. Titres are expressed as the Mean±SD of the log of the highest dilution of serum that gave a positive staining.

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**Figure S5** Antibody reactivity to dried methanol-fixed sporozoites induced by immunization with irradiated sporozoites. IgG response is exclusively directed against the CS. Individual serum samples from groups of mice immunized with the sporozoites from the different parasite lines were analyzed by IFAT against dried and methanol-fixed sporozoites to detect the total CS and other antigens content using secondary antibodies specific to IgG. Titres are expressed as the Mean±SD of the log of the highest dilution of serum that gave a positive staining.

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**References**

1. Hoffman SL, Geh LM, Lake TG, Schneider I, Le TP, et al. (2002) Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. J Infect Dis 185: 1153–1164.
2. Nussenzweig RS, Vanderberg JP, Spitalny GL, Rivera-Ortiz C, Orton CG, et al. (1972) Sporozoite induced immunity in mammalian malaria. A review. Am J Trop Med Hyg 22: 722–728.
3. Drulhe P, Renia L, Fidock DA (1998) Immunity to Liver Stages. In: Sherman IW, ed. Malaria: Parasite Biology, Pathogenesis, and Protection. Washington, D.C.: ASM Press, pp 513–543.
4. Zavala F, Tam JP, Masuda A (1986) Synthetic peptides as antigens for the detection of humoral immunity to Plasmodium falciparum sporozoites. J Immunol Methods 93: 55–61.
5. Romero P, Maryasi JL, Corradin G, Nussenzweig RS, Nussenzweig V, et al. (1989) Cloned cytotoxic T cells recognize an epitope on the circumsporozoite protein and protect against malaria. Nature 341: 323–325.
6. Rodrigues MM, Cordey AS, Arteaga G, Corradin G, Romero P, et al. (1991) CD8+ cytolytic T clonot clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria. Int Immunol 3: 579–585.
7. Renia L, Grillot DA, Marussig M, Corradin G, Mältigen F, et al. (1993) Efferct functions of circumsporozoite peptide-primed CD4+ T cells clones against Plasmodium yoelii liver stages. J Immunol 150: 1471–1478.
8. Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, et al. (2006) The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. Nature 444: 937–940.
9. Hoffman SL (2006) Malaria: a protective parasite. Nature 444: 824–827.
10. Gruner AC, Masuth M, Tewari R, Romero JP, Depiny N, et al. (2007) Sterile Protection against Malaria Is Independent of Immune Responses to the Circumsporozoite Protein. PLoS ONE 2: e1371.
11. Sedegah M, Weiss WR, Hoffman SL (2007) Cross-protection between attenuated Plasmodium berghei and P. yoelii sporozoites. Parasite Immunol 29: 559–563.
12. Weiss WR, Berzofsky JA, Houghten RA, Sedegah M, Hellingdale MR, et al. (1992) A T cell clone directed at the circumsporozoite protein which protects mice against both Plasmodium yoelii and Plasmodium berghei J Immunol 149: 2103–2109.
13. Tewari R, Rathore D, Grisanti A (2005) Motility and infectivity of Plasmodium berghei sporozoites expressing avian Plasmodium gallinaceum circumsporozoite protein. Cell Microbiol 7: 697–707.
14. Nardin EH, Nussenzweig V, Nussenzweig RS, Collins WE, Harinasuta T, et al. (1982) Circumsporozoite proteins of human malaria parasites Plasmodium falciparum and P. vivax. J Exp Med 156: 19–20.
15. Marussig M, Renia L, Motard A, Mältigen F, Petrou P, et al. (1997) Linear and multiple antigen peptides containing defined T and B epitopes of the Plasmodium yoelii circumsporozoite protein: antibody-mediated protection and boosting by sporozoite infection. Int Immunol 9: 1017–1024.
16. Migliorini P, Bierschort B, Corradin G (1993) Malaria vaccine: immunization of mice with a synthetic T cell helper epitope alone leads to protective immunity. Eur J Immunol 23: 582–585.
17. Sedegah M, Heshtrong RC, Hobart P, Hoffman SL (1994) Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. Proc Natl Acad Sci U S A 91: 9866–9870.
18. Zavala F, Tam JP, Barr PJ, Romero P, Ley V, Nussenzweig RS, Nussenzweig V (1987) Synthetic peptide vaccine confers protection against murine malaria. J Exp Med 166: 1591–1596.
19. Keiter KE, McKinney DA, Torneipoorth N, Okekehouse CF, Heppner DG, et al. (2001) Efficacy of recombinant circumsporozoite protein vaccine regimen against experimental Plasmodium falciparum malaria. J Infect Dis 183: 640–647.
20. Keiter KE, Cummings JF, Okekehouse CF, Nielsen R, Hall BT, et al. (2000) Phase 2a trial of 0, 1, and 3 month and 0, 1, and 28 day immunization schedules of malaria vaccine RTS,S/AS02 in malaria-naïve adults at the Walter Reed Army Institute of Research. Vaccine 26: 2191–2202.
21. Stoute JA, Slaoui M, Heppner DG, Jr., Monin P, Keiter KE, et al. (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med 336: 86–91.
22. Alonso PL, SaelardJ, Aponte JJ, Leach A, Macete E, et al. (2004) Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. Lancet 364: 1411–1420.
23. Bojang KA, Mälligan PJM, Pinder M, Vigeron I, Allouche A, et al. (2001) Efficacy of RTS,S/AS02A malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet 358: 1927–1934.
24. Snounou G, Gruner AC, Muller-Graf CD, Mzayer D, Renia L, et al. (2005) The Plasmodium sporozoite survives RTS,S vaccination. Trends Parasitol 21: 456–461.
25. Alshulla S, Oberholzer R, Junna O, Kubboja S, Machera F, et al. (2008) Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. N Engl J Med 359: 2533–2544.
26. Bejon PA, Lusingu J, Okuto A, Leach A, Lienou M, et al. (2008) Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med 359: 2521–2532.
27. Mikolajczak SA, Aly AS, Kappe SH (2007) Preerythrocytic malaria vaccine development. Curr Opin Infect Dis 20: 461–466.
28. Clyde DF, McCarthy VC, Miller RM, Hornick RB (1973) Specificity of protection of man immunized against sporozoite-induced *falciparum* malaria. Am J Med Sci 266: 398–403.

29. Belnoue E, Costa FTM, Frankenberg T, Vigario AM, Voza T, et al. (2004) Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. J Immunol 172: 2487–2495.

30. Belnoue E, Voza T, Costa FTM, Gruner AC, Mauduit M, et al. (2008) Vaccination with Live *Plasmodium yoelii* Blood Stage Parasites under Chloroquine Cover Induces Cross-Stage Immunity against Malaria Liver Stage. J Immunol 181: 8552–8558.

31. Mueller AK, Labaied M, Kappe SH, Matuschewski K (2005) Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. Nature 433: 164–167.

32. Renia L, Gruner AC, Mauduit M, Snounou G (2006) Vaccination against malaria with live parasites. Expert Rev Vaccines 5: 473–481.

33. van Dijk MR, Douradinha B, Franke-Fayard B, Heusler VT, van Dooren MW, et al. (2005) Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. Proc Natl Acad Sci U S A 102: 12194–12199.

34. Luke TC, Hoffman SL (2003) Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated *Plasmodium falciparum* sporozoite vaccine. J Exp Biol 206: 3803–3808.

35. Janse CJ, Mons B, Rouwenhorst RJ, van der Klooster PJF, Overdulve JP, et al. (1985) In vitro formation of ookinetes and functional maturity of *Plasmodium berghei* gametocytes. Parasitology 91: 19–29.

36. Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M, et al. (2004) A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol Biochem Parasitol 137: 23–33.

37. Hulier E, Petour P, Snounou G, Nivez MP, Miltgen F, et al. (1996) A method for the quantitative assessment of malaria parasite development in organs of the mammalian host. Mol Biochem Parasitol 77: 127–135.

38. Preiser PR, Khan SM, Costa FTM, Jarra W, Belnoue E, et al. (2002) Stage-specific transcription of distinct repertoires of a multigene family during *Plasmodium* life cycle. Science 295: 342–345.

39. Bruna-Romero O, Hafalla JC, Gonzalez-Aseguinolaza G, Sano G, Tsuji M, et al. (2001) Detection of malaria liver-stages in mice infected through the bite of a single *Anopheles* mosquito using a highly sensitive real-time PCR. Int J Parasitol 31: 1499–1502.

40. Grillot DA, Michel M, Muller I, Tougue C, Renia L, et al. (1990) Immune responses to defined epitopes of the circumsporozoite protein of the marine malaria parasite, *Plasmodium yoelii*. Eur J Immunol 20: 1213–1222.

41. Weiss WR, Mellonk S, Houghten RA, Sedegah M, Kumar S, et al. (1990) Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. J Exp Med 171: 763–773.

42. Lopez JA, Renggli J, Eberl G, Corradin G, Roggero MA, et al. (1996) Immunogenicity of synthetic peptides corresponding to the nonrepeat regions of the *Plasmodium falciparum* circumsporozoite protein. In: Vaccines 96, Cold Spring Harbor Laboratory Press. pp 253–260.

43. Renia L, Miltgen F, Charovnivt Y, Ponsudharai T, Verhave JP, et al. (1998) Malaria sporozoite penetration: a new approach by double staining. J Immunol Methods 112: 201–205.

44. Silvie O, Greco C, Franetich JF, Dubart-Kupperschmitt A, Hanzoun L, et al. (2006) Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the *Plasmodium* species. Cell Microbiol 8: 1134–1146.

45. Renia L, Mattei DM, Goma J, Pied S, Dubois P, et al. (1990) A malaria heat shock like protein epitope expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by non-parenchymal liver cells. Eur J Immunol 20: 1445–1449.