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Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to *Plasmodium falciparum* circumsporozoite protein (CSP) and α DEC-CSP in Non Human Primates

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Abstract

Development of a fully effective vaccine against the pre-erythrocytic stage of malaria infection will likely require induction of both humoral and cellular immune responses. Protein based vaccines can elicit such broad-based immunity depending on the adjuvant and how the protein is formulated. Here to assess these variables, non-human primates (NHP) were immunized three times with *Plasmodium falciparum* (Pf) circumsporozoite protein (CSP) or CSP cloned into MG38, a monoclonal antibody that targets DEC-205 (α DEC-CSP), an endocytic receptor on dendritic cells (DCs). Both vaccines were administered with or without poly(I:C) as adjuvant. Following three immunizations, the magnitude and quality of cytokine secreting CD4+ T cells were comparable between CSP + poly(I:C) and α DEC-CSP + poly(I:C) groups with both regimens eliciting multi-functional cytokine responses. However, NHP immunized with CSP + poly(I:C) had significantly higher serum titers of CSP-specific IgG antibodies and indirect immunofluorescent antibody (IFA) titers against Pf sporozoites. Furthermore, sera from both CSP or α DEC-CSP + poly(I:C) immunized animals limited sporozoite invasion of a hepatocyte cell line (HC04) *in vitro*. To determine whether CSP-specific responses could be enhanced, all NHP primed with CSP or α DEC-CSP + poly(I:C) were boosted with a single dose of 150,000 irradiated Pf sporozoites (PfSPZ) intravenously. Remarkably, boosting had no effect on the CSP-specific immunity. Finally, immunization with CSP + poly-ICLC reduced malaria parasite burden in the liver in an experimental mouse model. Taken together, these data showing that poly(I:C) is an effective adjuvant for inducing potent antibody and Th1 immunity with CSP based vaccines offers a potential alternative to the existing protein based pre-erythrocytic vaccines.

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Keywords

Pre-erythrocytic vaccines; Poly(I:C); Circumsporozoite protein (CSP)

1. Introduction

Malaria is an infectious disease of tropical regions infecting approximately 500 million people and causing 1–2 million deaths each year [1]. Adults living in malaria endemic region acquire a form of adaptive immunity over time which provides protection against clinical disease, but is dependent upon continuous exposure to the parasite for its maintenance [2]. Among the five species of *Plasmodium* known to infect humans, *P. falciparum* (Pf) is the leading cause of morbidity and mortality. While public health measures such as insecticide treated bed nets and anti-malarial therapy have significant effects on morbidity and mortality, vaccines offer the most compelling intervention for effective and durable prevention of this infection.

At present, the most clinically advanced pre-erythrocytic malaria vaccine candidate uses circumsporozoite protein (CSP), which is expressed abundantly on the surface of the sporozoite stage of the parasite [3,4]. CSP-specific antibodies [5], CD8+ and CD4+ T cells [6–8] have been shown to elicit protective immunity in mouse models of malaria. In humans it seems clear that antibodies against CSP may be necessary but not entirely sufficient for the protection seen. Indeed, CSP-specific Th1 responses have also been suggested to correlate with protection in humans following vaccination or natural infection [9,10]. Based on these findings there has been substantial effort to develop vaccines using CSP as an antigen. Currently, a phase III efficacy trial is underway using the RTS,S vaccine. RTS,S is a complex formulation comprised of two polypeptide chains of Pf CSP (amino acid 207 to 395) linked to hepatitis B surface antigen (HBsAg) to form a particle. This is then mixed with the TLR4 ligand, MPL and QS-21 in an oil-in-water (AS02A) or liposome (AS01B) formulation. Immunization of malaria naïve individuals with RTS,S and AS02A or AS01B induces CSP-specific CD4+ T cells and humoral immune responses with ~ 30–50% efficacy [11]. Importantly, Th1 and CSP-specific humoral immunity are increased with AS01B compared to AS02A [11–14] suggesting that the vaccine formulation may have a critical role in optimizing immunity. Finally, protective immunity induced following immunization with RTS,S appears to wane over time and is not boosted upon natural infection [15]. Thus, developing alternative CSP based vaccines with improved adjuvants, formulations or both may improve the durability of humoral and T cell immunity and enhance protection.

In terms of formulations, recombinant proteins can be administered as soluble antigens or as a particle such as the RTS,S vaccine. Alternatively, more efficient processing and presentation of proteins with increase in immunogenicity can be achieved by targeting the protein directly to dendritic cells (DCs) through monoclonal antibodies against cell surface receptors [16]. In this regard, DEC-205, an endocytic receptor expressed at high levels on lymphoid tissue DCs has been extensively characterized for targeting protein antigens in mice [16–18]. Indeed, DEC-205 mediated delivery of protein antigens improves the induction of both Th1 and CD8+ T cell responses in mouse models [16,19]. T cell immunity with α DEC-205 requires poly(I:C) as an adjuvant [17]. Poly(I:C), a synthetic double stranded RNA, is a potent inducer of IL-12 and type I IFNs through activation of innate immunity via endosomally expressed TLR3 and the cytoplasmic receptor MDA-5 [20]. Moreover, poly(I:C) through induction of type I IFNs enhances DC maturation and B cell activation leading to induction of potent CD4+ T cell and humoral immune responses, respectively, in mice with protein antigens [21–23]. Finally, type I IFN is critical for cross presentation of protein antigens to generate CD8+ T cell responses in mice [24,25].

Collectively, these data strongly support poly(I:C) as an adjuvant for improving humoral and cellular immunity with protein based vaccines. While the ability of poly(I:C) to induce broad-based immunity in mice has been established with protein and α DEC vaccines, there is only initial data on the potency of poly(I:C) as an adjuvant in NHP [26]. As innate immune mechanisms are far more similar between humans and NHP than mice, evaluation of NHP may provide a more predictive model for what would be observed in humans.

The primary aim of this study was to compare the adaptive immune responses generated in NHP following immunization with CSP or α DEC-CSP (CSP cloned into the carboxyl terminus of the heavy chain of mAb against DEC-205) with or without poly(I:C) as adjuvant. In addition, as prime-boost immunization with heterologous vaccine formulations has been shown to enhance immunity in a variety of experimental settings compared to either vaccine modality alone, all animals immunized with CSP or α DEC-CSP with or without poly(I:C) were boosted with irradiated PfSPZ. As irradiated PfSPZ are the gold standard for eliciting complete protection in humans, this study provides the first assessment of how they would influence an existing CSP-specific response in NHP. Finally, to determine whether this vaccine approach could have a biologic effect *in vivo*, C57BL/6 mice were immunized with CSP with or without poly-ICLC [26] and challenged with chimeric *P. berghei* sporozoites expressing Pf CSP (Pb-Pf) [27]. Overall, this study shows that poly(I:C) is an effective adjuvant for inducing potent multi-functional CD4+ T cells and antibody responses in mice and NHP with CSP. Thus, poly(I:C) offers a promising adjuvant for application in humans as part of a pre-erythrocytic Pf vaccine regimen with protein based vaccines.

2. Materials and Methods

2.1 Animals

Lab bred Indian rhesus macaques were stratified into comparable groups based on age, weight and sex of the animals. Animals were maintained at the animal facility of BIOQUAL Inc. (Rockville, MD). Female C57BL/6 mice were obtained from The Jackson Laboratory. Mice were maintained in the Vaccine Research Center Animal Care Facility (Bethesda, MD) under pathogen-free conditions. All experiments were conducted according to the guidelines of the National Research Council, under protocols approved by the Institutional Animal Care and Use Committee at the National Institutes of Health.

2.2 Immunizations

Groups of NHP (n=4 to 5/group) were immunized three times s.c. in the axillary region with 400 μ g of recombinant Pf circumsporozoite protein (CSP) with or without 4 mg of poly(I:C) (Invivogen, San Diego, CA) or poly(I:C) alone at two separate sites. Additional groups of animals were immunized three times with 400 μ g of α DEC-CSP (CSP cloned into the carboxyl terminus of heavy chain of α DEC-205 mAb clone MG38) with or without 4 mg of poly(I:C) given in a total volume of 2 ml divided into two sites s.c. [28,29]. Injections were done at weeks 0, 5 and 13. At week 27 all the animals were boosted with 150,000 irradiated PfSPZ i.v.. For mouse experiments, C57BL/6 mice were immunized with 20 μ g of CSP with or without 50 μ g of poly-ICLC (Oncovir, Washington, DC) or poly-ICLC alone at weeks 0, 3 and 6. All injections were done s.c.

2.3 Reagents

The Escherichia Coli derived recombinant Pf CSP comprises of amino acid 123–411 from the T4 isolate [18,30]. This construct contains 3 repeats NANPNVDP and 21 repeats NANP plus the carboxyl terminus domain. For generation of α DEC-CSP, the exact same CSP sequence comprising of amino acid 123–411 was cloned in fusion with the carboxyl

terminus of the heavy chain of MG38 antibody (IgG1) [18]. Approximately 30% of the weight of the α DEC-CSP construct comprises of CSP. Poly(I:C) was purchased from Invivogen (San Diego, CA) and Poly-ICLC from Oncovir, Inc. (Washington, DC). Poly-ICLC is poly(I:C) stabilized with poly lysine and carboxymethylcellulose to prevent rapid inactivation *in vivo* by natural enzymes (Oncovir, Inc., Washington, DC). Radiation attenuated (150 Gy) Pf sporozoites strain NF54 (PfSPZs) were provided by Sanaria, Inc. (Rockville, MD). Pf CSP 15-mer peptides overlapping by 11 amino acids and spanning the entire length of the protein were synthesized by Henry Zebroski in the Rockefeller University proteomics facility.

2.4 Preparation of PBMCs

PBMCs were isolated from fresh blood by Ficoll density centrifugation using Leucosep™ tubes (greiner bio-one). PBMCs were used immediately or after cryopreservation for ELISPOT analysis or intracellular FACS staining.

2.5 Detection of CSP-Specific IFN- γ Producing PBMCs by ELISPOT Assay

The frequency of IFN- γ producing cells from PBMCs was determined by ELISPOT assay as previously described [31]. Briefly, 2×10^5 PBMCs were added in triplicate to 96-well plates coated with anti-human IFN- γ (Bender MedSystems, Vienna, VA). Pf CSP pooled peptides (2 μ g of 15-mer peptides overlapping by 11 amino acids spanning the entire protein) were added per well and incubated for 18 h at 37°C. The number of spot-forming cells was determined by using the Axioplan 2 imaging system (Zeiss, Germany).

2.6 Polychromatic Flow Cytometry

2×10^6 PBMCs were stimulated in complete RPMI 1640 for 5 h with 5 μ g/ml α -CD28-Ax680, α -CD49d (BD Pharmingen, San Diego, CA) and Brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO) at 10 μ g/ml each with or without 2 μ g/ml CSP pooled peptides (15-mer peptides overlapping by 11 amino acids). After stimulation, cells were stained as described previously [32]. Briefly, cells were surface stained for CCR7 at 37°C for 20 min. This was followed by 15 min surface staining at room temperature for CD4, CD8, CD95 (BD Pharmingen, San Diego, CA) and CD45RA (Beckman Coulter, Chaska, MN). In addition, Aqua Blue (Molecular Probes, Carlsbad, CA) was added to identify dead cells. After washing, fixing, and permeabilizing, cells were stained intracellularly for IFN- γ , IL-2, TNF- α , and CD3 (all purchased from BD Pharmingen, San Diego, CA). Approximately 1×10^6 cells were acquired on a BD LSR II (BD Biosciences) and FACS data was analyzed using FlowJo software (Tree Star) and SPICE. Antibodies specific for CD28 (clone CD28.2), CCR7 (clone 150503), CD4 (clone M-T477), and CD8 (clone OKT8) were labeled with fluorescent conjugates at Molecular Probes. Multi-parameter flow cytometry for mouse spleen cells was performed as described previously [33]. Antibodies specific for CD3, CD4, IFN- γ , IL-2 and TNF- α were purchased from BD Pharmingen (San Diego, CA). Anti-CD8 antibody and cell viability dye ViViD were purchased from Biolegend (San Diego, CA) and Molecular Probes (Carlsbad, CA) respectively.

2.7 Detection of Serum Antibodies

Pf CSP-specific IgG antibodies were detected in plasma or serum of immunized animals at indicated time points. Ninety-six well plates were coated with CSP overnight. Plasma or serum from immunized animals were added in serial dilutions to CSP coated plates and incubated for 2 h. Following 1 hour incubation with horseradish-peroxidase-conjugated anti-monkey IgG antibody at room temperature (BD Pharmingen, San Diego, CA), plates were developed using 3,3',5,5' – tetramethylbenzidine substrate-chromogen (DAKO, Carpinteria, CA).

2.8 Inhibition of Liver Stage Development Assay

To measure the functional capacity of monkey sera to block *Pf* sporozoite invasion of a hepatocyte cell line, an inhibition of liver stage development assay (ILSDA) was performed. HC-04 (1F9) cells (NMRC, Silver Spring, MD) were seeded in 8-well LabTek chamber slides (Thermo Fisher Scientific, Waltham, MA) at a concentration of 40,000 cells/well. Twenty-four hours later, 25,000 irradiated *Pf* sporozoites were added to each well, in triplicate, with or without the specified serum diluted 1:20. MAb anti-PfCSP [3,34] (100 µg/ml) was used as a positive control. After 3 hours, wells were washed 3 times with medium and incubated for 72 hours with daily medium changes. At the end of the 3-day incubation, cells were fixed with ice-cold methanol, and stained with rabbit anti-Pf Liver Stage Antigen-1 (LSA-1) sera (Sanaria Inc., Rockville, MD), followed by Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). The total number of liver stage parasites per well were counted using a BX51 fluorescence microscope (Olympus) at 400× magnification.

2.9 Indirect Immunofluorescent Antibody (IFA) Assay

Sporozoite specific IgG from monkeys were measured by adding serially diluted sera to air dried *P. falciparum* sporozoites mounted on slides, followed by a fluorescein conjugated anti-monkey IgG antibody. The endpoint titer was defined as the greatest serum dilution that yielded a positive fluorescence above the pre-immune control levels.

2.10 Challenging Mice With Chimeric *P. berghei* Sporozoites Expressing Pf CSP (Pb-Pf)

Immunized mice were challenged with 10,000 chimeric *P. berghei* sporozoites expressing Pf CSP comprising of the repeat domain (Pb-Pf). These parasites were generated as described elsewhere [27]. 40 hours after challenge with sporozoites, the mice were euthanized and the liver excised to evaluate parasite development. For this purpose, total liver RNA was purified and the presence of malaria specific 18S rRNA measured by using real time PCR, as described [35]. The total number of 18S rRNA copies were determined using a standard curve obtained with purified plasmodial rRNA.

3. Results

3.1 Pf CSP or αDEC-CSP + Poly(I:C) Induce Comparable IFN-γ Responses in NHP

Based on the established role that CSP-specific humoral and cellular immunity has on protection in mice and humans [4–8,36–38], we compared two distinct protein based vaccine platforms using CSP and poly(I:C) as an adjuvant in NHP. Animals were immunized three times with CSP or αDEC-CSP with or without poly(I:C) or poly(I:C) alone. The magnitude of CSP-specific IFN-γ producing cells in peripheral blood was assessed throughout the course of the study by ELISPOT analysis. As shown in Fig. 1, there was a progressive increase in the frequency of IFN-γ producing cells in animals immunized with CSP or αDEC-CSP + poly(I:C) after three immunizations. The magnitude of IFN-γ producing cells was comparable between animals immunized with CSP + poly(I:C) or αDEC-CSP + poly(I:C) at 2 weeks post each immunization. In terms of durability, both vaccine groups had sustained IFN-γ responses albeit at low frequencies when assessed at week 26 (Fig. 1). These data demonstrate that poly(I:C) is an effective adjuvant for generating strong CSP-specific IFN-γ producing cells in NHP with CSP or αDEC-CSP.

3.2 Irradiated PfSPZ Boosting Does Not Increase IFN-γ Responses Induced by CSP or αDEC-CSP + Poly(I:C) Immunization

Vaccination with irradiated PfSPZ is the current gold standard for complete protection in humans [39–41]. Since multiple immunizations are required to elicit protective immunity, our aim was to determine whether a single dose of irradiated PfSPZ could boost CSP-

specific cellular and humoral immunity as well as induce responses to other malarial antigens. For this study, we obtained irradiated PfSPZ that had been isolated from the mosquito for the first time under GMP conditions. This provided a unique opportunity to assess their immunogenicity in NHP. All animals immunized with CSP or α DEC-CSP with or without poly(I:C) were boosted at week-27 (12 weeks after the 3rd immunization) via the intravenous route with 150,000 irradiated PfSPZ. An additional group of naïve NHP was added and received only the irradiated PfSPZ intravenously thereby allowing a comparison between prime-boost and boost alone. Interestingly, there was no significant increase in the frequency of IFN- γ producing cells in the PBMCs of any of the vaccine groups following the irradiated PfSPZ boost compared with the last assessment done at week-26. Moreover, a single dose of irradiated PfSPZ did not generate measurable CSP-specific IFN- γ producing cells (Fig. 1). To determine whether the irradiated PfSPZ immunization elicited malarial specific T cell responses to non-CSP antigens, irradiated PfSPZ themselves were used for re-stimulation *in vitro* in ELISPOT and ICS assays. Using this assay we were unable to detect any malaria specific responses following a single immunization with irradiated PfSPZ (data not shown). As this assay is able to measure malaria specific T cell responses in NHP and humans that had received multiple immunizations with the same irradiated PfSPZ (manuscript in preparation) it suggests that, a single immunization of irradiated PfSPZ is not sufficient in NHP to boost CSP-specific responses or elicit detectable responses to other malarial antigens.

3.3 CSP or α DEC-CSP + Poly(I:C) Immunization Generates Multifunctional CD4+ T Cell Responses

To establish whether the IFN- γ producing cells detected in Fig. 1 by ELISPOT assay were CD4+, CD8+ T cells or both, PBMCs were analyzed by multi-parameter flow cytometry. In addition to IFN- γ , this analysis included assessment of IL-2 and TNF- α as well as cell surface markers to delineate their phenotype into central and effector memory cells. At 2 weeks after the third immunization, there were comparable frequencies of CSP-specific IFN- γ , IL-2, and TNF- α producing CD4+ T cells in CSP or α DEC-CSP + poly(I:C) groups with no cytokines detected in CSP or α DEC-CSP immunized animals (Fig. 2A). We could not detect CSP-specific CD8+ T cells in any vaccine group with this immunization regimen. We next determined the quality of the response as defined by any combination of IFN- γ , IL-2 and TNF- α at the single cell level [32,33]. The actual frequency of each of the potential combinations of cytokines is shown in Fig 2B and C for CSP or α DEC-CSP + poly(I:C) respectively. The relative proportion of such responses are depicted by pie charts in Fig. 2D and E from individual animals with the mean for all 5 animals highlighted in the box. The mean proportion of CD4+ T cells producing IFN- γ /IL-2/TNF- α is ~ 25–35% following immunization with CSP + poly(I:C) or α DEC-CSP + poly(I:C). Of note, IFN- γ producing CD4+ T cells were split among CCR7– and CCR7+ effector and central memory T cells while IL-2 and TNF- α were higher in CCR7+ cells (data not shown). Thus, both CSP and α DEC-CSP administered with poly(I:C) elicited multi-functional central and effector memory CD4+ T cell responses.

3.4 Immunization With CSP + Poly(I:C) Generates High Titer of CSP-Specific IgG Antibody

A notable feature of CSP is a central region comprised of a repetitive sequence of four amino acids (NANP) forming a major B cell epitope [4,42,43]. Antibodies specific against this region are believed to be a central component of the protection conferred by the RTS,S vaccine [11]. Thus, we assessed the CSP-specific humoral immune responses generated in NHP following immunization with CSP or α DEC-CSP with or without poly(I:C). As shown in Fig. 3A and B, significantly higher titers of CSP-specific IgG antibodies were measured following the third immunization with CSP + poly(I:C) compared to α DEC-CSP + poly(I:C). Two doses of either vaccine were sufficient to attain the peak titers of CSP-

specific IgG antibodies with no further boosting upon third immunization (Fig. 3C). A gradual decline in the level of CSP-specific antibodies was observed following the third immunization with either vaccine formulation (Fig. 3D and E). However, measurable CSP-specific IgG antibodies were still detected even at week 38 post primary immunization with CSP + poly(I:C) or α DEC-CSP + poly(I:C) (Fig. 3E).

We next assessed whether irradiated PfSPZ immunization would boost CSP-specific IgG antibodies generated following immunization with CSP or α DEC-CSP + poly(I:C). Consistent with the data in Fig. 1, there was no increase in CSP-specific IgG antibodies following immunization with irradiated PfSPZ in any of the primed vaccine groups or when given alone (Fig. 3D and E). Of note, in our other NHP study, we did detect CSP-specific IgG antibodies after two immunizations i.v. (manuscript in preparation). Therefore, a single dose of PfSPZ injected i.v. does not boost CSP-specific CD4+ T cell and humoral immune responses.

3.5 Immunization With CSP or α DEC-CSP Plus Poly(I:C) Induces Antibodies That Neutralize *In Vitro*

We next determined the functional properties of CSP-specific antibodies generated following immunization with CSP or α DEC-CSP plus poly(I:C) by two assays. As shown in Table IA, there was a four-fold increase in indirect immunofluorescent antibody (IFA) titers against Pf sporozoites from pooled sera collected at week-4 after the 3rd immunization with CSP + poly(I:C) compared to α DEC-CSP + poly(I:C). Moreover, IFA titers against Pf sporozoites from sera of individual animals immunized with CSP + poly(I:C) show mean titers of ~ 400,000 at week-4 post third immunization which declined to ~ 25,000 by week 13 (Table IB). It should be noted that the assessment of IFA titers in Tables IA and B were done on separate days thus accounting for a modest discrepancy in titers from pooled sera and individual sera from CSP + poly(I:C) immunized animals 4 weeks after the 3rd immunization. Nevertheless, such animals had the highest titers on both days. To assess the ability of CSP-specific antibodies to block Pf sporozoite entry into a liver cell line *in vitro*, a functional assay to inhibit liver stage development (ILSDA) was performed. At 4 weeks after the 3rd immunization, pooled sera from CSP or α DEC-CSP + poly(I:C) immunized NHP inhibited Pf sporozoite entry into the hepatocyte cell line by 56% and 43% respectively whereas sera from CSP, α DEC-CSP or poly(I:C) immunized animals showed only minimal inhibition in this assay (Table II). Additional analysis using sera from individual animals immunized with CSP + poly(I:C) showed inhibition of Pf sporozoite invasion in a range between 33.9 – 71.34 % at week-4 following 3rd immunization (Table III). These data show that the magnitude of CSP specific IgG antibodies and IFA titers against Pf sporozoites were significantly higher in CSP + poly(I:C) compared to α DEC-CSP + poly(I:C) immunized animals. In addition, antibodies generated by either vaccine platform could inhibit invasion of a hepatocyte cell line by Pf sporozoites *in vitro* in a functional assay.

3.6 Immunization With CSP + Poly-ICLC Reduces Malaria Parasite Burden in Mice Upon Challenge

As Pf CSP + poly(I:C) appears to be effective for eliciting functional antibody and CD4+ T cell responses, we next assessed whether this vaccine approach could limit malaria parasite burden in the liver *in vivo*. As NHP are susceptible to *Plasmodium knowlesi* but not Pf, we used a mouse challenge model using chimeric *Plasmodium berghei* sporozoites expressing Pf CSP (Pb-Pf) [27]. C57BL/6 mice were immunized three times at three-week intervals with Pf CSP with or without poly-ICLC or poly-ICLC alone. It should be noted that for these studies, poly-ICLC which is poly(I:C) formulated with poly lysine and carboxymethylcellulose, was used based on recent studies showing greater potency than the poly(I:C) used in the NHP study (data not shown). In order to assess protection at a memory

time-point, all animals were challenged with 10,000 Pb-Pf i.v. two months following the last immunization.

As shown in Fig. 4A high titers of CSP-specific IgG antibodies were generated in the serum of CSP + poly-ICLC immunized animals as compared to CSP or poly-ICLC alone groups. CSP-specific T cell responses were assessed in the spleen 40 hrs post-challenge following *ex vivo* stimulation of splenocytes with overlapping peptides. As shown in Fig 4B, a high frequency of CSP-specific CD4⁺ T cells producing IFN- γ , IL-2 and TNF- α were present in mice immunized with CSP + poly-ICLC even 2 months after the 3rd immunization but not in CSP or poly-ICLC alone groups. In terms of T cell quality, Fig. 4D shows the frequency of CD4⁺ T cells producing various combinations of cytokines and Fig. 4C depicts a pie chart representation of the relative proportion of such responses for individual animals. The mean for all 5 animals shows that ~ 30% of the responses were multi-functional secreting all three cytokines (Fig. 4C). Thus, immunization with CSP + poly-ICLC elicits high titers of CSP-specific IgG antibodies as well as multi-functional CD4⁺ T cells which are durable. Similar immunogenicity data was observed in BALB/c mice (data not shown). Finally, following challenge with 10,000 Pb-Pf parasites i.v, a significant reduction in parasite burden was assessed in the livers of CSP + poly-ICLC immunized animals as compared with CSP only group (Fig. 4E). Based on the detection of parasite 18S rRNA in the liver it would not be expected that sterilizing immunity would be observed with this high challenge dose and route. Nevertheless, these data provide evidence that this vaccine regimen can have an effect on parasite burden *in vivo*.

4. Discussion

Fully effective vaccines against malaria will likely require generation of broad-based humoral and cellular immunity. Thus, developing non-live vaccine formulations that can elicit potent antibody and T cell responses offers a platform that can itself induce substantial protection such as the RTS,S vaccine, and also be used with other vaccines to further optimize responses and protection. In this report, we compare the immunogenicity of Pf CSP as a soluble antigen versus targeting the CSP to DCs through the DEC-205 receptor using poly(I:C) as adjuvant in NHP. The data show that targeting CSP to DCs through DEC-205 receptor does not enhance the magnitude or alter the quality of CSP-specific CD4⁺ T cells generated post-immunization compared to CSP with the same adjuvant. In addition, we failed to detect measurable CSP-specific CD8⁺ T cells in NHP immunized with CSP or α DEC-CSP + poly(I:C). However, significantly higher titers of Pf sporozoite binding CSP-specific IgG antibodies were generated in NHP immunized with CSP + poly(I:C) as compared to α DEC-CSP + poly(I:C) group. Antibodies from both vaccine groups showed neutralizing activity by limiting Pf sporozoite entry into a hepatocyte cell line in an *in vitro* assay. Taken together, the data show that CSP delivered as soluble antigen elicits comparable Th1 immunity and more potent antibody response as compared to CSP targeted to DCs through DEC-205 receptor when adjuvanted with poly(I:C).

The lack of demonstrable differences in Th1 immunity with CSP compared to the α DEC-205 targeting approach and the failure to elicit CD8⁺ T cell responses differs from data obtained in mice [16,18]. Several explanations may explain these findings. First, in this study we used a high dose (400 μ g) of CSP to immunize NHP. Mouse studies reveal that very low amounts of α DEC-antigen are far more efficient than substantially higher amounts of protein [16]. It is possible that a dose response of CSP and α DEC-CSP might have revealed a difference in their relative immunogenicity. Second, the lack of CD8⁺ T cell responses by the DEC targeting construct may reflect differences between mouse and NHP or a requirement of anti-CD40 antibody that was used in the mouse study [16,18]. In addition, there may be a limited number of Pf CSP CD8⁺ T cell epitopes in NHP. Third, the

avidity of the α DEC antibody may be critical for optimizing its effects. In this regard, clone MG38 used in this study has a much lower binding affinity for DEC-205 receptor as compared to the newer generation mAb clone 3G9 (data not shown). Indeed, in ongoing experiments in NHP using a higher affinity α DEC-205 (clone 3G9) with an HIV Gag protein administered with Poly-ICLC shows induction of CD8+ T cells that is not observed with HIV Gag protein and Poly-ICLC (manuscript in preparation). Thus, future studies using a higher affinity α DEC-205 antibody will assess whether DC targeting of CSP is advantageous for the development of a pre-erythrocytic vaccine against malaria.

An important aspect of any vaccine study is the durability of the responses. In this regard, low frequency CSP-specific CD4+ T cells were detected in the peripheral blood of both CSP or α DEC-CSP + poly(I:C) immunized groups at approximately 6 months post primary immunization. Moreover, there was also a decline in the titers of CSP-specific IgG antibodies over time. These results are similar to data obtained following immunization with adjuvanted RTS,S [15]. Therefore, a CSP based vaccination regimen may require annual boosting to maintain a sufficient threshold of antibody to mediate protection. Accordingly, CSP-specific CD4+ T cell responses were boosted upon an additional immunization with a single dose of CSP + poly-ICLC at week-73 post primary immunization (data not shown). Finally, in terms of having a biologic effect *in vivo*, we show that mice immunized with CSP + poly-ICLC had a decrease in the parasite burden in the liver upon i.v. challenge with a high dose of 10,000 chimeric Pb-Pf parasites two months following the last immunization. These data establish that a truncated Pf CSP with poly-ICLC as an adjuvant, has some protective effect by limiting parasite burden in the liver upon Pf infection. Ongoing studies using a full-length Pf CSP and poly-ICLC have shown more substantial reduction in parasite burden in the liver (manuscript in preparation). In comparing this study with other platforms, a prior report using QS-21 as an adjuvant with a multiple antigen peptide based vaccine conferred sterilizing immunity in mice using a mosquito challenge with Pb-Pf parasites. In this study, transfer of serum from immunized animals into naïve mice was sufficient to mediate sterile protection upon challenge with infected mosquito bite [27]. Thus, it will be of interest to assess whether our vaccine regimen used here would have a greater effect on protection with this challenge model rather than with a high dose of parasites administered i.v..

The other major aim of this study was to assess whether a heterologous prime-boost immunization with CSP + poly(I:C) and irradiated PfSPZ could enhance immunity compared to either vaccine modality alone. Multiple immunizations with radiation attenuated or genetically modified *Plasmodium* sporozoites generate sterilizing immunity across multiple species [39–41,44–46]. Thus, establishing a combination vaccine regimen that could limit the number of immunizations with irradiated PfSPZ may have important clinical application. Here we sought to determine how boosting with a single immunization with irradiated PfSPZ influenced existing CSP-specific antibody and Th1 responses. CSP-specific CD4+ T cells and humoral immune responses generated following immunization with CSP/ α DEC-CSP + poly(I:C) were not boosted by a single dose of 150,000 irradiated PfSPZ administered intravenously. Moreover, we failed to detect any CSP-specific antibody or T cell responses to a single immunization with irradiated PfSPZ. These data suggest that more than one boost with irradiated PfSPZ may be required to enhance the CSP-specific humoral and cellular responses. Indeed, data from human vaccine studies show that several immunizations with irradiated PfSPZ showed only a modest induction of CSP-specific IFN- γ producing cells [47]. Similarly, we have shown that CSP-specific antibodies and malaria-specific T cell responses can be induced in NHP with irradiated PfSPZ but this required at least two immunizations given i.v. (manuscript in preparation). Additional possibilities to explain the lack of boosting by irradiated PfSPZ are that pre-existing CSP-specific antibodies from the primary immunization may bind to the irradiated PfSPZ thus effectively

neutralizing any boosting effect. In summary, the inability of irradiated PfSPZ to boost CSP-specific CD4+ T cell and humoral immune responses generated in NHP following immunization with CSP or α DEC-CSP + poly(I:C) suggest that either these heterologous vaccine platforms do not optimize immunity, the order of prime-boosting needs to be changed or there needs to be a greater number of immunizations with irradiated PfSPZ.

In conclusion, this NHP immunogenicity study shows that CSP-specific antibody and CD4+ T cell responses can be elicited using an unformulated Pf CSP with poly(I:C). Thus, poly(I:C) offers a potential alternative to AS01B as an adjuvant with protein based vaccines. A critical question is whether the efficacy seen with the RTS,S/AS01B vaccine in humans is due to antibodies, CD4+ T cells or both. If poly(I:C) is a more effective adjuvant for inducing CD4+ T cell responses than AS01B and such responses have a direct effector role or lead to enhanced antibody responses, it offers the potential to improve upon the clinical efficacy of RTS,S/AS01B. In this regard, the data presented here in NHP with CSP and a non optimized formulation of poly(I:C) induced a significantly higher number of CSP-specific ELISPOT responses than detected in another NHP study following three immunizations with RTS,S/AS01B [48]. The frequency of multi-functional CSP-specific CD4+ T cells secreting IFN- γ , IL-2 and TNF- α reported here is also higher than observed in NHP immunized with RTS,S/AS01B [14]. While the role of multi-functional Th1 responses in malaria infection is unclear, a recent study reported a strong association of multi-functional CSP-specific CD4+ T cells with a better outcome upon malaria challenge following immunization with RTS,S/AS01B in a phase 2 clinical trial [11]. In terms of humoral immunity, the antibody responses generated in this study are measured differently than the other NHP studies with RTS,S/AS01B. However, they are clearly robust and functional. An important caveat to making such comparisons amongst different studies is the variability associated with NHP studies and the assays used to measure adaptive immunity. Ultimately, a direct comparative study would be required to assess differences amongst RTS,S and CSP with poly(I:C) as adjuvant. Finally, since induction of CD8+ T cells has been shown to have a major role in controlling liver stage of malaria infection [6,7] optimization of the protein and poly(I:C) formulation may be required to enhance cross-priming. As poly-ICLC used in the mouse studies is a more potent formulation than the poly(I:C) used in the NHP study, it is possible that it would enhance priming for CD8+ T cells. The induction of such responses may require additional formulation of the CSP, which is being evaluated. Overall, having a new non-live protein based platform that can induce potent antibody and Th1 responses combined with a recombinant live viral vector vaccine to induce CD8+ T cell responses could provide improved immunity and have a major impact upon the development of a pre-erythrocytic vaccine.

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References

1. Breman JG, Plowe CV. A Malaria Vaccine for Control: More Progress. *J Infect Dis.* 2009 Aug 1; 200(3):317–20. [PubMed: 19569963]
2. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. *Clin Microbiol Rev.* 2009 Jan; 22(1):13–36. Table of Contents. [PubMed: 19136431]
3. Nardin EH, Nussenzweig V, Nussenzweig RS, Collins WE, Harinasuta KT, Tapchaisri P, et al. Circumsporozoite proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. *J Exp Med.* 1982 Jul 1; 156(1):20–30. [PubMed: 7045272]

4. Nardin EH, Nussenzweig RS. T cell responses to pre-erythrocytic stages of malaria: role in protection and vaccine development against pre-erythrocytic stages. *Annu Rev Immunol.* 1993; 11:687–727. [PubMed: 8476576]
5. Potocnjak P, Yoshida N, Nussenzweig RS, Nussenzweig V. Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *J Exp Med.* 1980 Jun 1; 151(6):1504–13. [PubMed: 6991628]
6. Romero P, Maryanski JL, Corradin G, Nussenzweig RS, Nussenzweig V, Zavala F. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature.* 1989 Sep 28; 341(6240):323–6. [PubMed: 2477703]
7. Rodrigues MM, Cordey AS, Arreaza G, Corradin G, Romero P, Maryanski JL, et al. CD8+ cytolytic T cell clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria. *Int Immunol.* 1991 Jun; 3(6):579–85. [PubMed: 1716146]
8. Renia L, Grillot D, Marussig M, Corradin G, Miltgen F, Lambert PH, et al. Effector functions of circumsporozoite peptide-primed CD4+ T cell clones against Plasmodium yoelii liver stages. *J Immunol.* 1993 Feb 15; 150(4):1471–8. [PubMed: 7679428]
9. Sun P, Schwenk R, White K, Stoute JA, Cohen J, Ballou WR, et al. Protective immunity induced with malaria vaccine, RTS,S, is linked to Plasmodium falciparum circumsporozoite protein-specific CD4+ and CD8+ T cells producing IFN-gamma. *J Immunol.* 2003 Dec 15; 171(12):6961–7. [PubMed: 14662904]
10. Reece WH, Pinder M, Gothard PK, Milligan P, Bojang K, Doherty T, et al. A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural Plasmodium falciparum infection and disease. *Nat Med.* 2004 Apr; 10(4):406–10. [PubMed: 15034567]
11. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, et al. Randomized, Double-Blind, Phase 2a Trial of Falciparum Malaria Vaccines RTS,S/AS01B and RTS,S/AS02A in Malaria-Naive Adults: Safety, Efficacy, and Immunologic Associates of Protection. *J Infect Dis.* 2009 Aug 1; 200(3):337–46. [PubMed: 19569965]
12. Polhemus ME, Remich SA, Ogutu BR, Waitumbi JN, Otieno L, Apollo S, et al. Evaluation of RTS,S/AS02A and RTS,S/AS01B in adults in a high malaria transmission area. *PLoS One.* 2009; 4(7):e6465. [PubMed: 19649245]
13. Stewart VA, McGrath SM, Walsh DS, Davis S, Hess AS, Ware LA, et al. Pre-clinical evaluation of new adjuvant formulations to improve the immunogenicity of the malaria vaccine RTS,S/AS02A. *Vaccine.* 2006 Oct 30; 24(42–43):6483–92. [PubMed: 16904798]
14. Mettens P, Dubois PM, Demoitie MA, Bayat B, Donner MN, Bourguignon P, et al. Improved T cell responses to Plasmodium falciparum circumsporozoite protein in mice and monkeys induced by a novel formulation of RTS,S vaccine antigen. *Vaccine.* 2008 Feb 20; 26(8):1072–82. [PubMed: 18258343]
15. Stoute JA, Kester KE, Krzych U, Welde BT, Hall T, White K, et al. Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. *J Infect Dis.* 1998 Oct; 178(4):1139–44. [PubMed: 9806046]
16. Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H, et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med.* 2004 Mar 15; 199(6):815–24. [PubMed: 15024047]
17. Trumpfheller C, Caskey M, Nchinda G, Longhi MP, Mizenina O, Huang Y, et al. The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proc Natl Acad Sci U S A.* 2008 Feb 19; 105(7):2574–9. [PubMed: 18256187]
18. Boscardin SB, Hafalla JC, Masilamani RF, Kamphorst AO, Zebroski HA, Rai U, et al. Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. *J Exp Med.* 2006 Mar 20; 203(3):599–606. [PubMed: 16505139]
19. Trumpfheller C, Finke JS, Lopez CB, Moran TM, Moltedo B, Soares H, et al. Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J Exp Med.* 2006 Mar 20; 203(3):607–17. [PubMed: 16505141]
20. Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell.* 2006 Jun 9; 22(5):561–9. [PubMed: 16762830]

21. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4⁺ Th1 immunity with poly IC as adjuvant. *J Exp Med*. 2009 Jul 6; 206(7):1589–602. [PubMed: 19564349]
22. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity*. 2001 Apr; 14(4):461–70. [PubMed: 11336691]
23. Le Bon A, Thompson C, Kamphuis E, Durand V, Rossmann C, Kalinke U, et al. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J Immunol*. 2006 Feb 15; 176(4):2074–8. [PubMed: 16455962]
24. Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, Gewert D, et al. Cross-priming of CD8⁺ T cells stimulated by virus-induced type I interferon. *Nat Immunol*. 2003 Oct; 4(10):1009–15. [PubMed: 14502286]
25. Le Bon A, Durand V, Kamphuis E, Thompson C, Bulfone-Paus S, Rossmann C, et al. Direct stimulation of T cells by type I IFN enhances the CD8⁺ T cell response during cross-priming. *J Immunol*. 2006 Apr 15; 176(8):4682–9. [PubMed: 16585561]
26. Stahl-Hennig C, Eisenblatter M, Jasny E, Rzehak T, Tenner-Racz K, Trumpfheller C, et al. Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. *PLoS Pathog*. 2009 Apr. 5(4):e1000373. [PubMed: 19360120]
27. Persson C, Oliveira GA, Sultan AA, Bhanot P, Nussenzweig V, Nardin E. Cutting edge: a new tool to evaluate human pre-erythrocytic malaria vaccines: rodent parasites bearing a hybrid *Plasmodium falciparum* circumsporozoite protein. *J Immunol*. 2002 Dec 15; 169(12):6681–5. [PubMed: 12471098]
28. Guo M, Gong S, Maric S, Misulovin Z, Pack M, Mahnke K, et al. A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. *Hum Immunol*. 2000 Aug; 61(8):729–38. [PubMed: 10980384]
29. Granelli-Piperno A, Pritsker A, Pack M, Shimeliovich I, Arrighi JF, Park CG, et al. Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin/CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell stimulation of the mixed leukocyte reaction. *J Immunol*. 2005 Oct 1; 175(7):4265–73. [PubMed: 16177066]
30. Cerami C, Frevert U, Sinnis P, Takacs B, Clavijo P, Santos MJ, et al. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. *Cell*. 1992 Sep 18; 70(6):1021–33. [PubMed: 1326407]
31. Wille-Reece U, Flynn BJ, Lore K, Koup RA, Kedl RM, Mattapallil JJ, et al. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8⁺ T cell responses in nonhuman primates. *Proc Natl Acad Sci U S A*. 2005 Oct 18; 102(42):15190–4. [PubMed: 16219698]
32. Lamoreaux L, Roederer M, Koup R. Intracellular cytokine optimization and standard operating procedure. *Nat Protoc*. 2006; 1(3):1507–16. [PubMed: 17406442]
33. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med*. 2007 Jul; 13(7):843–50. [PubMed: 17558415]
34. Zavala F, Cochrane AH, Nardin EH, Nussenzweig RS, Nussenzweig V. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. *J Exp Med*. 1983 Jun 1; 157(6):1947–57. [PubMed: 6189951]
35. Bruna-Romero O, Hafalla JC, Gonzalez-Aseguinolaza G, Sano G, Tsuji M, Zavala F. 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*. 2001 Nov; 31(13):1499–502. [PubMed: 11595237]
36. Nardin EH, Herrington DA, Davis J, Levine M, Stuber D, Takacs B, et al. Conserved repetitive epitope recognized by CD4⁺ clones from a malaria-immunized volunteer. *Science*. 1989 Dec 22; 246(4937):1603–6. [PubMed: 2480642]
37. Weiss WR, Mellouk S, Houghten RA, Sedegah M, Kumar S, Good MF, et al. Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. *J Exp Med*. 1990 Mar 1; 171(3):763–73. [PubMed: 1689762]

38. Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, et al. The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. *Nature*. 2006 Dec 14; 444(7121):937–40. [PubMed: 17151604]
39. Clyde DF, McCarthy VC, Miller RM, Hornick RB. Specificity of protection of man immunized against sporozoite-induced falciparum malaria. *Am J Med Sci*. 1973 Dec; 266(6):398–403. [PubMed: 4590095]
40. Clyde DF, Most H, McCarthy VC, Vanderberg JP. Immunization of man against sporozoite-induced falciparum malaria. *Am J Med Sci*. 1973 Sep; 266(3):169–77. [PubMed: 4583408]
41. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis*. 2002 Apr 15; 185(8):1155–64. [PubMed: 11930326]
42. Zavala F, Tam JP, Hollingdale MR, Cochrane AH, Quakyi I, Nussenzweig RS, et al. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. *Science*. 1985 Jun 21; 228(4706):1436–40. [PubMed: 2409595]
43. Good MF, Berzofsky JA, Miller LH. The T cell response to the malaria circumsporozoite protein: an immunological approach to vaccine development. *Annu Rev Immunol*. 1988; 6:663–88. [PubMed: 2968085]
44. Nussenzweig RS, Vanderberg J, Most H, Orton C. Protective immunity produced by the injection of x-irradiated sporozoites of *plasmodium berghei*. *Nature*. 1967 Oct 14; 216(5111):160–2. [PubMed: 6057225]
45. Clyde DF. Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971–75. *Bull World Health Organ*. 1990; 68(Suppl):9–12. [PubMed: 2094597]
46. Mueller AK, Labaied M, Kappe SH, Matuschewski K. Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature*. 2005 Jan 13; 433(7022):164–7. [PubMed: 15580261]
47. Doolan DL, Southwood S, Freilich DA, Sidney J, Graber NL, Shatney L, et al. Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data. *Proc Natl Acad Sci U S A*. 2003 Aug 19; 100(17):9952–7. [PubMed: 12886016]
48. Stewart VA, McGrath SM, Dubois PM, Pau MG, Mettens P, Shott J, et al. Priming with an adenovirus 35-circumsporozoite protein (CS) vaccine followed by RTS,S/AS01B boosting significantly improves immunogenicity to *Plasmodium falciparum* CS compared to that with either malaria vaccine alone. *Infect Immun*. 2007 May; 75(5):2283–90. [PubMed: 17307942]

Figure 1. Frequency of IFN- γ producing cells after immunization with CSP or α DEC-CSP + poly(I:C)

NHP were immunized three times with CSP or α DEC-CSP with or without poly(I:C) at weeks 0, 5 and 13. As controls, animals were immunized with poly(I:C) alone. PBMCs were analyzed for production of IFN- γ by ELISPOT assay at weeks 2, 7, 15 (two weeks after each immunization) and 26 post primary immunization. At week-27 all animals were boosted with 150,000 irradiated PfSPZ i.v.. As controls for the boost 5 animals were immunized with irradiated PfSPZ alone. At week-2 post irradiated PfSPZ boost (week-29) PBMCs were analyzed for production of IFN- γ by ELISPOT assay. Shown are the IFN- γ SFC/10⁶ PBMCs for individual animals in each group (4–5 animals/group). The # denotes p<0.05 using non-parametric Wilcoxon Rank test comparing CSP with CSP + poly(I:C) and α DEC-CSP with α DEC-CSP + poly(I:C) immunized groups respectively.

Figure 2. Magnitude and quality of CSP-specific CD4⁺ T cell responses following immunization with CSP or α DEC-CSP + poly(I:C)

PBMCs were analyzed by multi-parameter flow cytometry at week-2 following the 3rd immunization (week-15). The frequency of total cytokine producing CD95⁺CCR7^{hi} and lo CD4⁺ T cells (IFN- γ , IL-2 or TNF- α) was analyzed (A). Data are shown for individual animals in each group. Using SPICE analysis the cytokine producing CD4⁺ T cell responses were divided into 7 distinct subpopulations producing any combination of IFN- γ , IL-2, and TNF- α . The total frequency of combination cytokine producing CD4⁺ T cells were analyzed for groups immunized with CSP + poly(I:C) (B) and α DEC-CSP + poly(I:C) (C). Pie chart representation of distinct combinations of cytokine producing CD4⁺ T cells is shown for individual animals from CSP + poly(I:C) immunized group (D) and α DEC-CSP + poly(I:C) group (E). The pie charts highlighted in boxes show the mean frequency for each group. The # denotes $p < 0.05$ using non-parametric Wilcoxon Rank test comparing CSP with CSP + poly(I:C) and α DEC-CSP with α DEC-CSP + poly(I:C) immunized groups respectively.

Figure 3. Immunization with CSP + poly(I:C) elicits high titer of CSP-specific IgG antibodies
 CSP-specific IgG antibodies were assessed in the plasma or serum of animals immunized with CSP or α DEC-CSP with or without poly(I:C) at indicated time points post-immunization. Data is shown for 5 individual animals at 4 weeks following the 3rd immunization (week-17) with CSP + poly(I:C) (A) and α DEC-CSP + poly(I:C) (B). A comparison of CSP-specific antibodies was assessed pre-immunization and 4 weeks following the 2nd and 3rd immunization with CSP or α DEC-CSP with or without poly(I:C) (C). At week-27 post primary immunization NHP were boosted with 150,000 irradiated PfSPZ i.v.. CSP-specific IgG antibodies were assessed one week prior to irradiated PfSPZ boost and at weeks 2 and 11 post-boost in the indicated groups (D). The kinetics of CSP-specific IgG antibodies were determined in the plasma of animals immunized with CSP or α DEC-CSP with or without poly(I:C) and boosted with irradiated PfSPZ or irradiated PfSPZ

only at indicated time points (E). Data shown in C, D and E is the mean $\pm$ S.D. for 4–5 animals/group. The asterisks denote $p < 0.05$ using the unpaired student's t-test with Welch's correction.

Figure 4. Immunization with CSP + poly-ICLC reduces malaria parasite burden in mice upon challenge

C57BL/6 mice were immunized with 3 doses of CSP with or without poly-ICLC or poly-ICLC alone. At week-3 following the 3rd immunization CSP-specific IgG antibodies were assessed in the serum of all immunized animals as well as naïve controls (A). Data shown represents the mean $\pm$ S.D. for 4 – 8 animals/group. Two months following last immunization all animals were challenged with 10,000 chimeric Pb-Pf intravenously. At 40 hrs post-challenge the total frequency of CSP-specific cytokine producing CD4⁺ T cell responses (IFN- γ , IL-2 or TNF- α) were measured in the spleen using multi-parameter flow cytometry as described in Fig. 2(B). The total frequency and pie chart representation of combination cytokine producing CD4⁺ T cells are shown for individual animals immunized with CSP + poly-ICLC (C and D). The pie chart highlighted in the box shows the mean frequency for the group (C). *P. berghei* 18S rRNA copies were measured in the liver extracts of immunized animals at 40 hrs post-challenge (E). The asterisks denote $p < 0.05$ using the unpaired student's t-test with Welch's correction. The # denotes $p < 0.05$ using the non-parametric Wilcoxon Rank test.

IFA titers from immunized NHP sera against Pf sporozoites

Table 1

NHPs were immunized 3 times with CSP or aDEC-CSP with or without poly(I:C) or poly(I:C) alone. At week-4 following the 3rd immunization, sporozoite specific IgG were measured by adding serial dilutions of pooled sera to air dried *P. falciparum* sporozoites mounted on slides, followed by a fluorescein conjugated anti-monkey IgG antibody (A). IFA titers were measured for individual animals immunized with CSP + poly(I:C) at indicated time points (B). The endpoint titer was defined as the greatest serum dilution that yielded a positive fluorescence above the pre-immune control levels.

A				
Experimental group (pooled sera)		IFA pre-bleed titer		IFA endpoint titer
CSP		<1:50		1:6,400
CSP + Poly(I:C)		<1:50		1:102,400
aDEC-CSP		<1:50		1:200
aDEC-CSP + Poly(I:C)		<1:50		1:25,600
Poly(I:C)		<1:50		<1:50

B					
IFA Titers From CSP + Poly(I:C) Immunized NHP Sera Against Pf Sporozoites					
NHP ID	Pre-Serum	4 Wks after 3 rd dose	13 Wks after 3 rd dose	2 wks after Irra. PTSPZ boost	11 wks after Irra. PTSPZ boost
CG6P	<50	409,600	25,600	25,600	12,800
CG8T	<50	819,200	204,800	409,600	51,200
CG6B	<50	102,400	25,600	25,600	12,800
CG6H	<50	409,600	25,600	25,600	12,800
CG6X	<50	409,600	12,800	12,800	6,400

Sera from immunized NHP blocks sporozoite invasion and development in a human hepatocyte cell line. HC-04 cells were added to 8-well chamber slides. Twenty four hours later, *P. falciparum* sporozoites were added with or without the indicated sera, in triplicate. Seventy-two hours later, cells were fixed and stained for PfLSA-1 (liver stage antigen-1) and counted by microscopy. The total number of liver stage parasites per well are shown, and percent inhibition is relative to pre-sera.

Table II

		number of liver stage parasites/well				% inhibition compared to pre-sera
		well 1	well 2	well 3	mean $\pm$ SD	
medium	no sera	211	234	197	214.00 $\pm$ 18.7	-
α CSP mAb	(100 μ g/mL)	20	17	10	15.67 $\pm$ 5.1	-
CSP	pre-sera	190	176	180	182.00 $\pm$ 7.2	-
	4 weeks after 3rd dose	184	160	152	165.33 $\pm$ 16.7	9.16
CSP+ Poly(I:C)	pre-sera	224	205	192	207.00 $\pm$ 16.1	-
	4 weeks after 3rd dose	102	90	81	91.00 $\pm$ 10.5	56.04
α DEC-CSP	pre-sera	228	202	191	207.00 $\pm$ 19.0	-
	4 weeks after 3rd dose	189	172	179	180.00 $\pm$ 8.5	13.04
α DEC-CSP + Poly(I:C)	pre-sera	217	207	186	203.33 $\pm$ 15.8	-
	4 weeks after 3rd dose	125	113	107	115.00 $\pm$ 9.1	43.44
Poly(I:C)	pre-sera	197	209	178	194.67 $\pm$ 15.6	-
	2 weeks after 2nd dose	203	183	170	185.33 $\pm$ 16.6	4.79

Sera from immunized NHP blocks sporozoite invasion and development in a human hepatocyte cell line. HC-04 cells were added to 8-well chamber slides. Twenty four hours later, *P. falciparum* sporozoites were added with or without the indicated sera, in triplicate. Seventy-two hours later, cells were fixed and stained for PfLISA-1 (liver stage antigen-1) and counted by microscopy. The total number of liver stage parasites per well are shown, and percent inhibition is relative to wells with no sera. The asterisks denote $p < 0.05$ using the unpaired student's t-test with Welch's correction.

Table III

	well #1	well #2	well #3	mean $\pm$ s.d.	% inhibition (95% CI)
no sera	290	256	274	273.3 $\pm$ 17.0	N/A
anti-PfCSP mAb	15	13	8	12.0 $\pm$ 3.6	95.61% (85.4%–100%)
CSP+Poly(I:C) pre-bleed	257	241	228	242.0 $\pm$ 14.5	11.46% (0%–26.5%)
CSP+Poly(I:C)	162	171	138	157.0 $\pm$ 17.1*	42.56% (26.4%–58.8%)
4 weeks after 3 rd dose	88	69	78	78.33 $\pm$ 9.5*	71.34% (58.3%–84.4%)
CG6B	197	174	171	180.67 $\pm$ 14.2*	33.9% (19.0%–48.8%)
CG6H	144	120	111	125.0 $\pm$ 17.1*	54.27% (38.1%–70.5%)
CG6X	145	137	153	145.0 $\pm$ 8.0*	46.95% (29.9%–64.0%)
CSP					
4 weeks after 3 rd dose	243	268	244	251.67 $\pm$ 14.2	7.93% (0%–22.8%)