Published as: *Vaccine*. 2010 June 23; 28(29): 4644–4652.

Immunogenicity and protective efficacy of a recombinant yellow fever vaccine against the murine malarial parasite *Plasmodium yoelii*

Cristina T. Stoyanov^a, Silvia B. Boscardin^{b,†}, Stephanie Deroubaix^b, Giovanna Barba-Spaeth^{a,‡}, David Franco^c, Ruth S. Nussenzweig^d, Michel Nussenzweig^b, and Charles M. Rice^{a,*}

^a Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, NY 10065

^b Laboratory of Molecular Immunology, The Rockefeller University, New York, NY 10065

^c Aaron Diamond AIDS Research Center, The Rockefeller University, New York, NY 10016

^d Department of Medical and Molecular Parasitology, Department of Pathology, New York University School of Medicine, New York, NY 10016

Abstract

The live-attenuated yellow fever vaccine (YF17D) is one of the safest and most effective vaccines available today. Here, YF17D was genetically altered to express the circumsporozoite protein (CSP) from the murine malarial parasite *Plasmodium yoelii*. Reconstituted recombinant virus was viable and exhibited robust CSP expression. Immunization of naïve mice resulted in extensive proliferation of adoptively transferred CSP-specific transgenic CD8⁺ T cells. A single immunization of naïve mice with recombinant YF17D resulted robust production of IFN- γ by CD8⁺ T cells and IFN- γ and IL-2 by CD4⁺ T cells. A prime-boost regimen consisting of recombinant virus followed by a low dose of irradiated sporozoites conferred protection against challenge with *P. yoelii*. Taken together, these results show that recombinant YF17D can efficiently express CSP in culture, and prime a protective immune response *in vivo*.

Keywords

YF17D; malaria; protection

1. Introduction

Malarial parasites infect an estimated 250 million people each year, resulting in up to 1 million deaths annually, mostly children less than 5 years of age. The morbidity, mortality, and socioeconomic burden of endemic malaria make it one of the most devastating infectious diseases and an urgent public health problem. Additionally, malaria is a significant threat to

* Corresponding author: Charles M. Rice: ricec@rockefeller.edu, phone (212) 327-7046, fax (212) 327-7048.

† Current Address: Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brasil 05508-900

‡ Current Address: Laboratory of Structural Virology, Institut Pasteur, 75015 Paris, France

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

individuals traveling to endemic countries. No vaccine is available, and emerging resistance to antimicrobial drugs and insecticides is cause for increasing concern.

Current vaccine efforts aim to intervene at various stages of the parasite life cycle. Pre-erythrocytic vaccines, such as irradiated sporozoites, endeavor to prevent initial infection. Since the late 1960s, irradiated sporozoites have been known to confer sterile immunity to mice and remain the gold standard for immunogenicity studies [1]. In the 1970s, analogous studies of irradiated *P. falciparum* and *P. vivax* sporozoite vaccination in humans yielded promising results [2]. Unfortunately, effective immunization requires administration of large numbers of the inactivated parasites delivered intravenously or by mosquito bites. Since a practical route of delivery has yet to be developed, irradiated sporozoite immunization is still experimental.

The main protective antigen of irradiated sporozoites is the circumsporozoite protein (CSP) [3], which coats the surface of the sporozoite and it is thought to be involved in parasite development and binding to hepatocytes [4,5]. Several attempts have been made to induce immunity against *Plasmodium spp.* by delivery of CSP or CSP-derived peptides. The most promising candidate is a recombinant protein vaccine, named RTS,S/AS02A (GlaxoSmithKline Biologicals [GSK]) [6]. RTS,S/AS02A consists of a portion of CSP fused to the hepatitis B virus surface antigen and is formulated with the AS02A adjuvant (GSK), an oil-in-water emulsion containing the immunostimulants QS21 (a triterpene glycoside purified from the bark of *Quillaja saponaria*) and 3D-MPL (3-deacylated monophosphoryl lipid A [MPL]) [6,7]. RTS,S/AS02A recently completed Phase 2b clinical trials with a vaccine efficacy against new infection of 65.9% [8,9]. The RTS,S/AS02A vaccine is still under evaluation, and the results from Phase III trials remain to be determined.

Unlike malaria, a highly safe and effective vaccine exists to protect against yellow fever virus (YFV). The YFV vaccine, a live attenuated virus termed YF17D, has been used to successfully immunize more than 400 million people since 1937 [10]. The vaccine has minimal side effects and appears to provide life-long immunity in the majority of recipients after a single subcutaneous dose; neutralizing antibodies can be detected in some individuals more than 30 years after vaccination [11]. The success of YF17D as a vaccine and vaccine vector is thought to be due to the ability of the attenuated virus to infect and activate professional antigen presenting cells (APCs) at the site of inoculation. YF17D productively infects human dendritic cells (DCs), the most specialized APCs, and these DCs can process and present foreign antigens to CD8⁺ T-cells [12-14]. YF17D is also known to activate DCs by signaling through multiple tolllike receptors (TLRs), specifically TLR 2, 7, 8, and 9 [12,13]. Activated DCs then travel to the nearest lymph nodes, where they stimulate T-lymphocytes to initiate adaptive immune responses.

Previous studies have shown that YF17D can be engineered to express heterologous antigens and can induce protective immune responses against the inserted epitopes. Clinical studies of YF17D-based chimeric vaccine candidates targeting dengue type 2 virus, Japanese encephalitis virus (JEV), and West Nile virus (WNV) are currently underway [15-19]. Recombinant YF17D has also been used to express malarial sequences. YF17D encoding a single short *Plasmodium* T-cell epitope is a potent inducer of cytotoxic T lymphocyte (CTL) responses and can significantly reduce parasite burden in the mouse liver when followed by a large dose (10^4) of irradiated sporozoites [20].

Here, we have attempted to enhance CTL and humoral responses against *Plasmodium* by further exploring the potential of the YFV vaccine vector. A new recombinant YF17D virus expressing the *P. yoelii* CSP, including several B- and T-cell epitopes, was constructed and characterized *in vitro* and *in vivo*. Here, CSP was inserted in a previously unexplored site within the YF17D genome. Immunization of adult mice with the recombinant YFV resulted in robust

CTL responses in the liver and spleen of immunized animals and, when followed by a low-dose boost with irradiated sporozoites, conferred protection to homologous challenge.

2. Materials and methods

2.1 Plasmid constructs

Plasmids were constructed using conventional molecular cloning techniques. Constructs were verified by restriction enzyme digestion and sequencing of PCR-amplified segments. Primers and vector sequences are available upon request. To create the YF17D vaccine vector, the full-length viral genome cDNA [21] was cloned into the copy control pCC1 (blunt cloning-ready) vector (Epicentre Biotechnologies) according to the manufacturer's recommendations. The resulting pCC1-YF17D plasmid is maintained as a single copy in *Escherichia coli*, strain EPI300, and upon addition of an induction agent, the clone is amplified to high copy numbers (Epicentre Biotechnologies). pCC1-YF17D was then modified for insertion of foreign sequences in frame with the YF17D open reading frame (ORF). First, a PCR product containing the restriction sites *SrfI*, *SacI*, *KpnI*, *HpaI* and *NsiI* was generated and cloned into pNEB193 (New England Biolabs) via *SfoI* and *PmeI* to construct a shuttle plasmid, pNEB193/link. An 887 base pair (bp) sequence including the 5'NTR and the first 75 bp of the capsid gene was amplified from pCC1-YF17D and cloned into pNEB193/link via the *SrfI* and *SacI* restriction sites to generate pNEB-C25, this plasmid contains a conserved region of YF17D that is important for genome cyclization during virus RNA replication [22]. Second, a 273 bp segment encoding an *AgeI* site, followed by foot and mouth disease virus (FMDV) 2A and mammalian ubiquitin (Ubi) sequence, was assembled with a 1548 bp region of YF17D, encompassing the capsid, prM, and part of the envelope gene to the *NsiI* site. The first 75 bp of capsid in this context was recoded using assembly PCR to prevent duplication of the cyclization sequence. The cassette was cloned into pNEB-C25 using *AgeI* and *NsiI* to create pNEB-C25/2A-Ubi/mdC, a final shuttle plasmid harboring *SacI* and *AgeI* cloning sites for insertion of foreign sequences in frame between the truncated capsid sequence and FMDV 2A-Ubi cassette. A mammalian codon-optimized version of the circumsporozoite (CS) gene from *Plasmodium yoelii*, encompassing nucleotides (nt) 172-1034, was amplified from pDEC-PyCS [23] with the addition of *SacI* and *AgeI* sites, and inserted into pNEB-C25/2A-ub/mdC. From pNEB-C25/2A-Ubi/mdC, the YF17D/PyCS cassette was removed and cloned into pCC1-YF17D via *SrfI* and *NsiI* to generate pCC1-YF17D/PyCS.

2.2 Animals and cells

Five to six week old adult female BALB/c mice were obtained from Jackson Laboratories. Transgenic H2K^{b/d} -mice expressing CD8⁺ T cells restricted for the *P. yoelii* CSP epitope SYVPSAEQI were kindly provided by Dr. Fidel Zavala [24]. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at The Rockefeller University.

Baby hamster kidney (BHK J) cells were maintained at 37°C and 5% CO₂ in minimum essential medium (MEM) containing 7.5% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, and 100U penicillin-100 µg streptomycin (GIBCO). Bone marrow-derived dendritic cells (BmDC) were generated as described previously [25]. Briefly, tibias and femurs were cleared of connective tissue, and bone marrow was flushed into complete medium (RPMI 1640 containing 5% FCS, 50 µM 2-ME, 2 mM glutamine, 0.1 mM nonessential amino acids, and 100U penicillin-100 µg streptomycin) (GIBCO) using a 27G needle and a 10 ml syringe. Cells were centrifuged for 5 min at 1500 rpm and resuspended in 1 ml (per mouse) of red blood cells lysis buffer (GIBCO). After incubation for 2 min at room temperature, 25 ml of complete medium were added and the cells were centrifuged for an additional 5 min at 1500 rpm. Cells were resuspended in 3 ml of complete medium, filtered through a 40 µm filter and counted.

The cells were cultured in 24-well plates (2×10^6 cells per ml) in the presence of murine GM-CSF (20 ng/ml) (Peprotech) and IL-4 (3 ng/ml) (R&D System). Fresh cytokines and media were added on day 2 and on day 4. On day 7, most of the cells had acquired typical dendritic morphology, and these cells were used as the source of DC. Splenocytes and liver lymphocytes were maintained in RPMI 1640 containing 5% FCS, 50 μ M 2-ME, 2 mM glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES, and 0.1 mM sodium pyruvate.

2.3 Viruses and parasites

Parental pCC1-YF17D and recombinant pCC1-YF17D/PyCS plasmid DNA were purified by alkaline lysis and CsCl banding. Linearized template was created by *Xho*I digestion, and was used for *in vitro* transcription by SP6 RNA polymerase to generate 5' capped viral genomic RNA (Ambion). Five micrograms of the resulting RNA were electroporated into BHK J cells as previously described [26]. The specific infectivity of the viral RNA was measured by seeding serial dilutions of electroporated cells on monolayers of untransfected BHK J cells, overlaying with 0.6% agarose in 2 $\times$ medium, and counting viral plaques after 96 hours. Virus stocks were harvested at 48 hours post-electroporation (hpe) (YF17D) or 72 hpe (YF17D/PyCS). Single use aliquots were stored frozen at -80°C until use.

Expression of CSP and YF antigens was assessed by immunofluorescence with anti-PyCS (3F11 monoclonal) and anti-YF E (5E3 monoclonal [27]), and by Western blot analysis with anti-YF NS4 (C-12 polyclonal [28]) in addition to the above mentioned antibodies.

P. yoelii (17 $\times$ NL strain) was maintained by alternate cyclic parasite passage in *Anopheles stephensi* and infected blood transfer to mice. Sporozoites were obtained by dissection of salivary glands of *A. stephensi* and collected in medium RPMI 1640 at 4°C.

2.4 Viral growth and plaque assay

BHK J cells were infected at a multiplicity of infection (MOI) of 1. One hour post-infection (hpi), media was replaced. Supernatants were collected at 24, 48, 72, and 96 hpi and stored frozen at -80°C. Virus-containing supernatants were analyzed by plaque assay as previously described [26]. Briefly, serial 10-fold dilutions were used to infect monolayers of BHK J cells. After 1 hour, cells were overlayed with 0.6% agarose in 2 $\times$ medium. Plaques were fixed with 7.4% formaldehyde and stained with 0.3% crystal violet at 72 hpi.

2.5 Reverse transcriptase polymerase chain reaction (RT PCR)

For RT PCR, virus was passaged consecutively in BHK J cells with a MOI of 1. After each passage, 150 μ l of virus-containing supernatant was harvested and RNA was purified using QIAmp Viral RNA mini kit following the manufacturer's recommendations (Qiagen). RNA was analyzed using the SuperScript III One-Step RT-PCR System (Invitrogen). Passages 1 and 6 were analyzed by sequencing (Geneart).

2.6 Immunizations and challenge

Mice were primed subcutaneously (s.c.) in 200 μ l volume with 2×10^5 PFU YF17D/PyCS, 2×10^5 PFU YF17D, or 1 $\times$ phosphate buffered saline (PBS) solution, or intravenously (i.v.) with 50,000 irradiated sporozoites. Boost consisted of 5,000 irradiated sporozoites given i.v. four weeks following primary immunization or 2×10^5 PFU YF17D/PyCS given s.c. Challenge was performed by i.v. injection of 250 viable sporozoites at three weeks following boosting. Blood smears were prepared from the tail of infected animals on days 3-10 following the challenge injection to measure parasitemia. Groups that became parasitemic by day 6 were not analyzed further and were considered non-protected. The number of infected red blood cells

(iRBC) in a total count of 1000 RBCs was determined by staining with Giemsa (SIGMA). Animals with 0 iRBCs were considered protected.

2.7 Liver lymphocyte isolation

Mice were euthanized and perfused *in situ* at room temperature using PBS. The livers were then harvested and placed on cold PBS+2%FBS (Invitrogen). After the removal of the gall bladders, livers were cut into small pieces and pressed gently through a 70 μ m cell-strainer (BD Biosciences). The cell suspension was washed twice with PBS+2% FBS and centrifuged at 500 $\times$ g for 5 min at 4 °C. A maximum of two livers were resuspended in 25 ml of a room temperature 37.5% Percoll solution (Amersham-Pharmacia) and then centrifuged at 700 $\times$ g for 15 min at room temperature. The cell pellet was washed once with PBS+2%FBS and then lysed in 1-2 ml of red blood cell lysis buffer (GIBCO) for 5 min at room temperature. The remaining cells were washed twice with PBS+2%FBS and concentrations were adjusted to 4 $\times$ 10⁶ cells/ml.

2.8 Peptide libraries

A 15-mer overlapping peptide library for the *P. yoelii* CS protein (CSP) was synthesized by the Proteomics Resource Center, The Rockefeller University [23].

2.9 Extracellular and intracellular cytokine staining

Intracellular IFN- γ and TNF- α production by CD4⁺ and CD8⁺ T cells was evaluated using bulk splenocytes and lymphocytes derived from the livers of immunized animals. Splenocytes and liver lymphocytes were incubated with 2 μ M of each CSP peptide pool [23], the CD8 immunodominant peptide (SYVPSAEQI) or medium alone in the presence of 2 μ g/ml of costimulatory α CD28 antibody (clone 37.51). Cells were cultured for 12 hours in the presence of brefeldin A (BD Biosciences), incubated with anti-CD16/CD32 antibody to block Fc- γ receptors, and stained with anti-mouse CD4 (clone GK 1.5), CD8 (clone 53-6.7), and CD3 (clone 145-2C11) antibodies for 15 min at 4°C. After fixation with Cytofix/Cytoperm Plus (BD Biosciences), cells were stained for intracellular IFN- γ (XMG1.2) for 15 min at room temperature.

Surface expression of mouse CD11c, CD86 and intracellular expression of *P. yoelii* CSP were quantified using Pacific Blue-conjugated anti-CD11c (clone N418), PE-conjugated anti-CD86 (clone PO3.1), and monoclonal 488-conjugated anti-PyCS (clone 3F11 conjugated with Alexa Fluor 488, Molecular Probes) antibodies, respectively. For flow cytometry, BmDCs were stained with the antibodies for 15 min at 4°C and after fixation with Cytofix/Cytoperm Plus, cells were stained for intracellular PyCS for 15 min on ice.

All conjugated antibodies were purchased from BD Biosciences or eBioscience, and diluted according to manufacturers recommendations. Data were collected using LSRII or FACS Calibur and analyzed using FlowJo software (Tree Star).

2.10 Cloning and production of recombinant PyCS protein

The *P. yoelii* CS sequence (nt 172-1034) was subcloned into pET21a (Novagen) *Hind*III and *Xho*I sites to create a C-terminally His-tagged PyCS expression construct, termed pET21a+PyCS. Tuner competent bacteria (Novagen) were transformed with pET21a+PyCS, and a single colony was picked and grown overnight at 37°C in LB-ampicillin (100 μ g/ml), followed by induction (when D.O._{600nm} reached 0.5) with 2 mM IPTG for 4 hours at 30°C. Cells were collected by centrifugation and resuspended in 5 ml of lysis buffer (50 mM Tris, 200 mM NaCl, 10% glycerol, pH 8.0) per 125 ml culture pellet. PMSF to a final concentration of 1 mM was added and the bacterial suspension was sonicated 5 times for 1 min with 1 min intervals. The

lysate was clarified by centrifugation at 28,000 ×g for 45 min and the supernatant was incubated under rotation with 5 ml Ni-NTA resin (Qiagen), which had been previously equilibrated in lysis buffer, for 2 hours at 4°C. After 4 washes with 10 ml wash buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 30 mM imidazole, pH 8.0), protein was eluted from the Ni-NTA matrix using 5 ml of elution buffer (50 mM Tris, 1M NaCl, 10% glycerol, 500 mM imidazole, pH 8.0) for 1 hour at 4°C. Purified CSP was then dialyzed into PBS, filtered in 0.2 µm filter, and stored at -80.

2.11 ELISA

For the detection of CSP-specific and YFV-specific antibodies, high-binding ELISA plates (Costar) were coated overnight with 5 µg/ml recombinant CSP in PBS or with 50 µl of virus-containing supernatant derived from infected BHK J cells, respectively. Plates were then washed three times with PBS containing 0.02% Tween-20 and blocked with PBS-BSA 1% for 1 hour at room temperature. Serial dilutions of sera in PBS-BSA 0.25% were incubated for 2 hours at room temperature before addition of goat anti-mouse IgG Fc-specific antibody conjugated to horseradish peroxidase (1:2,000; Jackson ImmunoResearch Laboratories). ELISA were developed using horseradish peroxidase substrate (Biorad), and OD_{405nm} was measured using a VERSAmax microplate reader (Molecular Devices). Titers represent the highest dilution of serum showing an OD₄₀₅ >0.1 over background. The results are presented as the log₂ antibody titer of each group of mice ± standard deviation.

2.12 Proliferation of transgenic T cells

CD8⁺ T cells from Thy 1.1⁺ SYVPSAEQI-specific TCR transgenic mice were purified and carboxyfluorescein succinimidyl ester (CFSE) labeled as described previously [29]. 3-5 × 10⁶ cells were transferred i.v. to naive BALB/c mice, followed by s.c. infection with YF17D/PyCS or YF17D at 24 hours-post transfer. Cell division was measured in the spleen and liver by CFSE dilution 2 days later. Transgenic T cells were gated using anti-Thy 1.1 (clone OX-7) and anti-CD8 (clone 53-6.7) antibodies (BD Biosciences).

2.13 Infection of BmDCs

BmDCs were prepared as described above and infected with YF17D/PyCS at a MOI of 5 or exposed to LPS (500 ng per 2 × 10⁶ cells) in a 24 well culture plate (Costar). Forty-eight hours following infection, most of the media was removed and cells were harvested with cold PBS for FACS analysis as described above.

3. Results

3.1 Construction and in vitro characterization of recombinant yellow fever 17D virus

A recombinant YF17D cDNA was modified to encode the *Plasmodium yoelii* circumsporozoite (CS) sequence, encompassing nucleotides 172-1034. This sequence was chosen deliberately to include the immunodominant repeated peptides QGPGAP and QQPP, and the well-described class I CTL epitope SYVPSAEQI [30-33]. The CS sequence was inserted in frame into the YF17D cDNA following nucleotides 1-75 of the capsid gene, which contain the genome cyclization sequence required for replication. The foot-mouth disease virus (FMDV) 2A peptide and the mammalian ubiquitin (Ubi) coding sequences were inserted directly following CSP to ensure efficient cleavage from the downstream YF polyprotein (Figure 1A). The CSP-FMDV2A-Ubi cassette was followed by the full-length capsid gene and the remainder of the YFV genome. The resulting recombinant cDNA, pCC1-YF17D/PyCS, was *in vitro* transcribed to generate infectious viral RNA.

To determine if recombinant YF17D/PyCS was viable and expressed the inserted transgene, infectious RNA was transfected into BHK J cells. Immunofluorescent staining and Western blot analysis of electroporated cells revealed that the recombinant virus expressed accurately processed YF envelope (E) protein and non-structural protein 4B (NS4B), as well as the *P. yoelii* CSP (Figure 1B-C). To investigate the ability of the recombinant genome to produce infectious virus, the specific infectivity of the transfected RNA was measured and single-step growth curves were generated. YF17D/PyCS remained cytopathic but yielded plaques that were distinctly smaller than those of the parental YF17D virus at 72 hours post infection (hpi) (Figure 2A). Although the recombinant virus had slower kinetics following initial infection, it reached titers approaching those of the parental virus by 96 hpi (Figure 2B). The specific infectivity of RNA derived from the YF17D/PyCS construct was approximately 2-fold lower than the parental YF17D RNA (Figure 2C). To determine if the recombinant genome was genetically stable, YF17D/PyCS was passaged consecutively in BHK J cells at MOI of 1. The presence of the insert was verified by RT PCR and sequencing, and could be detected intact up to 6 passages; PyCS expression became undetectable by passage 7, when we were also unable to amplify the PyCS insert from infected cells (Figure 2D and data not shown). These results demonstrate that a monocistronic YF17D genome encoding a large heterologous gene insertion in the capsid region is viable in cell culture but is genetically stable for only a limited number of passages.

3.2 Cellular immune responses in mice immunized with YF17D/PyCS

The ability of the recombinant YFV to stably express CSP in culture encouraged us to examine immunogenicity *in vivo*. To determine if YF17D/PyCS could effectively prime an immune response, we developed a prime-boost immunization regimen using YF17D/PyCS alone or in combination with irradiated sporozoites. Groups of five mice received 2×10^5 plaque forming units (PFU) of YF17D/PyCS alone or followed by a boost with 5000 irradiated sporozoites or with 2×10^5 PFU of YF17D/PyCS four weeks later. Control groups were immunized with PBS, or with one (5000) or two doses of irradiated sporozoites (50000 followed by 5000 parasites). Nine days post-boosting, intracellular levels of IFN- γ and TNF- α production by peptide-stimulated T cells were measured in three mice from each group. Synergistic induction of IFN- γ was observed in liver-derived CD8⁺ T cells following the YF17D/PyCS-prime and sporozoite-boost regimen, resulting in cytokine production that was approximately 2-3 fold greater than after two doses of irradiated sporozoites. Furthermore, immunization with single dose of YF17D/PyCS resulted in higher levels of IFN- γ -production than with a single dose of irradiated sporozoites. A second dose of YF17D/PyCS did not significantly increase the production of IFN- γ by CD8⁺ T cells. IFN- γ -production in CD4⁺ T cells derived from the liver was also greater in YF17D/PyCS-immunized mice than in irradiated sporozoite-immunized animals. Similar results were observed for T cells isolated from the spleen (data not shown). In addition, a small percentage of CD4⁺ T cells produced IFN- γ , IL-2 or TNF- α following immunization with YF17D/PyCS (Figure 3). These data indicate that the YF17D/PyCS-based regimen can activate T cell effector functions at levels comparable or superior to high-dose immunization with sporozoites.

3.3 Proliferation of SYVPSAEQI-specific CD8⁺ T-cells in mice immunized with YF17D/PyCS

T cells are essential for the development of immunity against malaria, and CD8⁺ T cells targeting *P. yoelii* CSP are known to eliminate liver stage parasites in an antigen-specific manner [34]. YF17D can infect antigen-presenting cells leading to activation of T cells [12-14], suggesting that the recombinant YF17D virus may be a potent inducer of anti-parasite immunity. We therefore investigated the *in vivo* proliferation of CSP-specific CD8⁺ T cells following immunization with YF17D/PyCS. T cells specific for CSP epitope SYVPSAEQI were isolated from a previously described T cell receptor (TCR) transgenic mouse model [24]. Purified T cells were labeled before adoptive transfer into naïve BALB/c mice. Following

immunization of the recipient mice with YF17D/PyCS, SYVPSAEQI-specific T cells proliferated extensively in both the liver and the spleen, as determined by decreased CFSE fluorescence intensity in daughter cells. This proliferation was not observed in mice immunized with the parental YF17D (Figure 4). These results suggest that delivery of the CSP by YF17D/PyCS can lead to expansion of T cells specific for malarial epitopes.

3.4 Humoral immune responses in mice immunized with YF17D/PyCS

Humoral responses in the immunized animals were measured by ELISA for CSP- and YFV-reactive antibodies at fourteen days following initial immunization (pre-boost) and at nine days following the boost (post-boost) (Tables 1 and 2). To determine if the recombinant virus was immunogenic in mice, groups of three mice were immunized with 2×10^5 PFU of YF17D/PyCS- or YF17D- prime followed by a boost with 5,000 irradiated sporozoites. Control mice were immunized with PBS. Specific anti-YFV antibodies were found in mice that received either the recombinant YF17D/PyCS or YF17D alone but not in control mice (Table 1).

To determine if the recombinant virus induced anti-CSP antibodies, we immunized groups of three mice with 2×10^5 PFU YF17D/PyCS alone or followed by a boost with 5,000 irradiated sporozoites. Control groups were immunized with PBS, or with one (5000) or two doses of irradiated sporozoites (50000 followed by 5000 parasites). Low levels of anti-CSP antibodies were detected following primary immunization with YF17D/PyCS, and an increase in antibody titers was observed following the boost with irradiated sporozoites (>2.5 fold). Antibody levels induced following the YF17D/PyCS-prime, irradiated sporozoite-boost regimen, however, were not significantly different when compared to titers induced after a single immunization with 5000 irradiated sporozoites alone ($p > 0.01$, One-way ANOVA). Immunization with two doses of irradiated sporozoites yielded antibody titers that were significantly higher than those observed after one immunization with YF17D/PyCS followed by irradiated sporozoites or irradiated sporozoites alone ($p < 0.01$, One-way ANOVA) (Table 2). These results indicate that YF17D/PyCS induces only low levels of anti-CSP antibodies and that a heterologous boost is critical to augment the antibody response in mice.

3.5 Protection against sporozoite challenge conferred by immunization with YF17D/PyCS and a low-dose of irradiated sporozoites

To examine the potential of recombinant YFV to confer protection against challenge, we repeated the immunization schedules described above with groups of 10 mice and challenged the mice with 250 viable sporozoites three weeks following the final boost. Blood smear analysis of the immunized mice at 10 days post-challenge showed that all ten animals receiving 2×10^5 PFU of YF17D/PyCS followed by a boost with 5,000 irradiated sporozoites were completely protected against homologous challenge. Four out of ten animals administered a low dose (5000) of irradiated sporozoites alone were protected and all ten animals receiving two doses of irradiated sporozoites (50000 followed by 5000 sporozoites) were protected. None of the animals inoculated with a single dose of YF17D/PyCS or PBS alone were protected (Table 3). These results show that the YF17D/PyCS-prime, irradiated sporozoite-boost regimen is as effective as multiple doses of irradiated sporozoites at conferring immunity to *P. yoelii* challenge in mice.

3.6 Upregulation of CD86 in mouse bone marrow derived dendritic cells (BmDC) after exposure to YF17D/PyCS virus

Previous studies have shown that YF17D is capable to productively infect human DCs *ex vivo*, and infected DCs can process and present foreign antigens to CD8⁺ T-cells [12-14]. To determine if mouse DCs were susceptible to YF17D/PyCS infection or activation, we prepared BmDCs and inoculated them with a MOI of five. DCs were also exposed to LPS as a positive control. Thirty-six hours after infection, BmDC were analyzed by flow cytometry with anti-

CD11c DC marker, anti-CD86 DC activation marker, and anti-CSP (3F11) antibodies. Even though noticeable infection was not observed, upregulation of CD86 was apparent in cells that had been exposed to YF17D/PyCS or LPS (Figure 5). These results suggest the recombinant YF17D/PyCS is an effective activator of antigen presenting cells.

4. Discussion

Here we describe the construction and characterization of a recombinant YF17D virus expressing CSP (amino acids 57-344) from *Plasmodium yoelii*. A novel insertion site within the YF17D genome was explored, allowing creation of a stable recombinant virus. Previous studies have shown that manipulation of the YF17D capsid is well tolerated as long as the 5' cyclization sequence, which forms an essential interaction with a region at the 3' end of viral genome, is unperturbed [22,35,36]. We maintained the cyclization sequence upstream of the inserted *P. yoelii* gene, and recoded the duplicated N-terminal portion of the capsid to ablate the second cyclization site. Processing of PyCS from the YF17D polyprotein was achieved by insertion of the FMDV 2A peptide followed by ubiquitin, as has previously been described for hepatitis C virus (HCV) reporter genomes [37]. We determined that the YF17D/PyCS genome exhibited slightly delayed replication but was genetically stable throughout 6 passages in cell culture. Since we were able to harvest YF17D/PyCS virus with titers comparable to that of YF17D, we hypothesize that the altered kinetics may result from the increased length of the recombinant genome, or from unpredicted factors such as aberrant RNA secondary structures. Although, these recombinant viruses were genetically stable for only a limited number of passages in vitro, and *in vivo* stability can be unpredictable, virus stocks suitable for vaccination can be generated directly from electroporated cells without passaging to minimize loss of the CSP sequences.

We investigated the ability of the recombinant virus to elicit anti-CSP immune responses and protect against sporozoite challenge in adult mice. Inoculation of mice with 2×10^5 PFU of YF17D/PyCS, followed by boosting with 5000 irradiated sporozoites conferred protection against infection with live *P. yoelii*, while neither of the regimen components alone were completely protective. Previously, YF17D expressing a single CTL H2K^d restricted epitope derived from *P. yoelii* CSP was shown to induce long-lived IFN- γ -producing CD8⁺ T cells, and achieved sterile immunity in 9 out of 10 mice immunized with 5×10^5 PFU of virus followed by 10000 irradiated sporozoites [20]. In the present study, half the dose of recombinant YFV and half the number of irradiated sporozoites resulted in increased efficacy against challenge with three times the number of viable sporozoites. The higher protective efficacy of the YF17D/PyCS genome observed in our study may be a result of the broader repertoire of epitopes represented by the larger CSP insert. In addition to CD8⁺ T cell epitopes, such as immunodominant aa 280-288 [31,34] and subdominant aa 58-67, we included defined CD4⁺ T cell epitopes (aa 57-70 and aa 59-79), all of which have been associated with reduced parasitemia [39-41]. Given that adult mice are generally resistant to YF17D replication [42], the low level of immunity induced by the recombinant alone is not surprising. We speculate that experimental immunization of rhesus macaques, a broadly accepted model for yellow fever studies, would yield an even more pronounced anti-CSP response.

We observed induction of IFN- γ -producing CD4⁺ and CD8⁺ T cells after YF17D/PyCS immunization, which was further enhanced by a low dose of irradiated sporozoites. Extensive proliferation of CSP epitope-specific T cells from TCR-transgenic mice suggested the expansion included cell subsets targeting CS sequences. Notably, the IFN- γ response to the YF17D/PyCS-based regimen was remarkably superior to that induced by two doses of irradiated sporozoites in our study. High doses of irradiated or attenuated sporozoites, which elicit sterile immunity in experimentally infected rodents, primates, and human volunteers, mediate protection primarily through CD8⁺ T cell activation and the production of IFN- γ .

[43-45]. The presence of CD8⁺ T cells is similarly essential for immunity against liver-stage parasites in murine models of malaria [43,46]. A critical role for IFN- γ producing CD4⁺ T cells has also been highlighted in murine and in human malaria. For instance, adoptive transfer of a CD4⁺ T-cell clone that recognizes an epitope of *P. yoelii* CSP protects mice against sporozoite challenge [40,47]. Likewise, elevated levels of IFN- γ producing CD4⁺ T cells have been associated with protection from placental malaria [48]. IFN- γ is thought to inhibit parasite development thereby reducing re-infection, and high levels of IFN- γ have been associated with protection from parasitemia, clinical malaria, and anemia [49-51]. Finally, in contrast to the irradiated sporozoite immunizations, we observed a multifunctional response to YF17D/PyCS vaccination, in which IL-2 and IFN- γ -producing CD4⁺ T-cells were detected. IL-2 has also been associated with reduced parasitemia in infected individuals [52]. The strong role of the T cell response in anti-*Plasmodium* immunity was emphasized by the observation that the YF17D/PyCS-based regimen conferred protection despite significantly low-levels anti-CSP antibodies as compared to inoculation with irradiated sporozoites.

The efficient priming ability of the recombinant YF vaccine may reflect the activity of DCs. Although YF17D is known to productively infect human DCs [12,20], productive infection of mouse DCs or macrophages has not been reported. Mouse BmDCs, however, appeared to become activated upon exposure to the recombinant YFV, as measured by the upregulation of CD86. Previous studies have shown that exposure to YFV induces upregulation of CD86 in infected human DCs and in DCs that are not productively infected [12]. Activation of exposed but uninfected mouse BmDCs might be the result of abortive viral entry or binding to an unknown viral receptor.

In conclusion, we have developed a recombinant YF17D capable of inducing a strong T-cell mediated immune response targeting *P. yoelii* in adult mice. Effective priming by YF17D/PyCS substantially reduces the number of irradiated sporozoites required to confer immunity to mice. Fewer doses of a vaccine could result in a more cost-effective schedule, could reduce the number of vaccines in already crowded childhood vaccine schedules and may be especially beneficial for developing countries that have difficulty delivering a complete multiple-dose schedule. This reduction and potential elimination of the highly immunogenic sporozoite boost may hasten development of a much-needed malaria vaccine.

Acknowledgments

We thank Catherine Murray, John Schoggins, Svetlana Marukian, Maryline Panis, Anesta Webson, David Bowman, and Megan Holz for technical and editorial assistance.

This research was supported by funds from Foundation for the National Institutes of Health through the Grand Challenges in Global Health initiative, Marie-Josée and Henry Kravis Fellowship at The Rockefeller University, Greenberg Medical Research Institute, and The State of São Paulo Research Foundation (FAPESP).

References

1. Nussenzweig RS, Vanderberg JP, Most H, Orton C. Specificity of protective immunity produced by x-irradiated *Plasmodium berghei* sporozoites. *Nature* 1969 May 3;222(5192):488–9. [PubMed: 5768632]
2. Clyde DF. Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. *Am J Trop Med Hyg* 1975 May;24(3):397–401. [PubMed: 808142]
3. Nussenzweig V, Nussenzweig RS. Rationale for the development of an engineered sporozoite malaria vaccine. *Adv Immunol* 1989;45:283–334. [PubMed: 2665440]
4. Singh AP, Buscaglia CA, Wang Q, Levay A, Nussenzweig DR, Walker JR, et al. Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite. *Cell* 2007 Nov 2;131(3):492–504. [PubMed: 17981117]

5. Frevert U, Sinnis P, Cerami C, Shreffler W, Takacs B, Nussenzweig V. Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J Exp Med* 1993 May 1;177(5):1287–98. [PubMed: 8478608]
6. Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. RTS,S Malaria Vaccine Evaluation Group. *N Engl J Med* 1997 Jan 9;336(2):86–91. [PubMed: 8988885]
7. 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]
8. 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]
9. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. *Lancet* 2007 Nov 3;370(9598):1543–51. [PubMed: 17949807]
10. Theiler M, Smith HH. The use of yellow fever virus modified by in vitro cultivation for human immunization. *J Exp Med* 1937;65:787–800. [PubMed: 19870634] *Rev Med Virol* 2000 Jan-Feb;10 (1):6–16. discussion 3-5. [PubMed: 10654001]
11. Lefevre A, Marianneau P, Deubel V. Current Assessment of Yellow Fever and Yellow Fever Vaccine. *Curr Infect Dis Rep* 2004 Apr;6(2):96–104. [PubMed: 15023271]
12. Querec T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R, et al. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med* 2006 Feb 20;203(2):413–24. [PubMed: 16461338]
13. Barba-Spaeth G, Longman RS, Albert ML, Rice CM. Live attenuated yellow fever 17D infects human DCs and allows for presentation of endogenous and recombinant T cell epitopes. *J Exp Med* 2005 Nov 7;202(9):1179–84. [PubMed: 16260489]
14. Palmer DR, Fernandez S, Bisbing J, Peachman KK, Rao M, Barvir D, et al. Restricted replication and lysosomal trafficking of yellow fever 17D vaccine virus in human dendritic cells. *J Gen Virol* 2007 Jan;88(Pt 1):148–56. [PubMed: 17170447]
15. Guirakhoo F, Kitchener S, Morrison D, Forrat R, McCarthy K, Nichols R, et al. Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity: effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. *Hum Vaccin* 2006 Mar-Apr;2(2):60–7. [PubMed: 17012873]
16. Dean CH, Alarcon JB, Waterston AM, Draper K, Early R, Guirakhoo F, et al. Cutaneous delivery of a live, attenuated chimeric flavivirus vaccine against Japanese encephalitis (ChimeriVax-JE) in non-human primates. *Hum Vaccin* 2005 May-Jun;1(3):106–11. [PubMed: 17012854]
17. Monath TP, Liu J, Kanesa-Thasan N, Myers GA, Nichols R, Deary A, et al. A live, attenuated recombinant West Nile virus vaccine. *Proc Natl Acad Sci U S A* 2006 Apr 25;103(17):6694–9. [PubMed: 16617103]
18. Arroyo J, Miller C, Catalan J, Myers GA, Ratterree MS, Trent DW, et al. ChimeriVax-West Nile virus live-attenuated vaccine: preclinical evaluation of safety, immunogenicity, and efficacy. *J Virol* 2004 Nov;78(22):12497–507. [PubMed: 15507637]
19. Brandler S, Brown N, Ermak TH, Mitchell F, Parsons M, Zhang Z, et al. Replication of chimeric yellow fever virus-dengue serotype 1-4 virus vaccine strains in dendritic and hepatic cells. *Am J Trop Med Hyg* 2005 Jan;72(1):74–81. [PubMed: 15728870]
20. Tao D, Barba-Spaeth G, Rai U, Nussenzweig V, Rice CM, Nussenzweig RS. Yellow fever 17D as a vaccine vector for microbial CTL epitopes: protection in a rodent malaria model. *J Exp Med* 2005 Jan 17;201(2):201–9. [PubMed: 15657290]
21. Rice CM, Grakoui A, Galler R, Chambers TJ. Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. *New Biol* 1989 Dec;1(3):285–96. [PubMed: 2487295]

22. Hahn CS, Hahn YS, Rice CM, Lee E, Dalgarno L, Strauss EG, et al. Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. *J Mol Biol* 1987 Nov 5;198(1):33–41. [PubMed: 2828633]
23. 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]
24. Sano G, Hafalla JC, Morrot A, Abe R, Lafaille JJ, Zavala F. Swift development of protective effector functions in naive CD8(+) T cells against malaria liver stages. *J Exp Med* 2001 Jul 16;194(2):173–80. [PubMed: 11457892]
25. Lutz MB, Kukulski N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999 Feb 1;223(1):77–92. [PubMed: 10037236]
26. Amberg SM, Rice CM. Mutagenesis of the NS2B-NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. *J Virol* 1999 Oct;73(10):8083–94. [PubMed: 10482557]
27. Schlesinger JJ, Walsh EE, Brandriss MW. Analysis of 17D yellow fever virus envelope protein epitopes using monoclonal antibodies. *J Gen Virol* 1984 Oct;65(Pt 10):1637–44. [PubMed: 6208313]
28. Chambers TJ, McCourt DW, Rice CM. Yellow fever virus proteins NS2A, NS2B, and NS4B: identification and partial N-terminal amino acid sequence analysis. *Virology* 1989 Mar;169(1):100–9. [PubMed: 2922923]
29. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* 2001 Sep 17;194(6):769–79. [PubMed: 11560993]
30. Vaughan K, Blythe M, Greenbaum J, Zhang Q, Peters B, Doolan DL, et al. Meta-analysis of immune epitope data for all Plasmodia: overview and applications for malarial immunobiology and vaccine-related issues. *Parasite Immunol* 2009 Feb;31(2):78–97. [PubMed: 19149776]
31. Franke ED, Corradin G, Hoffman SL. Induction of protective CTL responses against the *Plasmodium yoelii* circumsporozoite protein by immunization with peptides. *J Immunol* 1997 Oct 1;159(7):3424–33. [PubMed: 9317141]
32. Romero P, Maryanski JL, Cordey AS, Corradin G, Nussenzweig RS, Zavala F. Isolation and characterization of protective cytolytic T cells in a rodent malaria model system. *Immunol Lett* 1990 Aug;25(1-3):27–31. [PubMed: 1704348]
33. Lal AA, de la Cruz VF, Welsh JA, Charoenvit Y, Maloy WL, McCutchan TF. Structure of the gene encoding the circumsporozoite protein of *Plasmodium yoelii*. A rodent model for examining antimalarial sporozoite vaccines. *J Biol Chem* 1987 Mar 5;262(7):2937–40. [PubMed: 3102479]
34. 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]
35. Molenkamp R, Kooi EA, Lucassen MA, Greve S, Thijssen JC, Spaan WJ, et al. Yellow fever virus replicons as an expression system for hepatitis C virus structural proteins. *J Virol* 2003 Jan;77(2):1644–8. [PubMed: 12502883]
36. Khromykh AA, Meka H, Guyatt KJ, Westaway EG. Essential role of cyclization sequences in flavivirus RNA replication. *J Virol* 2001 Jul;75(14):6719–28. [PubMed: 11413342]
37. Tscherne DM, Jones CT, Evans MJ, Lindenbach BD, McKeating JA, Rice CM. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J Virol* 2006 Feb;80(4):1734–41. [PubMed: 16439530]
38. Bonaldo MC, Mello SM, Trindade GF, Rangel AA, Duarte AS, Oliveira PJ, et al. Construction and characterization of recombinant flaviviruses bearing insertions between E and NS1 genes. *Virol J* 2007;4:115. [PubMed: 17971212]
39. Grillot D, Michel M, Muller I, Toungne C, Renia L, Mazier D, et al. Immune responses to defined epitopes of the circumsporozoite protein of the murine malaria parasite, *Plasmodium yoelii*. *Eur J Immunol* 1990 Jun;20(6):1215–22. [PubMed: 1695152]

40. Renia L, Marussig MS, Grillot D, Pied S, Corradin G, Miltgen F, et al. In vitro activity of CD4+ and CD8+ T lymphocytes from mice immunized with a synthetic malaria peptide. *Proc Natl Acad Sci U S A* 1991 Sep 15;88(18):7963–7. [PubMed: 1680235]
41. Del Giudice G, Grillot D, Renia L, Muller I, Corradin G, Louis JA, et al. Peptide-primed CD4+ cells and malaria sporozoites. *Immunol Lett* 1990 Aug;25(1-3):59–63. [PubMed: 1704350]
42. Zisman B, Wheelock EF, Allison AC. Role of macrophages and antibody in resistance of mice against yellow fever virus. *J Immunol* 1971 Jul;107(1):236–43. [PubMed: 4326399]
43. Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V. Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature* 1987 Dec 17-23;330(6149):664–6. [PubMed: 3120015]
44. Malik A, Egan JE, Houghten RA, Sadoff JC, Hoffman SL. Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *Proc Natl Acad Sci U S A* 1991 Apr 15;88(8):3300–4. [PubMed: 1707538]
45. 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]
46. Weiss WR, Sedegah M, Beaudoin RL, Miller LH, Good MF. CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc Natl Acad Sci U S A* 1988 Jan;85(2):573–6. [PubMed: 2963334]
47. 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]
48. Othoro C, Moore JM, Wannemuehler KA, Moses S, Lal A, Otieno J, et al. Elevated gamma interferon-producing NK cells, CD45RO memory-like T cells, and CD4 T cells are associated with protection against malaria infection in pregnancy. *Infect Immun* 2008 Apr;76(4):1678–85. [PubMed: 18250175]
49. Dodo D, Omer FM, Todd J, Akanmori BD, Koram KA, Riley EM. Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *Plasmodium falciparum* malaria. *J Infect Dis* 2002 Apr 1;185(7):971–9. [PubMed: 11920322]
50. D'Ombrain MC, Robinson LJ, Stanisic DI, Taraika J, Bernard N, Michon P, et al. Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children. *Clin Infect Dis* 2008 Dec 1;47(11):1380–7. [PubMed: 18947328]
51. Luty AJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, et al. Interferon-gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *J Infect Dis* 1999 Apr;179(4):980–8. [PubMed: 10068595]
52. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* 2009 Jul 30;361(5):468–77. [PubMed: 19641203]

Figure 1. Construction and viability of YF17D/PyCS

Schematic representation of recombinant YF17D/PyCS (A). Primers used to verify the integrity of the PyCS insert by RT PCR are depicted by arrows. Expression of *P. yoelii* CSP and various YFV proteins by recombinant and parental viruses as assessed by immunofluorescence (B) and by Western blot analysis (C).

Figure 2. In vitro characterization of YF17D/PyCS

A representative plaque assay of a virus kinetics experiment with samples harvested at 72 hours post-infection (hpi) (A) and a single-step growth curve depicting the growth of YF17D versus YF17D/PyCS (B). Infectivity of viral RNA as measured by an infectious center assay (C). RT PCR of viral RNA after 10 consecutive passages in BHK J cells at MOI=1. PCR primers are indicated in schematic of Figure 1A. Figures 2A is representative of three independent experiments. Figures 2B and 2C show the mean and the standard deviation of three experiments.

Figure 3. Induction of INF- γ , IL-2, and TNF- α -producing T-cells after immunization with YF17D/PyCS followed by a boost vaccination with irradiated sporozoites

BALB/c mice were injected with one or two doses of irradiated sporozoites, one or two doses of YF17D/PyCS, YF17D/PyCS plus irradiated sporozoites, or PBS. At day 37, lymphocytes were isolated from livers and spleens and assayed for the production of IFN- γ , IL-2 and/or TNF- α by flow analysis. Cells were gated based on the CD4⁺ and CD8⁺ phenotype and we used Boolean combinations of the three cytokines gates to discriminate responding cells based on their functionality with respect to cytokine production. Shown are the frequencies of Boolean-gated populations. Data shown is representative of three independent experiments with n=5 per experiment.

Figure 4. Proliferation of transgenic (tg) T-cells as a result of vaccination with YF17D/PyCS
 Shown are CFSE-labeled $CD8^+$ $Thy1.1^+$ lymphocytes and their mean fluorescent intensity is depicted on the left. This experiment was carried out with duplicate samples and the results are representative of two independent experiments.

Figure 5. Increased surface expression of murine CD86 activation marker after exposure to YF17D/PyCS

BmDCs were prepared from BALB/c mice and infected with an MOI of 5. Forty-eight hours following infection, BmDCs were analyzed for CD11c, PyCS, for CD86 expression. Induction of CD86 by LPS is shown as a positive control. Red line represents mock-infected cells, blue line represents YF17D/PyCS-infected cells, and green line represents LPS exposed cells.

Table 1
Humoral responses against yellow fever following prime (pre-) and boost (post-) immunizations

Groups	anti-YFV antibodies ^a	
	pre-	post-
YF17D/PyCS + IrrSpz	12.40 +/- 1.0	13.31+/-0.58
YF17D + IrrSpz	12.31 +/- 1.15	12.64+/-1
PBS	0	0

^aThe average reciprocal antibody dilution (LOG 2) +/- standard deviation.

Table 2
Humoral responses against *P.yoelii* following prime (pre-) and boost (post-) immunizations

Groups	anti-CSP antibodies ^a	
	pre-	post-
YF17D/PyCS	3.21+/- 2.83	3.21+/- 2.83
YF17D/PyCS + IrrSpz	3.21 +/-2.83	8.98 +/- 0.58
PBS + IrrSpz	0	7.31 +/- 0.58
PBS	0	0
IrrSpz + IrrSpz	12.64 +/- 1.00	15.32 +/- 0.59

^aThe average reciprocal antibody dilution (LOG 2) +/- standard deviation. This experiment is representative of three independent experiments.

Table 3
Parasitemia in immunized mice following challenge infection with 250 viable sporozoites

Groups	Parasitemia ^a	Protection (%)
YF17D/PyCS	20/20 ^a	0
YF17D/PyCS + IrrSpz	0/20	100
PBS + IrrSpz	8/20	60
PBS	20/20	0
IrrSpz + IrrSpz	0/20	100

^aThe number of parasitemic animals/total number of animals. This experiment is the sum of two independent experiments