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A prime-boost immunization regimen based on a simian adenovirus 36 vectored multi-stage malaria vaccine induces protective immunity in mice

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ABSTRACT

Malaria remains a considerable burden on public health. In 2015, the WHO estimates there were 212 million malaria cases causing nearly 429,000 deaths globally. A highly effective malaria vaccine is needed to reduce the burden of this disease. We have developed an experimental vaccine candidate (PyCMP) based on pre-erythrocytic (CSP) and erythrocytic (MSP1) stage antigens derived from the rodent malaria parasite *P. yoelii*. Our protein-based vaccine construct induces protective antibodies and $CD4^+$ T cell responses. Based on evidence that viral vectors increase $CD8^+$ T cell-mediated immunity, we also have tested heterologous prime-boost immunization regimens that included human adenovirus serotype 5 vector (Ad5), obtaining protective $CD8^+$ T cell responses. While Ad5 is commonly used for vaccine studies, the high prevalence of pre-existing immunity to Ad5 severely compromises its utility. Here, we report the use of the novel simian adenovirus 36 (SAd36) as a candidate for a vectored malaria vaccine since this virus is not known to infect humans, and it is not neutralized by anti-Ad5 antibodies. Our study shows that the recombinant SAd36*PyCMP* can enhance specific $CD8^+$ T cell response and elicit similar antibody titers when compared to an immunization regimen including the recombinant Ad5*PyCMP*. The robust immune responses induced by SAd36*PyCMP* are translated into a lower parasite load following *P. yoelii* infectious challenge when compared to mice immunized with Ad5*PyCMP*.

1. Introduction

Malaria remains a considerable burden on public health. In 2015, there were an estimated 212 million cases of malaria and 429,000 deaths, most of which occurred in children under 5 years of age, making it a leading cause of death in this population [1]. A vaccine against malaria is a research priority and an essential tool for control and elimination efforts.

The development of an effective malaria vaccine has been a challenge due to the complex *Plasmodium* life cycle [2]. The pre-erythrocytic stage is initiated by the injection of sporozoites into the dermis by *Anopheles* spp mosquitos. The sporozoites then travel via the circulatory system to invade hepatocytes, where the parasite then differentiates and produces tens of thousands of infectious merozoites per infected hepatocyte that burst to release the parasites into the circulatory system where they invade erythrocytes. Within the erythrocyte, the parasite again undergoes a cycle of growth and proliferation, ultimately resulting in the bursting of the erythrocyte and the release of new infectious merozoites to continue the erythrocytic stage of infection, producing the clinical symptomatology associated with malaria.

Several key proteins are responsible for the motility and invasion of the infectious forms into their respective target cells. During the pre-erythrocytic stage, the circumsporozoite protein (CSP) and the thrombospondin-related adhesive protein (TRAP) are responsible for the gliding motility and infectivity of the sporozoite [3,4]. Similarly, the merozoite surface protein 1 (MSP1) is involved in the invasion of the merozoite into the erythrocyte, and antibodies targeting $MSP1_{19}$ have been found to inhibit merozoite invasion of erythrocytes in humans [5].

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Many malaria vaccine candidates have therefore aimed to target the pre-erythrocytic antigens to prevent hepatocyte infection and the erythrocytic antigens to prevent clinical manifestations. Radiation-attenuated sporozoites have been used to produce sterilizing immunity, but this method remains impractical for widespread use due to logistical constraints [2]. Furthermore, the most advanced malaria vaccine, RTS,S/AS01, has failed to produce long-lived efficacy [6,7], likely due to lack of $CD8^+$ T cell responses induced and its design based on a single pre-erythrocytic stage antigen target, CSP [8], as a single sporozoite that evades immune responses induced against CSP can produce tens of thousands of blood stage merozoites [2,9,10]. Moreover, preclinical murine studies on a vaccine candidate based on two pre-erythrocytic-stage antigens, CSP and the thrombospondin-related adhesive protein (TRAP), have not shown increased efficacy compared to single antigen vaccines [11]. Therefore, we hypothesize that a malaria vaccine targeting multiple *Plasmodium* stages is necessary for optimal induction of protective immunity.

We have designed *P. yoelii* chimeric recombinant protein-based vaccines, constructed by binding of cognate promiscuous T cell epitopes (i.e. capable of binding to 10 or more MHC class II molecules) to well characterized B-cell epitopes, representing CSP and the erythrocytic-stage antigen merozoite surface protein 1 (MSP1) [12,13]. We also have expressed a hybrid protein by genetic fusion of the chimeric CSP and MSP-1 proteins [14], designated *P. yoelii* Chimeric Multistage Protein (PyCMP). This vaccine protected mice from experimental challenge through induction of $CD4^+$ T cells and antibodies [14]. However, the lack of induction of protective $CD8^+$ T cells led us to pursue an adenovirus-vectored malaria vaccine, and we reported that an Ad5 prime and two proteins boosts significantly increased the PyCMP protective effect [15].

Despite the relevance of the Ad5-based vector as promising vaccine platform, adult populations exhibit a high prevalence of pre-existing anti-Ad5 neutralizing antibodies, limiting its effectiveness [16,17]. Simian adenoviruses provide a promising alternative, as they maintain the same safety profile as Ad5 [16,18] and the level of anti-vector neutralizing activity of human sera has been found to be low [19]. In addition, the use of simian adenoviruses in Ebola Virus [20,21], HIV [18], HCV [22], and malaria [23–26] vaccine candidates provides further support for the safety and utility of these vectors.

Here we evaluated the immunogenicity and protective efficacy of a heterologous Ad prime – protein boost vaccination regimen, testing three different doses of the simian adenovirus 36 (SAd36), a vector resistant to neutralizing anti-Ad5 antibodies [19]. This vector was engineered to express the synthetic PyCMP gene. We show that immunization regimens including SAd36-*PyCMP* improves immunogenicity and efficacy in comparison to Ad5 vectored PyCMP, making SAd36 a promising vector for the development of an effective malaria vaccine.

2. Materials and methods

2.1. Viral vectors

The replication incompetent Ad5*PyCMP* vector was constructed using the E1-deleted Ad5 backbone as we previously described [15,27]. To construct the genome of simian adenovirus 36 SAd36 from species E containing the *PyCMP* transgene cassette in place of the deleted E1A/B genes we used the strategy originally described by Roy et al. [19]. We employed the E1-deleted molecular clone pC36.000.CMV.PI.EGFP.BGH (p1411) of an SAd36 vector expressing eGFP and a pShuttle plasmid that were kindly provided by Dr. James M. Wilson (Penn Vector Core – Gene Therapy Program, University of Pennsylvania). The *PyCMP*-coding sequence was cloned into the pShuttle plasmid between the CMV promoter and BGH polyadenylation signal. The expression cassette was excised with I-CeuI and PI-SceI restriction enzymes and ligated to plasmid DNA containing the SAd36 genome, which was linearized using unique I-CeuI and PI-SceI restriction sites introduced in place of E1 region. The ligated DNA was transformed into *E. coli* strain, XL10-Gold (Stratagene), to select the plasmid containing viral genome carrying the CMV-driven PyCMP transgene. The constructed genome was released from plasmid DNA by digestion with PacI and transfected into HEK293 cells to rescue the replication incompetent SAd36*PyCMP* vector. Both SAd36*PyCMP* and SAd36-GFP vectors were upscaled in HEK293 cells and then purified using double cesium chloride gradient centrifugation as described [28]. The purified vector preparations were dialyzed against PBS containing 10% glycerol, and viral particle (vp) titers were determined based on absorbance at 260 nm as described by Maizel et al. [29].

2.2. Chimeric vaccine, immunization regimens, and parasites

The *PyCMP* gene is a 1242 bp gene encoding a chimeric antigen based on the *P. yoelii* CSP genetically linked to a chimeric *P. yoelii* MSP1. The transgene expression and purification have been previously described [14].

Female CB6F1/J ($H\text{-}2^{d/b}$) mice, 6–8 weeks of age, were purchased from Jackson Laboratory. This strain was selected based on the response of syngeneic mice to chimeric antigens [31] and to characterize T cells response via the $H\text{-}2K^d$/SYVPSAEQI/APC tetramer (Tetramer Core Facility, Emory University) which includes the CTL epitope of the *P. yoelii* CSP included in PyCMP. Mice (n = 10 per group) were primed intramuscularly at day 0 with recombinant SAd36 at a dose of 10^6 (Low dose), 10^7 (Intermediate dose), or 10^{10} v.p. (High dose), or the previously protective recombinant Ad5 10^7 v.p. dose [15,30]. All mice received two boosting immunizations with 20 μg of PyCMP emulsified in Montanide ISA 51 (Seppic, Fairfield, NJ) at days 30 and 60. Naïve mice (n = 10) were used as a control (Table 1). Mice were bled 20 days after each

Table 1
Immunization groups. Mice were immunized intramuscularly at day 0 with adenovirus in PBS at the dose corresponding to their group. Mice were then boosted subcutaneously at days 30 and 60 with 20 μg of the *P. yoelii* chimeric multistage protein (PyCMP) emulsified in Montanide ISA 51 VG in a 1:1 volume ratio. Control group mice received no immunizations ($n = 10$ per group). Mice were bled for analysis of antibody titers via ELISA 20 days after each immunization. PBMCs were obtained from mouse whole blood samples at days 10, 20, 40, 50, and 70 post priming and were processed for flow cytometry.

Immunization group	Priming, Day 0		Protein Boost 1, Day 30	Protein Boost 2, Day 60
	Ad-*transgene*	Dose		
SAd36 10^6	SAd36-*PyCMP*	10^6 v.p.	PyCMP	PyCMP
SAd36 10^7	SAd36-*PyCMP*	10^7 v.p.	PyCMP	PyCMP
SAd36 10^{10}	SAd36-*PyCMP*	10^{10} v.p.	PyCMP	PyCMP
Ad5 10^7	Ad5-*PyCMP*	10^7 v.p.	PyCMP	PyCMP
Control	No immunization		No immunization	No immunization

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