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Inclusion of the murine IgGκ signal peptide increases the cellular immunogenicity of a simian adenoviral vectored *Plasmodium vivax* multistage vaccine

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ABSTRACT

Introduction: Cellular and humoral immune responses are both involved in protection against *Plasmodium* infections. The only malaria vaccine available, RTS,S, primarily induces short-lived antibodies and targets only a pre-erythrocytic stage antigen. Inclusion of erythrocytic stage targets and enhancing cellular immunogenicity are likely necessary for developing an effective second-generation malaria vaccine. Adenovirus vectors have been used to improve the immunogenicity of protein-based vaccines. However, the clinical assessment of adenoviral-vectored malaria vaccines candidates has shown the induction of robust *Plasmodium*-specific CD8+ but not CD4+ T cells. Signal peptides (SP) have been used to enhance the immunogenicity of DNA vaccines, but have not been tested in viral vector vaccine platforms.
Objectives: The objective of this study was to determine if the addition of the SP derived from the murine IgGκ light chain within a recombinant adenovirus vector encoding a multistage *P. vivax* vaccine candidate could improve the CD4+ T cell response.
Methods: In this proof-of-concept study, we immunized CB6F1/J mice with either the recombinant simian adenovirus 36 vector containing the SP (SP-SAd36) upstream from a transgene encoding a chimeric *P. vivax* multistage protein or the same SAd36 vector without the SP. Mice were subsequently boosted twice with the corresponding recombinant proteins emulsified in Montanide ISA 51 VG. Immunogenicity was assessed by measurement of antibody quantity and quality, and cytokine production by T cells after the final immunization.
Results: The SP-SAd36 immunization regimen induced significantly higher antibody avidity against the chimeric *P. vivax* proteins tested and higher frequencies of IFN-γ and IL-2 CD4+ and CD8+ secreting T cells, when compared to the unmodified SAd36 vector.
Conclusions: The addition of the murine IgGκ signal peptide significantly enhances the immunogenicity of a SAd36 vectored *P. vivax* multi-stage vaccine candidate in mice. The potential of this approach to improve upon existing viral vector vaccine platforms warrants further investigation.

1. Introduction

The life cycle of *Plasmodium* parasites is known for its complexity, and as a result, immunity to malaria infections in vertebrates relies on both humoral and cellular immune responses. Early passive transfer experiments demonstrated the protective role of IgG antibodies derived from malaria immune adults when used as a therapeutic intervention [1]. Clinical trials of sporozoite inoculation have revealed that IFN-γ producing T cells are associated with protection against malaria [2]. Based on this evidence, a multistage vaccine capable of eliciting both cytophilic antibodies and antigen-specific T cells would likely enhance the protective efficacy of a comprehensive vaccination strategy.

The RTS,S/AS01 vaccine represents a significant breakthrough as the first *P. falciparum* malaria vaccine that has completed Phase 3

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clinical trials [3]. However, RTS,S has reported low efficacy due in part to protection based primarily on antibodies against the circumsporozoite protein (CSP) central repeat region [4] present in pre-erythrocytic stage forms, which wane rapidly and require boosting immunizations to maintain efficacy [5]. The inclusion of erythrocytic stage targets to control parasites that evade liver clearance and enhancing cellular immunogenicity are likely necessary for developing an effective second generation of malaria vaccines.

Adenoviral vectored malaria vaccines have been able to improve the immunogenicity of protein-based vaccines [6–9] and induce protective *Plasmodium*-specific CD8+ T cells in pre-clinical and clinical studies [10–12], but low induction of CD4+ T cell suggests further improvements to adenoviral vectors should be investigated [11]. Recent studies examining the induction of CD4+ T cells following vaccination with an Ad5 vector have shown that significantly lower frequencies of antigen-specific CD4+ T cells are induced when compared to acute infection, an effect that could be attributed to lower IL-2 signaling [13]. Increasing secretion or altering post-translational modifications of adenoviral transgene products might result in improved presentation of vaccine antigens to CD4+ T cells.

Signal peptides (SP), also referred to as signal sequences, are short peptides (~20–30 residues) that can influence the targeting pathway of the protein and promote protein secretion or specific post-translational modifications such as glycosylation [14]. As a result, SP from highly secreted proteins have been used to improve protein secretion levels of recombinant proteins in cell lines [15–17], as well as for ectopic expression of endogenous adenoviral genes [18]. Recently, the inclusion of an SP into a DNA vaccine targeting HPV oncogenes was found to induce potent cellular and humoral immune responses that protected against tumor challenge [19]. Of the signal peptides used to improve transgene expression, the sequence derived from the murine immunoglobulin kappa (IgGκ) light chain (METDTLLLWVLLLWVPGSTG), is one of the most well characterized [15–17].

We hypothesized that the addition of the signal peptide derived from murine IgGκ light chain upstream of a transgene delivered via a recombinant adenovirus vector would improve the CD4+ T cell response to the transgene product in comparison to vaccination with the same recombinant vector without the signal peptide [10]. Here we demonstrate that the addition of the murine IgGκ SP improves the immunogenicity of an adenoviral vectored *P. vivax* multistage vaccine [20,21] in mice by significantly increasing IFN-γ and IL-2 secretion by CD4+ T cells, and improving antibody avidity. To our knowledge, this is the first report of the insertion of a signal peptide sequence as part of an adenoviral transgene with the goal of improving the immunogenicity of an adenoviral vectored vaccine candidate.

2. Material and methods

2.1. Viral vectors

The DNA sequence encoding the hybrid cPvCSP/cPvMSP1 protein containing the C-terminal six-His tag was codon-optimized for mammalian expression and incorporated into a pShuttle plasmid between the CMV promoter and BGH polyadenylation signal. The constructed plasmid was further modified to introduce the N-terminal SP into the hybrid cPvCSP/cPvMSP1 protein. The oligonucleotide duplex encoding IgGκ light chain SP was cloned into KpnI restriction site upstream of the cPvCSP/cPvMSP1 transgene resulting in additional three amino acids (Tyr-Pro-Thr) introduced between the signal peptidase consensus cleavage site and the first Met start codon of the cPvCSP/cPvMSP1 transgene. Both cPvCSP/cPvMSP1 or SP-cPvCSP/cPvMSP1 expression cassettes were excised with I-CeuI and PI-SceI restriction enzymes and ligated to plasmid carrying the SAd36 genome using unique I-CeuI and PI-SceI restriction sites introduced in place of E1 region, as previously described [10,22].The ligated DNA was transformed into *E. coli* strain, XL10-Gold (Stratagene), to select the plasmids containing viral genomes carrying the CMV-driven cPvCSP/cPvMSP1 and SP-cPvCSP/cPvMSP1 expression cassettes. The constructed genomes were released from the plasmids by digestion with PacI restriction enzyme and were then transfected into HEK293 cells to rescue the replication incompetent SAd36 vector derivatives as described elsewhere [10]. Both vectors were upscaled in HEK293 cells and purified using double cesium chloride gradient centrifugation as previously described [23]. 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. [24].

2.2. In vitro viral vector culture and western blot analysis

To assess the expression levels of cPvCSP/cPvMSP1 and SP-cPvCSP/cPvMSP1 transgenes, monolayers of A549 cells grown in 6-well plates were incubated for 1 h with either vector at the multiplicity of infection (MOI) of 2500 vp/cell. Infection medium (DMEM/F-12, 1:1) containing 2% FBS was replaced with fresh culture medium containing 5% FBS and cells were incubated at 37 °C and 5% CO_2 for at least 48 h to allow transgene expression. The samples of cell lysates and culture medium supernatants were collected and analyzed by Western blot using anti-six-His tag mAb Penta-His (QIAGEN) and polyclonal IgG purified from sera of rabbits immunized with the cPvCSP or the cPvMSP1 chimeric proteins (Covance Inc.).

2.3. Chimeric protein vaccine design and peptide pools

We have previously described the synthetic genes encoding the chimeric *P. vivax* CSP (cPvCSP) [20] and the chimeric merozoite surface protein 1 (cPvMSP1) [21]. These chimeric proteins include several promiscuous T cell epitopes (PTE) capable of binding to multiple human HLA alleles and at least one B cell epitope, with each region separated by GPGPG spacers to enhance stability. cPvCSP contains (1) two PTE from the C-terminal region of *P. vivax* CSP; (2) the conserved region I of *P. vivax* CSP; (3) VK210 type 1 repeat sequence variants, and (4) three copies of the 9-mer peptide representing the VK247 type 2 repeat sequence variant [20]. cPvMSP1 includes (1) five PTE from PvMSP1; (2) an extended $PvMSP1_{19}$ fragment that includes two T helper epitopes derived from $PvMSP1_{33}$; and (3) six copies of the CSP repeat region NANP derived from *P. falciparum* included as a purification tag [21]. Production of the transgenes and proteins have been described previously [20,21].

Peptide libraries containing 15-mer synthetic peptides, overlapping by 11 residues each and spanning the complete sequence of both cPvCSP and cPvMSP1 were commercially synthesized (Sigma-Aldrich), and used to characterize T cell reactivity to specific protein regions as described [20,21]. The cPvCSP peptide library was separated into 4 pools, with pool A representing the first PTE in cPvCSP; pool B representing the second PTE and the region I; pool C representing the VK210 repeat sequences; and pool D representing the VK247 repeat sequences [20]. The two cPvMSP1 peptide pools represented the cPvMSP1 PTE (pool 1) and the PTE derived from $PvMSP\text{-}1_{33}$ and the $PvMSP\text{-}1_{19}$ protein fragment (pool 2) [21].

2.4. Mouse immunizations

Female CB6F1/J ($H\text{-}2^{d/b}$) mice, aged 6–8 weeks were obtained from the Jackson Laboratory and housed in micro-isolation cages. We have previously assessed the immune response of six inbred

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