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14. ABSTRACT

Proposed vaccine principle relies on secreted gp96-lg chaperoning PfCSP and PfAMA 1 sporozoite proteins that are efficiently taken up and cross presented by activated DC via MHC I to CD8 CTL, thereby stimulating an avid, antigen specific, cytotoxic T cell response. This vaccine principle has been used successfully in murine models of cancer, in non-human primates for SIV vaccination and is in clinical trials for the treatment of non-small cell lung cancer patients. The generation of a powerful, cytotoxic anti sporozoite CD8 CTL response by the vaccine is expected to provide prophylactic immunity for malaria by removing infected liver cells before sporozoites can replicate and spread to the erythrocyte stage causing parasitemia.

In the second year, we performed and completed all mouse immunogen icity experiments that addressed the effect of primary 293-gp96-lg PfAMA 1-PfCSP immunization as well as effect of different route of immunization on the gp96-induced immunogenicity. We found that gp96 delivered subcutaneously, induces very strong antigen specific immune response systemically as well as liver-infiltrating effector CD8 + T cells. We have also started manufacturing GMP-grade vaccine material for use in non-human primate studies.

15. SUBJECT TERMS- Malaria, Plasmodium Falciparum, circumsporozoite protein (CSP), apical membrane antigen-1, vaccine (AMAI), heat shock proteins, gp96-lg, cytotoxic T cells, cell mediated immunity

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1. INTRODUCTION:

We have previously shown that cell-based vaccines secreting heat shock protein gp96-Ig (for short from here on: **gp96**) are safe for use in humans and represent the most efficient vaccine approach studied to date for stimulating multi-epitope specific cytotoxic T cells. In the proposed studies, we will adapt this vaccine approach to stimulate cytotoxic T cells against malaria antigens and investigate the optimal vaccination route to target these T cells to the liver. To accomplish these studies, we are collaborating with experts in the malaria vaccine field, Capt. Eileen F. Villasante, M.D., Ph.D., Head Malaria Department Infectious Diseases Directorate at Naval Medical Research Center and Lt. Kimberly A. Edgel, PhD, Deputy Head, Malaria Department Infectious Diseases Directorate at Naval Medical Research Center. By conducting head-to-head studies to another promising malaria vaccine, these studies will help to set clinical priorities based on the most effective pre-clinical data in animal models.

2. KEYWORDS:

Malaria, Plasmodium Falciparum, circumsporozoite protein (CSP), apical membrane antigen-1, vaccine (AMA1), heat shock proteins, gp96-Ig, cytotoxic T cells, cell mediated immunity

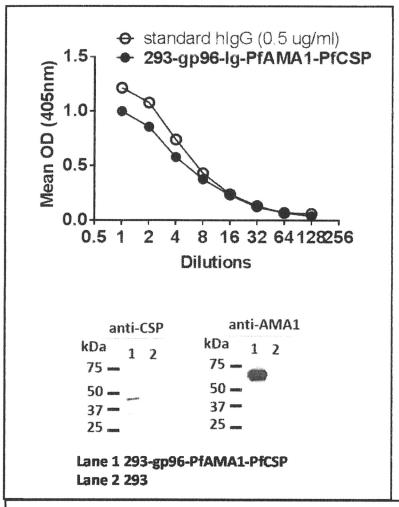
3. OVERALL PROJECT SUMMARY:

The goal of our project is to combine the *Plasmodium falciparum* (Pf) antigens circumsporozoite protein (CSP) and apical membrane antigen-1 (AMA1) with a novel method of immunization that is based on the gp96-Ig vaccine platform to enable production of a strong, protective, cell-mediated immunity (CMI) response (interferon gamma [IFN-γ]-positive CD8+ cytotoxic T cells).
This will be accomplished through three specific aims: (1) construction of the 293-gp96-$Ig^{PfAMA1\text{-}PfCSP}$ and $293^{PfAMA1\text{-}PfCSP}$ vaccine cell lines; (2) determination of the safety and immunogenicity of the 293-gp96-$Ig^{PfAMA1\text{-}PfCSP}$ vaccine in mice; and (3) determination of the safety and immunogenicity of the 293-gp96-$Ig^{PfAMA1\text{-}PfCSP}$ vaccine in rhesus macaques.

Summary of Current Objectives: During the last year we have been intensively working on the experiments related to **Specific Aim 2: Determination of the safety and immunogenicity of the 293-gp96-$Ig^{PfAMA1\text{-}PfCSP}$ vaccine in mice and Specific Aim 3, Task 3a:** Manufacturing of GMP-grade vaccine material for use in non-human primate studies (Task 3b).

Summary of Results:

To determine the potential protective efficacy of the gp96-Ig vaccine in future studies, we first determined the **immunogenicity of 293-gp96-$Ig^{PfAMA1\text{-}PfCSP}$ in mouse model** and performed experiments proposed in **Task 2a.** Our previous studies established methods for primary immunization with gp96-Ig vaccines that lead to CD8+ T cell proliferation which peaks at 4-5 days after immunization and can be detected in blood, spleen and different tissues (gut, reproductive tract).
We generated 293-gp96-$Ig^{PfAMA1\text{-}PfCSP}$ and as shown in Figure 1, confirmed that vaccine cells produce 0.5 μg/ml gp96-Ig and express plasmodium falciparum (Pf) antigens CSP and AMA1. Production of gp96-Ig was measured by established ELISA protocol using supernatant form $1x10^6$ cells that are cultured in 1 ml for 24h. Western blotting with anti-PfCSP and anti-PfAMA1 as primary antibody and anti IgG-HRP as secondary labeled antibody confirmed expression of PfCSP and PfAMA1 protein (Figure 1).



B6 mice were vaccinated with 293-gp96-Ig$^{PfAMA1\text{-}PfCSP}$ by intraperitoneal route (Figure 2). We also used two Mock controls: 293-gp96-Ig and PBS. Four days after immunization, mice were sacrificed, spleen was collected and AMA1 and CSP specific CD8 T cells responses were measured by intracellular cytokine staining (ICS) assay. Capt. Eileen F. Villasante, M.D., Ph.D. and Lt. Kimberly A. Edgel, PhD provided us with the two pools of overlapping CSP and AMA1 peptides that we used to stimulate lymphocytes obtained from spleen *in vitro* and measure the production of IFN-γ and TNF-α, by intracellular cytokine staining and flow cytometry to assess the specificity of the gp96-Ig induced CD8 T cells. Only in animals that were vaccinated with 293-gp96-Ig$^{PfAMA1\text{-}PfCSP}$, PfCSP and PfAMA1 specific CD8+ T cells were observed

Figure 1. 293-gp96-Ig-PfAMA1-PfCSP ELISA for gp96-Ig production and WB for PfCSP and PfAMA1 expression. One million 293-gp96-Ig-PfAMA1-PfCSP cells were plated in 1 ml for 24 h and gp96-Ig production in the supernatant was determined by ELISA using anti-human IgG antibody for detection with human IgG1 as a standard. Western blot of 293-gp96-Ig-PfAMA1-PfCSP: 293- gp96-Ig-PfAMA1-PfCSP cells were analyzed by SDS-PAGE and Western blotting with anti-PfCSP and anti-PfAMA1 as primary antibody and anti IgG-HRP as secondary labeled antibody.

(Figure 2 A and B) while their frequencies were at the background level for the animals that received Mock control (293-gp96-Ig or PBS, Figure 2A). We found both, PfCSP and PfAMA1 specific CD8+ T cells that produce IFNγ and TNFα (Figure 2 B).
Since the objective of the Task 2a was to find the most effective immunization route before

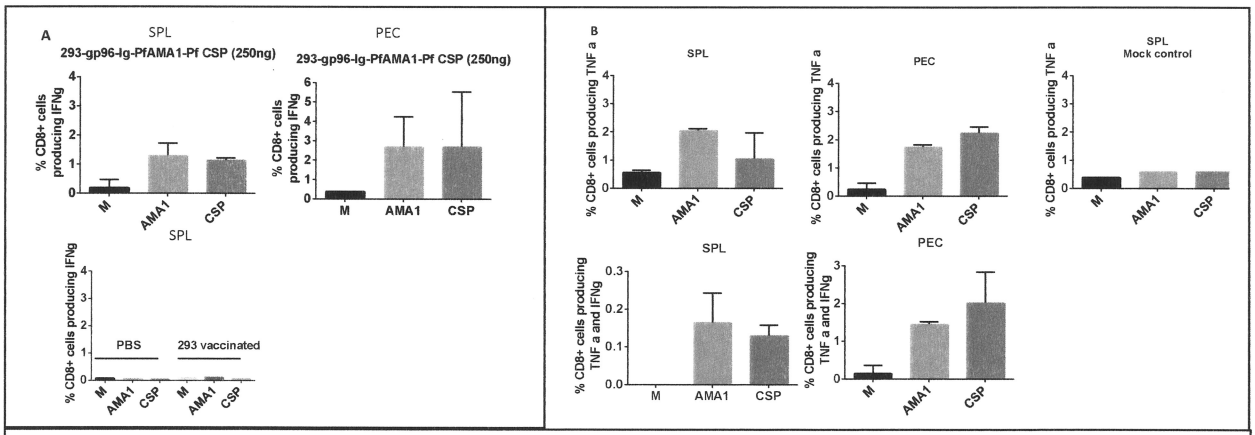


Figure 2. 293- gp96-Ig-PfAMA1-PfCSP vaccine induces PfAMA1- and PfCSP- CD8 specific immune responses. 293-gp96-Ig and 293-gp96-Ig-PfAMA1-PfCSP cells or PBS were injected in B6 mice by intraperitoneal route. Four days later, mice were sacrificed and frequency of PfAMA1 and PfCSP specific CD8 T cell responses in the spleen (SPL) and peritoneal cavity (PEC) were measured by intracellular cytokine staining (ICS) assay and analyzed by flow cytometry. Total SPL or PEC cells were cultured overnight in medium only or with pool of overlapping PfAMA1 and PfCSP peptides. Cells were stained for surface CD3, CD8 and intracellular cytokines IFNg and TNFa . Cells were analyzed on flow cytometer and bar graph shows percentage of CD3+CD8+ cells that produce IFNg (A) or TNFa (B) or both (B).

advancing to Task 2b, we set up experiments shown in Figure 3. We found that the best route of vaccination for induction of AMA1 and CSP specific CD8 T cell responses was intraperitoneal and subcutaneous route (Figure 3). Since the translation of intraperitoneal route of vaccination to the clinical settings could present a big hurdle, we decided to pursue subcutaneous delivery of gp96-Ig vaccine in all future immunogenicity experiments (mouse and nonhuman primate in vivo experiments). Furthermore, we confirmed that subcutaneous route of vaccination induces dramatic increase in the liver-infiltrating CD8+ T cells (Figure 4). We found that after gp96-Ig immunization, 80% of all CD3+ cells in the liver are CD8 + T

lymphocytes. These liver-infiltrating CD8+ T, in contrast to CD4+ T cells, are mostly effector memory cells (CD44+CD62L- cells) (Figure 4).

To facilitate progression from these studies to nonhuman primate studies (Task 3b) and eventually to IND and Phase I clinical testing of 293-gp96-Ig$^{PfAMA1\text{-}PfCSP}$, we will utilize GMP-grade vaccine material. In our previous Phase I clinical trial testing the safety of a gp96-Ig vaccine in NSCLC patients, GMP grade material was produced at the University of Miami GMP manufacturing facility. Thus, the first objective of specific aim 3 is to manufacture GMP-grade vaccine material. Vaccine cells that were generated in our laboratory (Figure 1) have been significantly improved regarding the gp96-Ig production (Figure 5). In order to establish the vaccine cell line that can be manufactured in GMP facility, we went through the process of single cell cloning to select for the vaccine cells with the highest production of gp96-Ig as well as AMA1 and CSP protein expression (Figure 5). We developed vaccine cell 293-gp96-Ig$^{PfAMA1\text{-}PfCSP}$ that produced app. 2μg/ml gp96-Ig in a standardized ELISA assay (Figure 5. Clone H5, D5, D7, D8) and Clone D7 and D8 are currently been assessed for sterility in order to be transferred to GMP facility for manufacturing.

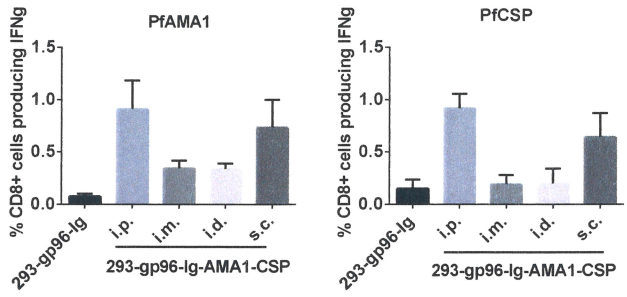


Figure 3. Induction of PfAMA1 and PfCSP specific CD8 T cell responses by different route of 293-gp96-Ig-PfAMA1-PfCSP vaccination. One million 293-gp96-Ig-PfAMA1-PfCSP cells were injected in B6 mice by intraperitoneal (i.p.), intra muscular (i.m.), intradermal (i.d.) and subcutaneous (s.c.) route. Five day later mice were sacrificed and frequency of PfAMA1 and PfCSP specific CD8 T cell responses in the spleen (SPL) were measured after in vitro peptide stimulation by intracellular cytokine staining (ICS) assay and analyzed by flow cytometry. Control mice were injected with one million of 293-gp96-Ig cells by i.p. route. Bar graph shows percentage of PfAMA1 and PfCSP specific CD3+CD8+ cells that produce IFNg

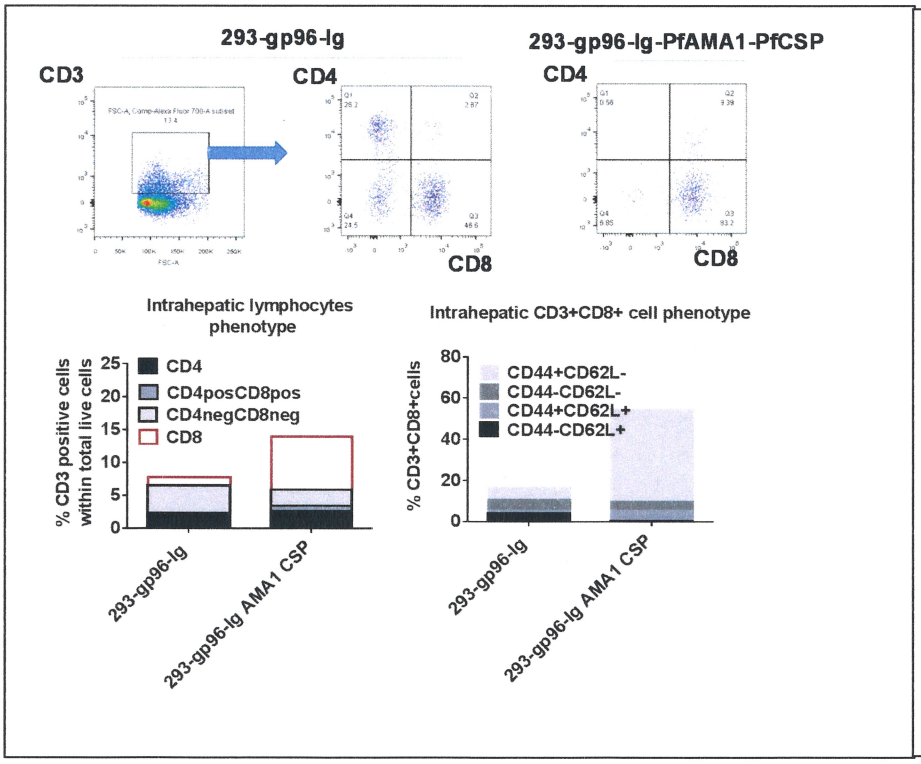


Figure 4. Subcutaneous route of vaccination induces dramatic increase in the liver-infiltrating CD8+ T cell. One million 293-gp96-Ig and 293-gp96-Ig-PfAMA1-PfCSP cells were injected by s.c. route in B6 mice. Five day later mice were sacrificed, intrahepatic lymphocytes were isolated and frequency of PfAMA1 and PfCSP specific intrahepatic CD8 T cell responses were measured after in vitro peptide stimulation by intracellular cytokine staining (ICS) assay and analyzed by flow cytometry. Bar graphs shows percentage of intrahepatic CD3+ subsets and phenotype of intrahepatic CD3+CD8+ cells regarding the CD44 and CD62L surface expression.

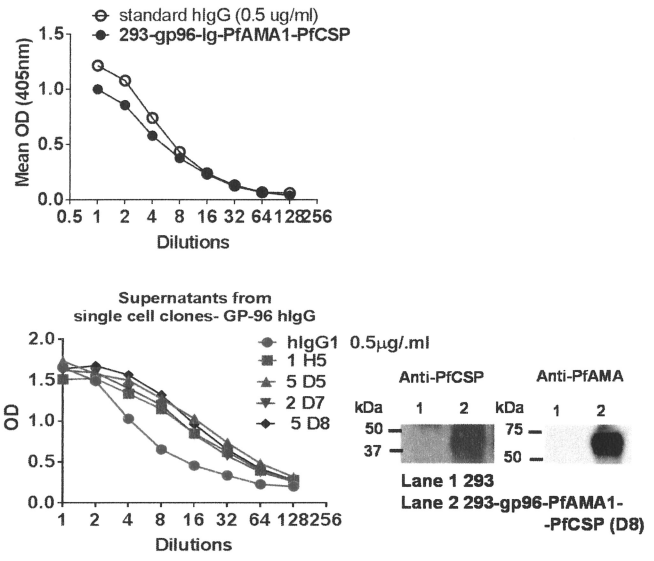


Figure 5 Single cell cloning of gp96-Ig malaria vaccine. : One million of 293-gp96-Ig-PfAMA1-PfCSP cells and different single cell clones (H5, D5, D7, D8) were plated in 1 ml for 24 h and gp96-Ig production in the supernatant was determined by ELISA using anti-human IgG antibody for detection with mouse IgG1 as a standard. **Western blot of 293- gp96-Ig-PfAMA1-PfCSP.** 293- gp96-Ig-PfAMA1-PfCSP cells (single cell clone D8) were analyzed by SDS-PAGE and Western blotting with anti-PfCSP and anti-PfAMA1 as primary antibody and anti IgG-HRP as secondary labeled antibody

Summary of Progress and Accomplishment with Discussion:

We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine cell line is immunogenic in the mouse model. We found that subcutaneous route of vaccination induces dramatic increase in the liver-infiltrating CD8+ T cells. Importantly, the magnitude of malaria antigen-specific CD8+ T cell responses is believed to be the best measure of immunity targeting the hepatic stages of infection and the failure of the RTS,S vaccine to stimulate CD8+ cytotoxic T cell immunity was a significant weakness in the approach. Our findings are strongly supportive of the novel gp96-Ig malaria vaccine as unique systemic and liver-homing, sporozoite specific CD8 CTL vaccine strategy.

Following successful completion of our milestones, we have already proceed to experiments under Specific Aim 2b (side by side comparison of memory responses induced by 293-gp96-IgPfAMA1-PfCSP vaccine cell and NMRC-M3V-D/Ad-PfCA) and we will have results in the following weeks.

4. KEY RESEARCH ACCOMPLISHMENTS:

We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine cell line is immunogenic in the mouse model and characterized subcutaneous route of vaccination as the appropriate immunization route for optimal PfAMA1 and PfCSP specific CD8+ responses within the liver.

We generated vaccine cells that produce/secrete high level of gp96-Ig and plasmodium falciparum antigens (Pf) AMA1 and CSP and starting the manufacturing of GMP-grade vaccine material for use in non-human primate studies (Task 3b).

CONCLUSION:

Our approach to vaccine development is to develop a multi-antigen malaria vaccine by generating high levels of multi-epitope, plasmodium-antigen specific CD8 cytotoxic T lymphocytes, mimicking the radiation attenuated whole parasite. Our experience documents that the cell based gp96-Ig approach is highly effective in generating high levels of antigen specific CD8 CTL which is effective in stimulating high-frequencies of poly-antigen specific CTL in both human cancer patients and SIV-specific CTL in rhesus macaques and which is safe. We adapted this vaccine strategy to malaria, and we transfect HEK-293 cells with the Plasmodium Falciparum circumsporozoite protein (PfCSP) and apical membrane antigen 1 (PfAMA-1) and with gp96-Ig and generated vaccine cells line 293-gp96-$Ig^{PfAMA1\text{-}PfCSP}$.
Our immunogenicity studies in mice are designed to enable a nonhuman primate immunogenicity studies and will provide a head-to-head comparison to another promising malarial vaccine candidate, NMRC-M3V-Ad-PfCA. The ultimate goal is to develop a universal vaccine that is highly effective and practical, which is in line with the DoD area of research interest. Our findings are strongly supportive of the novel gp96-Ig malaria vaccine strategy as unique **systemic and liver-homing, sporozoite specific CD8 CTL vaccine strategy.**

Our work in the next 2-3 months will include completion of GMP vaccine production.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Nothing to report

5. **INVENTIONS, PATENTS AND LICENSES:**

Nothing to report

6. **REPORTABLE OUTCOMES:**

Nothing to report

7. **OTHER ACHIEVEMENTS:**

- We have developed 293-gp96-IgPfAMA1-PfCSP cell line

8. **REFERENCES:**

Nothing to report

9. **APPENDICES:**

Nothing to report