The innovative approach taken by our laboratory, relies on secreted gp96-Ig chaperoning antigenic proteins that are efficiently taken up by activated APCs and cross presented via MHC I to CD8 CTL, thereby stimulating an avid, antigen specific, cytotoxic CD8 T cell response. Here we developed malaria vaccine that relies on secreted gp96-Ig chaperoning Plasmodium falciparum antigenic sporozoite proteins CSP and AMA1. 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 fourth year, we completed all proposed mouse immunogenicity experiments that addressed the effect of secondary 293-gp96-Ig PfAMA1-PfCSP immunization and induced memory responses as well as we compared the immunogenicity of the 293-gp96-Ig PfAMA1-PfCSP vaccine to the immunogenicity of NMRC-M3V-D/Ad-PfCA vaccine. We found that gp96-Ig vaccination provided stronger antigen specific CD8 T cell responses in the liver and uterus compared to NMRC vaccine. Since we have already completed manufacturing of GMP-grade vaccine material, we are ready for non-human primate studies.

Malaria, Plasmodium Falciparum, circumsporozoite protein (CSP), apical membrane antigen-1, vaccine (AMA1), heat shock proteins, gp96-Ig, 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, Eileen F. Villasante, Ph.D., 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. **ACCOMPLISHMENTS:**

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<sup>AMA1-PfCSP</sup> and 293<sup>AMA1-PfCSP</sup> vaccine cell lines; (2) determination of the safety and immunogenicity of the 293-gp96-Ig<sup>AMA1-PfCSP</sup> vaccine in mice; and (3) determination of the safety and immunogenicity of the 293-gp96-Ig<sup>AMA1-PfCSP</sup> vaccine in rhesus macaques.

**Summary of Current Objectives:** Last year, together with our collaborators at NMRC, we were working on the animal protocol for the start of nonhuman primates studies (Specific Aim 3. GMP Production, Safety and Immunogenicity analysis of 293-gp96-Ig<sup>PfAMA1-PfCSP</sup> in Rhesus Macaques, Task 3b). Experiments in Aim 3. Task 3b are scheduled for total of 36 weeks.

During the last year we have also performed additional analyses on previously collected uterine tissue specimens from experiments related to Specific Aim 2b: Determine non-inferiority at the time of the vaccine ‘memory’ response by comparative vaccination with the 293-gp96-IgPfAMA1-PfCSP vaccine versus the NMRC-M3V-D/Ad-PfCA vaccine.

**Summary of Results:**

In our previous studies during past four years, we generated 293-gp96-IgPfAMA1-PfCSP vaccine cell line and demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine cell line is immunogenic in the mouse model. In addition, 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 completed all experiments under Specific Aim 1, 2a and 2b (side by side comparison of memory responses induced by 293-gp96-

![Secondary immune responses to gp96 and DNA/Ad5 vaccines in the liver](image)

**Figure 1.** 293- gp96-Ig-PfAMA1-PfCSP vaccine induces superior PfAMA1- and PfCSP- CD8 specific immune responses in the liver compared to NMRC-M3V-D/Ad-PICA vaccine. A) One million of 293-gp96-Ig and 293-gp96-Ig-PfAMA1-PfCSP cells were injected in B6 mice by subcutaneous route at week 0, 4 and 12. Plasmid VR1020 containing AMA1 gene (VR2571) and plasmid VR1020 containing CSP gene (VR2577) (100ug each) were injected by intramuscular route at week 0, 4 and 8 and on week 12 animals were boosted with adenovirus5-vectorized vaccine encoding CSP and AMA1 (AdPfCSP and AdPfAMA1) (1x10⁸ PU/administration) by intramuscular route. Five days after the last boost, all mice were sacrificed and frequency of PfAMA1 and PfCSP specific CD8 T cell responses in the liver was measured by intracellular cytokine staining (ICS) assay and analyzed by flow cytometry. Total intrahepatic lymphocyte were cultured overnight in medium only or with pool of overlapping PfAMA1 and PfCSP peptides. B) Cells were stained for surface CD3, CD8 and intracellular cytokine IFNg. Cells were analyzed on flow cytometer and bar graph shows percentage of CD3+CD8+ cells that produce IFNg

PfAMA1-PfCSP vaccine cell and NMRC-M3V-D/Ad-PICA) as reported in last year annual report and summarized in Figure 1.

In addition, we have expanded our analysis on reproductive tissue (uterus). Malaria in pregnancy poses a great health risk to mother and her fetus and results into complications, such as abortion, still birth, intra uterine growth retardation, and low birth weight. Vaccines that increase maternal malaria specific CD8+ T cell responses in uterus could be detrimental in the context of protection from *P. falciparum* infections during pregnancy.

B6 mice were vaccinated with 293-gp96-IgPfAMA1-PfCSP by subcutaneous route and with DNA/AD5 vaccine by intramuscular route according to the vaccine regiment shown on Figure 1A. We also used Mock controls: 293-gp96-Ig. Five days after the last boost, mice were sacrificed, spleen, mesenteric lymph nodes, liver and uterus were collected and AMA1 and CSP specific CD8 T cells responses were measured by intracellular cytokine staining (ICS) assay. Two pools of overlapping CSP and AMA1 peptides that we used to stimulate lymphocytes obtained from liver and uterus *in vitro* and measure the production of IFN-γ, by
intracellular cytokine staining and flow cytometry to assess the specificity of the gp96-Ig induced CD8 T cells. In animals that were vaccinated with 293-gp96-Ig\textsuperscript{PfAMA1-PfCSP}, we observed the highest frequency of PfCSP and PfAMA1 specific CD8+ T cells (Figure 2) while their frequencies were at the background level for the animals that received Mock control (293-gp96-Ig). We found higher frequency of PfAMA1 specific CD8+ T cells that produce IFN\textgreek{y} compared to PfCSP specific CD8+ T cells (Figure 2).

We confirmed that gp96-Ig vaccine represents a superior vaccination regimen for induction of AMA1 and CSP specific CD8 T cell memory responses not only in liver but also in reproductive tract.

**Summary of Progress and Accomplishment with Discussion:**

We performed an additional head-to-head immunogenicity comparison of gp96-Ig vaccine to the protective, T cell immunity-based NMRC-M3V-D/Ad-PfCA vaccine (DNA prime/adenovirus boost regimen). We demonstrated that 293-gp96-IgPFAMA1-PfCSP vaccine cell line is immunogenic and can induce superior memory responses compared to NMRC-M3V-D/Ad-PfCA vaccine in reproductive tissue, uterus. **Superior treatment difference as measured by CD8+ IFN\textgreek{y} responses comparing 293-gp96-IgPFAMA1-PfCSP and NMRC-M3V-D/Ad-PfCA for CSP or AMA1 met the "go criteria" for moving forward to NHP studies (Specific Aim 3b).**

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**Figure 2.** 293-gp96-Ig-PfAMA1-PfCSP vaccine induces superior PfAMA1- and PfCSP- CD8 specific immune responses in the uterus compared to NMRC-M3V-D/Ad-PfCA vaccine. A) One million of 293-gp96-Ig and 293-gp96-Ig-PfAMA1-PfCSP cells were injected in B6 mice by subcutaneous route at week 0, 4 and 12. Plasmid VR1020 containing AMA1 gene (VR2571) and plasmid VR1020 containing CSP gene (VR2577) (100ug each) were injected by intramuscular route at week 0, 4 and 8 and on week 12 animals were boosted with adenovirus5-vectored vaccine encoding CSP and AMA1 (AdPfCSP and AdPFAMA1) (1x10\textsuperscript{8} PU/administration) by intramuscular route. Five days after the last boost, all mice were sacrificed and frequency of PfAMA1 and PfCSP specific CD8 T cell responses in the uterus was measured by intracellular cytokine staining (ICS) assay and analyzed by flow cytometry. Total uterine lymphocyte (obtained after collagen D digestion) were cultured overnight in medium only or with pool of overlapping PfAMA1 and PfCSP peptides. B) Cells were stained for surface CD3, CD8 and intracellular cytokine IFN\textgreek{y}. Cells were analyzed on flow cytometer and bar graph shows percentage of CD3+CD8+ cells that produce IFN\textgreek{y}.
Because of our additional analysis of gp96-Ig vaccine-induced CD8 T cell responses in reproductive tract, we will include the analysis of reproductive tract as a part of immune response analysis in proposed non-human primate experiments (Specific Aim 3). Our findings are strongly supportive of the novel gp96-Ig malaria vaccine as unique liver- and uterus-homing, sporozoite specific CD8 CTL vaccine strategy. A vaccine to prevent pregnancy associated malaria (PAM) would represent a valuable addition to the current set of tools available (long-lasting insecticide-impregnated nets (LLINs) and use of the anti-malarial drug combination sulfadoxinepyrimethamine (SP). Following successful completion of our final “go criteria” milestones, we proceeded to experiments under Specific Aim 3.

Our collaborator, Eileen F. Villasante, Ph.D., Head Malaria Department Infectious Diseases Directorate at Naval Medical Research Center is in the process of finalizing the IACUC protocol for nonhuman primate immunogenicity studies (Task 3b).

4. **IMPACT:**

We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine is non-inferior at the time of the vaccine memory response in the liver and uterus by comparative vaccination with 293-gp96-Ig PfAMA1-PfCSP vaccine versus the NMRC-M3V-D/Ad-PfCA.

We generated vaccine cells that produce/secrete high level of gp96-Ig and *Plasmodium falciparum* antigens (Pf) AMA1 and CSP 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 human cancer patients, SIV-specific CTL in rhesus macaques and recently, we have generated ZIKA-specific CTL in pregnant mice. We adapted this vaccine strategy to malaria, and we transfect HEK-293 cells with the *P. falciparum* circumsporozoite protein (PfCSP) and apical membrane antigen 1 (PfAMA-1) and with gp96-Ig and generated vaccine cells line 293-gp96-IgPfAMA1-PfCSP.

Our immunogenicity studies in mice were designed to enable a nonhuman primate immunogenicity study. We have provided a head-to-head comparison to another promising malarial vaccine candidate, NMRC-M3V-Ad-PfCA and confirmed gp96-Ig induces superior memory responses compared to the DNA/Ad5 vaccine regimen. 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- and uterus-homing, malaria antigen specific CD8 CTL vaccine strategy.
5. CHANGES/PROBLEMS;

Principal Investigator (PI), LCDR Kimberly Edgel received Permanent Change of Station orders and has transferred from the Naval Medical Research Center (NMRC) to the US Naval Medical Research Unit No. 2 in Phnom Penh, Cambodia and therefore, was unable to serve as PI. Replacement of the Award PI from LCDR Kimberly Edgel to Dr. Eileen Villasante was approved in September 2017.

6. PRODUCTS:

- We have developed and manufactured 293-gp96-IgPfAMA1-PfCSP cell line that will be used in Specific Aim 3.

7. PARTICIPANTS&OTHER COLLABORATING ORGANIZATIONS

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Nothing to report

9. INVENTIONS, PATENTS AND LICENSES:

Nothing to report

10. REPORTABLE OUTCOMES:

Nothing to report

11. REFERENCES:

Nothing to report

12. APPENDICES:

Nothing to report