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14. ABSTRACT

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 $^{PfAMA1\text{-}PfCSP}$ immunization and induced memory responses as well as we compared the immunogenicity of the 293-gp96-Ig$^{PfAMA1\text{-}PfCSP}$ 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.

15. SUBJECT TERMS

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-$Ig^{PfAMA1\text{-}PfCSP}$ 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: 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-IgPfAMA1-PfCSP in Rhesus Macaques, Task 3b**). Experiments in Aim 3. Task 3b are scheduled for total of 36 weeks. NHP studies are planned to start in October 2018.

During the last year we have also performed additional immunogenicity experiments as well as additional analyses on previously collected liver 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-IgPfAMA1-PfCSP vaccine cell and NMRC-M3V-D/Ad-PfCA) as reported in last year annual report.

Liver CD8+ tissue resident memory T (Trm) cells were recently shown to be critical for memory-stage CD8+ T cell-mediated protection against PE Plasmodium infection (Fernandez-Ruiz 2016. Immunity). Immunogenicity data from our study provide a translational pathway for development of prime-boost secreted gp96-Ig vaccines designed to harness such Trm

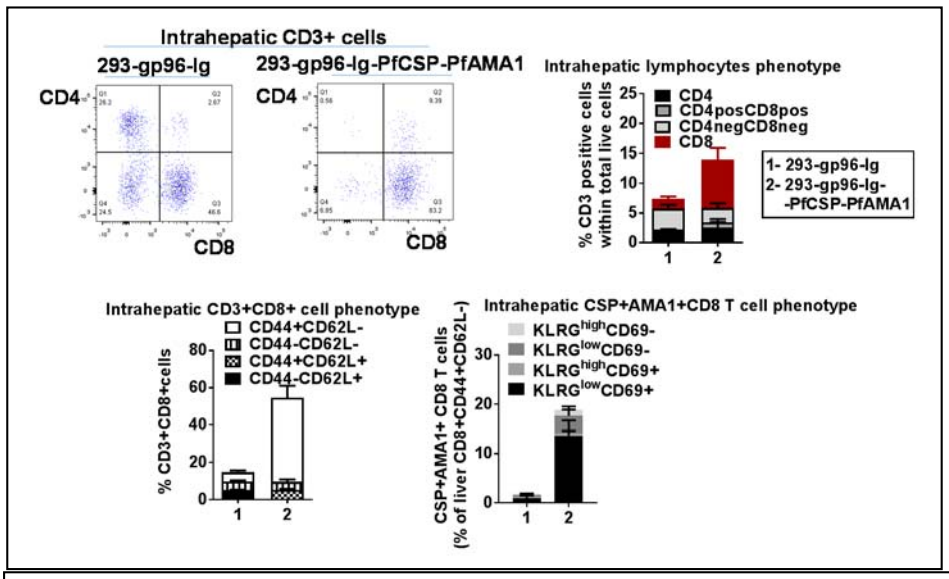

Figure 4. Subcutaneous route of vaccination induces high-frequency of memory CD8+ T cell in the liver. One million 293-gp96-Ig and 293-gp96-Ig-PfAMA1-PfCSP cells were injected by s.c. route in B6 mice. Five days later mice were sacrificed, intrahepatic lymphocytes were isolated and frequency of intrahepatic CD3+ lymphocytes and phenotype of intrahepatic CD3+CD8+ cells regarding the CD44 and CD62L surface expression as well as KLRG1 and CD69 was analyzed.

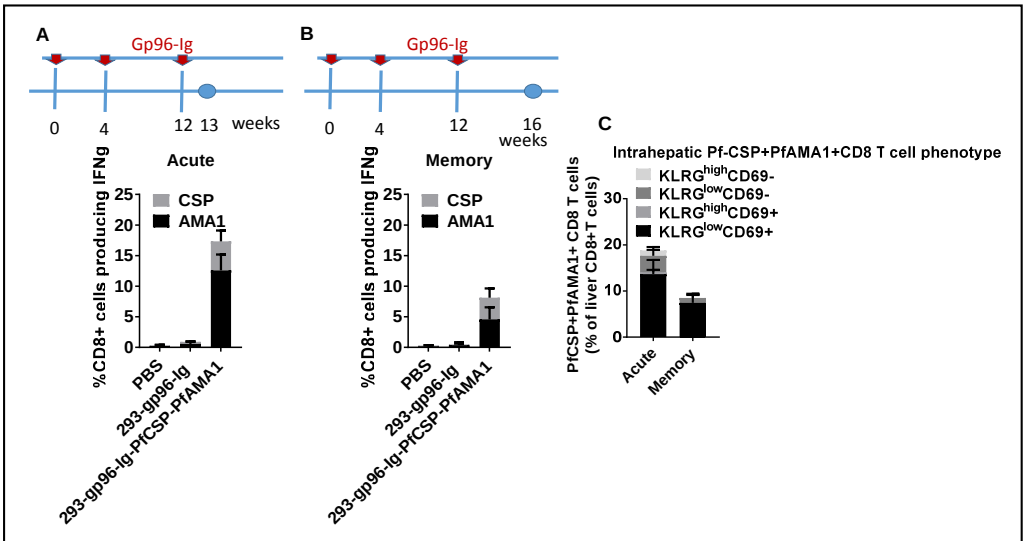

Figure 5. S.c. route of vaccination induces high-frequency of effector and memory CD8 T cells in the liver. One million 293-gp96-Ig-PfAMA1-PfCSP cells were injected in B6 mice at week 0, 4 and 12 by subcutaneous (s.c.) route. Five days (week 13) (A) or 28 days (week 16) (B) later mice were sacrificed and frequency of PfCSP and PfAMA1 specific CD8 T cell responses in the liver 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 and PBS by s.c. route. Bar graph shows percentage of PfCSP and PfAMA1 specific CD3+CD8+ cells that produce IFNg as well as the frequency of KLRG1 and CD69 expressing intrahepatic PfCSP and PfAMA1 CD3+CD8+ cells.

cells. We confirmed previously that subcutaneous route of vaccination induces dramatic increase in the liver-infiltrating CD8+ T cells. We found that after gp96-Ig immunization, 80% of all CD3+ cells in the liver are CD8 + T lymphocytes. These liver-infiltrating CD8+ T are mostly effector

memory cells (CD44+CD62L- cells) (Fig 1). Interestingly, these effector memory cells, are highly activated and express CD69, activation marker that has been confirmed on Trm cells in different tissues and in liver. In addition, intrahepatic CD8 T cells after gp96-Ig vaccination have a very low expression of Killer cell lectin-like receptor subfamily G member 1 (KLRG1), identifying them as proliferation-competent long-lived memory CD8 T cells. More detail Trm phenotyping (CXCR6) and gene signature will be included in the further studies in order to confirm this exciting data about gp96-Ig vaccine induced memory T cell population in the liver. Also, we analyzed the frequency of Pf-specific CD8 T cells after 5 days (acute, effector time point) vs 28 d after (memory time point) (Fig 2). We found that gp96-Ig dramatically induces Pf-specific CD8+ T cells at effector/acute time point after vaccination (Fig 2A). Even though frequency of Pf-specific CD8 T cells decreases over time (day 28 after vaccination/ memory time point), vaccine induced Pf-memory cells consist of almost exclusive population of CD69+KLRG1low cells (Fig 2C). KLRG1low memory cells retain high cytotoxic and proliferative capacity distinct from other populations, which contributes to effective anti-influenza and anti-tumor immunity (Herndler-Brandstetter, D. 2018, Immunity). Our work demonstrates that gp96-Ig vaccine induces KLRG1lowCD8+ T cells and uniquely promotes functionally versatile memory cells and long-term protective immunity.

In addition, we have expanded our analysis on other T cells, including GD T cells. Gp96 targets different cell types and alter the nature, intensity and kinetics of innate and inflammatory immune response: NK, NKT, γδ-T cells, macrophages and DC in addition to adaptive immune responses. We have evidence that gp96-SIV /HIV vaccines induced the recruitment and activation of large numbers of DC as well as the recruitment of antigen specific CD8 and CD4 cells to local vaginal and intestinal tissues. We have found that protected, gp96-SIV-vaccinated animal has significantly increased frequency of γδ T cells in the rectal lamina propria and those γδT cells produce the majority of IL-17 (unpublished data form previous gp96-Ig-SIV study, published by Strbo et al. J. Immunol. 2013). Moreover, one week post infection, animals from vaccinated group, gp96SIV+gp120, have significantly higher frequencies of γδT cells in LPL than mock vaccinated animals, implicating their role in the protection. γδ T cells have been shown to contribute to protection against PE malaria in mouse models (Tsuji, M 1994, PNSA), and these T cells expand in humans following whole-SPZ immunization Teirlinck, A.C. , 2011, PloS Path) and the correlation between γδ T cell frequency and CHMI outcome has also been reported Ishizuka, A.S , 2016 Nat Med). Our additional data analysis suggests that Pf-gp96-Ig Vaccine induces immunity not only through induction of conventional CD8 and CD4 T cell responses but also through induction of γδ T cells (Fig 3). We found increased numbers of GDT cells in the peritoneal cavity 5 days after vaccination. Phenotype of vaccine –induced

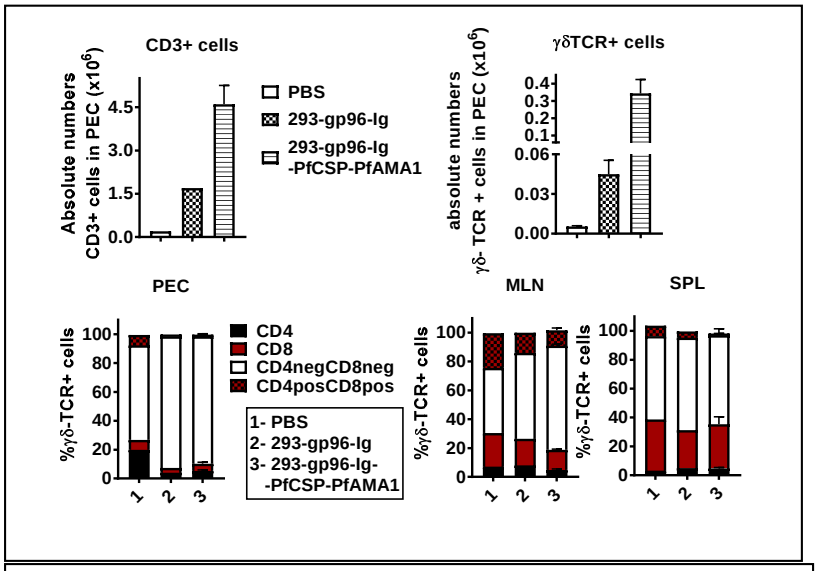

Figure 3. High levels of gdTCR+ cells in 293-gp96-Ig-PfCSP-PfAMA1 vaccinated mice. Number of CD3 + and gd-TCR+ cells within peritoneal cavity excaudate cells (PEC) in control (PBS and 293-gp96-Ig) or vaccinated mice (293-gp96-Ig-PfCSP-PfAMA1) 5 days after vaccination. Phenotype of gd-TCR+ cells inPEC, MLN and SPL.

GD T cells in peritoneal cavity (PEC) and mesenteric lymph nodes (MLN) was different from GD T cells in non-vaccinated animals, with majority of the GD T cells been CD4-CD8- cells. Change in the phenotype was not observed among SPL GD T cells.

Summary of Progress and Accomplishment with Discussion:

We performed an additional analysis of cellular immune responses induced by gp96-Ig. We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine cell line is immunogenic and can induce long-lived memory responses in liver (Fig 1 and 2).
Superior treatment difference as measured by CD8+ IFNγ 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).
Because of our additional analysis of gp96-Ig vaccine-induced CD8 T cell responses in the liver, future analysis of immune response in upcoming non-human primate experiments will include more detailed analysis of vaccine induced memory responses (Specific Aim 3).
Our findings are strongly supportive of the novel gp96-Ig malaria vaccine as unique liver- -homing, sporozoite specific CD8 CTL vaccine strategy.
Following successful completion of our final "go criteria" milestones, we proceeded to experiments under Specific Aim 3 that.
Our collaborator, Eileen F. Villasante, Ph.D., Head Malaria Department Infectious Diseases Directorate at Naval Medical Research Center will start NHP experiments in October 2018.

4. **IMPACT:**

We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine inducecs Pf-specific memory CD8 T cell responses in the liver as well as GD T cells. Overall, broad-based T cell responses that are induced by live-attenuated vaccines also occur after immunization with the secreted gp96-Ig vaccines, highlighting one of many important similarities between these approaches.

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 memory CD8 CTL which is effective in stimulating high-frequencies of poly-antigen specific CTL in human cancer patients, SIV-specific CTL in rhesus macaques, HIV- specific in humanized mice 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-Ig$^{PfAMA1\text{-}PfCSP}$.
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-homing, malaria antigen specific CD8 CTL vaccine strategy.**

5. CHANGES/PROBLEMS;

Nothing to report

6. PRODUCTS:

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

INVENTIONS, PATENTS AND LICENSES:

Invention disclosure has been filed at University of Miami, Jul 23, 2018. (UM-D2019-0019 Title: Secreted gp96-Ig vaccine for Malaria Prophylaxis)

7. PARTICIPANTS&OTHER COLLABORATING ORGAANIZATIONS

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES:

Nothing to report