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TITLE: Immune Therapeutics That Combine Fast-Acting Monoclonal Antibody and a Vaccine for Long-Lasting Protection Against *Plasmodium falciparum* Malaria

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14. ABSTRACT

The WHO reports more than 600,000 yearly deaths due to Plasmodium falciparum malaria. In addition to highly effective anti-malarial compounds and seasonal chemoprevention, vaccines (RTS,S and R21) have been licensed for the control of malaria in pediatric populations. Despite these advances, P. falciparum is rapidly developing resistance to multiple antimalarial drugs, and vaccine efficacy remains moderate and short-lived. As such, we have proposed a two-component immune intervention for adult, military, and traveler markets. Component 1 will be a fast-acting monoclonal antibody (mAb) against P. falciparum's circumsporozoite protein (CSP), while component 2 will be a longer-lasting tobacco mosaic virus-based vaccine displaying the immunodominant CSP NPNA epitope. In the first year, we have concentrated on development of component 1, a monoclonal antibody optimized for longer half-life. During the primary screening and down selection of our first candidate, mAb 317 was found to have off-target binding; consequently, we proceeded with another candidate, mAb 311, for the half-life selection studies. Half-life mutants of mAb 311 were produced and screened using in vitro assays and were observed to retain wild-type antigen binding with improved FcRn binding dynamics. The in vivo studies to determine the half-life of the mAb 311 component are underway. In parallel, development of the component 2 vaccine for cGMP large-scale manufacture is almost complete. The overall project progress is satisfactory.

15. SUBJECT TERMS

None listed.

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1. **INTRODUCTION:** *Plasmodium falciparum* malaria remains a significant burden to sub-Saharan African pediatric populations despite recent advances in vaccine (GSK's RTS,S/AS01 and Oxford University's R21/Matrix M) and prophylactic monoclonal antibody (NIH's CIS43LS and L9LS) implementation. These first-generation malaria vaccines are administered seasonally and their induced-antibodies decay rapidly, necessitating yearly boosting and co-administration with seasonal chemoprevention. A combinatorial approach wherein a monoclonal antibody is administered before vaccination remains unaddressed. In this 3-year TTDA proposal, we proposed a mAb therapeutic candidate that can be given in combination with a malaria vaccine to provide immediate protection following passive transfer. This two-component anti-malaria therapeutic can be administered to travelers and troops immediately before entry into the endemic area. The two components will be acting in a serial manner to provide both short- and long-lasting protection against malaria.

2. **KEYWORDS:** Malaria, *falciparum*, monoclonal, antibody, half-life, extension, vaccine, combinatorial

3. **ACCOMPLISHMENTS: Development of Component 1 (YEAR 1)**

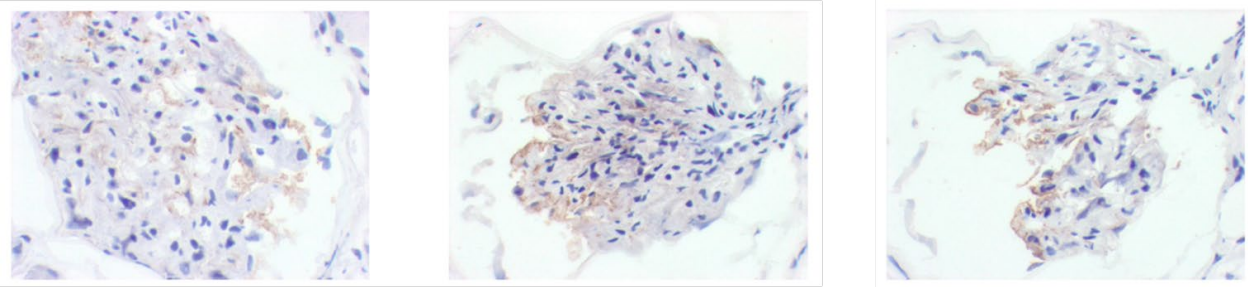
Optimization of mAb serum half-life

- Subtask 6: Immunohistochemistry test to poly-reactivity or auto-reactivity of mAb EXT-317 – **COMPLETED**
- Subtask 1: Produce extended half-life variants of mAb 317 (WT, YTE, KF, LS, QVV, and DHS variants) **– COMPLETED**
- Subtask 2: Expression and purification of variant mAbs **– COMPLETED**
- Subtask 3A: hFcRn transgenic mice protocol submission and approval (IACUC and ACURO) – **COMPLETED**
- Subtask 3B: Comparative evaluation of half-life of the variants (ELISA-based, bio-layer interferometry-based assay **– COMPLETED**
- Subtask 4: Biological screening of mAb variants (NANPx6 peptide, whole sporozoite, and avidity index ELISAs, Western blot, immunofluorescence assay, ILSDA) – **PARTIALLY COMPLETED**
- Subtask 5: Down-selection of half-life of mAb EXT-317 using *in vivo* testing in hFcRn transgenic mice – ***NOT YET STARTED***

Achievements, activities, results, outcomes: In order to assess off-target risks before investing in the development of candidate mAb 317, we performed immunohistochemical staining. This also allowed for additional investigation for other mAb candidates if mAb 317 did not meet criteria for progression. The results below are presented in chronological order of performance. We explain how the outcomes have changed the scope of subsequent inquiries.

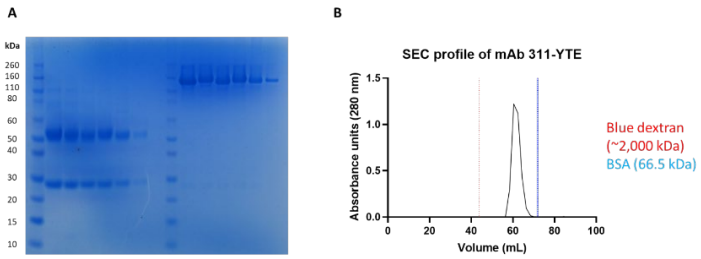
3.1 Immunohistochemical analysis of mAb 317 binding to human tissues: As part of Aim 1, we determined the potential off-target binding of mAbs to human tissues. Immunohistochemistry staining indicated that mAb 317 (our original camdidate) reacted with extracellular granular material (intravascular and/or extravascular) in the kidneys, mainly in glomerular tufts **(Figure 1)**. As such, we elected to proceed with a different mAb candidate (mAb 311) that showed no such off-target binding. MAb 311 is a well-profiled monoclonal antibody against *Plasmodium falciparum* that binds to the same antigen, circumsporozoite protein (CSP), as mAb 317 does. In mouse efficacy studies, mAb 311 has performed similarly to mAb 317 (Livingstone *et al*., *Scientific Reports* 2021). Recent structural data shows that it can effectively bind CSP repeat region epitopes at very high density (Martin *et al*., *Nature Communications* 2023).

Figure 1. Binding of mAb 317 from 3 independent sources to extracellular granular material in a kidney glomerular tuft (10 µg/mL) as indicated by red staining.



3.2 Mutagenesis and production of half-life extended mutants of mAb 311: Plasmids corresponding to the above-described half-life mutants (WT, YTE, KF, LS, QVV, and DHS variants) were commercially synthesized by ATUM (Newark, CA) and co-expressed with the light-chain of mAb 311 at a small scale in Expi293 cells (ThermoFisher, Waltham, MA). Products were purified according to the manufacturer's instructions using Cytiva's Protein G Sepharose 4 Fast Flow Resin (Marlborough, MA), and were found to be highly pure and possessing identical biophysical character per SDS-PAGE and SEC **(Figure 2)**.

Figure 2. A) Representative SDS-PAGE gel of purified 311 half-life mutants, both reduced (left) and non-reduced (right). B) Size-exclusion chromatography analysis of representative monoclonal mAb311-YTE using Cytiva's HiLoad Superdex 200, 16-600, with protein standards indicated by dashed lines (red: blue dextran; blue: bovine serum albumin).



3.3 Activity of mAb 311 half-life extension mutants: The affinity of each half-life variant towards both the major repeat epitope, NANPx6, as well as the full-length CSP was measured by ELISA. We showed that epitope binding of mAb 311 to the repeat region was unimpacted by the half-life mutations in the Fc region, with all monoclonal mutants demonstrating an $OD_{415} > 1$ using 100 ng/mL concentration **(Figure 3)**. Preliminary analyses indicated that FL-CSP affinity may be impacted by these mutations in Fc, perhaps due to interference with homotypic interactions, although this requires additional verification that we will perform. Bio-layer interferometry (BLI) assessment of FcRn binding of the mAb 311 half-life mutants was conducted at pH 6. Increased association of mAb 311 to a membrane-bound FcRn in the low-pH endosomal compartment would increase scavenging and consequently half-life of this antibody. Indeed, our data revealed a consistent rank order and increased affinity of the mutants to recombinant FcRn as compared to the wild-type **(Figure 4)**. Half-life kinetics, however, are also reliant on how efficacious the antibody-FcRn complex dissociates at the cell-surface (~pH 7.4). Based on both increased affinity relative to the WT at pH 6 and efficient release at pH 7.4 as determined by BLI **(Figure 4)**, as well as preservation of critical FcγR binding necessary for functional activity from literature sources, we have selected the LS mutant as our most promising variant.

Figure 3. Binding of mAb 311 variants to CSP antigens (NANPx6 and full-length CSP) as assessed by ELISA. One-way ANOVA analyses indicated no significant differences between groups for NANPx6, but moderate differences between certain groups for FL-CSP; noticeably, this preliminary data requires additional replicates to confirm this.

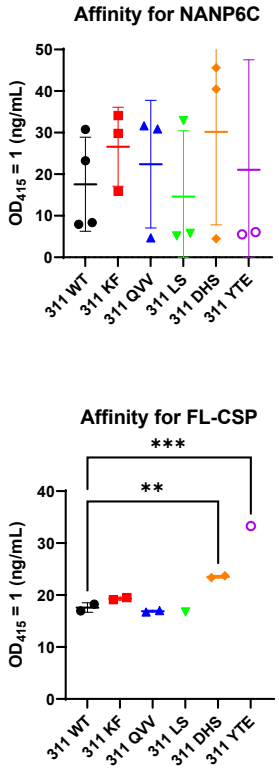
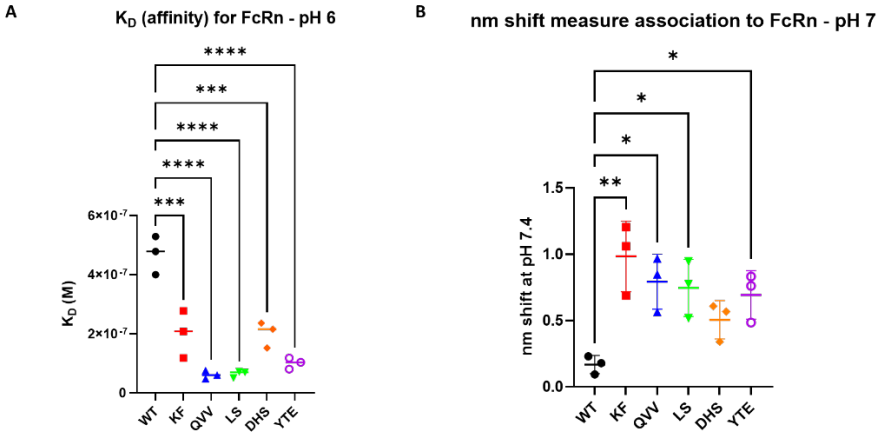


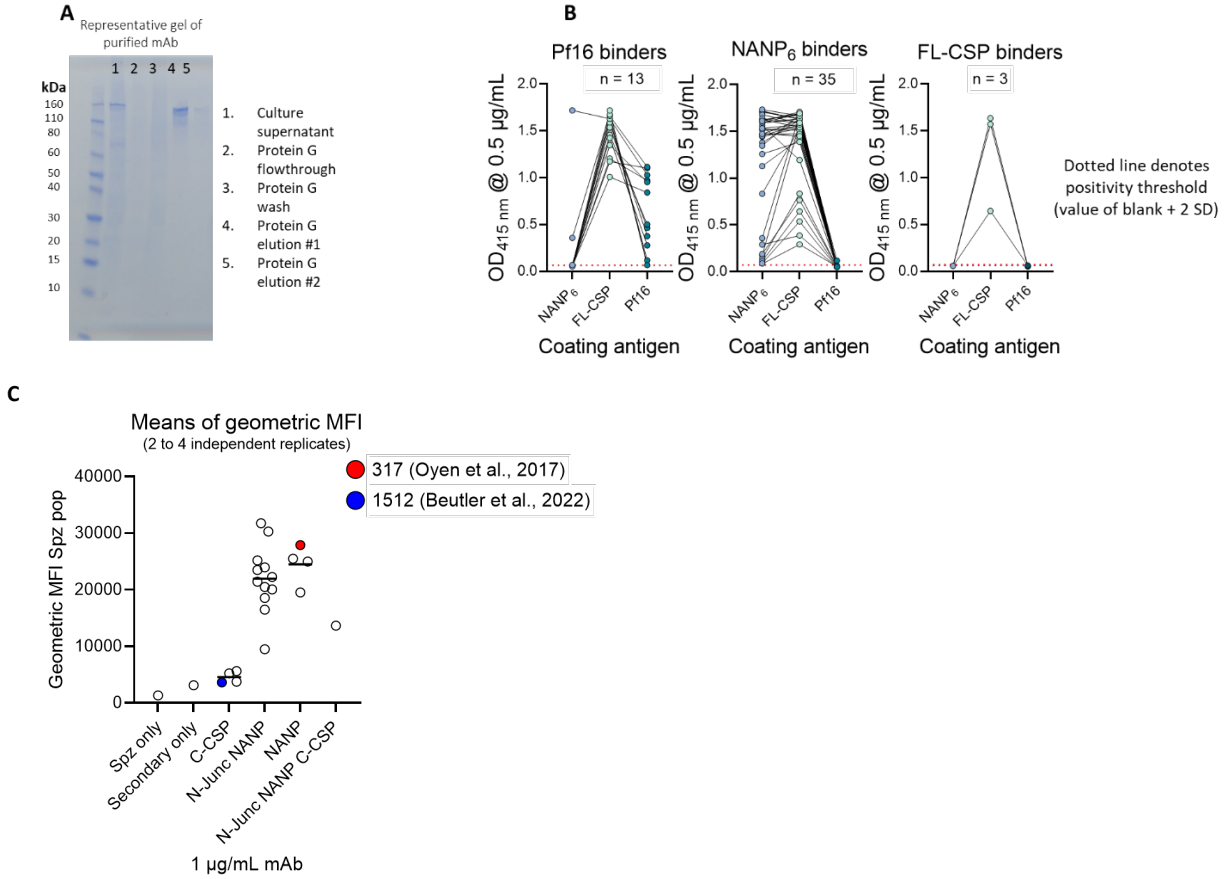
Figure 4. A) FcRn binding of half-life variants at pH 6 reveals a consistent rank-ordering, with all variants demonstrating a higher affinity for FcRn than the wild-type. B) FcRn binding of half-life variants at pH 7.4 indicates DHS has the most efficacious release. One-way ANOVA was used to analyze the data.



3.4 Novel mAb screening: Given potential intellectual property concerns with using a monoclonal antibody derived from a commercial vaccine, we have also invested time in developing a candidate obtained from a clinical trial with our first-generation vaccine candidate, FMP013 (a separately funded effort through the Gates Foundation). Of nearly 50 monoclonals identified in collaboration

with the German Cancer Research Center, we have down-selected 23 due to their superior binding to CSP antigens or live sporozoites **(Figure 5)**. Currently, larger scale purifications of monoclonals from HEK293 cultures are proceeding as described above for a planned study to assess their efficacy in murine challenge experiments. Based on our data for mAb 311, we do not anticipate the LS half-life mutation will impact its reactivity to CSP antigens, and our previous work developing mAb 311 variants will be easily translatable should a novel candidate emerge.

Figure 5: A) Representative gel showing purification of 50 selected monoclonal antibodies. B) Identification of class-specific monoclonal antibodies with their ELISA reactivity at a limiting concentration (0.5 µg/mL). C) Based on the preliminary outcomes in B, monoclonals were selected for live binding to sporozoites using flow cytometry.



3.5 hFcRn transgenic mice protocol submission and approval (IACUC and ACURO): Amendment to ACURO protocol PR211833.e001 entitled, "Developing immunotherapies against malaria and SARS-CoV-2 infections in mice (Mus musculus)", is approved under IACUC protocol number 22-13-BrAD and expires 05/15/2025. The protocol allows us to test the half-life of the mAbs in hFcRn transgenic mice.

What opportunities for training and professional development has the project provided?

The project has afforded many opportunities for professional development and training. Ms. Emma Ryan has gained exposure to the expression, purification, and *in vitro* assessment of monoclonal antibodies and FcRn-anitbody interactions through ELISA and BLI methodologies. She has been able to apply this to train Ms. Amber Ballard-Sims, who has achieved similar wetlab expertise and learned

laboratory management skills. Mr. Dallas Brown has also utilized his mammalian experience with expression and purification of monoclonal antibodies to train Ms. Ryan and Ms. Ballard-Sims. Ms. Ryan was also able to attend the American Society of Tropical Medicine and Hygiene conference in Chicago.

How were the results disseminated to communities of interest? *Nothing to Report.*

What do you plan to do during the next reporting period to accomplish the goals: In year 2, we hope to accomplish a 2-4 fold extension of serum half-life of either mAb 311 or the candidate that emerges from FMP013 monoclonals. This resulting EXT mAb will be combined with the TMV-NPNA vaccine to initiate in vivo studies.

Subtask 1: C57Bl/6 mouse study to optimize an adjuvant formulation for the TMV-NPNAx5 vaccine (Component 2).
Subtask 2: Determine the ID50 for mAb EXT-317 at T1/2
Subtask 3: Testing combination of EXT-317 and TMV-NPNAx5
Subtask 4: Apply for Phase 2 TTDA funding to CDMRP to fund process development of the mAb and the vaccine.

4. **IMPACT:** The project is ongoing "nothing to report".

What was the impact on the development of the principal discipline(s) of the project? *"Nothing to Report."*

What was the impact on other disciplines? *Nothing to Report*

What was the impact on technology transfer? - *Nothing to Report*

What was the impact on society beyond science and technology? *"Nothing to Report."*

5. CHANGES/PROBLEMS:

5.1 Screening of additional mAbs: Since monoclonal antibody 317 did not meet the epitope specificity criteria (off-target binding), we selected another closely related monoclonal antibody 311 for the half-life extension studies for Component-1 in the YEAR 1. In parallel we are also screening a set of novel monoclonal antibodies against CSP using *in vitro* assays. This is being done to ensure that we take the best possible monoclonal antibody as Component 1 to conduct the *in vivo* studies propose in YEAR 2. There is no significant change in project aims and objectives.

5.2 Changes in approach and reasons for change: There is no significant change in the approach to develop a 2-component malaria intervention.

5.3 Actual or anticipated problems or delays and actions or plans to resolve them: There have been significant issues finding appropriately trained post-doctoral scientist for this project. We continue to advertise and conduct interviews but the work is being currently kept on schedule by hard working technicians and research fellows.

Changes that had a significant impact on expenditures: No change

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: No Change

Significant changes in use or care of human subjects: No Change

Significant changes in use or care of vertebrate animals: No Change

Significant changes in use of biohazards and/or select agents: No Change

6. PRODUCTS:

- **Publications, conference papers, and presentations:** Poster presented at the American Society of Tropical Medicine and Hygiene 2023, Chicago. Title "*Comparison of junctional-, minor repeat-, and major repeat-focused circumsporozoite vaccines using the tobacco mosaic virus epitope display platform"*
- **Journal publications** *Nothing to Report*
  Books or other non-periodical, one-time publications - *Nothing to Report*

  Other publications, conference papers and presentations. Nothing to Report.
- **Website(s) or other Internet site(s)**: Nothing to Report.
- **Technologies or techniques:** Nothing to Report.
- **Inventions, patent applications, and/or licenses:** Nothing to Report.
- **Other Products - N/A**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	***Sheetij Dutta***
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Dutta provides day to day guidance to the lab staff*
Funding Support:	*Government and CDMRP (30%)*

Name:	***Dallas Brown***
Project Role:	*Lab Manager/Lab instructor*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*12*
Contribution to Project:	*Ensures lab staff are trained and all equipment and supplies are ready at the time of experiments; expressed and purified monoclonals; assessed live sporozoite binding of monoclonals*

Funding Support:	*USAID*
Name:	***Emma Ryan***
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*12*
Contribution to Project:	*Expressed and purified monoclonals; assessed FcRn binding as well as binding of all monoclonals to antigens; assessed live sporozoite binding of monoclonals*
Funding Support:	*CDMRP*
Name:	***Amber Sims***
Project Role:	*Technician 1*
Researcher Identifier (e.g. ORCID ID):	*n/a*
Nearest person month worked:	*12*
Contribution to Project:	*Amber Sims expressed and purified monoclonals; ensures all supplies are ready at time of experiments*
Funding Support:	*CDMRP*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? *"Nothing to Report."*

What other organizations were involved as partners? *Nothing to Report."*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *Nothing to report*

QUAD CHARTS: *See attached*

9. **APPENDICES:** *Nothing to report.*