AWARD NUMBER: W81XWH-15-1-0401; PR140044

TITLE: Malaria Prevention by a New Technology: Vectored Delivery of Antibody Genes

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REPORT DATE: **October 2019**

TYPE OF REPORT: **Annual**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2019	Annual	8 SEP 2018 - 7 SEP 2019

4. TITLE AND SUBTITLE

Malaria Prevention by a New Technology: Vectored Delivery of Antibody Genes

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0401

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
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Baltimore MD 21218

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
FORT DETRICK, MARYLAND 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT. Malaria has proven refractory to conventional immunization approaches. We are exploring a novel route to induction of antimalaria immunity: adeno associated virus (AAV) vectored introduction of genes encoding known protective monoclonal antibodies (MAbs) into whole animals. Using a technology originally applied to expression of HIV antibodies [1], we demonstrated that mice can be protected from *Plasmodium* infection by vector-driven expression of a monoclonal antibody (2A10) against circumsporozoite protein, an antigen found on the surface of the form of the parasite injected by mosquitoes [2]. Building on that observation, this project has two overall specific aims: 1. identification and evaluation of additional, potentially more effective, MAbs in the murine system, and 2. tests of protective efficacy of MAbs delivered by AAV vectors in a non-human primate (NHP; *Aotus nancymaae*) model of *P. falciparum* infection. Efforts in this period have been directed primarily at Aim 2, assessing protection against malaria sporozoite infection conferred by MAb expression in NHPs. As noted in earlier Quarterly reports and in detail below, published procedures for challenging *Aotus* in order to determine protection, which rely upon development of parasitemia post-challenge, are not reproducible in our hands. Therefore, we have explored an alternative method of determining efficacy based on measurement of liver parasite load. Assessment of protection using the new protocol is underway.

15. SUBJECT TERMS
Malaria, monoclonal antibody, immunization, vaccine, gene transfer, adeno associated virus, AAV, *Plasmodium falciparum*, sporozoite murine challenge model, non-human primate challenge model, *Aotus*, parasite liver burden, qPCR

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. Introduction. Malaria is caused by parasites of the genus *Plasmodium* and is responsible for about 500,000 deaths per year, mostly in sub-Saharan Africa and mostly induced by infection with *P. falciparum*. In addition to the burden it imposes on residents of endemic areas, malaria poses a significant threat to US service personnel serving in Africa and other malaria-endemic areas. An effective vaccine would be of enormous value in relieving the toll exacted by malaria in both populations. However, extensive efforts to develop malaria vaccines using conventional approaches have been largely unsuccessful and no satisfactory malaria vaccine exists. The long-term objective of this project is to assess the promise of a novel immunization technology termed vectored immunoprophylaxis (VIP) in inducing protective immunity to malaria. VIP employs adeno associated virus (AAV) vectors to deliver genes encoding monoclonal antibodies (MAbs) to animals. Mice transduced by VIP vectors that encode monoclonal antibodies directed against the *P. falciparum* circumsporozoite protein (CSP) rapidly develop high serum levels of the MAb and are protected from experimental infection by a transgenic rodent parasite that expresses *P. falciparum* CSP. This project will assess in more depth the potential of VIP technology in malaria immunization. It has two specific aims: 1. to use the murine challenge model to identify additional MAbs with potential in the VIP system and optimize their expression *in vivo*, and 2. to test the most promising MAbs for protective efficacy in a non-human primate model of *P. falciparum* infection that employs *Aotus nancymaae* new-world monkeys.

2. Keywords: Malaria, monoclonal antibody, immunization, vaccine, vectored immunoprophylaxis, gene transfer, virus vector, adeno associated virus, AAV, *Plasmodium falciparum,* sporozoite, murine challenge model, non-human primate challenge model, *Aotus*

3. Accomplishments.

A. Major Goals **Timeline (months)**

Projected

Completed(%)

Goal 1: VIP vector development **Completed**

1. Prepare, purify and sequence new MAbs
2. Construct first-round vectors
3. Optimize MAb expression in new vectors

Milestone: Selection of candidates for mouse experiments.

Goal 2: Evaluate candidate vectors in mice **Completed**

1. Local IRB/IACUC Approval
2. Assess protection by VIP vectors; IV challenge
3. Assess protection by VIP vectors; mosquito bite challenge
4. Determine mouse dose-responses; mosquito bite challenge
5. Assess protection by vector pairs; mosquito bite challenge

Milestones: Selection of VIP vectors for *Aotus* studies.

Goal 3: Determine *Aotus* dose response **Completed**

1. Local IRB/IACUC Approval
2. Dose response in *Aotus*

Goal 4: *Aotus* challenge 1 (mAb 2A10)	**Underway;** Anticipated completion 12/19*
Goal 5: *Aotus* challenge 2 (mAbs TBD)	Will not be performed*

* Please see **E.5. Challenges and Problems**, below

B. What was accomplished under these goals (Previous and current reporting periods, as noted)

Goal 1: VIP vector development*. 1. Prepare, purify and sequence new MAbs.* It was initially anticipated that it would be necessary to determine the amino acid sequences of candidate MAbs in order to prepare synthetic MAb-encoding genes for incorporation into VIP vectors. The publication of the amino acid sequences of a series of potent anti-CSP human mAbs [3,4], sequences kindly furnished to us prior to publication by PATH-MVI, and a sequence provided by Leidos, has eliminated the need for determination of sequence by us for the seven MABs described below. Determination of the sequence for an eighth MAb, against CelTOS, a protective antigen described by E. Angov of WRAIR, has been suspended due to time constraints.

2. Construct first-round vectors (previous and current periods). Seven vectors encoding distinct anti-CSP MAbs have been prepared: anti-CSP mAbs 2A10, 2C11 (prior to this award), 5D5, 2H8, 667 (previous reporting periods), CIS 43, and MGU12 (current period).

3. Optimize MAb expression in new vectors (previous periods). Vector-driven MAb expression is influenced by the amino acid sequence of the framework portions of the MAb variable regions. Alterations in the framework generally do not affect antibody binding, and so framework modifications can be used to modulate expression independently of antibody specificity and affinity. In an effort to maximize mAb expression from vectors encoding the new mAbs CIS43 and MGU12 , framework sequences from our highest-expressing MAb (2A10) were incorporated into a vector that retains the specificity-determining regions of those antibodies. Disappointingly, mAb expression from the modified vectors was reduced compared to that from the original CIS43 and MGU12 vectors. Importantly, while it is clear from our published mouse data that high expression levels enhance protective efficacy, extravagant levels of expression of a potent MAb may not be needed to confer protection. Therefore, pursuit of enhanced MAb expression is not considered an essential element of the project and further optimization efforts will not be made.

Goal 2: Evaluate candidate vectors in mice. *1. Assess protection by new VIP vectors; intravenous (IV) challenge (previous periods).* During previous funding periods, three MAbs were assessed for protective efficacy by both IV injection of sporozoites and exposure to infected mosquito bites. One is protective in both assays and one is protective in neither. The remaining MAb protects in mosquito bite challenge, but not IV challenge. Because mosquito bites represent the route of natural infection and seem from these results to provide a more sensitive indication of protection, use of IV challenge as a measure of efficacy for new vectors in mice will not be performed.

3. Assess protection by new VIP vectors; mosquito bite challenge (previous and current periods). Mosquito bite challenge experiments have been completed for all seven anti-CSP MAbs: 2A10, 2C11 (prior to this award), 5D5, 2H8, 667 (prior funding period), CIS 43 and MGU12 (this period). The MAb showing the highest protective efficacy in mice at the onset of this project, 2A10, is included as a positive control in the experiments presented in Figure 1. Notably, three of the newly characterized MAbs are more effective in protecting mice from infection than 2A10, especially MGU12. MGU 12 therefore is the current leading candidate for NHP studies when those can be resumed with further funding.

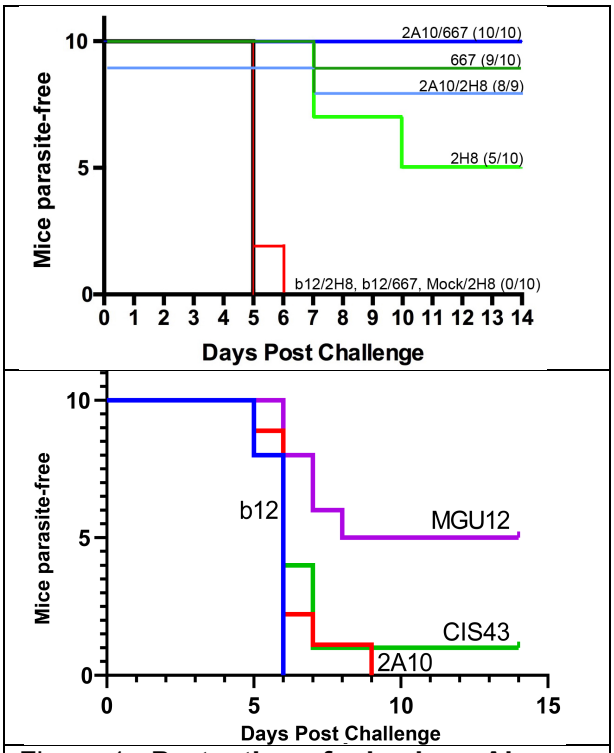

Figure 1. **Protection of mice by mAbs 2H8, 667, CIS43, and MGU12.** AAV8 vectors encoding each mAb as well as positive (2A10) and negative (b12) control mAbs were used to transduce 9 or 10 mice. Transduced mice were challenged by bites of mosquitoes infected with a transgenic *P. berghei* parasite that displays the *P. falciparum* CSP. Mice remaining parasite-free are plotted vs. day post-challenge. Mice that are parasite-free 14 days post-challenge are considered protected. These experiments were done at different times, and positive and negative controls for each therefore are included. The top panel is reproduced from the 2018 Annual Report.

4. Determine mouse dose-responses; mosquito bite challenge. No studies were conducted this funding period.

5. Assess protection by vector pairs; mosquito bite challenge (previous funding periods). One study, which included the 2A10 MAb and MAb 5D5, was completed in the previous funding period. As reported in the 2017 Technical Progress Report, this pair was chosen because the two MAbs target distinct epitopes: the CSP central repeat (2A10) and a conserved epitope in CSP that lies near the site of a proteolytic cleavage that is required for cell invasion by sporozoites (5D5). 2A10 is protective in about 70% of animals, while 5D5 is not detectably protective alone. The combination had efficacy indistinguishable from that of 2A10 alone, indicating that in this case, no synergy occurs (data not shown).

Goal 3: Determine *Aotus* dose response. 1. *Local IRB/IACUC and ACURO Approva*l has been obtained.

2. *Dose response in Aotus (pevious funding periods).* These studies were completed in the previous funding period with a malaria-irrelevant mAb (this was misreported in the 2017 report as anti-CSP mAb 2A10), using two doses based on literature values for related vectors. (The irrelevant mAb, against HIV gp120, was used in anticipation of subsequent use of these animals in challenge studies.) Three of the four transduced animals produced the mAb, while one did not (Figure 2, top panel). Unexpectedly, the

lower dose tested (2 x 10^{12} genome copies [GC] per monkey) proved to yield serum MAb levels equal to that of the higher dose (10^{13} GC/monkey) in responding monkeys. Thus, the system seems to be saturated with respect to the inoculum of AAV at these doses. Ultimately, it may be desirable to test lower doses to determine the minimum amounts of vector that produces a protective response in preparation for clinical trials. However, that must await the successful development of the challenge system (see below).

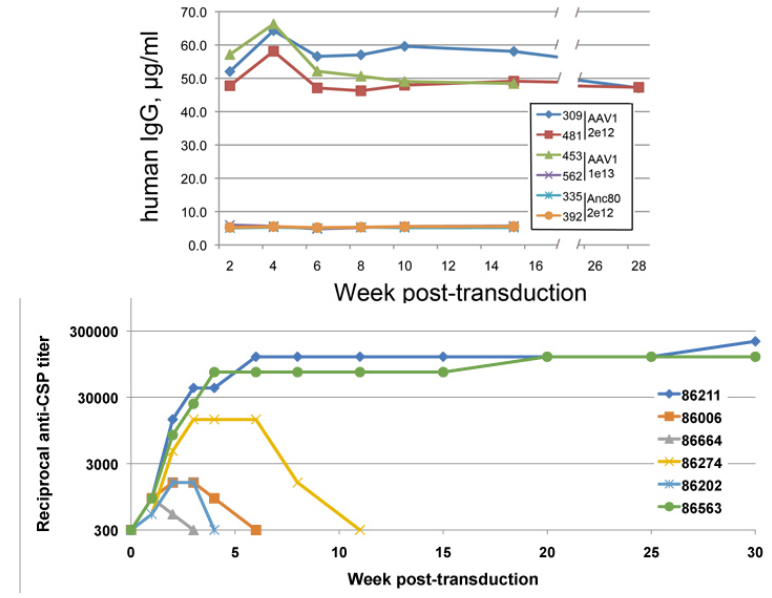

Figure 2. MAb expression after transduction of *Aotus*. Top: *Aotus* were transduced with AAV1-b12 at the indicated doses. Human IgG expression was measured by ELISA. 'Anc80' refers to a novel AAV capsid type used in two monkeys (see text). Bottom: Six *Aotus* were transduced with 5e12 AAV1-2A10 each. Anti-CSP was quantified by ELISA

An additional vector, Anc80, with a capsid based on *in vitro* analyses of AAV capsid genes and projected to be insensitive antibodies to existing AAV types, was included in this study but was ineffective in producing mAb.

Goal 4: *Aotus* challenge 1 (mAb 2A10).

1. Transduction of Aotus *for challenge (current funding period).* At the end of the previous funding period, six additional monkeys were transduced with vectors expressing mAb 2A10. Expression data for those animals is presented in Figure 2 (bottom panel). Two of these animals display persistent high level expression of MAb2A10, while four expressed the MAb only transiently. This is consistent with experience with *Aotus* transduced with b12 earlier (3 of 4 monkeys expressed b12; see Figure 2). The reason for this variable 'take' is not clear. All animals are screened and confirmed to be negative for AAV1 neutralizing antibody prior to purchase, ruling out pre-existing humoral immunity to AAV1. Successful MAb expression of half of transduced animals is not an insurmountable difficulty in challenge experiments, although it will increase the number of animals that will be required to demonstrate efficacy. It would, of course, be unacceptable in immunization in humans. The basis of the phenomenon therefore will be explored in concluding work on this project.

2. Development of a liver burden assay for infection (current period). As detailed below, exhaustive efforts to conduct challenge experiments according to published procedures have been unsuccessful. Briefly, the published protocol relies on progression of the sporozoite challenge infection to blood stage parasitemia as an endpoint. In our hands, however, sporozoite infection is extremely irreproducible, and in addition, the strains reported to be required for the challenge produce sporozoites poorly in our mosquito populations (*An stephensi,* as published).

As an alternative to progression to patency as an endpoint, we are attempting to use the presence of parasites in the liver after sporozoite injection as a measure of infection and, potentially, protection. After inoculation by the mosquito, malaria sporozoites travel to the liver, where they develop for about 10 days to produce blood-stage merozoites, which go on to replicate in red blood cells. Our prophylaxis is intended to prevent liver infection by neutralizing sporozoites prior to their arrival there, and if successful, should reduce the number of parasites that can be detected in the liver (liver burden). In a natural infection, very few sporozoites are injected and reach the liver, and it would likely not be possible to detect them reliably. However, challenge with a large enough dose of sporozoites may give rise to measureable liver burdens (this is routinely done in mice), and we have chosen to explore this possibility in *Aotus*.

In these experiments, we use the NF54 laboratory strain of *P. falciparum* as a source of sporozoites. We have shown that NF54 is not capable of progressing to blood stage disease in *Aotus*, but it produces very large numbers of sporozoites (20,000 or more per mosquito). This enables us to challenge with vast numbers of sporozoites (10^6) in each animal, and calculations indicated that at this level of inoculation, parasites in the liver would be easily detectable by qPCR, accounting even for substantial losses in infectivity during sporozoite preparation.

Since ACURO approval of a protocol amendment to permit repeated liver biopsies, one experiment has been done that included three naïve (non-transduced) *Aotus*. Pre-inoculation and 5 days post-challenge with 10^6 NF54 sporozoites injected intravenously, approximately 100mg liver biopsies were obtained by laparotomy. Total RNA was extracted, and parasite 18S RNA was quantified by TaqMan RT-qPCR, using cellular actin mRNA as an internal control. In two of three animals, parasite RNA was readily detected; in the third it was not. The reason for variability is not clear, however, larger liver samples to account for potential uneven parasite distribution in the organ and may increase reliability. We conclude that measurement of liver burden may provide a route to assessing infection in challenge experiments.

Five of the six *Aotus* shown in Figure 2 are available for challenge under this protocol (one has died for reasons not apparently related to the study). We therefore plan to proceed with a large-scale challenge/liver burden experiment in the next few weeks.

Goal 5: *Aotus* challenge 2 (mAbs TBD). Time constraints will prevent this work.

C. Opportunities for training and professional development. One Master's student and one postdoctoral fellow received training under this grant during this funding period.

D. How results were disseminated. Nothing to report

E. Plans for next reporting period

1. A final challenge experiment will be performed with assessment of infection and protection by quantitation of parasite 18s RNA in the liver.

2. Measurements *Aotus* antibodies against the AAV-delivered 2A10 MAb in transduced monkeys will be made to address the possibility that the decline in MAb expression seen in some animals is due to immune responses to the vector-produced MAb.

3. Data describing murine studies of all MAbs examined will be published, as will data on *Aotus* transduction by VIP vectors. The liver burden assay for sporozoite infection may be incorporated, deoending on results from the ongoing experiment.

4. Impact. Nothing to report.

5. Changes/Problems (updated from the Annual report of 2018). A protocol for the assessment of pre-erythrocytic vaccine efficacy using sporozoite challenge in *Aotus nancymaae* monkeys has been published [5,6]. Briefly, splenectomized *Aotus* monkeys are challenged by IV injection of *Plasmodium falciparum* sporozoites, and are then monitored for development of parasitemia. Protection reduces the proportion of the challenged *Aotus* that become parasitemic. Published reports achieve a success rate of about 70% in infection of naive *Aotus* with the optimal parasite stain (Santa Lucia, see below). Despite extensive efforts, we have been unable to reproduce these results and this has delayed initiation of the next phase of the project, challenges in *Aotus*.

The experimental endpoint in this procedure – parasitemia – imposes a requirement for efficient parasite growth in *Aotus*. Most *P. falciparum* strains do not satisfy this requirement, probably due to poor growth in the *Aotus* liver, and thus few *P. falciparum* isolates have been identified as suitable for the published sporozoite infection protocol [3,4]. The isolate used in most of the published studies is Santa Lucia, a Honduran *P. falciparum* isolate. As reported in the 2017 Annual Technical Progress Report, the strain was located at the NIH and provided to us by Thomas Wellems. As received, St. Lucia grew well in human RBCs in culture and gametocyte production could be induced by methods routinely used in the Johns Hopkins Malaria Research Institute (JHMRI) parasite core. However, *An. stephensi* mosquitoes fed on the gametocyte cultures produced oocysts but did not produce sporozoites, preventing sporozoite challenge.

It is not uncommon for malaria strains passed in culture to lose infectivity for mosquitoes, but infectious parasites can sometimes be selected from such populations by passage through animals. Therefore, two splenectomized *Aotus* (309, 481) were inoculated with blood-stage parasite cultures produced *in vitro*. Both *Aotus* became parasitemic and both developed gametocytes. *An stephensi* mosquitoes were fed on both monkeys. These mosquitoes developed oocysts and sporozoites, and multiple sporozoite preparations were made and injected IV into four naïve splenectomized *Aotus*. One of the four sporozoite-inoculated monkeys (453) developed parasitemia. Blood was drawn from this animal and aliquots were preserved. This parasite (SL453), when amplified in culture, was infectious for mosquitoes and infected mosquitoes produce sporozoites, although in modest numbers. Thus, at least infectious sporozoite production was restored by animal passage.

Insufficient sporozoites for challenge experiments (50,000 per animal in the published protocol) were obtained from mosquitoes fed on SL453 blood differentiated *in vitro*. Therefore, banked 453 blood was used to infect another animal (670), which became parasitemic and was used to feed mosquitoes. Again, insufficient sporozoites were produced to permit IV inoculation. However, these mosquitoes were allowed to feed on two animals, one of which (392) became parasitemic. These parasites (SL453/392) thus had been twice passaged through mosquitoes and to *Aotus via* sporozoite infection (once by injection and once by mosquito bite). Mosquitoes were fed on gametocytes produced *in vitro* from cultures infected with SL453/392 and became infected, but produced only modest sporozoite yields upon dissection. Because insufficient

sporozoites for the published IV inoculation were available from these mosquiroes, six *Aotus* were exposed tp SL453/392 by mosquito bite. After 60 days, none had become parasitemic and the experiment was terminated by pre-emptively treating all animals with chloroquine.

An additional literature search revealed a single reference to use of a different *P. falciparum* strain, GB4, for sporozoite infection of *Aotus*. We obtained that strain, again from Thomas Wellems at the NIH. GB4 grows well in culture and produced parasitemia in an blood culture-inoculated *Aotus*. However, mosquitoes fed on that monkey or on GB4 cultures differentiated *in vitro* produced few or no sporozoites.

Our consistent inability to obtain high sporozoite yields in our mosquitoes (An. stephensi [repeatedly], An. gambiae [twice], and An. albopictus [once]), and the unreliability of transmission to *Aotus* by mosquito bites has forced us to conclude that using the published parasite, mosquito, and *Aotus* strains/species we will be unable to conduct the challenge experiments needed to evaluate our immunization approach using the published assay.

Infectivity by parasites in mice and protective efficacy of immunization regimens can be measured by quantitation of parasite RNA in the liver of challenged animals by RT-qPCR. Although this has not been reported in monkeys, preliminary data (see above) suggests that liver burden can be used to detect infection *Aotus* and a final experiment under this award will test use of liver burden assays to assess parasite infection in a fgroup of 5 transduced and 1 naïve monkey. If promising, data from that experiment will form the basis for applications for extension of the project.

6. Products Nothing to report

7. Participants and collaborating Organizations.

Personnel

Gary Ketner Ph.D. No change

Robert J. Adams. DVM. No change

Gloria Shin, PhD. Postdoctoral Fellow. Anticipating the end of this award (August 31, 2018), Dr. Shin left the laboratory for a position in an academic laboratory at Johns Hopkins.

Suk Namkung, ScM student. Full time, no DoD support. Mr Namkung joined the laboratory in May, 2017 graduated in May, 2019.

Funding support: This award

Changes in active other support. Nothing to report

Organizations

PATH/MVI
2201 Westlake Avenue, Suite 200, Seattle, WA 98121

Furnished anti-CSP monoclonal antibody sequences

Walter Reed Army Institute of Research
503 Robert Grant Avenue
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Furnished anti CelTOS monoclonal antibodies on a collaborative basis

Leidos
5202 Presidents Court
Frederick, MD 21703
Furnished 5D5 MAb sequence

8. Special reporting requirements. None

9. Appendices. None

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