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TITLE: CONTROLLED RELEASE OF NKT CELL AGONIST AND NON-REPLICATING PATHOGEN FOR SINGLE-DOSE VACCINATION

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

1) Colorado School of Mines, 1500 Illinois Street, Golden, CO, 80401

2) University of Florida, 207 Grinter Hall, Gainesville, FL 32611

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14. ABSTRACT
The purpose of this application is to extend the duration of protection after a single vaccination, using novel approaches for controlled release. We hypothesized that targeting a specialized subset of immune cells called NKT-cells will significantly improve the longevity of single-dose non-replicating vaccines which has scope for enhancing immunity against a wide variety of microbial pathogens of concern for military operations. Further improvements in longevity will be achieved via extended release systems. We have formulated vaccines for delivery with or without control release based on particles, for comparing multiple doses with an extended release dose. We have performed two vaccination studies to test the adjuvant effects of different NKT cell activation agents. The first study demonstrated that NKT-cells do indeed enhance immunity against an influenza virus vaccine. The second study was terminated prematurely due to the CoV19 crisis. For the controlled release of vaccine, PLGA microparticles have been synthesized using various types of PLGA. These have been synthesized to encapsulate fluorescein (as a model for α-GalCer), gold nanoparticles (as a model for vaccine), and then for α-GalCer and H1N1 vaccine. So far, we have achieved an initial release of therapeutic agents followed by minimal passive release. α-GalCer release was shown to have an initial "burst" release followed by minimal diffusional release for 80 days. Release of other molecules, vaccine and α-GalCer is still ongoing. The technology being developed should extend current knowledge on using controlled release devices for increasing the durability and longevity of vaccine-induced immunity for influenza. The same controlled-release vaccine concepts should be transferrable to vaccines against other types of human pathogens.

15. SUBJECT TERMS
Controlled release, Natural killer T cell, Adjuvant, Influenza A virus, Vaccine, Prime-boost, NKT-cell agonist, Glycolipid, PLGA particles

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1. Introduction

This application focuses on basic research for extending the duration of protection after a single vaccination, by improving the efficacy of the vaccine, combined with novel approaches for controlled release. Specifically, we hypothesize that targeting NKT-cells will significantly improve the longevity of single-dose non-replicating vaccines against a wide variety of microbial pathogens of concern for military operations, and further improvements in longevity will be achieved via extended release systems. Our application explores using the powerful adjuvant effects of NKT-cells to extend protective immunity of single-dose vaccines. We will formulate vaccines for intramuscular delivery with control release based on particles for comparing multiple doses with an extended release dose. We will specifically focus on comparing multiple formulations with the same total dose but varying the extended release characteristics of the formulation and the frequency of the injections. In vitro tests will first be conducted to design formulations with controllable extended release profiles. Our target will be to control the release duration from 3 months to 9 months. We will start with the systems in which we have preliminary data comprising of PLGA particles. The particle sizes will be varied from 100 nm to 5 microns to achieve a range of release durations ranging from a few weeks to a month. We will also prepare core-shell particles by using a water-oil-water emulsions which will allow further increase in the release durations to a few months. By combining an NKT-cell adjuvant approach with extended release, we aim to generate multi-year protection, which will significantly benefit both the military and the public.

2. Keywords:

Controlled release,
Natural killer T cell,
Adjuvant,
Influenza A virus,
Vaccine,
Prime-boost,
NKT-cell agonist,
Glycolipid,
PLGA particles

3. Accomplishments

- **What were the major goals of the project?**

  Specific Aim 1. To compare the longevity of humoral and cellular immunity induced by different NKT cell agonists when formulated as part of a single-dose vaccine. (*Performed at the University of Florida under the supervision of Dr. Driver*)

  Specific Aim 2. To develop PLGA particle-based systems to achieve pulsatile release of the inactive virus and NKT cell-agonists. (*Performed at the Colorado School of Mines under the supervision of Dr. Chauhan*)

  Specific Aim 3. To investigate whether particle-based delivery systems enhance and extend long-term protection of NKT cell-adjuvanted single-dose vaccine. (*Performed at the University of Florida under the supervision of Dr. Driver*)

- **What was accomplished under these goals?**

  Specific Aim 1. To compare the longevity of humoral and cellular immunity induced by different NKT cell agonists when formulated as part of a single-dose vaccine. (*Performed at the University of Florida under the supervision of Dr. Driver*)

  - Major activities: We performed 2 vaccination studies to examine the adjuvant effects of different glycolipid agonists when formulated as part of an influenza vaccine.

- Significant results of key outcomes: In the first study C57BL6 mice were vaccinated with UV-killed A/PR/8/34 (kPR8) influenza virus administered in combination with 1 μg α-GalCer or vehicle. We used this study to train Ms. Wen, a graduate student, and set up the assays needed to complete the project. Thus, only one NKT cell agonist was tested, α-GalCer. Blood samples were collected every 15 days after vaccination and analyzed for the concentration of virus-specific Abs and T cells by ELISA and PR8-H2b tetramers that stain PR8-specific T cells (available from the NIH tetramer core), respectively. Virus-specific Abs were measured in blood by ELISA. We found that α-GalCer significantly increased virus specific Abs and IgG2 concentrations specifically, indicating that the adjuvant effects of α-GalCer promoted a T helper 2 response to the vaccine. We were unable to clearly identify PR8-specific T cells using PR8-specific tetramer despite careful attempts to optimize this reagent. Thus, we decided to use IFNγ-ELISpot assays for future vaccination studies, which is a robust assay that we have plenty of experience using.

- Our second vaccination study began in February of this year but was prematurely terminated due to the CoV19 crisis. The design was similar to the first study, except that we tested other NKT cell agonists, including 7DW8-5, C-glycoside, and B12. We were able to collect plasma samples up until day 30 post vaccination, after which we were requested to terminate the study. We were unable to necropsy the mice and collect tissue samples or measure cellular responses because the study was designated non-essential.

  Specific Aim 2. To develop PLGA particle-based systems to achieve pulsatile release of the inactive virus and NKT cell-agonists. (*Performed at the Colorado School of Mines under the supervision of Dr. Chauhan*).

- Major activities: PLGA microparticles were synthesized using various types of PLGA (50:50, 65:35, 75:25, and 85:15). They have been synthesized to encapsulate fluorescein (as a model for alpha-GalCer), gold nanoparticles (as a model for vaccine), and then for α-GalCer and H1N1 vaccine.

- Significant results of key outcomes: Microparticles were shown to load and release all molecules when using 0.25% pluronic F68 as a stabilizer in the double emulsion. Microparticles were approximately 1-5 μm in size. Release profiles following an initial release of therapeutic followed by minimal passive release. All of the fluorescein was released within 130 days. Release of other molecules, vaccine and alpha-GalCer is still ongoing. For microparticles loaded with gold nanoparticles, the amount of gold nanoparticles loaded in the W1 phase was shown to not affect loading significantly. 0.5% pluronic F68 in the W2 phase resulted in the highest loading. Release for gold nanoparticles was not shown in the 60 days it was measured, indicating that the particles will not release any gold nanoparticles until they are fully degraded. α-GalCer release was shown to have an initial "burst" release followed by minimal diffusional release for 80 days.

- Vaccine release was shown to also have an initial "burst" release followed by minimal diffusional release for 120 days. SEM images showed that pores begin to form in the microparticles after 65 days of release. After initial release profiles are acquired, the microparticle synthesis will be optimized using different PLGA ratios or drug loading to achieve desired release profiles of burst release, followed by another burst release after 1 month. Optimized microparticle formulations will be tested *in vivo*.

  Specific Aim 3. To investigate whether particle-based delivery systems enhance and extend long-term protection of NKT cell-adjuvanted single-dose vaccine. (*Performed at the University of Florida under the supervision of Dr. Driver*)

- No work has begun on SA3 because this aim requires the completion of SA1 and SA2.

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

- The project has enabled Ms. Yuhan Wen, a graduate student with Dr. Driver, to develop the skills and expertise to perform vaccine studies and train as an immunologist. She has become proficient in flow cytometry and a variety of immunological assays. The study has given her the opportunity to increase her knowledge about general immunology as well as vaccinology. She has also learned a great deal about natural killer T cells and their potential therapeutic applications. Ms. Wen presents her results in lab meetings but has not yet had the opportunity to attend conferences since the project began in October 2019 and her project was delayed by the CoV19 crisis.

- Dr. Olivia Lanier who is a postdoctoral fellow with Dr. Chauhan was trained on many new pieces of equipment such as HPLC, LC-MS/MS, UV-Vis, DLS, and SEM. Additionally, she has gained experience in data processing and statistical analysis. She has also been able to research and read more about the field of drug delivery, and thus broadened her overall knowledge of the field in preparation for a faculty position. In addition, she trained 4 undergraduate students to help with this project which enabled her to develop time management and leadership skills. For professional development, Dr. Lanier has submitted abstracts about this project to national conferences BMES and AIChE and hope to present to them in the Fall.

- **How were the results disseminated to communities of interest?**

- Dr. Lanier has submitted abstracts about this project to national conferences BMES and AIChE and hope to present to them in the Fall.

- **What do you plan to do during the next reporting period to accomplish the goals?**

- We plan to complete the assessment of different glycolipid antigens in SA1 that was cut short by the CoV19 pandemic. A vaccination study for this purpose is due to commence within 2 weeks of this submission. (*Performed at the University of Florida under the supervision of Dr. Driver*)

- We will continue to optimize our particle delivery systems for the controlled release of both vaccine and glycolipid NKT cell agonists for use in the in vivo studies. We plan to create microparticles that have a burst release and then passive release until 30 days at which point, the particles should start to degrade and produce another burst release. So far, the second burst release is suboptimal, and the particle degradation has been gradual over ~100 days. We plan to send optimized particles for *in vivo* studies. (*Performed at the Colorado School of Mines under the supervision of Dr. Chauhan*)

- We plan to test cellular and humoral immunity generated by the particle vaccines in in vivo vaccine studies. (*Performed at the University of Florida under the supervision of Dr. Driver*)

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

- The PLGA particles being developed should extend current knowledge on using controlled release devices for increasing the durability and longevity of vaccine-induced immunity for influenza.

- **What was the impact on other disciplines?**

- The same controlled-release vaccine concepts that we are developing for influenza should be transferrable to vaccine against other types of human pathogens.

- **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

- **Changes in approach and reasons for change**

  - Our application proposed analyzing for the concentration of virus-specific T cells using PR8-H2b tetramers that stain PR8-specific T cells that we obtained from the NIH tetramer core. However, we were unable to clearly identify PR8-specific T cells using this reagent despite careful attempts to optimize the staining. Thus, we decided to use IFNγ-ELISpot assays for future vaccination studies which is a robust assay that we have plenty of experience using.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  - It is possible we won't be able to achieve a second burst release using PLGA. After we have analyzed all variations of PLGA and determine which parameters induce changes in the release profile, we may have to change our approach to a different polymer.

- **Changes that had a significant impact on expenditures**

  - Our expenditures will be affected by the vaccine study that was lost during the CoV19 shutdown. To mitigate this loss, we will repeat the study using fewer mice and if necessary, we will apply to redistribute funds from material costs to animal expenses.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  - **Significant changes in use or care of human subjects**

    - Nothing to report

  - **Significant changes in use or care of vertebrate animals.**

    - We have fewer animals available for SA1 due to loss of a vaccine study during the CoV19 shutdown. To mitigate this loss, we will repeat the study using fewer mice, so that we do not have to request more animals. However, we will request more mice if there is too much variability in the results to obtain conclusive results.

  - **Significant changes in use of biohazards and/or select agents**

    - Nothing to report

6. Products

- **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**
  - Nothing to report

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name:	John Driver
Project Role:	PI
Researcher Identifier	0000-0002-6714-3335
Nearest person month worked	1
Contribution to Project	Dr. Driver managed all aspects of the project and analyzed and interpreted the data

Name:	Anuj Chauhan
Project Role:	Co-PI
Researcher Identifier	0000-0002-1920-2900
Nearest person month worked	1
Contribution to Project	Dr. Chauhan managed the production of PLGA particles and analyzed and interpreted the data

Name:	Yuhan Wen
Project Role:	Graduate Student
Researcher Identifier	0000-0001-8789-2555
Nearest person month worked	5
Contribution to Project	Ms. Wen performed work in the area of testing NKT cell adjuvants and influenza A virus vaccines

Funding Support	University of Florida Graduate Student Fellowship

Name:	Olivia Lainier
Project Role:	Postdoctoral fellow
Researcher Identifier	0000-0002-6401-0465
Nearest person month worked	7
Contribution to Project	Ms. Lanier performed work in encapsulating adjuvants in colloids and subsequent incorporation into microparticles for controlled release

Name:	Sadie Auer
Project Role:	Biological Scientist
Researcher Identifier	N/A
Nearest person month worked	3
Contribution to Project	Ms. Auer assisted in work in the area of testing NKT cell adjuvants and influenza A virus vaccines
Funding Support	This award/NIH

- **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?**
  - Microparticles are being produced at the Colorado School of Mines

    Organization Name: Colorado School of Mines

    Location of Organization: 1500 Illinois Street, Golden Colorado, 80401

    Partner's contribution to the project Development of controlled release particles

    Financial support; None;

    In-kind support; partner makes controlled release particles;

    Facilities partner uses their facilities to make particles;

    Collaboration partner's staff work with project staff on the project;

    Personnel exchanges None; and

    Other. None

8. Special Reporting Requirements

- This is a duplicative report. Tasks are marked with the responsible PI and research site.

9. Appendices

- Nothing to report