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

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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 a vaccination study to examine the adjuvant effects of different glycolipid agonists when formulated as part of an influenza vaccine.

  - Significant results of key outcomes: We completed analyzing the second of two vaccination studies (Subtasks 3 & 4). This study was prematurely terminated in the previous funding period due to the CoV19 crisis. The study involved vaccinating mice with inactivated influenza virus vaccine and different NKT cell agonists, including α-galactosylceramide (α-GalCer), 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. Since, resuming normal research activities, we found that influenza-specific antibody titers were similar among the different NKT cell agonist recipients. Furthermore, none of the agonists substantially increased antibody levels beyond those found in mice that received the vaccine alone.

  - We selected the NKT cell agonist α-GalCer to adjuvant our vaccines.

    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: We experimented with various formulation parameters to optimize the release profiles of our particles. In preparation for these studies, we compared several protein-based methods to measure virus release from particles and selected an ELISA approach as the most accurate method (Major task 2.1 - Subtask 1). We tested various types of PLGA formulations to obtain an efficient vaccine release profile (Major task 2.1 - Subtask 2). Initially, these release studies were performed using gold nanoparticles, which did not load efficiently. Consequently, we switched to fluorescein protein and demonstrated that the particles released all of the encapsulated fluorescein within 130 days. Particles generated for these exploratory studies were approximately 1-5 μm in size.

- After establishing the release characteristics of our particles, we prepared virus loaded PLGA particles with UV-inactivated virus particles to determine the effect of different formulations on virus release profiles (Major task 2.1 - Subtask 3). Our first attempts resulted in minimal diffusional release after 120 days. Consequently, we experimented with different PLGA ratios and drug loading methods. We discovered two approaches that greatly enhanced vaccine release. The first was incorporation NaOH inside the vaccine particle which increased degradation of the particle from within, which resulted in substantial vaccine release over 7 weeks. This was through the creation of large voids through which the vaccine could diffuse. The second approach used a new formulation of PLGA known to degrade at a higher rate than our previous PLGA formulations. This PLGA has a lower molecular weight which can improve drug loading for molecules of a particular size. Vaccine release profiles from this new formulation are similar to the NaOH particles.

- We experimented with formulations to achieve different particle sizes in order to optimize vaccine incorporation and extend the length of time that particles are retained in the muscle to deliver the vaccine. We have been able to develop particles of between 15 and 170 μm in size that are capable of efficiently releasing vaccine.

- We have explored encapsulating the NKT cell agonist α-GalCer into PLGA particles, including in combination with the vaccine (Major task 2.2. - Subtask 2). Surprisingly, we found that α-GalCer-vaccine co-encapsulation significantly increased the particle loading efficiency as well as vaccine release from the particle (Major task 2.2. - Subtask 3). A study is planned to determine the loading efficiency of the α-GalCer using mass spectrometry (Major task 2.2. - Subtask 1).

- We are formulating PLGA particles with DiR, a lipophilic, near infrared fluorescent cyanine dye ideal for staining the cytoplasmic membrane. We have applied to perform an additional study to determine how long particles are retained in the body and whether they remain at the injection site (thigh muscle) of move to other locations (Major task 3.3.). This has important implications for the safety and efficacy of our approach. Particle distribution and degradation will be subsequently measured by performing in vivo imaging on the mice at two-week intervals for a period of 4 months.

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*)

- We have initiated in vivo studies to evaluate the efficacy of vaccine particles for immunizing mice compared to mice vaccinated with soluble vaccine. Mice injected with standard and NaOH-encapsulated versions of the PLGA vaccine particles were subsequently monitored for the development of influenza-specific antibodies every 15 days (Major task 3.1 – Subtasks 1-3).

- Mice injected with soluble the vaccine quickly developed influenza-specific antibodies as expected. However, mice injected with particles with a slow in vitro vaccine release profile did not develop any antibody response.

- Mice injected with NaOH-loaded vaccine particles with a high in vitro release profile induced large amounts of influenza-specific total IgG antibodies, which were comparable to mice that received the soluble vaccine. However, when we examined the antibody isotypes produced, we found a stark contrast in the ratio of IgG1 and IgG2a antibodies between particle and soluble vaccine recipients. The particle vaccine recipients produced high levels of IgG1 and undetectable levels of IgG2a, whereas mice that received the soluble vaccine produced mostly IgG2a and very little IgG1. This is significant as IgG1 has more functionality than IgG2a and is considered more important for antiviral responses.

- Mice immunized with the NaOH-loaded vaccine particles are being aged in the mouse vivarium at UF and will be challenged with influenza after 6 months, which is in 3 months time (Major task 3.2 – Subtasks 1-3).

- **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 has had the opportunity to be trained on a number of instruments that she will use for this project including various flow cytometers and cell sorters as well as the IVIS in vivo system.

- 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 presented abstracts about this project to national conferences BMES and AIChE. Dr. Lanier was recently accepted into a preeminent postdoctoral fellowship program at the University of Texas, Austin, which transitions postdoctoral fellows to faculty positions.

- The project has trained Zachery Sparks, an PhD student with Dr. Chauhan to formulate PLGA vaccine particle beads. Mr. Sparks has been instrumental in improving the release profile of PLGA vaccine particles and has taken the project over from Dr. Lanier.

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

- Dr. Lanier submitted abstracts about this project to national conferences BMES and AIChE and presented them in Fall 2020. She was selected to present this project at BMES 2020 Virtual Annual Meeting on October 14-17, 2020.

  Abstract Title: **Controlled Release of Non-Replicating Pathogen and NKT-Cell Agonist for a Single Dose Vaccination**
  Authors: Olivia Lanier, Yuhan Wen, Sadie Auer, John Driver, Anuj Chauhan

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

- 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. (*Performed at the Colorado School of Mines under the supervision of Dr. Chauhan*)

- We plan use the optimized vaccine particles for *in vivo* studies. Mice vaccinated with particles and soluble vaccine will be compared for cellular and humoral immunity. We will also challenge mice with live virus to test whether the particle vaccine offers longer lasting protection than soluble vaccine (*Performed at the University of Florida under the supervision of Dr. Driver*).

- A project modification has been submitted to inject mice with PLGA particles loaded with DiR, a lipophilic, near infrared fluorescent cyanine dye ideal for staining the cytoplasmic membrane. This will enable us to determine how long the particles are retained in the body and whether they remain at the injection site (thigh muscle) or move to other locations. This has important implications for the safety and efficacy of our approach. One obstacle for this technology is whether beads could be removed if adverse effect arose.

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.

  - We are discovering that particle encapsulated vaccines induce humoral immune responses that are qualitatively very different to soluble vaccines which has important implications for using particle-based vaccines to protect against influenza and other virus infections.

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

  - The same controlled-release vaccine concepts that we are developing for influenza should be transferrable to vaccines against other 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**

  - We have submitted an updated SOW to perform an additional task to inject mice with controlled-release particles containing fluorescent cell tracker dye particles. Particle distribution and degradation will be subsequently measured by performing in vivo imaging on the mice at two-week intervals for a period of 4 months. This will enable us to determine how long the particles are retained in the body and whether they remain at the injection site (thigh muscle) or move to other locations. This has important implications for the safety and efficacy of our approach. One obstacle for this technology is whether beads could be removed if adverse effect arose.

  - We are increasing the size of the particles we inject so that they are retained longer in vivo.

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

  - Our recent break throughs in particle development have delayed some of the in vivo studies as we decided that potential benefits were worth the postponement. We anticipate injecting mice with our most advanced particles by the end of May. Our plan was to challenge mice with live virus 6 months following vaccination. This will be a month over the project end date. To resolve this issue, we may request and additional NCE or challenge mice with live virus a month earlier than planned.
  -
- **Changes that had a significant impact on expenditures**

  - Nothing to report.

- **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**
    - We have applied to the funding agency for 10 more mice to image particles in vivo.
- **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 may repeat the study using fewer mice, so that we do not have to request more animals.
- **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:	PhD 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	3
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:	MS student
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

Name:	Zachery Sparks
Project Role:	PhD Student
Researcher Identifier	N/A
Nearest person month worked	3
Contribution to Project	Mr. Sparks performed work in encapsulating adjuvants in colloids and subsequent incorporation into microparticles for controlled release
Funding Support	Dr. Chauhan's research funds

- **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?**
  - Vaccine particles 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