Appendix 1 Syllabi for Asynchronous or Synchronous Immunology Lab

BIOL504 Immunology Lab: Research Skills and Immunologic Applications – Fall 2020

Instructors: Dr. Dyan Morgan

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Your instructors are available to answer questions via email, phone, and/or video conferencing.

Course Goals and Objectives:

This course is designed to provide students an understanding of human immune responses and how these are assessed experimentally. The course has a focus on research skills, experimental design, and specific methods for evaluating immune responses. This year, we will focus on COVID-19 and the components that are going into the vaccine development. We will consider many aspects such as correlates of immunity, antigen selection, assessment of immune responses, and vaccine approval and success evaluation. These lessons will then be applied to a student project in developing their own novel vaccine against their microbe of choice. This project will serve as the students final.

Course Prerequisite: Concurrent or prior enrollment in BIOL503

Textbook and Technology Requirements:

- Kuby Immunology, 8th Edition (2019) by Punt, Stranford, Jones, and Owen. You should purchase the format of textbook (e-book, loose-leaf, hardcover, etc) that suits your preferences and budget. The textbook is available to purchase from the KU Bookstore or directly from the publisher.
- Access to the internet. All course materials will be delivered and received via Blackboard (courseware.ku.edu), so you will need a reliable internet connection. This KU webpage provides suggestions that may be helpful if you are struggling to access the internet: https://remote.ku.edu/access

Course Format:

This course will be conducted **asynchronously** meaning that you have the maximum level of flexibility for completing coursework. All materials will be posted on Blackboard with deadlines for each assignment.

Each week we will have:

- Assigned reading or activity
  o Textbook (Kuby), research papers, or activity
- A pre- or in-"class" assignment (10 points) generally due by Thursdays at 11:59pm
- Recorded discussion led by Drs. Dyan Morgan or Scott Hefty to be posted Wednesday evening
- A post-"class" assignment (40 points) generally due by the following Wednesday at 2pm
  Towards the end of the course, we will have two assessments of students' Vaccine Project
  Final Exam is the Vaccine Project to be submitted before Dec 11th at 4 p.m.

Grading:

A = 90-100%	Pre-/in-class assignments	= 10% (11 assignments, can drop one)
B = 80-89%	Post-class assignments	= 40% (11 assignments, can drop one)
C = 70-79%	Pre-project reports	= 20% (each worth 10%)
D = 60-69%	Final Project	= 30% (Dec 11th by 4 p.m.)
F = <60%		

Academic Integrity: Academic integrity is a central value in higher education. It rests on two principles: first, that academic work is represented truthfully as to its source and its accuracy, and second, that academic results are obtained by fair and authorized means. "Academic misconduct" occurs when either of these principles is knowingly violated.

Academic Misconduct: Breaches of academic integrity (academic misconduct) have been rare in this class. However, we take academic misconduct very seriously. In this class, the most obvious kind of academic misconduct would be providing or receiving improper help on pre- or post-class assignments and/or plagiarizing sources for the vaccine project. Sanctions for charges of academic misconduct may include censure or written warning, reduction of grade for the work, reduction of grade for the course, a transcript notation, expulsion from the course, and/or expulsion from the university depending on the severity of the offense and the number of prior incidences on a student's record. Additionally, many medical, graduate, dental, business, and other professional schools ask applicants about charges of academic misconduct on the application form. If you find yourself underprepared for an assignment, it is always better to opt for a low grade than to cheat.

Academic Accommodations: The Student Access Center (SAC) coordinates academic accommodations and services for all eligible KU students with disabilities. If you have a disability for which you wish to request accommodations and have not contacted SAC, please do so as soon as possible. They are located in 22 Strong Hall and can be reached at 785-864-4064 (V/TTY). Information about their services can be found at www.access.ku.edu. Please contact us privately in regard to your needs in this course.

Religious observances: Please review the course schedule during the first week of class. Any student whose religious observances would prevent them from taking part in class activities should contact us during the first week of class so that we can make alternative arrangements.

Intellectual Property: All course materials prepared by the instructors and provided to you are the property of the instructors. Unless explicit permission is obtained from the instructor, course materials may not be modified nor transferred or transmitted to any other person, whether or not that individual is enrolled in the course.

General Course Schedule

	Topics	Points
Aug 26th	Orientation and Introduction into Experimental Design	
	- Pre- and post-class assignment	10/40
Sept 2nd	Experimental Design – Scientific Foundations	
	- Pre- and post-class assignment	10/40
Sept 9th	Research Development – Building on a Solid Foundation	
	- Pre- and post-class assignment	10/40
Sept 16th	Correlates of Immunity	
	- Pre- and post-class assignment	10/40
Sept 23rd	Antigen Selection and Vaccine Development I	
	- Pre- and post-class assignment	10/40
Sept 30th	Antigen Selection and Vaccine Development II	
	- Pre- and post-class assignment	10/40
Oct 7th	Methods for Measuring Immunity	
	- Pre- and post-class assignment	10/40
Oct 14th	Methods for Evaluating Neutralizing Antibodies	
	- Pre- and post-class assignment	10/40
Oct 21st	Methods for Cell Mediated T-cells	
	- Pre- and post-class assignment	10/40
Oct 28th	Methods for Cytotoxic T-cells	
	- Pre- and post-class assignment	10/40
Nov 4th	Initial Vaccine Project Review and Assessment	
	- Vaccine Project Submitted	10%
Nov 11th	Key Aspects for Assessing Correlates of Immunity	
	- Pre- and post-class assignment	10/40
Nov 18th	Open Review for Vaccine Project (you bring the questions)	
	- Vaccine Project Submitted	10%
Dec 11th	Final Project due by 4 p.m.	30%

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                                          864-5392 or 840-5695 (Cell)

Course meeting: Wednesdays 2-4 p.m. CST
                             Online Synchronous via Zoom (https://kansas.zoom.us/)
                             https://kansas.zoom.us/j/93961663336
                             Meeting ID: 939 6166 3336
                             Passcode: 2020

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Course Format:

We will meet weekly at 2 p.m. on Wed. and may go until 4 p.m.
Each week we will have;

- Assigned reading or activity to be completed prior to class ○ Textbook (Kuby), research papers, or activity
- A pre- or in-class assignment (10 points)
- Discussion led by Drs. Dyan Morgan or Scott Hefty
- A post-class assignment (40 points)

Towards the end of the course, we will have two assessments of students' Vaccine Project
Final Exam is the Vaccine Project to be submitted before Dec 11th at 4 p.m.

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A = 90-100%	Pre-/in-class assignments	= 10% (11 assignments, can drop one)
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F = <60%		

Important Aspects for Fall Online Course:

- It is different! – but the scientific and educational excellence is still there for you!
  We want the experience to be as interactive and engaging as possible. There will still be lecture components. We highly encourage questions! We will use the hand-raise in zoom as well as the chat function to ask questions.

- Strive to find the best and stable learning environment for you – quiet and no distractions.
- Importantly, your environment is just that – yours! You don't need to have your video on or you can use a background (appropriate content).

- Be in the moment! It is easy to be online at home, but not paying close attention. Phone, TV, other distractions - Turn em' off! It is your education and success that you have invested in.

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| Dec 11th | Final Project due by 4 p.m. | 30% |

Appendix 2 Assignment Prompts

Week 1: Pre-class/In-class (10 points)

This syllabus assignment helps us all ensure that we're starting the semester with a clear understanding of the plan and expectations for the semester. Please review the syllabus and submit this assignment prior to 2pm CST on Wednesday August 26th. You can complete this assignment as many times as needed - so should be able to earn the full points possible.

Week 1: Post-class (40 points)

Please complete the Labster Experimental Design Module found in "Labster Simulations."

Week 2: In-class Poll question (10 points)

1. An experiment describes the acquisition of cell supernatant from a single well of Jurkat T-cells cultured after 24 hours of treatment. This supernatant is used to evaluate the presence and levels of IL-2 cytokine using an enzyme linked immunosorbent assay. The supernatant is added to three ELISA wells and IL-2 levels are measured.
    This experiment measuring the levels of IL-2 from a single well sample in triplicate would be considered (2 points):

    A. Technical Replicates

    B. Biologic Replicates

    C. Experimental Replicates

    D. What the heck is an ELISA?

    E. IL-what?

    F. Did you make up Jurkat?

2. An experiment describes the acquisition of cell supernatant from three individual wells of Jurkat T-cells cultured after 24 hours of treatment. This supernatant is used to evaluate the presence and levels of IL-2 cytokine using an enzyme linked immunosorbent assay. Supernatant from each well is added to single ELISA wells and IL-2 levels are measured.
    This experiment measuring the levels of IL-2 from triplicate samples would be considered (2 points):

    A. Technical Replicates

    B. Biologic Replicates

    C. Experimental Replicates

    D. Still don't know what this ELISA you speak of

    E. IL-who?

    F. Did you mean Meerkat?

3. An experiment describes the acquisition of cell supernatant from wells of Jurkat T-cells cultured after 24 hours of treatment. This supernatant is used to evaluate the presence and levels of IL-2 cytokine using an enzyme linked immunosorbent assay. Supernatant from each well is added to individual ELISA wells and IL-2 levels are measured. The same experiment is repeated as similarly as possible but on different days.
    This experiment measuring the levels of IL-2 from samples taken on different days would be considered (2 points):

A. Technical Replicates

B. Biologic Replicates

C. Experimental Replicates

D. Are you going to tell us about ELISAs eventually?

E. IL-how?

F. Do Jurkat's really have culture?

4. An experiment describes the acquisition of cell supernatant from wells of Jurkat T-cells cultured after 24 hours of treatment with the known T-cell stimulant Phytohaemagglutinin. This supernatant is used to evaluate the presence and levels of IL-2 cytokine using an enzyme linked immunosorbent assay. Supernatant from each well is added to individual ELISA wells and IL-2 levels are measured.
This use of a known stimulant for IL-2 production in Jurkat T-cells would be considered (2 points):

A. Technical Replicates

B. Random addition

C. Experimental Replicates

D. Positive control

E. Negative control

F. You're just making up words now -Phyto huh?

5. An experiment describes the acquisition of cell supernatant from wells of Jurkat T-cells cultured after 24 hours of treatment with the same volume and solution used to add the known T-cell stimulant Phytohaemagglutinin – but without the compound. This supernatant is used to evaluate the presence and levels of IL-2 cytokine using an enzyme linked immunosorbent assay. Supernatant from each well is added to individual ELISA wells and IL-2 levels are measured. This use of the same volume and solution without compound for IL-2 production in Jurkat T-cells would be considered (2 points):

A. Technical Replicates

B. Biologic Replicates

C. Experimental Replicates

D. Positive control

E. Negative control

F. Phytohaemagglutinin seems beanie

Week 2: Post-class (40 points)

Use PubMed to identify 3 publications that strongly support the need for a vaccine candidate that you have identified. Your vaccine should not be a currently available vaccine or COVID-19.

Week 3: In-class Poll question (10 points)

1. I have had a formal course in statistics and ...... (3 points)
   a. I have a great grasp of statistical applications for Biology.
   b. I got through the course but have little idea how stats/concepts are applied.

     c.   I recall some concepts and have recognized their applications occasionally.
     d.   There is a dark hole in my soul due to the pain this course caused me.
     e.   I haven't taken a course in statistics and not looking forward to it.
     f.   I haven't taken a formal course in statistics and can't wait!

2.   I could explain it to someone else..... (3 points)
     a.   How a t-test is performed and the key factors
     b.   What standard deviation and reflects and how it is calculated
     c.   What p-value means and what level is typically acceptable (and why)
     d.   What 'Power' in a Power analysis means
     e.   How a Power analysis is performed and application for a sample size selection

3.   Tell us what vaccine target you selected and what evidence you found that this vaccine is needed. (4 points)

Week 3: Post-class Assignment (40 points)

Write 2 -3 complete and well-written paragraphs on the significance of your vaccine target selection using a total of 7-10 references (you can include your 3 previously identified references in this group).
Please include the following information:
1. Public health impact and significance of the pathogen
2. Populations affected
3. Transmissibility and biology of the organism

Week 4: In-class Assignment (10 points)

1.   Scientific Bias is (5 points):
a.   when you use Jedi mind control to get an experiment to work
b.   when you show only selective data in a report to convince my audience
c.   the unintentional influence on experimental design, execution, and interpretation
d.   when you throw out results because they don't match your expectations.
e.   when you throw out results because they don't match your expectations
f.   not an issue in Immunology because we don't involve human subjects (i.e., psych studies)

2.   Scientific Bias can be addressed by (5 points):
a.   Going to the dark side to really influence your experiments.
b.   asking your lab mates to watch you perform your experiments and look for issues with your technique.
c.   have two scientists perform the experiments and then compare data sets.
d.   have two scientists work together but separate experimental activities (one obtains samples - one analyzes samples)
e.   Randomize samples and perform analyses without knowing what the specific for individual samples.

Week 4: Post-class Assignment (40 points)

Consider and research three different vaccine formats and the potential pros and cons to utilizing each format to develop a vaccine for your target. Then write a 1-page report with a paragraph describing each of these three possible formats and the pros and cons for each format for use in developing a vaccine against your chosen target. Make sure you can justify your choices with in-text citations and a list of references cited. Upload your report to Blackboard as a Word doc or pdf.

Week 5: In-class Assignment (10 points)

1.   What is scientific transparency? (3 points)

a. Saying 'Boo' when you enter the lab
b. A complete and comprehensive description of how experiments were performed
c. Discussing with other scientists how experiments were performed, data acquired, presented, any manipulation, and interpreted
d. Sharing reagents and methods with other scientists

2. Why is transparency critical for rigorous science and experimental design? (3 points)
   a. All dead Nobel prize winners are ghosts (but not all ghosts are Nobel prize winners)
   b. Transparency is key for effective reproducibility.
   c. Transparency allows for alternate interpretations.
   d. Transparency enables scientific progress.
   e. Transparency (and asking important questions) affects your scientific reputation and your credibility.

3. Why is effective documentation important for rigorous science and experimental design? (4 points)
   a. Who will believe in ghosts unless you document it?
   b. We are legally required to maintain a lab notebook.
   c. It is cathartic to write about experiments that fail
   d. It is the key factor for (yours and others) science to be reproducible
   e. Regular (written) reminders of what we are focused on and explaining how experiments are addressing this is an effective strategy for scientific efficiency
   f. Poor documentation of experimental details could lead you to inaccurate results and conclusion
   g. Poor documentation could lead to months and years of effort being wasted (reputation too)

Week 5: Post-class Assignment (40 points)

Read the NEJM paper (Phase 1-2 Trial of a SARS-CoV-2 Recombinant Spike Protein Nanoparticle Vaccine) assigned in class and answer the questions that follow.
When you are explaining something please feel free to add citations and references, if it supports your point.
Some questions may have a breakdown of total points in brackets to help you to answer.

1. Identify and mention 10 terms (that you came across while reading the paper) that are unfamiliar and research 3 of those 10 terms and explain those here. (5+3points)
2. Describe 3 specific experimental design components that were discussed (in class so far) that are present in the paper. You can also highlight components that were discussed but are apparently absent in the paper. (6 points)
3. How was immunogenicity assessed?   (4 points)
4. Focus on Fig 3 and associated methods (page 5-7 of method supplement). Now:
   a) Describe ELISA and microneutralization methods as you would to a non-science friend (a layperson; 5+5 points).
   b) For ELISA, what is titer, and what is EU? (3 points)
   c) For Microneutralization: What is CPE and is it Qualitative or Quantitative? (3 points)
5. How are these individual experiments informing us about the effectiveness of the vaccine candidate? (6 points)

Week 6: In-class Assignment (10 points)

1. How did you know this was a research paper that included rigorous and significant science? (3 points)
   a. Dr. Hefty said so......
   b. Anything from New England has to be good.
   c. Experimental design details included critical components that support rigorous research.
   d. Impact factor of NEJM is < 2.
   e. Impact factor of NEJM is < 40.
   f. Impact factor of NEJM is < 80.
   g. Anything Corona is significant now.

2. What countries were the contributing scientists from? (3 points)
   a. USA
   b. England
   c. Australia
   d. Sweden
   e. Japan

3. The ELISA methods indicated the inclusion of a positive and negative control. What were these? (3 points)
   a. PC – convalescence serum, NC – negative patient serum
   b. PC – human serum against another virus protein, NC – uninfected human serum
   c. PC and NC were not identified.
   d. PC –serum from a rabbit immunized with spike protein, NC – serum from a rabbit not immunized.
   e. Defining controls are not critical for high impact journals (trust us, we did it correctly)

4. Reading this paper was... (1 point)
   a. Incredibly difficult
   b. Moderately difficult
   c. Better than what I thought it would be.
   d. Not Applicable

Week 6: Post-class Assignment (40 points)

You have already chosen your pathogen of choice. Now based on your choice write a 3-4 paragraph report to answer the following question.

What is known regarding correlates of immunity for my pathogen?

Use the following questions to guide your response to this question:

- Are people who are initially infected with your pathogen protected from subsequent infections? If so, what immune response is correlated with this protection?
- Is there less disease upon subsequent infections? If so, what immune response is correlated with this decrease?
- Have vaccine studies indicated that eliciting specific immune response provides some level of protection (or decreased disease)?
- If little or no protective responses are observed, what immune responses have been evaluated?

Make sure you have more than 4 references, and they are cited properly in-text and at the end when you make your reference list.

Week 7: In-class Assignment (10 points)

Upload your peer-reviewed feedback comments of the last assignment from the breakout session here. This assignment will be counted as in-class points for Week 7.

Week 7: Post-class Assignment (40 points)

Complete the Labster Simulation Exercise on ELISA and answer the questions in the module.

Week 8: In-class Assignment (10 points)

After analyzing the data provided to you via the excel file, upload the excel file with your names and the names of your partners by the end of class.

Week 8: Post-class Assignment (40 points)

You have the data from your in-class excel file that indicates the presence of antibodies following immunization. So, the next question is: are the antibodies generated following immunization functional for neutralizing the virus?

To explain, prepare a 1-page document. Write a method for viral neutralization (not ELISA) with appropriate experimental design components (for example: replicates, controls, etc.) included. Generate a 'result' with images that convey the expected level of cytopathic effect. Use images from published research (include citations in the results section). Make sure to convey your expected results in words explaining the figures and include citations to support your points.

Week 9: In-class Assignment (10 points)

1. Which vaccine format would encourage the presentation of antigen by MHC class I (ENDOGENOUS)? (5 points)

      a.   Recombinant protein
      b.   DNA vaccine
      c.   Replicating viral vector
      d.   mRNA
      e.   Attenuated organism
      f.   Inactivated organism or toxid

2.Which vaccine format would encourage the presentation of antigen by MHC class II (EXOGENOUS)? (5 points)

      a.   Recombinant protein
      b.   DNA vaccine
      c.   Replicating viral vector
      d.   mRNA
      e.   Attenuated organism
      f.   Inactivated organism or toxoid

Week 9: Post-class Assignment (40 points)

Post-class assignment is to complete the Labster cell culture module.

Week 10: In-class Assignment (10 points)

1. Which vaccine format would encourage the presentation of antigen by MHC class I (ENDOGENOUS)? (5 points)

      a.   Recombinant protein
      b.   DNA vaccine
      c.   Replicating viral vector

d. mRNA
e. Attenuated organism
f. Inactivated organism or toxoid

2. Which vaccine format would encourage the presentation of antigen by MHC class II (EXOGENOUS)? (5 points)

a. Recombinant protein
b. DNA vaccine
c. Replicating viral vector
d. mRNA
e. Attenuated organism
f. Inactivated organism or toxoid

3.Flow cytometry....

a. uses light scattering to separate cells of different size and granularity.
b. can analyze the size and granularity of individual cells.
c. can determine the presence or absence of a specific fluorescent marker on individual cells.
d. can measure the intensity of a specific fluorescent marker.
e. can generate two dimensional (X v Y) profiles of cell samples based upon specific light or fluorescent properties.
f. can select a subset of cells with certain properties for additional cell marker analyses.
g. can evaluate the light and fluorescent properties of millions of individual cells in less than a minute
h. appears to be Dr. Hefty's favorite technique

Week 10: Post-class Assignment (40 points)

Write a 2-page report on experimental design and expected outcomes for assessing-

T-cell responses (either CD4 or CD8) to your vaccine/antigen

- Describe the vaccination format and composition (antigen selection)
- Sample isolation and culture details
- Describe basic steps in the method (just one is needed)
- Generate results using data from research publication (doesn't have to be a paper using your antigen/organism)
- Include the reference where you got the data. Ensure inclusion of appropriate experimental design components!

Week 11: Post-class Assignment (100 points)

Provide an outline of the research report (using the guideline provided - see Appendix 2).

List each of the questions/issues that need to be addressed. Add bullet point details with your specific information that addresses these questions.
- Include your current knowledge and what you haven't determined yet
- If you haven't determined something yet – list what you need to do/determine to address this 'gap'.

Leaving a particular topic blank or not addressing it will result in point deduction.

Week 12: In-class Assignment (10 points)

Please type/upload the name of your in-class reviewer and the comments provided by them.

Week 12: Post-class Assignment (40 points)

Write a first rough draft of your research report building from your outline. Make sure you have all the components needed although we know you are continuing to work on it as this is just a draft.
This should be a report style document and is <u>NOT</u> an outline.

Week 13: Post-class Assignment (100 points)

Submit your full vaccine project (Draft 2 of 3). This assignment is worth 100 points (10% of your final course grade). This assignment cannot be dropped. Feedback from your first draft should be addressed in this second draft.

Week 14: Post-class Assignment (300 points)

Submit your final vaccine project report. This assignment is worth 300 points (30% of your final course grade). This assignment cannot be dropped. Feedback from your previous drafts should be addressed. Please review the report requirements in the previous lecture folders and the guidelines provided.

Appendix 3 Final Vaccine Project Guidelines with Grading Notes (in red)

Final Research Report Guidelines - 300 points

Novel Vaccine Efforts for Selected Pathogen

The final project is a research report focused on the development of a novel vaccine against a selected pathogen. Each student has selected a pathogen early in the semester to learn about the basic biology, disease(s) caused, and public health impacts. This well-cited information serves as the rationale for the development of a novel vaccine. Students have learned about the known and unknown correlate(s) of immunity which informs them on the format and antigen(s) selection for novel vaccine development. Students have learned about vaccine formats and the immune responses that should be elicited. Students have also learned how key immune responses are evaluated technical and the type of expected results (data). Essential aspects of experimental design for the execution of rigorous scientific methods have also been described.

The final report should incorporate all of the components outlined below. Scientific references are required to support information provided. There is no specific page limit for this report. Ensure that you have sufficiently addressed each of the aspects listed below.

Why is a vaccine needed for your selected pathogen? **60 points** – divided equally between 5 subpoints, making sure the expected components are covered to give them maximum points.

1) Public health impact of a pathogen
2) Describe the basic biology of the organism
3) Describe how the organism is transmitted/acquired and how disease is caused.
4) Describe current strategies for treatment and prevention, if available.
5) Include description about the need and impact of a vaccine to public health

What is known about the immune responses to this pathogen, specific components (antigens) and attempted vaccines (if applicable). **60 points**- This division is variable depending on what is known about the immune response of the pathogen. So depending on the pathogen, the weightage can be changed.

1) From individuals infected with this pathogen, describe their level of protection from subsequent infection. Include the known correlates of immunity. If individuals are not protected, what immune responses are generated against the pathogen? If vaccines have been attempted, what has been learned about specific immune responses and aspects of protection?
2) Describe specific components of the pathogen that generate a strong immune response during infection. Describe components that are key to the infectious process for which a targeted immune response could prevent this activity.

3) Provide a description that combines the known immune responses, aspects of immunity, and specific antigens or infectious components that could be included in your vaccine. This serves as the scientific rationale for the selected vaccine antigen/component.

What vaccine format and antigen that should be utilized to elicit a potentially protective or disease decreasing vaccine? **50 points** – To earn full points all prompts must be discussed accurately.

1) Describe the rationale for the selected vaccine format and the specific antigen(s) that will be included in this vaccine. Include what immune effector functions are expected to be elicited based upon this vaccine. Discuss the advantages and disadvantages to the selected vaccine format.

How are you going to assess your candidate vaccine? **100 points** – This portion of the report is weighted most heavily as this is the area where we can assess 1) if students can design an experiment using the experimental design components, and 2) if all the 3 immune effector functions are assessed. The figures and results must be described and interpreted to show understanding.

1) Describe the appropriate experimental design, methods, and expected outcomes for evaluating your candidate vaccine. Ensure that you include assessment of three immune effector functions (antibody, T-helper, and cytotoxic T-cells). 15 points (if they have all 3 effector functions evaluated in the reports they earn 15 points)
2) Be very specific about the experimental design components (controls, replicates, bias, statistical analyses, reproducibility aspects, etc.) that need to be included – be specific, thorough, and clear with this description and components. 30 points (each section has 5-point weightage + 5 points for sample size justification)
3) Describe the methods in a step by step process and descriptions of the importance of various components. 25 points (method description for all 3-effector functions)
4) Include example data from published papers that reflect the expected outcomes. These data do not have to be specific to your organism, antigen, etc., but should be data from the methods that you are describing. Ensure that you fully describe what is in your 'results'. For example, what is on the X-axis, Y-axis, and what do the data included mean – especially as it pertains to your vaccine candidate. You can modify the figures to match your experiment and expected results. 30 points (figures are labelled according to the vaccine candidate and explained in the results with description- to ensure students can interpret data)
5) There is an assumption that your vaccine candidate will be successful, especially in eliciting certain immune effector functions.

Summarize the vaccine efforts and outcomes. **20 points**- This is mainly a conclusion section to ensure that the students have understood the key aspects of the project.

1) Briefly re-state the need for the vaccine to your organism
2) Briefly re-state the known details of immune responses to your organism
3) Highlight the vaccine format and antigen selected
4) Summarize the key observations from your experimental studies that supports the potential of your vaccine candidate.
10 points for accurately including references and in-text citations.

Appendix 4 Examples of Student Work

Introduction

       Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) and a member of the Retroviridae family. As a retrovirus, HIV contains an RNA genome that is reverse transcribed to DNA and integrated into host cells' genomes. The virus is transmitted in bodily fluids. Contaminated bodily fluids are most commonly exchanged via sexual intercourse but may be exchanged via other activities – such as needle sharing and childbirth – as well. (0) HIV has cumulatively infected ~75 million people worldwide, and AIDS-related infections have caused ~32 million deaths. (1) The HIV pandemic disproportionately affects resource-poor regions such as Africa. As of 2019, there were ~38 million HIV-positive individuals worldwide, ~2 million of which were infected that year. (2) Despite millions of people being HIV-positive, the death-toll in 2019 was ~690,000 – a figure much smaller than those of earlier years. (1,2) Improved patient prognosis is due to antiretroviral therapy (ART), the primary HIV management strategy, which slows the progression to AIDS by ultimately decreasing viral load. (3) Because transmission probability is dose-dependent and miniscule – following a single exposure to infected bodily fluids, only ~1 in 1000 people will contract the virus – ART-mediated reduction of viral load also lowers transmission probability to negligible levels. (3,4)

       Despite the success of ART at improving patient prognosis and preventing transmission, it has failed to completely curb the HIV pandemic, as evidenced by the millions of new cases in 2019 alone; with more individuals entering the HIV-positive population (new cases) than leaving it (deaths), the total HIV-positive population only grows. ART's failings run deeper than access issues in resource poor regions. The daily, multidrug cocktail regimen requires strict adherence, which only 40-60% of patients achieve. (5) Furthermore, antiretroviral drug-resistant (ADR) HIV strains have emerged, and it has been shown that ART escalates the mutation rate of these strains. Greater mutation rate heightens evolutionary potential by raising the probability of resistance-conferring mutations. (6) While studies on ADR-HIV prevalence and transmission are often limited to local populations, multiple studies have shown worrisome trends. The overall results of one such study, which utilized longitudinal data to analyze trends in ADR-HIV transmission over time in San Diego, suggest that it is trending upwards. (7) Emergence and increasing prevalence of ADR-HIV suggest our primary HIV management strategy may suffer widespread failure in the future.

       The global impact of HIV, in conjunction with current and potential ART-related challenges, begets the need for an alternative solution for managing the HIV pandemic. While an increase in patient adherence or a decrease in regime strictness – perhaps through advances in drug-delivery systems – may theoretically improve the state of the HIV pandemic, patient habits are often resistant to change, and it is unlikely that advancements will reduce adherence issues to clinically negligible levels. Development of new retroviral drugs would only be a temporary solution, as resistance to these new drugs would likely emerge – possibly in a shorter timeframe than previously observed due to increased mutation rates in strains exposed to the old drugs. A vaccine would avoid the above-mentioned pitfalls of ART, as vaccination is typically only necessary a small, set number of times and does not foster the same degree of within-host viral evolution. Furthermore, a mathematical model assuming realistic vaccine efficacy (moderate) predicts that vaccination is medically merited and financially feasible. (8) A vaccine is thus a much needed and reasonable solution to pursue.

Background & Rationale for Vaccine Design

HIV initially binds to CD4, a protein expressed by certain host immune cells, via the viral envelope glycoprotein gp120 and subsequently binds to a chemokine receptor. Such binding facilitates membrane fusion, which is mediated by the viral envelope glycoprotein gp41 and facilitates entry. (9) CD4-expressing immune cells include dendritic cells (DCs) and CD4+ T cells, both of which are productively infected by HIV. (4) HIV kills CD4+ T cells irregardless of their infection status. (10) Following viral entry into these immune cells, reverse transcriptase reverse transcribes the RNA genome to DNA. HIV then uncoats, releasing viral DNA into the cytoplasm. (11) This DNA localizes to the nucleus, where it integrates into the host cell's genome. Integrated viral DNA is replicated, transcribed, and translated by the host cell, enabling virion production. (9) Sustained virion production gradually increases viral load, and as viral load increases, so too does CD4+ T cell depletion.

HIV is quickly disseminated from its initial infection site – most commonly, the mucosa associated with sexual transmission – by DCs. Infected DCs transport the virus to the lymphoid tissues, where there is an abundance of the virus' preferred target: CD4+ T cells. (4) Normal DC interaction with CD4+ T cells facilitates the latter's infection, and HIV-infected CD4+ T cells serve as the virus' main replication site and reservoir within the body. (4,12) While the host's immune system attempts to mount a response, it is often "too little, too late" – by the time the troops have been rallied, the time for containment has passed. (4)

HIV undermines host immunity in a variety of ways. Because the virus is highly mutable, its initial replicative burst – which occurs while the host's immune system is still formulating its response – allows for the timely generation of escape mutants, or virions capable of evading the host immune response. (4,12,13) In addition to antigenic variation, escape mutants' surface proteins often exhibit a higher degree of glycosylation, which helps shield them from host immune cells. These escapees manage to avoid cell-mediated and humoral immune responses, both of which are present. (12) Host immunity is also hindered by severe HIV-mediated CD4+ T cell depletion. HIV replicates more aggressively in *activated* CD4+ T cells than in *resting* CD4+ T cells; the host immune response thus generates more favorable replicative conditions for the virus. Finally, HIV may go latent in resting CD4+ T cells, facilitating evasion of CD8+ T cells. (4) The result of this present yet undermined immune response is establishment of a viral set point, or stable viral load. HIV infection is lifelong, and the virus continually depletes CD4+ T cells, gradually degrading immune function. Eventually, CD4+ T cell losses are such that the host becomes vulnerable to opportunistic infections, marking the onset of AIDS. (12)

Though infection is lifelong, select hosts' immune systems demonstrate a greater degree of viral control than do average hosts' immune systems; such hosts are called controllers. The cell-mediated response precedes the humoral response, and its timing and effectiveness influences viral trajectory; quicker timing and greater effectiveness correlate with decreased viral load and thus greater CD4+ T cell preservation. The most conserved feature of controllers (with respect to cell-mediated immunity) is expression of particular MHC variants, which present select Gag peptides. CD8+ and CD4+ T cell profiles in controllers suggest that quality is the primary factor in establishing cell-mediated viral control: controller CD8+ T cells have been shown to mediate a more diverse set of effector functions with greater potency than non-controller CD8+ T cells. Meanwhile, CD4+ T cells provide vital support to CD8+ T cells.

Notably, CD4+ T cells expressing IL-21 dominate in controllers; IL-21 promotes expression of perforin which, along with granzyme B, is upregulated in controller CD8+ T cells and crucial for cellmediated toxicity. (14)

While the humoral response that follows the cell-mediated response is typically inadequate early on in the course of infection, certain hosts have been shown to produce broadly neutralizing antibodies (bNAbs) following years of infection. (14,15) bNAbs target select Env peptides and, unlike antibodies produced during the early humoral response, exhibit diversity in their Env binding capabilities; they are able to recognize and neutralize a variety of possible mutants. (15) Env is a precursor to both gp120 and gp41, which are both required for viral entry into host cells – hence the potential for neutralization. (12, 17) While there are various classes of bNAbs, with members of different classes binding different Env regions, each class is highly effective; for example, certain VRC01-class bNAbs are capable of neutralizing ≥90% of HIV virions. bNAbs' heavy and light chain hypervariable regions often feature high degrees of somatic hypermutation, and their heavy chain hypervariable regions are often elongated, possibly helping them work around virions' glycan shields. The prolonged time period required for bNAb development and their characteristic features – along with the various structural and conformational changes in Env through time – suggest intimate, within-host bNAb/HIV coevolution. (15)

No HIV vaccine attempts have elicited an immune response on par with that of controllers, and most have ended in complete failure. However, one candidate – RV144 – demonstrated efficacy, albeit subpar and waning: ~60% at one year and ~30% at 3 and a half years. Both priming injections and boosters were utilized, with the former consisting of a vector expressing Gag, Pro, and Env peptides, and the latter consisting only Env peptides. The vaccine stimulated humoral immunity, and despite suboptimal neutralizing capabilities, the IgG antibodies produced had effector functions – such as agglutination, complement-activation, and antibody-dependent cytotoxicity – that mediated viral control. While both anti-Env IgG and IgA were detected at varying levels among vaccinated trial participants, the former was inversely correlated with infection and the latter was directly correlated with infection; evidence suggests that IgA competes with IgG for HIV binding spots, but lacks the protective effector functions of IgG, thereby counteracting IgG's protective benefits. (16)

While the naturally occurring, protective immune responses observed in controllers are limited to small subsets of HIV-infected individuals, they provide a sort of proof of concept that control is possible. These responses further establish that Gag peptides have the potential to stimulate a severity-reducing cell-mediated immune response, and Env peptides have the potential to stimulate a severity-reducing humoral immune response. RV144 – despite its lackluster results – reinforces the potential of Env peptides. Gag is a precursor to viral inner structural proteins, and Env is a precursor to viral envelope proteins (gp120 and gp41). (17) Collectively, this motivates vaccine-targeting of both Gag and Env peptides. Including peptides of other precursors may hinder escape mutant generation, as mutations enabling evasion are less likely to occur in tandem, motivating vaccine-targeting of Pol peptides as well. Pol is a precursor of viral enzymes. (17) These peptides – from Gag, Env, and Pol – will be targeted by an RNA vaccine.

RNA vaccines utilize mRNA molecules engineered to encode viral peptides; targeted peptides from Gag, Env, and Pol will be encoded in separate mRNAs. These mRNAs will be delivered to host cells via liposomal carriers, which enhance immunogenicity. mRNA carries no risk of genomic integration and

need not cross the nuclear membrane, increasing safety and decreasing challenge of delivery. Once delivered into host cells' cytoplasm, mRNAs are translated by host cell machinery to produce endogenous peptides for presentation on MHCI. RNA vaccines thus facilitate cell-mediated immunity, making them well-suited against HIV. (18) Incorporation of certain signal sequences into peptide sequences may also direct presentation on MHCII, thereby facilitating humoral immunity. (19) Such sequences will be incorporated in a subset of each peptide's corresponding mRNA pool. Dual presentation of peptides on MHCI and MCHII is hoped to facilitate a T$_H$1 response, which is desirable for combatting viruses. (20) While host cells cannot replicate mRNA, mRNAs encoding a viral RNA polymerase will be included, allowing for self-amplification. (18)

Though RNA vaccines have many advantages – lack of risk for genomic integration, unnecessity of crossing the nuclear membrane, and self-amplification potential – mRNA is more unstable ex vivo, making its biological activity harder to maintain. (21) Furthermore, once in the cytoplasm, RNA faces the challenge of RNAses. (18) However, ex vivo stability may be improved by lyophilization, and RNAse degradation may be limited by ribonucleotide modifications and specialized carriers. (18,21) Adding to RNA vaccines' advantages is that they may be quickly adapted to rapidly evolving situations; because the process of mRNA synthesis is the same regardless of genes encoded, alterations in gene content and subsequent large-scale production of the altered mRNA may be accomplished on a smaller timescale. There is also flexibility with regards to which genes are encoded in the mRNA. (22) The short turnover between vaccine alteration and production, paired with the ability to fine-tune encoded gene sets, may prove useful against HIV, as the virus is highly mutable. This, in conjunction with the previously discussed advantages and remedies for disadvantages, make RNA vaccines a promising choice against HIV.

Vaccine Evaluation & Results

Antibody responses were evaluated via an ELISA, while cytotoxic and helper T cell responses were evaluated via ELISpots. The ELISA specifically evaluated anti-Env concentrations, while the ELISpots generally evaluated the numbers Gag-, Env-, and/or Pol-specific IFN-γ-secreting CD8+ or CD4+ T cells. IFN-γ was chosen for analysis in the assays because it is an effector cytokine secreted by both CTLs and T$_H$1 cells. (20) The same positive control – an assortment of antigens from measles, mumps, rubella, and varicella viruses – was used in each assay; these antigens are hereafter collectively denoted by Ag$_+$. Choice of positive control is motivated by the following:

   • Live attenuated vaccines against these pathogens are currently available and in use. (23)

   • Live attenuated vaccines against these pathogens – which are obligate intracellular viruses – are expected to stimulate both humoral and cell-mediated immunity, so antibodies, cytotoxic T cells, and T$_H$1 cells specific to some subset of Ag$_+$ are expected to be present in vaccinated individuals.
   (20)

   • These vaccines are included in the CDC-recommended vaccine schedule for children, so the necessary number of immunized individuals should be available for study participation. (24)

HIV-negative, immunocompetent participants with qualifying vaccination records (MMR, Varicella) were equally divided into experimental and control groups. Participants in the former were given the HIV RNA vaccine, while those in the latter were given a saline placebo. Participants in each group served as biological replicates within their group to account for natural biological variation. A triple blind study – in which participants, treatment administrators and sample collectors, and outcome evaluators were not aware of treatment allocation – was utilized to control for bias. Number codes were assigned to the two treatments and the various samples, with the number code key known to only a group of bystander researchers – those not directly involved in treatment administration and sample collection,

or outcome evaluation. Sample collection and assays were repeated 3 times at 1-week intervals starting week 4. These repeats served as experimental replicates to establish reproducibility.

Relevant statistical theory was obtained from (25) and (26) for what follows. Data from each assay was analyzed via a 2-sample t-test for samples with unequal variances (Welch's t-test) to determine whether results were statistically significant. The tests operated on the following hypotheses:

Null Hypothesis (H_o): The means of the control and experimental groups (μ_o and μ) are equal ($\mu_o = \mu$)

Alternative Hypothesis (H_1): $\mu > \mu_o$

A significance level (α) and test power ($1 - \beta$) of 0.05 and 0.95 were chosen – to consider results significant, we require that our probability of committing a type I error (p) be less than 0.05 ($p < \alpha$), and to achieve desired test power, we require that the probability of committing a type II error (β) be 0.05.
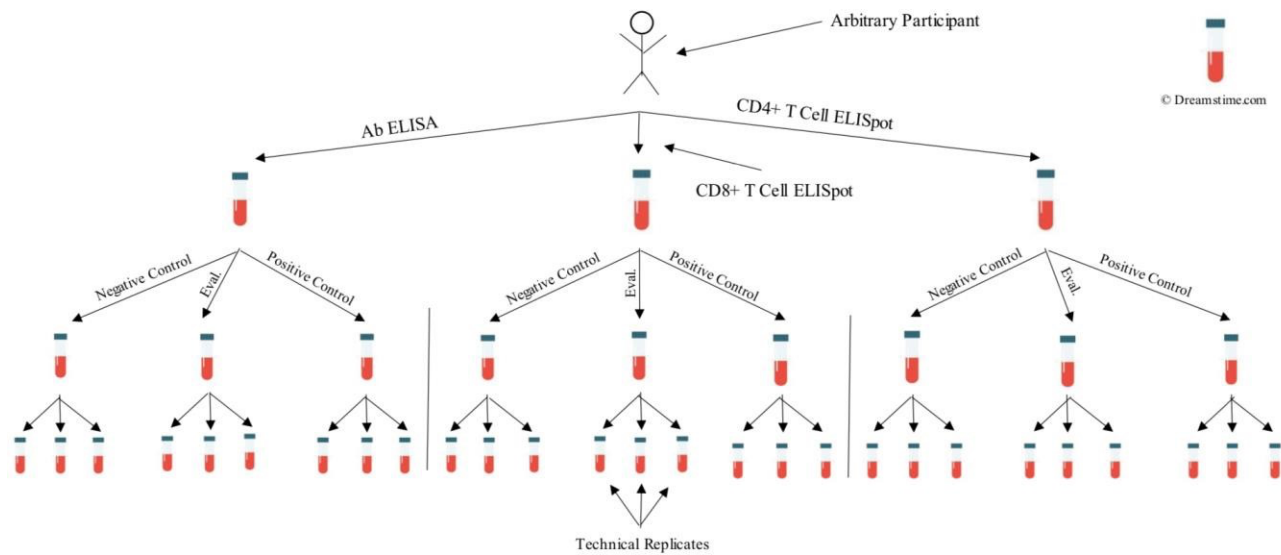
Type I Error: Accept that $\mu > \mu_o$ when $\mu = \mu_o$

Type II Error: Fail to reject that $\mu = \mu_o$ when $\mu > \mu_o$

With α = 0.05, $1 - \beta$ = 0.95, and an effect size (ε) of ~3.33282, a necessary sample size (n) of 6 – 3 in the control group and 3 in the experimental group – was determined via a power analysis using G*Power. While ~3.33282 is an a priori estimate of the effect size, it is a conservative one. Effect sizes corresponding to satisfactory results are expected to be higher; this justifies the choice of 6 participants, as n decreases with increasing ε. Retroactive justification will also be given once possible.

Three samples were consecutively collected from each participant on a given collection date, for a given set of assays – one for use in each assay.* Each sample was divided into three subsamples, creating three sets of subsamples – one set for use in the positive control, one set for evaluation, and one set for use in the negative control. Each subsample was further divided into three subsamples for use as technical replicates to monitor possible variations in laboratory equipment measurements. Henceforth, these subsamples will be referred to as samples. In what follows, let A, B, and C denote samples from individuals within the control group and D, E, and F denote samples from individuals within the experimental group.

* For ELISpot assays, PBMCs were isolated at this point, with PBMC samples replacing original samples in the rest of the sample division protocol.

Arbitrary Participant

Ab ELISA

CD4+ T Cell ELISpot

CD8+ T Cell ELISpot

© Dreamstime.com

Negative Control Eval. Positive Control

Negative Control Eval. Positive Control

Negative Control Eval. Positive Control

Technical Replicates

Assay Set 1, Antibody ELISA – Protocol & Results

The source used in this section was (26) unless otherwise indicated. MATLAB was utilized to perform data manipulation and generate graphical representations. Below, vaccine-targeted Env peptides are denoted as Ag_v.

- The wells of the... positive control plate were lined with Ag_+. evaluation plate were lined with

  Ag_v. negative control plate were not lined with antigens (bare wells).

  Antigen(s), when added, stick to the walls of the plate's wells and remain stuck there.

- Samples from the...

  positive control set were added to positive control plate wells.

  evaluation set were added to evaluation plate wells.

  negative control set were added to negative control plate wells.

 If a given sample contains antibodies against the antigen(s) lining their well – should there be any – these antibodies will bind to said antigens; antibodies within samples added to bare wells will have nothing to bind.

- Samples were washed away from each plate's wells. Any antigen-bound antibodies will remain and not be washed away.

- Anti-human, enzyme-conjugated antibody was added to each of the plates' wells. This antibody will bind to any antigen-bound antibodies. If there are no antibodies present, the antibodies will have nothing to bind.

- Unbound anti-human, enzyme-conjugated antibody was washed away, leaving only those antihuman, enzyme-conjugated antibodies bound to antigen-bound antibodies.

- The enzyme's substrate was added to each of the plates' wells. If anti-human, enzymeconjugated antibodies are present, the ensuing reaction will cause a color change. This color change indicates the presence (or, should there be no color change, absence) of antigen-specific antibodies.

- Optical density readings were obtained for each sample, and these readings were used to calculate antibody concentration in each sample.

Assuming all is well – everything worked properly, and the vaccine is a success – we expect the following.

- Because negative control wells were not lined with any antigens, there should be few antibodies in these wells; we expect little-to-no color change, or minimal concentrations of antibodies, across the board in these wells.

- Because evaluation wells were lined with Env antigens and we expect experimental group (vaccinated) individuals to have anti-Env antibodies, there should be numerous antibodies in wells containing samples from these individuals; we expect notable color change, or high concentrations of antibodies, in these wells. Because we do not expect control group (placebo) individuals to have anti-Env antibodies, there should be few antibodies in wells containing samples from these individuals; we expect little-to-no color change, or minimal concentrations of antibodies, in these wells.

- Because evaluation wells were lined with Ag_+ and we expect all individuals to have antibodies against some subset of Ag_+, there should be numerous antibodies in these wells; we expect notable color change, or high concentrations of antibodies, in these wells.

Below, the means and standard deviations of OD readings from technical replicates are reported in ordered pairs ($\mu_{TECH}, \sigma_{TECH}$), while each participant's antibody concentration (ζ) is reported in ng/mL.

Note: Raw data (appropriately manipulated to obtain presented data) was adapted from the excel activity data.

Negative Control	A	B	C	D	E	F
($\mu_{TECH}, \sigma_{TECH}$)	(0.1933, 0.0065)	(0.1433, 0.0022)	(0.2133, 0.0004)	(0.2033, 0.0042)	(0.2233, 0.0009)	(0.1667, 0.0006)
ζ	0.0080	0.0033	0.0107	0.0093	0.0122	0.0052

Evaluation	A	B	C	D	E	F
$(\mu_{TECH}, \sigma_{TECH})$	(0.4267, 0.0074)	(0.3733, 0.0020)	(0.6200, 0.0016)	(2.8267, 0.0052)	(2.8900, 0.0039)	(2.7400, 0.0073)
ζ	0.0139	0.0133	0.0709	16.9564	19.1864	15.0203
Positive Control	A	B	C	D	E	F
$(\mu_{TECH}, \sigma_{TECH})$	(2.8667, 0.0723)	(2.8700, 0.0624)	(2.7700, 0.0854)	(2.7967, 0.0723)	(2.9100, 0.0624)	(2.7100, 0.0854)
ζ	17.7228	18.7796	15.5492	16.3962	19.5989	14.5033

Concentrations are graphically depicted in the following:

Evaluation

Positive Control
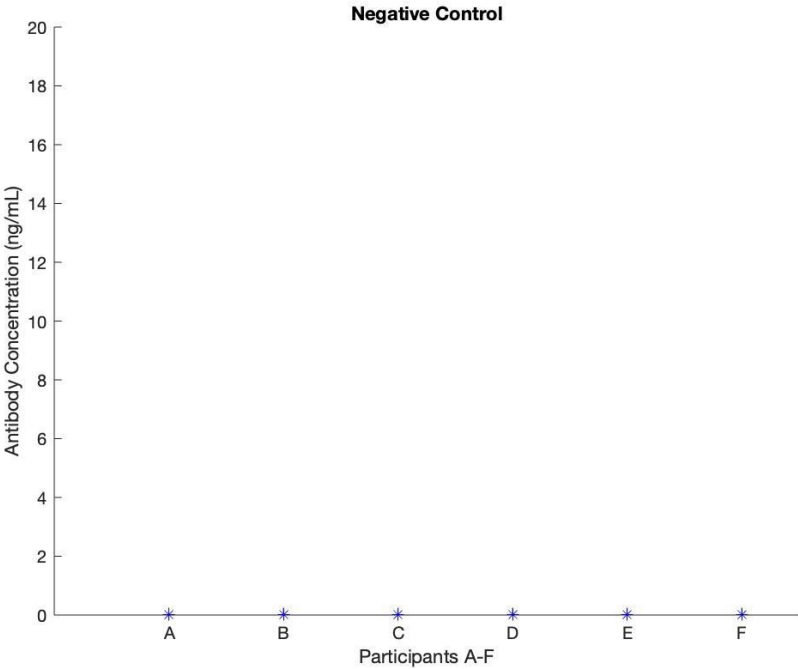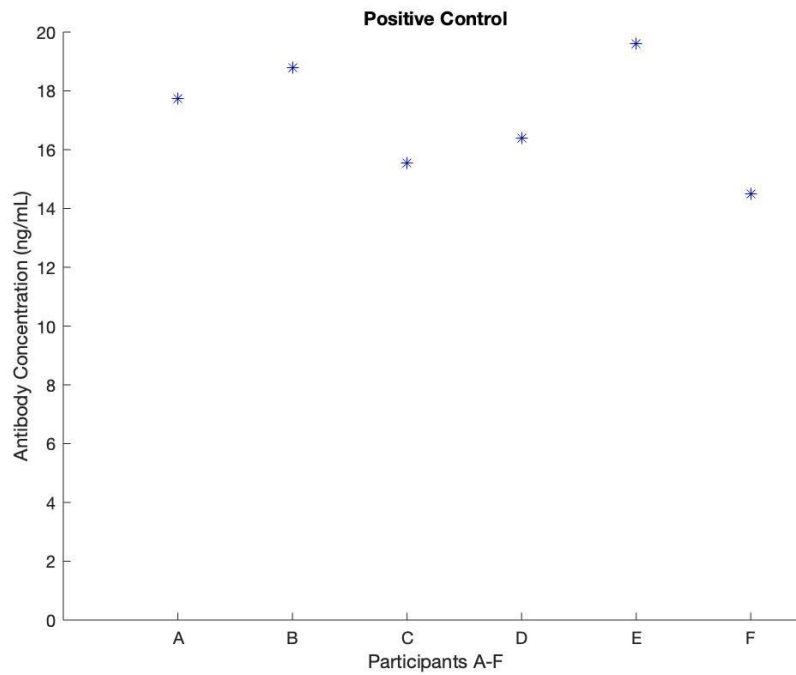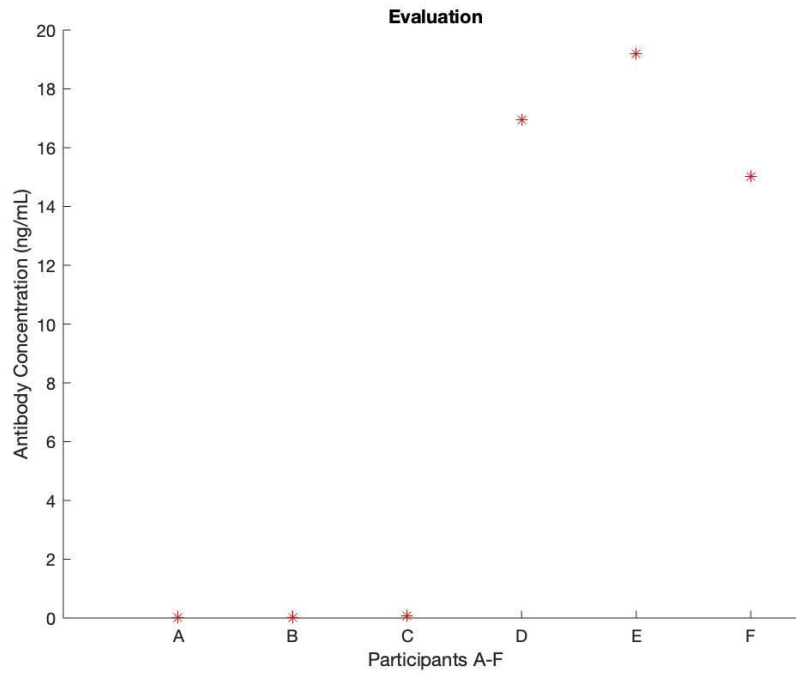
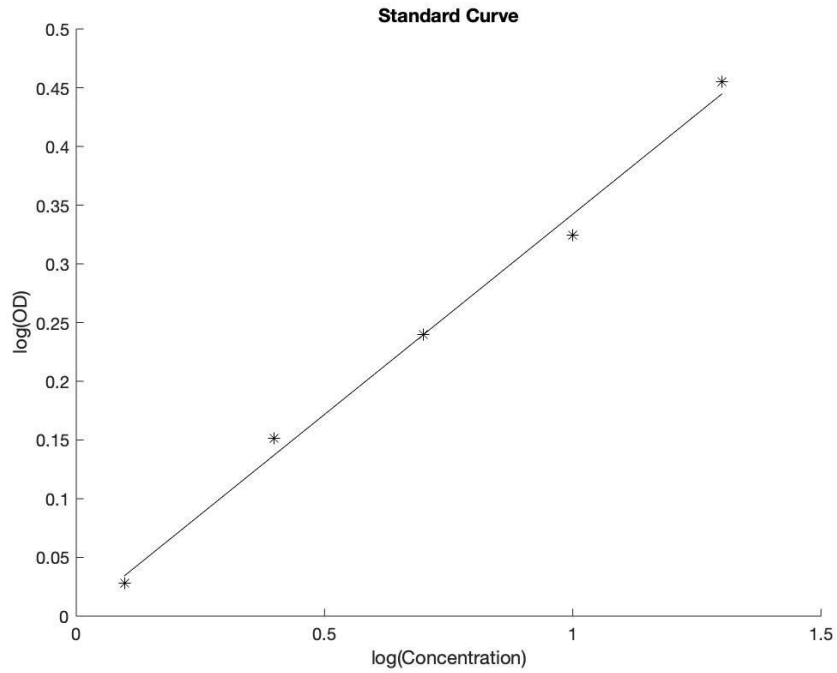Concentrations were calculated via the standard curve,

$$\log(OD) = 0.3410 \times \log(\text{Concentration}) + 0.0012,$$

which was obtained using the MATLAB polyfit function. The following displays the standard curve, as well as the standard concentrations and associated OD readings used to generate it (demarcated by asterisks):

Standard Curve

The curve was found to be a good fit, with R^2 = 0.9937 > 0.99. The experimental group mean, μ (obtained from antibody concentrations measured in participants given the vaccine), was tested against the control group mean, μ_o (obtained from antibody concentrations measured in participants given a placebo), using a Welch's t-test.

p = 0.0025	Experimental Group	Control Group
Sample Data	{16.9564, 19.1864, 15.0203}	{0.0139, 0.0133, 0.0709}
Sample Mean	17.0544	0.0327

Because p = 0.0025 < α = 0.05, we reject that $\mu = \mu_o$ and conclude $\mu > \mu_o$. So, the concentrations of antiEnv antibodies following vaccination are statistically significant – the vaccine stimulates humoral immunity. However, these antibodies may or may not be protective, their effector functions being as of yet unknown. Finally, the effect size for the above sample data was found to be ε^* = 11.5453 > $\varepsilon \approx$ 3.33282, justifying our choice of n = 6 (with regards to the ELISA assay).

Assay Set 1, APC / Cytotoxic T Cell Isolation & Cytotoxic T Cell ELISpot – Protocols & Results

The source used in this section is (26) unless otherwise noted. MATLAB was utilized to perform data manipulation and generate graphical representations. The following regards APC/cytotoxic T cell isolation.

• Samples were layered over Ficoll and fractionated via centrifugation.

• Antigen presenting cells (APCs) and T cells were isolated from each fractionated sample via extraction of the peripheral blood mononuclear cell (PBMC) layer.

- CD4+ T cells were removed from each PBMC-only sample as follows:

  - Biotinylated anti-CD4 antibodies were added to each sample. These antibodies bind to any CD4+ T cells present, forming biotinylated anti-CD4 antibody / CD4+ T cell complexes.
  - Streptavidin-conjugated magnetic beads were added to each sample. Streptavidin binds to biotin, forming biotinylated anti-CD4 antibody / CD4+ T cell / streptavidin-conjugated magnetic bead complexes.
  - Biotinylated anti-CD4 antibody / CD4+ T cell / streptavidin-conjugated magnetic bead complexes were removed using a magnet, to which the magnetic bead portions of the complexes are attracted. This removes CD4+ T cells from each sample, leaving only APCs and CD8+ T cells, as desired.

The following regards the ELISpot. Below, vaccine-targeted Gag, Env, and Pro peptides are denoted as Ag_v.

- Three sets of cell culture plates (positive control plates, evaluation plates, and negative control plates) were coated with anti-IFN-γ antibodies, washed with PBS, and blocked with serum albumin.

- PBMC samples were added to each set of cell culture plates; each cell culture plate was inoculated with PBMCs from a single sample.

- Ag_+ were added to the positive control cell culture plates, Ag_v were added to the evaluation cell culture plates, and no antigens were added to the negative control cell culture plates.

- Each set of cell culture plates were incubated to allow for possible CD8+ T cell interaction with and activation by APCs presenting whatever antigens may be present (should there be any). Should any antigen-specific CD8+ T cells be present and activated, they will secrete IFN-γ. The anti-IFN-γ antibodies coated on the bottom of each plate bind IFN-γ molecules in place (i.e. IFNγ will be bound in the vicinity of the CD8+ T cell that secreted it)

- Enzyme-conjugated anti-IFN-γ antibodies were added to each set of cell culture plates. The enzyme is chosen such that, when its substrate is added, a detectable color change occurs in the vicinity of any IFN-γ to which enzyme-conjugated anti-IFN-γ antibodies may be bound. This produces localized, colorized spots.

- As these local color spots correspond to local IFN-γ production, and local IFN-γ production corresponds to antigen-specific CD8+ T cell activation, they were used to approximate the number of antigen-specific CD8+ T cells present in each PBMV sample.

Assuming all is well – everything worked as expected, and the vaccine is a success – we expect the following. Images are courtesy of (27).

- Because antigens were not added to the negative control plates, there should be no IFN-γsecreting CTLs; we expect negligible spotting, or negligible numbers of IFN-γ-secreting CTLs, across the board in these plates.

(Student included modified figure from reference 27 here)

Negligible Spotting

- Because Gag, Env, and Pol antigens were added to the evaluation plates and we expect experimental group (vaccinated) individuals to have Gag-, Env-, and/or Pol-specific CD8+ T cells, there should be numerous IFN-γ-secreting CTLs in plates containing samples from these individuals; we expect extensive spotting, or high numbers of IFN-γ-secreting CTLs in these plates. Because we do not expect control group (placebo) individuals to have Gag-, Env-, and/or Pol-specific CD8+ T cells, there should be no IFN-γ-secreting CTLs in plates containing samples from these individuals; we expect negligible spotting, or negligible numbers of IFN-γ-secreting CTLs, in these plates.

(Student included modified figure from reference 27 here)

Extensive Spotting   Negligible Spotting

- Because Ag$_+$ were added to the positive control plates and we expect all individuals to have IFN-γ-secreting CD8+ T cells specific to some subset of Ag$_+$, there should be numerous IFN-γsecreting CTLs in plates containing samples from these individuals; we expect extensive spotting, or high numbers of IFN-γ-secreting CTLs, across the board in these plates.

(Student included modified figure from reference 27 here)

Extensive Spotting

Below, the means and standard deviations of IFN-γ-secreting CTL counts per 10^6 cells from technical replicates are reported in ordered pairs (μ_{TECH},σ_{TECH}).

Note: Raw data (appropriately manipulated to obtain presented data) was adapted from the IFN-γ-secreting CD8+ T cell ELISpot results in (27).
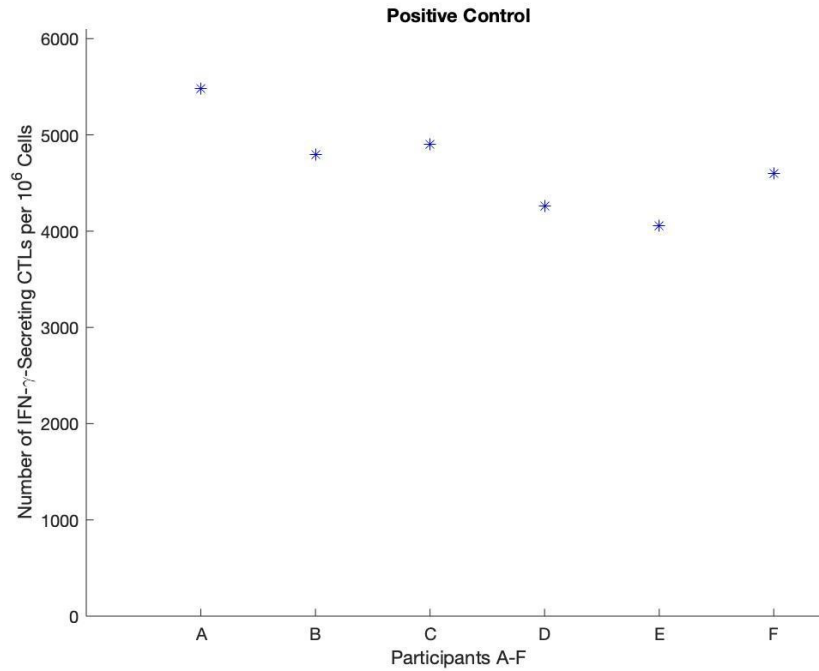
Negative Control	A	B	C	D	E	F
(μ_{TECH},σ_{TECH})	(43, 6)	(56, 10)	(59, 4)	(48, 8)	(54, 8)	(44, 5)

Evaluation	A	B	C	D	E	F
(μ_{TECH},σ_{TECH})	(52, 4)	(54, 6)	(52, 2)	(4656, 157)	(4587, 123)	(4853, 120)

Positive Control	A	B	C	D	E	F
(μ_{TECH},σ_{TECH})	(5477, 123)	(4798, 96)	(4901, 95)	(4263, 87)	(4059, 95)	(4597, 62)

IFN-γ-secreting CTL counts per 10^6 cells are graphically depicted by the following:
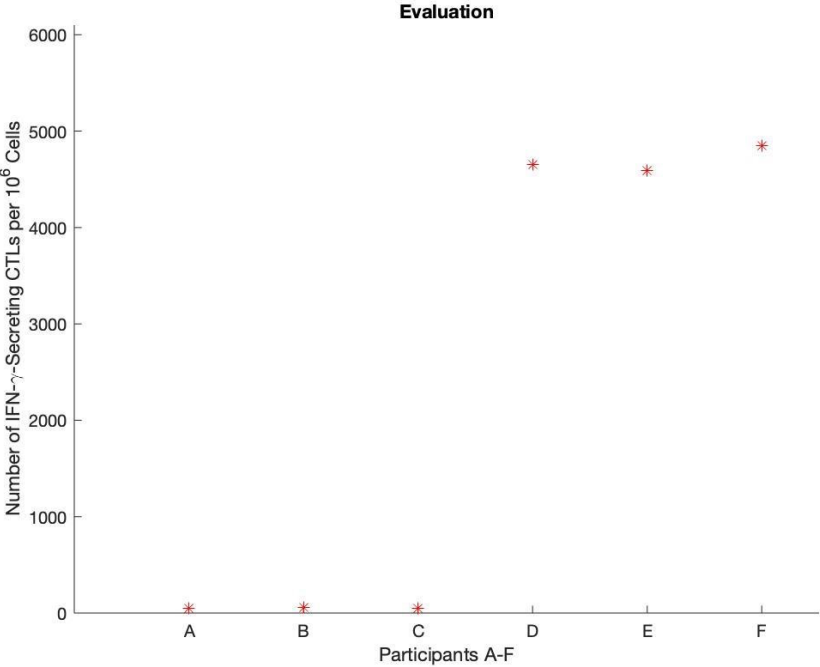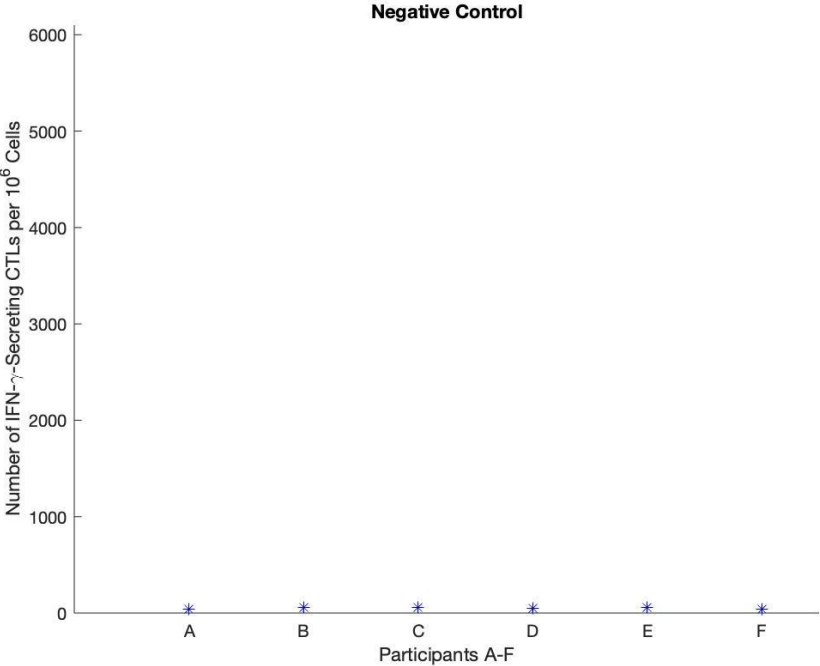
The experimental group mean, μ (obtained from IFN-γ-secreting CTL counts per 10^6 cells measured in participants given the vaccine), was tested against the control group mean, μ_0 (obtained from IFN-γsecreting CTL counts per 10^6 cells measured in participants given a placebo), using a Welch's t-test.

p = 0.00014693	Experimental Group	Control Group
Sample Data	{4656, 4587, 4853}	{52, 54, 52}
Sample Mean	4699	53

Because p = 0.00014693 < α = 0.05, we reject that $\mu = \mu_0$ and conclude $\mu > \mu_0$. So, the IFN-γ-secreting CTL counts per 10^6 cells following vaccination are statistically significant – the vaccine stimulates cellmediated immunity. However, these CTLs may or may not be protective, their effectiveness in response to a challenge in vivo being as of yet unknown. Finally, the effect size for the above samples was found to be $\varepsilon^* = 47.5981 > \varepsilon \approx 3.33282$, justifying our choice of n = 6 (with regards to this ELISpot assay).

APC / Helper T Cell Isolation & Helper T Cell ELISpot – Protocols & Results

The source used in this section is (26) unless otherwise noted. MATLAB was utilized to perform data manipulation and generate graphical representations. The following regards APC/Cytotoxic T cell isolation.

• Samples were layered over Ficoll and fractionated via centrifugation

• Antigen presenting cells (APCs) and T cells were isolated from each fractionated sample via extraction of the peripheral blood mononuclear cell (PBMC) layer.

- CD8+ T cells were removed from each PBMC-only sample as follows:

  - Biotinylated anti-CD8 antibodies were added to each sample. These antibodies bind to any CD8+ T cells present, forming biotinylated anti-CD8 antibody / CD8+ T cell complexes.
  - Streptavidin-conjugated magnetic beads were added to each sample. Streptavidin binds to biotin, forming biotinylated anti-CD8 antibody / CD8+ T cell / streptavidin-conjugated magnetic bead complexes.
  - Biotinylated anti-CD8 antibody / CD8+ T cell / streptavidin-conjugated magnetic bead complexes were removed using a magnet, to which the magnetic bead portions of the complexes are attracted. This removes CD8+ T cells from each sample, leaving only APCs and CD4+ T cells, as desired.

The following regards the ELISpot. Below, vaccine-targeted Gag, Env, and Pro peptides are denoted as Ag_v.

- Three sets of cell culture plates (positive control plates, evaluation plates, and negative control plates) were coated with anti-IFN-γ antibodies, washed with PBS, and blocked with serum albumin.

- PBMC samples were added to each set of cell culture plates; each cell culture plate was inoculated with PBMCs from a single sample.

- Ag_+ were added to the positive control cell culture plates, Ag_v was added to the evaluation cell culture plates, and no antigens were added to the negative control cell culture plates.

- Each set of cell culture plates was incubated to allow for possible CD4+ T cell interaction with and activation by APCs presenting whatever antigens may be present (should there be any). Should any antigen-specific CD4+ T cells be present and activated, they will secrete IFN-γ. The anti-IFN-γ antibodies coated on the bottom of each plate bind IFN-γ molecules in place (i.e. IFNγ will be bound in the vicinity of the CD4+ T cell that secreted it)

- Enzyme-conjugated anti-IFN-γ antibodies were added to each set of cell culture plates. The enzyme is chosen such that, when its substrate is added, a detectable color change occurs in the vicinity of any IFN-γ to which the antibodies may be bound. This produces localized, colorized spots.

- As these local color spots correspond to local IFN-γ production, and local IFN-γ corresponds to antigen-specific CD4+ T cell activation, they were used to approximate the number of antigenspecific CD4+ T cells present.

Assuming all is well – everything worked as expected, and the vaccine is a success – we expect the following. Images are courtesy of (27), and helper T cells are referred to has HTs.

- Because antigens were not added to the negative control plates, there should be no IFN-γsecreting HTs; we expect negligible spotting, or negligible numbers of IFN-γ-secreting HTs, across the board in these plates.

(Student included modified figure from reference 27 here)

• Because Gag, Env, and Pol antigens were added to the evaluation plates and we expect experimental group (vaccinated) individuals to have Gag-, Env-, and/or Pol-specific CD4+ T cells, there should be numerous IFN-γ-secreting HTs in plates containing samples from these individuals; we expect extensive spotting, or high numbers of IFN-γ-secreting HTs, in these plates. Because we do not expect control group (placebo) individuals to have Gag-, Env-, and/or Pol-specific CD4+ T cells, there should be no IFN-γ-secreting HTs in plates containing samples from these individuals; we expect negligible spotting, or negligible numbers of IFN-γ-secreting HTs, in these plates.

(Student included modified figure from reference 27 here)

Extensive Spotting   Negligible Spotting

• Because Ag+ were added to the positive control plates and we expect all individuals to have IFN-γ-secreting CD4+ T cells specific to some subset of Ag+, there should be numerous IFN-γsecreting HTs in plates containing samples from these individuals; we expect extensive spotting, or high numbers of IFN-γ-secreting HTs, across the board in these plates.

(Student included modified figure from reference 27 here)

Extensive Spotting

Below, the means and standard deviations of IFN-γ-secreting HT counts per 10^6 cells from technical replicates are reported in ordered pairs ($\mu_{TECH}, \sigma_{TECH}$).
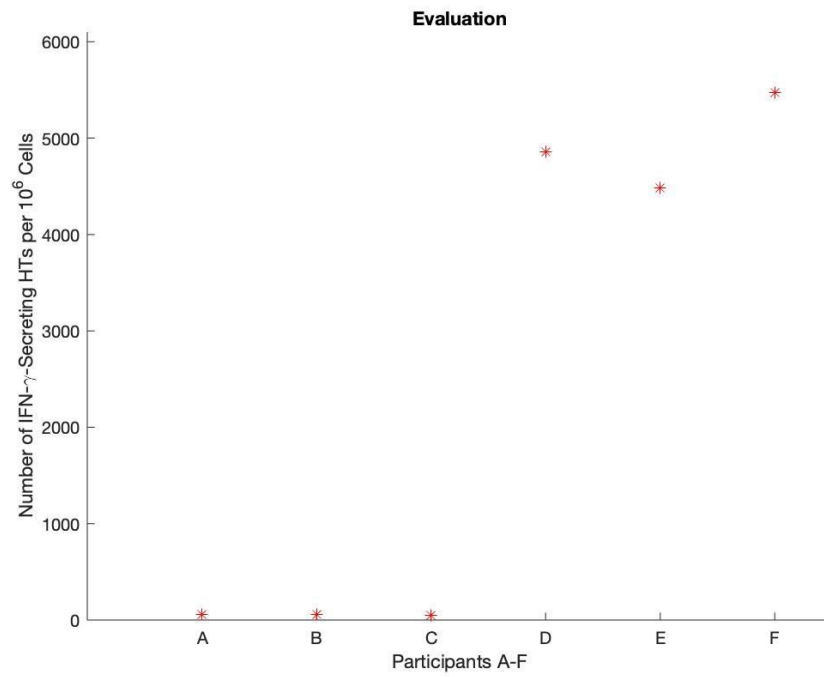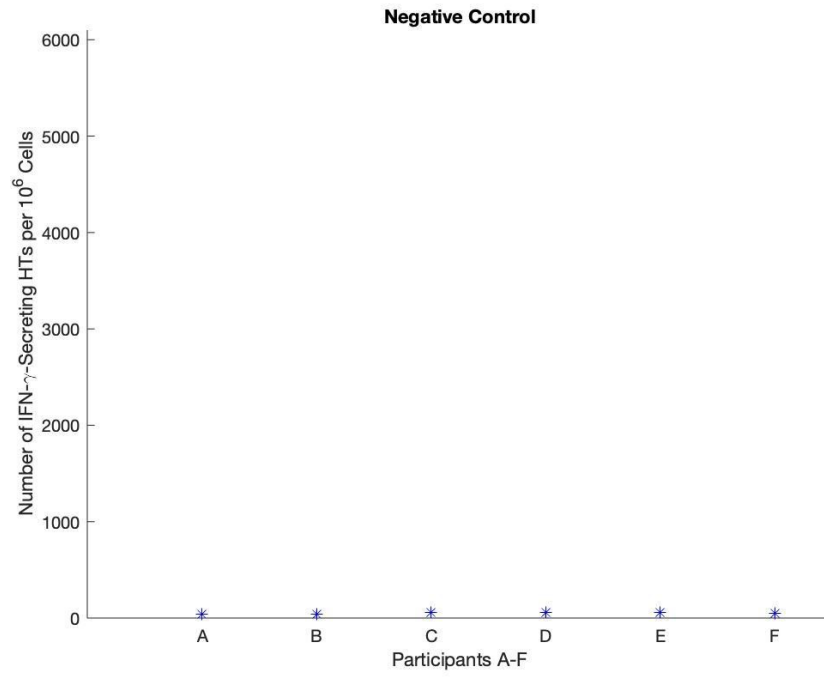
Note: Raw data (appropriately manipulated to obtain presented data) was adapted from the IFN-γ-secreting CD4+ T cell ELISpot results in (27).
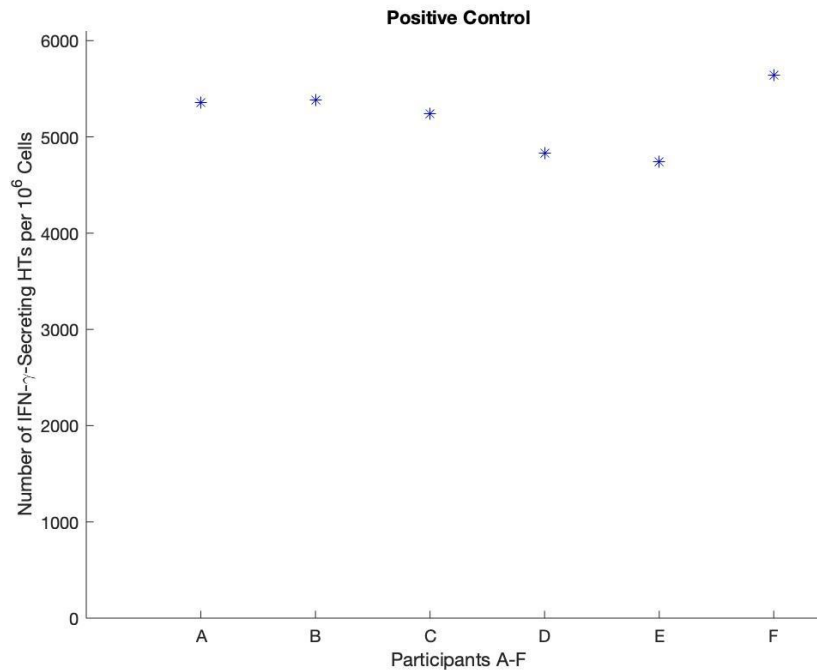
Negative Control	A	B	C	D	E	F
($\mu_{TECH}, \sigma_{TECH}$)	(44, 7)	(41, 9)	(59, 7)	(56, 8)	(56, 6)	(45, 6)

Evaluation	A	B	C	D	E	F
($\mu_{TECH}, \sigma_{TECH}$)	(61, 7)	(56, 7)	(53, 3)	(4855, 51)	(4480, 97)	(5473, 125)

Positive Control	A	B	C	D	E	F
($\mu_{TECH}, \sigma_{TECH}$)	(5354, 131)	(5379, 123)	(5241, 125)	(4829, 61)	(4745, 80)	(5642, 25)

IFN-γ-secreting HT counts per 10^6 cells are graphically depicted by the following:

Negative Control

Number of IFN-γ-Secreting HTs per 10^6 Cells

Participants A-F

Evaluation

Number of IFN-γ-Secreting HTs per 10^6 Cells

Participants A-F

Positive Control

The experimental group mean, μ (obtained from IFN-γ-secreting HT counts per 10^6 cell measured in participants given the vaccine), was tested against the control group mean, μ_o (obtained from IFN-γsecreting HT counts per 10^6 cells measured in participants given a placebo), using a Welch's t-test.

p = 0.0018	Experimental Group	Control Group
Sample Data	{4855, 4480, 5473}	{61, 56, 53}
Sample Mean	4936	57

Because p = 0.0018 < α = 0.05, we reject that $\mu = \mu_0$ and conclude $\mu > \mu_0$. So, the IFN-γ-secreting HT counts per 10^6 cells following vaccination are statistically significant – the vaccine supports both helper T cell dependent humoral and cell-mediated immunity and, moreover, stimulates a T_H1 response. However, these HTs may or may not be helpful, their effectiveness in response to a challenge in vivo being as of yet unknown. Finally, the effect size for the above samples was found to be $\varepsilon^* = 13.7601 > \varepsilon \approx 3.33282$, justifying our choice of n = 6 (with regards to this ELISpot assay).

Summary & Conclusion

HIV has infected ~75 million people worldwide to date and AIDS-related infections have caused ~32 million deaths – a staggering number of infections and devastating loss of life. As of 2019, there were ~38 million HIV-positive individuals worldwide, ~2 million of which were infected that year, indicating the pandemic is far from under control. Antiretroviral therapy (ART) is the lynchpin of HIV-management strategies, but antiretroviral drug-resistant (ADR) HIV strains have emerged, foretelling possible widespread failure of ART in the future. While HIV elicits both humoral and cell-mediated responses, these responses usually fail at arresting viral replication to manageable levels; only in select cases are they successful, and even then, infection is still lifelong. The most conserved feature of a

successful cellmediated response is expression of particular MHC variants, which present select Gag peptides; successful CD8+ and CD4+ T cell profiles suggest that quality is the primary factor in establishing cellmediated viral control. Meanwhile, a successful humoral response is characterized by production of broadly neutralizing antibodies (bNAbs) following years of infection; these bNAbs target select Env peptides and, unlike antibodies produced during the early humoral response, exhibit diversity in their Env binding capabilities. Together, these responses helped direct vaccine target selection.

Gag and Env peptides – along with Pol peptides – were chosen as RNA vaccine targets. The RNA vaccine included mRNAs encoding these peptides, as well as mRNA encoding a viral RNA polymerase for amplification of peptide-encoding mRNAs. The vaccine format naturally supports presentation of peptides on MHCI, and signal sequences directing presentation of peptides on MHCII were included in a subset of mRNAs. Taken together, peptide presentation on MHCI and MHCII is expected to facilitate both cell-mediated and humoral immunity. An antibody ELISA revealed high concentrations of Env-specific antibodies in vaccinated individuals, suggesting the vaccine may elicit a protective humoral immune response, and a cytotoxic T cell ELISpot revealed high counts of Gag-, Env-, and/or Pol-specific IFN-γ-producing CD8+ T cells, suggesting the vaccine may elicit a protective cellmediated immune response. Furthermore, a helper T cell ELISpot revealed high numbers of Gag-, Env-, and/or Pol-specific IFN-γ-producing CD4+ T cells, suggesting the vaccine may elicit a helper T cell response supportive of both cell-mediated and humoral immunity.

While the above results are promising, the effectiveness of these responses in vivo remains undetermined. Though a sterilizing immune response may be unlikely, given the unique challenges posed by HIV – especially the aggressive replication in *activated* CD4+ T cells, which helps generate escape mutants, and latency in *resting* CD4+ T cells, which facilitates immune system evasion – a functional immune response may be within reach. If the memory cells generated in response to vaccination are effective, this – paired with the speed at which a secondary immune response is mounted – may be sufficient to dampen HIV's initial replicative burst, making the generation of escape mutants less likely. With a lower peak in viral load and fewer escape mutants, the immune system may potentially maintain the viral load at a lower set point for some period time. As lower viral load corresponds to greater CD4+ T cell preservation and lower transmissibility, such an immune response may decrease disease severity and help break the chain of transmission, thereby improving HIV-positive individuals' quality of life and helping curb the pandemic. Though it is traditionally desirable for vaccination to prevent infections in the vaccinated population, reducing the probability of transmission from *vaccinated* HIV-positive individuals may achieve similar long-term results, should the reduction be great enough.

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* No issue indicated.

** Note issue or page numbers indicated.

Assessing IgM Antibody, CD4+ Helper T Cell, and CD8+ Cytotoxic T Cell Response to Whole Attenuated Malaria Vaccine Utilizing Microneutralization and Flow Cytometry

Introduction

Plasmodium is an ancient parasite that poses a threat to millions of people worldwide and is the leading cause of global vector-borne deaths. It's estimated that in 2016 alone, six species from the Plasmodium genus were responsible for roughly 216 million malaria cases globally with approximately 445,000 deaths (1). One such species, Plasmodium falciparum, is an obligate intracellular parasite that either presides in hepatocytes or erythrocytes. The parasite is disease causing only if it resides in erythrocytes. Once infected, humans may present with severe anemia and cerebral malaria (seizures and coma), which can lead to death (2). Disease symptoms manifest as a result of the interaction between the parasite and the host's pathophysiological response. In other words: disease severity is determined by the strength of the immune response (3) Furthermore, the parasite can be hard to eliminate from the human body. Plasmodium species can lay dormant in the hepatocytes in what is known as a hypnozoite stage. Control and eradiation of the disease is challenged by the failure to eliminate these hypnozoites (4). Parasite eradication is also being challenged by drug resistance to widely used drugs such as chloroquine (5).

The disease is transmitted to humans via an infected vector- most commonly being a mosquito (6). This parasite has the potential to infect anyone, but populations at risk are those who live in hot, humid climates where mosquitos thrive. Regions at risk include tropical Africa, Central America, and the Asian Oceana. However, human migration plays an important role in transmitting the parasite to non-endemic regions. For example, there have been large malaria outbreaks in developing Indian cities such as Mumbai, Goa, and Kolkata over the past three decades. These outbreaks have been linked to human migration of rural populations in search of construction work (7). In a more modern context, having extended airline networks has allowed previously isolated malarious regions to be connected to non-endemic regions. This has increased the rates of imported cases, local outbreaks in non-endemic areas, and the spread of drug resistance strains (8).

Those traveling to malaria endemic regions can prevent infection by using a drug called chemoprophylaxis. Unfortunately, chemoprophylaxis and most other antimalarials are unable to kill hepatic stage malaria. This presents a risk of relapse due to the perseverance of hypnozoites. However, there have been many antirelapse drugs developed to rid the host of these hypnozoites. Drugs like primaquine and tafenoquine kill liver stage malaria, and therefore eliminate the risk of relapse (9).

Populations hit hard by malaria can be challenged socially and economically. It has been shown that a 10% decrease in malaria incidence was correlated with an 0.3% increase in income per capita, and that overall industrial growth is slower in countries with higher malaria incidences. Thus, there is an inherent link between malaria and economic growth (10). The cost of caring for malaria can also be financially taxing to both effected families and the healthcare system. Increasing malaria prevention would reduce the burden on households and the healthcare system, which would improve overall productivity in endemic countries.

Malarial Correlates of Immunity

Maintaining a sufficient number of memory T and B cells is critical for mounting a quick and effective immune response to subsequent reinfections. However, immunologic memory to parasitic infections, such as malaria, are poorly understood. It was found that immunologic memory in individuals

from Malaria endemic regions is either poorly developed and simply not retained. Those who survived a natural malaria infection in their youth remain susceptible to recurrent infections. However, repeatedly inoculating individuals with attenuated strains seems to produce long lasting immunity against experimental malaria challenges (11).

Innate immunity is tricky in terms of malaria infections because it is important for clearing the infection. However, excessive activation contributes to the pathology of the disease and is therefore a key determinant to disease severity and outcome. The complement system initiates innate immune responses such as inflammation, opsonization, and coagulation – all of which have been observed in response to malaria infections. Excessive inflammation, endothelial activation, and activation of coagulation pathways can have disastrous effects. These mechanisms can lead to tissue hypoxia, altered blood-brain barrier, or neuronal injuries (12). However, as previously mentioned, there needs to be a balance. These same innate immune responses play a key role in developing protective immunity. The pro-inflammatory responses of the complement system will regulate antiparasitic Th1 development, which stimulate B cells to produce IgM (11).

Any form of memory is obviously associated with the adaptive immune system. In the case of malaria, it seems that memory is heavily mediated by T-cells, but also relies on memory B cells for blood stage *Plasmodium* infections. One study showed that IgM[+] memory B cells are critical for rapidly and effectively initiating the immune response to secondary infections. This may be because IgM antibodies are mediators for complement facilitated cellular lysis, which is important for control of blood stage infections. Furthermore, this study showed that both T-dependent and T-independent processes could contribute to a memory B cell response (13).

In addition, research has shown a correlation to immunity and the availability of helper CD4^{+} T cells and cytotoxic CD8^{+} T cells. T cell responses have the ability to form tissue-resident and long-lived memory populations against malaria infections. Unfortunately, the exact mechanism that governs the development and function of these T cell responses are poorly understood (14). Repeated exposure to malaria infections will generate disease limiting immunity against erythrocytic stage infections, but it will not generate sufficient immunity against hepatocytic stage infections; the exact reason for this is unknown. CD8^{+} T cells seem to play an important role in conferring protection at the liver stage of malaria infections. Multiple antimicrobial effector mechanisms are initiated upon activation of CD8^{+} T cells by recognition of pathogen peptides via surface MHC class I on antigen presenting cells. For example, IFNgamma is released, which has been shown to directly impair *Plasmodium* growth in human hepatocytic cell culture (15). As previously mentioned, too much inflammation can cause life threatening pathology to the host. Therefore, there needs to be a counter measure for reducing inflammation. IL-10 is a potent antiinflammatory cytokine that plays an essential role in limiting the immune response so it is not too severe (16).

Determining Vaccine Format

Live/attenuated vaccinations contain attenuated pathogens which are less virulent than the corresponding wildtype strain. Attenuation occurs by passing the pathogen through multiple series of *in vitro* cell cultures, most commonly utilizing chicken embryo cells. As the pathogen continues to replicate, it will get more efficient at infecting the cell culture and replicating. However, it will progressively become worse at infecting human hosts. Nonetheless, this vaccine type prevents the actual disease from manifesting in the host and will instead mimic the disease in a minor way. The wildtype antigens will still

be presented to the host, and this allows the host to develop an immune response which is comparable to the natural infection (17). An antibody response is induced and long-term immunity is acquired. Live/attenuated vaccines are advantageous because they can induce herd immunity through excretion of pathogenic particles, which may indirectly "vaccinate" surrounding individuals (18). Furthermore, they do not require an adjuvant because the attenuated pathogen will continue to replicate which self-enhances the immune response. In terms of parasitic vaccinations, multiple studies have shown that whole attenuated sporozoites will induce a powerful CD8$^+$ T-cell response with protection. Furthermore, it has been shown that whole-parasite vaccines consistently produce the best immunity by encouraging strong responses from both CD4$^+$ and CD8$^+$ T-cells (19). Despite these advantages, live/attenuated vaccines can pose great risks. Clinical disease can still occur following vaccination, however, the symptoms are usually milder than compared to a natural infection. Those who are pregnant risk fetal infections, while immunocompromised individuals are at risk for unregulated pathogen replication. Finally, this vaccine form can revert back to the wild-type disease causing form. The goal of this vaccine is to activate both T cells and B cells. The antigen should activate IgM$^+$ B cells to control for the blood stage malaria while simultaneously activating CD4$^+$ and CD8$^+$ T cells to control for the liver stage malaria.

Assessing Whole Attenuated Vaccine Efficacy

IgM Microneutralization Assay

Figure 1. Infection scheme for neutralization assay
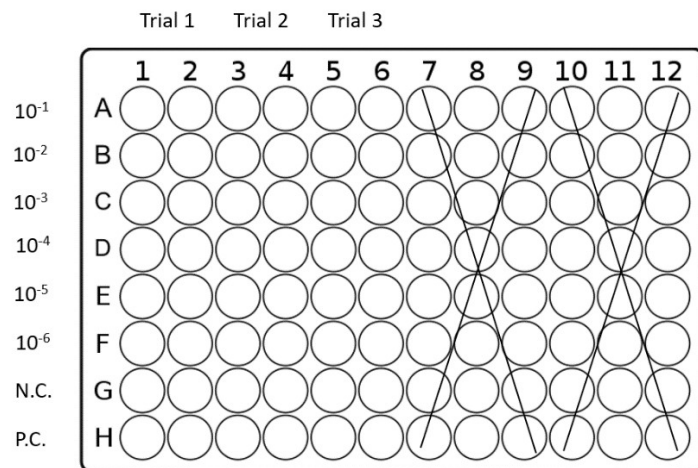
Figure 2. Cytopathic effects from neutralization assay (20)

(Student included modified figure from reference 20 here)

The IgM antibodies seem to have neutralizing effects against blood stage malaria. When the parasite concentration was too large, as seen in 10^{-1} through 10^{-3} samples, the parasite was able to infect the immortalized blood cell line which resulted in 100% plaque formation because the parasite destroyed the cell line (Table 1). However, as the parasite concentration became more manageable, the antibodies were able to neutralize. This can be determined by the decrease in plaque formation seen in the 10^{-4} through 10^{-6} samples (Figure 2). The results are verifiable because the controls produced expected results. The positive control, containing only malaria, showed complete plaque formation. The negative control, containing only antibody, showed no antibody formation. Each trial represents a biological replicate. Samples were collected on days 1, 14, and 28. Furthermore, a student's T test was performed to assess the variability between the 3 biological replicates. The resulting p value equaled 0.03, which indicates that the data is significant and not due to random chance. This means that there is only a 3% chance that these results are due to random chance.

Table 1. Quantitative Estimate of Average Cytopathic Effect Observed from Microneutralization Assay

Dilution	Average % CPE		
	Trial 1	Trial 2	Trial 3
10_{-1}	100	100	100
10_{-2}	100	100	100
10_{-3}	100	100	100
10_{-4}	100	75	15
10_{-5}	100	0	0
10_{-6}	50	0	0
Negative Control	0	0	0
Positive Control	98	100	100

CD8 $^+$ Cytotoxic T Cell Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) are critical components to the immune system. These cells are found within the blood, and give selective responses to the immune system. PBMCs consist of several types of cells such as dendritic cells, lymphocytes, NK cells, and monocytes. However, lymphocytes comprise of roughly 70% of PMBCs. They can be isolated to help assess T-cell responses – such as the response to the attenuated vaccine.

Flow cytometry will be used to quantify the specific amount of activated CD8$^+$ T cells which are secreting IFN-gamma within the PBMC population. Flow cytometry was chosen because it can analyze the size and granularity of each individual cell in this PMBC population in addition to determining how many of these cells are expressing the cytokine IFN-gamma. Flow cytometry consists of funneling cells through a nozzle that is one cell wide. These cells will pass an interrogation point, where the individual cell size, granularity, and fluorescence can be analyzed using a light beam. This data can be plotted on a twodimensional graph that gives a profile of each cell's granularity and size. The profile of interest for assessing T cell response would be the small and agranular cells – AKA the lymphocytes.

The PBMCs from the cell culture will be removed and transferred into a tube. They will then be washed to get rid of the media, serum, and antibiotics. Next, they will get fixed with paraformaldehyde which cross-links macromolecules in place, and will then be permeabilized with saponin. The saponin

allows the antibodies in the next step into the cells. In order to distinguish the CD8^{+} T cells from the other PBMCs, an anti-antibody will be added that contain fluorescent markers. Anti-CD8^{+} antibody labeled with FITC fluorescent marker will be added to the tubes. We can then take this population of lymphocytes and add Brefeldin A, which will block the vesicles that secrete IFN-gamma into the extracellular space. These cells can then be stained with anti-IFN-gamma for the presence of IFN-gamma. The tubes will be incubated to allow the antibodies to bind, and then they will be washed and subjected to flow cytometry.

Biological replicates were performed for this experiment. Whole blood samples were taken from a patient on days 1, 14, and 28. PBMCs were isolated from each sample, cultured, and subjected to flow cytometry. A negative control was used where no antigen was presented to the cultured cells. This was used as a baseline result to compare the level of CD8^{+} T cell activation after adding the antigen. In each case, the identity of the before vaccination and after vaccination samples were unknown to the experimenter to prevent bias. Furthermore, the final data was analyzed by a separate blinded experimenter. A student's T test was performed comparing the before vaccination and after vaccination group. The resulting p value equaled 0.02 (Table 2), which indicates that the data is significant and not due to random chance. This means that there is only a 2% chance that these results are due to random chance.

Figure 3. Flow cytometry detecting CD8^{+} T cells secreting IFN-gamma using FITC fluorescent marker (21) (Student included modified figure from reference 21 here)

Table 2. CD8^{+} T Cell Flow Cytometry Data of IFN-Gamma Response

	IFN-Gamma Response		
	Trial 1	Trial 2	Trial 3
Before Vaccination	10, 12, 13, 16, 23	8, 11, 15, 19, 22	9, 14, 15, 21, 28
After Vaccination	17, 53, 88, 140, 154	21, 36, 77, 91, 125	2, 45, 81, 113, 155
P Value	0.021	0.021	0.022
Mean P value	0.021		

Note: IFN-Gamma response values were randomly selected from the hundreds of responses given in each trial.

The before vaccination trials offer a baseline understanding of normal CD8^{+} T cells found within the blood of this patient. The Y axis indicates IFN-gamma while the X axis indicates the number of CD8^{+} T cells. T cell activation/differentiation was initiated after the blood stage attenuated malaria was added to the tissue culture (after vaccination). There was a large increase in CD8^{+} T cells within the tissue culture.

This is indicated by the increased number of dots (cells) found at the interrogation point during flow cytometry. Data was consistent throughout the three biological replicates on days 1, 14, and 28. This means that the blood stage attenuated malaria vaccine is properly activating a CD8^{+} T cell response within the body upon exposure.

CD4 [+] Helper T Cell Flow Cytometry

PMBCs were isolated and cultured as performed when assessing CD8^{+} activation. Flow cytometry will be used to quantify the specific amount of activated CD4^{+} T cells which are secreting IL-10 within the PBMC population. The PBMCs from the cell culture will be removed and transferred into a tube. They will then be washed to get rid of the media, serum, and antibiotics. Next, they were fixed with paraformaldehyde which cross-links macromolecules in place, and were then permeabilized with saponin. The saponin allows the antibodies in the next step into the cells. In order to distinguish the CD4^{+} T cells from the other PBMCs, an anti-antibody was added that contains fluorescent markers. Anti-CD4^{+} antibody labeled with FITC fluorescent marker was added to the tubes. We then took this population of lymphocytes and added Brefeldin A, which blocked the vesicles that secrete IL-10 into the extracellular space. These cells were then stained with anti-IL-10 for the presence of IL-10. The tubes were incubated to allow the antibodies to bind, and were then washed and subjected to flow cytometry.

Biological replicates were performed for this experiment. Whole blood samples were taken from a patient on days 1, 14, and 28. PBMCs were isolated from each sample, cultured, and subjected to flow cytometry. A negative control was used where no antigen was presented to the cultured cells. This was used as a baseline result to compare the level of CD4^{+} T cell activation after adding the antigen. In each case, the identity of the before vaccination and after vaccination samples were unknown to the experimenter to prevent bias. Furthermore, the final data was analyzed by a separate blinded experimenter. A student's T test was performed comparing the before vaccination and after vaccination group. The resulting p value equaled 0.04 (Table 3), which indicates that the data is significant and not due to random chance. This means that there is only a 4% chance that these results are due to random chance.

Figure 4. Flow cytometry detecting CD4^{+} T cells secreting IL-10 using FITC fluorescent marker (21)

(Student included modified figure from reference 21 here)

Table 3. CD4^{+} T Cell Flow Cytometry Data of IL-10 Response

	IL-10 Response		
	Trial 1	Trial 2	Trial 3
Before Vaccination	10, 14, 21, 26, 30	17, 21, 22, 29, 31	11, 14, 22, 27, 32
After Vaccination	8, 28, 33, 97, 144	13, 27, 33, 85, 125	15, 47, 52, 113, 155
P Value	0.041	0.043	0.033
Mean P value	0.039		

Note: IL-10 response values were randomly selected from the hundreds of responses given in each trial.

The before vaccination trials offer a baseline understanding of normal CD4^{+} T cells found within the blood of this patient. The Y axis indicates IL-10 while the X axis indicates the number of CD4^{+} T cells. T cell activation/differentiation was initiated after the blood stage attenuated malaria was added to the tissue culture (after vaccination). There was a large increase in CD4^{+} T cells within the tissue culture. This

is indicated by the increased number of dots (cells) found at the interrogation point during flow cytometry. Data was consistent throughout the three biological replicates on days 1, 14, and 28. This means that the blood stage attenuated malaria vaccine is properly activating a CD4^{+} T cell response within the body upon exposure.

Methods

<u>IgM Microneutralization Assay</u> (22)

The serum samples containing the IgM antibody were heat inactivated for 30minutes at 56°C. Following heat inactivation, the samples were allowed to equilibrate to room temperature. 900μL of DMEM was added to a tube of blood stage malaria parasite containing 100μL parasite suspension, and was subsequently labeled 10^{-1}. 10-fold serial dilutions were performed starting with the 10^{-1} tube until final dilution of 10^{-6} was obtained. 100uL of each sample was seeded into the 96-well plate following the scheme in figure 1. Dilutions were performed in DMEM, supplemented with 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine at 2mM. 100uL of serum sample was added to each well. For controls, the positive controls contained parasite only whereas the negative controls contained just antibodies and no parasite. The samples were transferred to a 96-well plate containing a confluent immortalized blood cell line (23). The 96-well plate was incubated at 37°C with 5% CO_2 for 1 hour with gentle rocking. Upon completing incubation, the media was dumped and replaced with 100uL of 1% methylcellulose overlay. The titer plate was incubated at 37°C with 5% CO_2 for 72 hours. The 96-well plate was then analyzed for plaque formation.

<u>Isolating, Surface Protein Staining, and Culturing PBMCs</u> (24)

Whole blood must be obtained from patients and transferred to a centrifuge tube. Because whole blood was being used, 2mL of Flow Cytometry Human Lyse Buffer was added to lyse the red blood cells. Then, a hydrophilic polysaccharide called ficoll was added. This helps separates the blood into layers where plasma is on the top followed by PBMCs just below the plasma (25). The sample was incubated for 10 minutes in a dark room. The cells were centrifuged at 1500rpm for 5 minutes and washed with PBS. The PBMCs were drawn from the vile and placed in their own tube. After obtaining the PBMCs, the cells were counted, and aliquots were made with 1×10^6 cells/100uL. Fc-block cells containing blocking IgG were added for 15 minutes at room temperature. 10uL/10^6cells conjugated primary antibody was added and the sample was vortexed. The cells were incubated for 30minutes at room temperature. Unbound antibody was washed away using 2mL of staining buffer. The sample was centrifuged at 1500rpm for 5 minutes, and the buffer as decanted. The cells were then resuspended with 2mL of staining buffer. After isolating and surface staining these PBMCs, they were tissue cultured for roughly 24hours to allow for adaptation and overall growth. They were incubated at 37°C with 5% carbon dioxide in RPMI media supplemented with fetal bovine serum and antibiotics to avoid both fungal and bacterial contamination. Following incubation, the blood stage attenuated malaria was added to the tissue culture, where incubation continued for another seven days with culture conditions. During this time, antigen presenting

cells such as dendritic cells presented the antigen to the T cells. A negative control sample was made where no blood stage attenuated malaria was added.

Isolating, Intracellular Staining, and Culturing PBMCs (24)

Surface protein staining was done before intracellular staining because permealization can reduce the availability of surface antigens. Whole blood was obtained from patients and transferred to a centrifuge tube. Because whole blood was being used, 2mL of Flow Cytometry Human Lyse Buffer was added to lyse the red blood cells. Then, a hydrophilic polysaccharide called ficoll was added. This helps separates the blood into layers where plasma is on the top followed by PBMCs just below the plasma. The sample was incubated for 10 minutes in a dark room. The cells were centrifuged at 1500rpm for 5 minutes.

The PBMCs were drawn from the vile and placed in their own tube. After obtaining the PBMCs, the cells were counted, and aliquots were made with 1×10^6 cells/100uL. 0.5mL of cold fixation buffer was added and the samples were vortexed followed by a 10minute incubation at room temperature. The sample was centrifuged at 1500rpm for 5 minutes, and the fixation buffer was decanted. The cells were then washed with 2mL PBS and resuspended in 200uL permeabilization buffer containing saponin. Fc-block cells with blocking IgG was added for 15minutes at room temperature. $10uL/10^6$ cells of conjugated antibody was added and the samples were vortexed followed by a 30minute incubation in a dark room. The cells were washed twice with permeabilization buffer containing saponin. Following the wash, the cells were resuspended in 400uL PBS. 100uL of the appropriate antibody labeled with fluorescent marker was added to the tubes: Anti-CD4$^+$ antibody labeled with FITC fluorescent marker or Anti-CD8$^+$ antibody labeled with FITC fluorescent marker. The cells were then subjected to 100uL of Brefeldin A, which blocked the vesicles that secrete cytokines into the extracellular space. These cells were then stained with anti-cytokine (either anti-IF-gamma for CD8$^+$ or anti-IL-10 for CD4$^+$) for the presence of the corresponding cytokine. The tubes were incubated for 24hours at 37°C with 5% carbon dioxide to allow the antibodies to bind, and were then washed and subjected to flow cytometry. After isolating and intracellularly staining these PBMCs, they were tissue cultured for roughly 24hours to allow for adaptation and overall growth. They were incubated at 37°C with 5% carbon dioxide in RPMI media supplemented with fetal bovine serum and antibiotics to avoid both fungal and bacterial contamination. Following incubation, the blood stage attenuated malaria was added to the tissue culture, where incubation continued for another seven days with culture conditions. During this time, antigen presenting cells such as dendritic cells presented the antigen to the T cells. A negative control sample was made where no blood stage attenuated malaria was added.

Discussion

Three biological replicates were used in each of the three experiments performed. Biological replicates are when experiments are performed with unique samples. In these experiments, unique samples were collected on days 1, 14, and 28. This specific number of three replicates was determined through a power analysis. In the field of research, achieving a P value of 0.05 or less is the gold standard. Larger sample sizes make it easier to obtain this level. However, larger sample sizes are often costly. Therefore, a power analysis can be conducted to help determine the smallest sample size suitable for achieving the significant 0.05 value. A power analysis incorporates the dynamic range and variability

between two groups. For example, "before vaccination" and "after vaccination". It is important to calculate the power analysis before collecting data as a way of reducing bias. In this case of these three experiments, the power analysis suggested using a sample size of three biological replicates.

An attenuated vaccine format was chosen because multiple studies have shown that whole attenuated malaria induces a powerful CD8$^+$ T-cell response with protection. Furthermore, it has been shown that whole-parasite vaccines consistently produce the best immunity by encouraging strong responses from both CD4$^+$ and CD8$^+$ T-cells. The results from this paper vindicate this observation. The whole attenuated malaria vaccine proved to substantially activate both CD8$^+$ and CD4$^+$ T cells as observed by the statistically significant increase in IFN-gamma and IL-10 respectively after vaccination. IFN-gamma will directly impair *Plasmodium* growth in human hepatocytic cell culture. IL-10 acts as a safety feature in this vaccine format by preventing too much inflammation. Excessive inflammation would assist in disease pathology. Furthermore, B cell activation occurred in which B cells produced IgM antibodies. These antibodies were capable of neutralizing the blood stage malaria and thereby prevent the parasite from entering an immortalized blood cell line. In conclusion, this vaccine format has shown promise in activating three essential correlates of immunity in regards to a *Plasmodium falciparum* infection.

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