Defining Skin Immunity of a Bite of Key Insect Vectors in Humans

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Principal Investigator

Matthew J. Memoli, MD, MS

Laboratory of Infectious Diseases

National Institute of Allergy and Infectious Diseases

National Institutes of Health

Tel: 301-443-5971

Email: memolim@mail.nih.gov

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Laboratory of Infectious Diseases

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National Institutes of Health

TEAM ROSTER

Principal Investigator: Matthew J. Memoli, MD, MS
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Tel: 301-443-5971
Email: memolim@mail.nih.gov

Associate Investigators: Jesus G. Valenzuela, PhD
Laboratory of Malaria and Vector Research
Vector Molecular Biology Section
National Institute of Allergy and Infectious Diseases
Tel: 301-761-5070
Email: jvalenzuela@niaid.nih.gov

Shaden Kamhawi, PhD
Laboratory of Malaria and Vector Research
Vector Molecular Biology Section
National Institute of Allergy and Infectious Diseases
Tel: 301-761-5081
Email: skamhawi@niaid.nih.gov

Alison Han, MD, MS
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
Tel: 301-480-5722
Email: alison.han@nih.gov

Luiz Fabiano Oliveira, MD, PhD
Laboratory of Malaria and Vector Research
Vector Molecular Biology Section
National Institute of Allergy and Infectious Diseases
Tel: 301-761-5073
Email: loliveira@niaid.nih.gov

Iliano V. Coutinho-Abreu, PhD
Laboratory of Malaria and Vector Research
Vector Molecular Biology Section
National Institute of Allergy and Infectious Diseases
Tel: 301-761-3977
Email: iliano.vieiracoutinhoabreugomes2@nih.gov

Joshua Lacsina, MD, PhD
Laboratory of Malaria and Vector Research
Vector Molecular Biology Section
National Institute of Allergy and Infectious Diseases
Tel: 301-275-2045
Email: joshua.lacsina@nih.gov

Luca Giurgea, MD
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
Tel: 301.538.5235
Email: luca.giurgea@nih.gov

Study Staff NIH:

Holly Ann Baus, RN, MSN
Laboratory of Immunoregulation
National Institute of Allergy and Infectious Diseases
Tel: 301-761-6800
Email: holly.baus@nih.gov

Lindsay M. Czajkowski, MSN, NP-C
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
Tel: 301-451-7705
Email: lindsay.czajkowski@nih.gov

Susan M. Reed, BA, CRC
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
Tel: 301-451-3354
Email: susan.reed@nih.gov

Rani S. Athota, PhD
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
Tel: 301-594-0803
Email: rani.athota@nih.gov

Institutional Review Board (IRB):

National Institute of Allergy and Infectious Diseases
Institutional Review Board
5601 Fishers Lane
Rockville, MD 20852

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LIST OF ABBREVIATIONS

AE	adverse event
CC	Clinical Center
cDNA	complementary DNA
CFR	Code of Federal Regulations
CRIMSON	Clinical Research Information Management System of the NIAID
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FDR	false discovery rate
GCP	Good Clinical Practice
GLM	general linear modelling
HRPP	Human Research Protection Program
ICH	International Council on Harmonisation
INR	international normalized ratio
IRB	institutional review board
LMVR	Laboratory of Malaria and Vector Research
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
PBMC	peripheral blood mononuclear cell
PI	principal investigator
RNA	ribonucleic acid
RNAseq	RNA sequencing
SAE	Serious Adverse Event
SCSU	Special Clinical Studies Unit
SUSAR	suspected unexpected serious adverse reaction
UP	unanticipated problem
UPnonAE	unanticipated problem that is not an AE

PROTOCOL SUMMARY

Full Title:	Defining Skin Immunity of a Bite of Key Insect Vectors in Humans
Short Title:	Skin immunity to vector bites
Sample Size:	N=90 participants (Cohort A n=45; Cohort B n=45)
Accrual Ceiling:	N=140 participants
Study Population:	Healthy volunteers 18 to 64 years of age
Accrual Period:	2 years
Study Duration:	Start Date: May 2018 End Date: May 2020 Length of individual subject participation is 7 days for Cohort A or 51 days for Cohort B.
Study Design:	This is a single-center, case-control study characterizing the innate immune response of human skin to insect bites. Participants will undergo vector feeding and have a skin biopsy taken from the site of vector bites (case) and from unbitten skin (control). Participants will be assigned to groups that differ by type of vector and number of feedings. Additionally, blood will be collected after selected feedings to evaluate systemic immune response.
Study Intervention Description:	Controlled feeding by 1 of 3 colony-reared vectors (*Aedes aegypti* mosquitoes, *Anopheles gambiae* mosquitoes, or *Lutzomyia longipalpis* sand flies). Participants will undergo either 1 feeding (Cohort A) or 4 feedings, each about 2 weeks apart (Cohort B), with the same vector type.
Primary Objectives:	The primary objectives of the study are: 1. To compare the early innate immune response in the skin of bitten (case) versus unbitten (control) skin for each of the three vector groups (*Aedes*, *Anopheles,* sand flies). 2. To chracterize the local skin adaptive immune response after multiple exposures over time to bites of each of the three vector groups (*Aedes*, *Anopheles*, sand flies).
Secondary Objectives:	The secondary objectives of this study are: 1. To analyze systemic immune response to vector salivary proteins in vector-naïve participants in each group (*Aedes, Anopheles*, sand flies).

2. To analyze systemic immune response to vector salivary proteins in vector-experienced participants in each group (*Aedes*, *Anopheles,* sand flies).

Exploratory Objective: The exploratory objective of this study is:

1. To evaluate gene expression after selected vector feedings to assess for reliable biomarkers of exposure.

Primary Endpoints:

1. Measurement of changes in the early innate immune response and cellular recruitment in the skin of bitten versus unbitten skin in each vector group in Cohort A by:
    a. immunohistochemistry of target proteins in vector-naïve participants for each of the three vector groups at 30 minutes and 4 hours after 1 feeding/exposure.
    b. Transcriptomics via deep sequencing and/or gene expression array of complementary DNA (cDNA) libraries made from skin ribonucleic acid (RNA) of vector-naïve participants for each of the three vector groups at 30 minutes and 4 hours after 1 feeding/exposure.
2. Measurement of changes in the adaptive immune response and cellular recruitment in the skin of bitten versus unbitten skin after the fourth and final feeding in each vector group in Cohort B by:
    a. immunohistochemistry of target proteins in vector-experienced participants for each of the three vector groups at 4 hours and 48 hours after 4 feedings/exposures.
    b. Transcriptomics via deep sequencing and/or gene expression array of cDNA libraries made from skin RNA of vector-experienced participants for each of the three vector groups at 4 hours and 48 hours after 4 feedings/exposures.

Secondary Endpoints:

1. Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) and cytokine analysis of plasma collected 24 hours after the one feeding in each vector group of vector-naïve participants in Cohort A.

2. Flow cytometry analysis of PBMCs and cytokine analysis of plasma collected 48 hours after the fourth and final feeding in each vector group of vector-experienced participants in Cohort B.

Exploratory Endpoint:

1. Comparison of gene expression of circulating white blood cells after the one feeding in each vector group of vector-naïve participants in Cohort A.
2. Comparison of gene expression of circulating white blood cells after the fourth and final feeding in each vector group of vector-experienced participants in Cohort B.

Précis

Vector-borne diseases continue to cause significant morbidity and mortality worldwide despite ongoing control efforts. Vectors like sand flies and mosquitoes deliver the pathogen into the skin of humans while taking a blood meal. Most vaccines under development ignore the importance of the complex infectious inoculum delivered by the vector and the local immune response that occurs at the site of the bite. In addition, many preclinical studies are carried out in animal models that do not replicate the natural route of infection, transmission by vector bites, and often bypass the skin interface altogether. As such, these studies do not evaluate what role the vector plays in the initiation of these infections. Further compounding this problem, many clinical studies are performed in naïve individuals who have never been exposed to the vector, while those living in endemic areas will have had long-term exposure to vectors through uninfected bites.

A cumulative body of evidence from animal models demonstrates that a variety of vector-derived components are co-delivered with the pathogen and may play an important role in the establishment of infection. There is limited knowledge of the effect of these vector-derived factors on the immune response in human skin and their potential impact on infection establishment.

In this protocol, we will examine the early skin immune response to bites of three arthropods: the mosquito *Aedes aegypti*, the vector of Zika, dengue, and chikungunya viruses; the mosquito *Anopheles gambiae*, the vector of malaria; and the sand fly *Lutzomyia longipalpis*, the vector of leishmaniasis. We will also explore how multiple vector bite exposures over time modulate future immune response at the bite site. Healthy participants will come to the National Institutes of Health (NIH) and undergo feeding by one of the three vectors, then have three skin punch biopsies performed by trained medical practitioners to evaluate local immune response. Participants in Cohort A will have one feeding; participants in Cohort B will have 4 feedings, each 2 weeks apart. Biopsies will be collected after the final feeding. Blood will be collected after the one feeding in Cohort A and after the fourth and final feeding in Cohort B to assess systemic immune response.

With the current rise of vector-borne diseases in the United States and around the world, we hope the results of this study contribute to future vaccine design and clinical development strategies for vector-borne diseases.

1 Background Information and Scientific Rationale

1.1 Background Information

Arthropod-borne diseases are emerging and re-emerging globally at a rapid pace [1]. Malaria, leishmaniasis, and dengue cause significant morbidity and mortality in many parts of the world, and the emergence of epidemics of Zika, chikungunya, and West Nile viruses show how quickly these diseases can spread [2]–[4]. Studies of pathogenesis and vaccine development for these diseases have mostly focused on the pathogens themselves, and little attention has been given to the role of their vectors. Many of these vectors deliver the pathogens into the skin of humans while taking a blood meal, and a cumulative body of evidence from animal models demonstrates that in the process of delivering a pathogen, a variety of vector-derived components are co-delivered that may play an important role in the establishment of infection [5]–[7].

The majority of vaccines developed against vector-borne diseases have not been effective in humans despite often showing some effect in certain animal models. Most developed vaccines ignore the importance of the complex infectious inoculum delivered by the vector and the local immune response that occurs at the bite site. In addition, many preclinical studies are carried out in animal models that do not replicate natural infection and do not evaluate what role the vector plays in these infections. As a consequence, many vaccine candidates fail during development. For example, Leishmania candidate vaccines have demonstrated protection in animals challenged artificially by introducing the parasite via a needle but not when challenged naturally by bites of infected sand flies [8].

We lack a clear understanding of the immune response in skin after insect bites and how that response affects disease initiation and efficacy of vaccines against these important pathogens. Furthermore, many clinical studies are performed in naïve individuals who have never been exposed to the vector, while people living in endemic areas will have long-term exposure to these vectors and different immune response to subsequent bites, which could interfere with vaccine effectiveness. These are likely important factors in why many of these vaccines fail once they move on to clinical trials.

In this protocol, we propose to perform a careful examination of the early skin immune response to bites of three arthropods: the mosquito *Aedes aegypti*, the vector of Zika, dengue, and chikungunya viruses; the mosquito *Anopheles gambiae*, the vector of malaria; and the sand fly *Lutzomyia longipalpis*, the vector of leishmaniasis. We will also explore how multiple vector bite exposures over time modulate future immune response at the bite site.

1.2 Scientific Rationale

Vector saliva contains a range of molecules including vasodilators, anticoagulants and immunomodulators whose purpose is to prevent the disruption of feeding by the host [9]. Vector saliva has been shown to inhibit T- and B-cell proliferation and downregulate the expression of interferon gamma (IFN-γ), a soluble T helper cell type 1 (Th1) pro-inflammatory immune mediator known to have in vivo therapeutic and prophylactic effects against other arthropod-transmitted diseases such as leishmaniasis [10]–[12]. With time and repeated exposure, individuals become desensitized to mosquito bites due to a reduction in T helper cell type 2 (Th2) cytokine mediators, thus allowing for the more effective development of pro-inflammatory Th1 responses [10].

When comparing the effect on parasite dissemination of needle infection vs. transmission of *Leishmania donovani* by sand fly bites to mice; mice only injected with virulent parasites demonstrated that the parasites did not visceralize to the spleens of the animals; in contrast, animals infected by the bite of a sand fly had spleen visceralization of the Leishmania parasite (Figure 1). The immune profile and cellular recruitment at the injection/bite site of animals infected by needle compared with those infected by bites of infected sand flies was also different. Animals bitten by *Leishmania*-infected sand flies activated the host inflammasome and displayed an increased recruitment of neutrophils that was sustained for more than 18 hours, a completely different innate response than animals who received parasite injected by needle (Figure 2). Pingen et al. also reported similar findings, showing that *Aedes aegypti* mosquito bites induced an inflammatory response in mice characterized by the presence of neutrophils, and this immune response augmented the severity of Semliki Forest and Bunyamwera virus infections [13]. All these results strongly suggest that a vector bite (mosquitoes or sand flies) alters the skin environment, and consequently, the establishment of the parasite or virus delivered by the vector. Our objective is to translate these findings in animal models to human studies, as well as to gain a better understanding of the immune responses generated in the skin of humans and how this information can help us to design or improve test vaccines targeting vector-borne diseases.

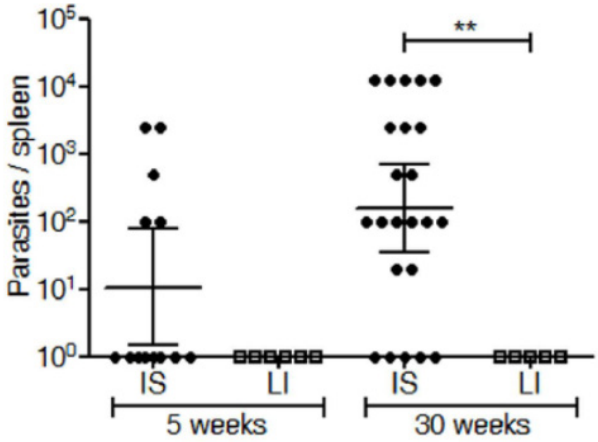

Figure 1. Parasite burden in mice after exposure to infected sand flies.
Parasite burden in individual mice spleens determined by serial dilution at 5 and 30 weeks after exposure to 20 infected sand flies (IS) or intradermal injection of 10^5 metacyclic Leishmania donovani *parasites (LI).*

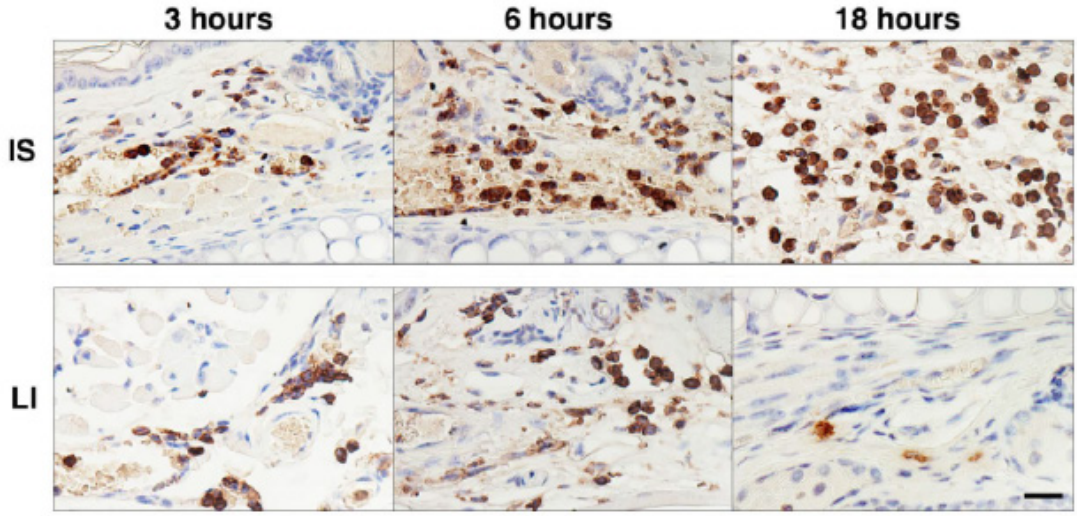

Figure 2. Histology time course after exposure to infected sand flies.
Mice ears 3-18 hours after exposure to 20 infected (IS) or intradermal injection with 10^5 metacyclic parasites (LI). Mice ear sections were stained with anti-Ly6G antibody. Pictures are representative of four samples per condition; scale bars indicate 20um.

Concerning adaptive responses after multiple exposures to vector saliva, various groups have shown that saliva of insects or bites of an insect can alter the immune response in rodents, dogs, and non-human primates [13]–[15]. In rodent models, Teixeira et al. have shown that the cytokine and chemokine environment in the skin changes significantly when the rodent was previously exposed to bites of sand flies compared to naïve animals [16]. In dogs, Collin et al. have shown that multiple sand fly bites generate an adaptive immune response that can be recalled by a sand fly bite [14]. In non-human primates and in human subjects living in a sand fly-prevalent area, a skin immune response to bites of uninfected sand flies was observed [15], [17]. These data show that

bites of vectors can generate a specific systemic immune response to vector-derived factors; we aim to investigate the nature of the adaptive immune response to vector bites in the skin of humans. This may elucidate how the immune response may affect the efficacy of vector-based vaccines currently in clinical trials.

This study will be the first to carefully examine the early immune response to bites of different vectors in humans and to explore how multiple exposures over time modulate future local skin responses, potentially affecting pathogen transmission and vaccine efficacy. We hypothesize that bites of arthropod vectors of disease egest specific vector-derived factors including saliva that modulate the innate immune response in naïve individuals not previously exposed to vector bites (Cohort A) and recall a saliva-specific adaptive immune response to the bite site in previously exposed individuals (Cohort B), thereby altering the outcome of disease caused by pathogens transmitted by these vectors and potentially the immune response to vaccines in endemic populations. Results from this study will provide a better understanding of the role vector-derived factors play in the establishment of vector-borne infections in humans. These findings may be useful in determining why many current vaccine candidates fail as they move further into clinical development. Additionally, the findings will be essential in informing future vaccine design and clinical development for both well-established vector-borne diseases like malaria and dengue as well as emerging threats requiring the rapid development of countermeasures like Zika and chikungunya viruses.

2 Study Objectives

2.1 Primary Objectives

The primary objectives of the study are:

1. To compare the early innate immune response in the skin of bitten (case) versus unbitten (control) skin for each of the three vector groups (*Aedes*, *Anopheles*, sand flies).
2. To chracterize the local skin adaptive immune response after multiple exposures over time to bites of each of the three vector groups (*Aedes*, *Anopheles*, sand flies).

2.2 Secondary Objectives

The secondary objectives of this study are:

1. To analyze systemic immune response to vector salivary proteins in vector-naïve participants in each group (*Aedes*, *Anopheles,* sand flies).
2. To analyze systemic immune response to vector salivary proteins in vector-experienced participants in each group (*Aedes, Anopheles*, sand flies).

2.3 Exploratory Objective:

The exploratory objective of this study is:

1. To evaluate gene expression after selected vector feedings to assess for reliable biomarkers of exposure.

3 Study Design

3.1 Description of the Study Design

This is a single-center, case-control study of healthy skin immune response to bites of three species of colony-reared arthropods not infected with any pathogen. Healthy participants will undergo vector feeding with one of the three types of vectors as described in Table 1. Participants will be assigned to a vector in a 1:1:1 ratio. Additionally, participants will be assigned to a cohort: Cohort A will undergo a single feeding and Cohort B will undergo 4 feedings, each about 2 weeks apart.

Table 1. Sample size estimate per cohort and vector group.

Vector	Cohort A (Vector-Naïve)	Cohort B (Vector-Experienced)	Total
Aedes aegypti	15	15	30
Anopheles gambiae	15	15	30
Lutzomyia longipalpis	15	15	30
Total	45	45	90

After the one vector feeding for Cohort A, participants will undergo skin punch biopsy at three sites: two at the sites of bites at 2 different time points (case) and one from unbitten skin (control). After the fourth and final vector feeding for Cohort B, participants will undergo skin punch biopsy at three sites: two at the sites of bites at 2 different time points (case), and one from unbitten skin (control). Skin biopsies will be used for research tests to evaluate local immune response to vector bites. Additionally, systemic immune response will be evaluated on blood collected after the one feeding for Cohort A and after the fourth and final feeding for Cohort B.

3.2 Study Endpoints

3.2.1 Primary endpoints

1. Measurement of changes in the early innate immune response and cellular recruitment in the skin of bitten versus unbitten skin in each vector group in Cohort A by:

a. immunohistochemistry of target proteins in vector-naïve participants for each of the three vector groups at 30 minutes and 4 hours after 1 feeding/exposure. Biopsies from eight participants within a group will be assigned for immunohistochemical analysis.

b. Transcriptomics via deep sequencing and/or gene expression array of cDNA libraries made from skin RNA of vector-naïve participants for each of the three vector groups at 30 minutes and 4 hours after 1 feeding/exposure. Biopsies from seven participants within a group will be assigned for transcriptomic analysis.

2. Measurement of changes in the adaptive immune response and cellular recruitment in the skin of bitten versus unbitten skin after fourth and final feeding in each vector group in Cohort B by:

a. immunohistochemistry of target proteins in vector-experienced participants for each of the three vector groups at 4 hours and 48 hours after 4 feedings/exposures. Biopsies from eight participants within a group will be assigned for immunohistochemical analysis.

b. Transcriptomics via deep sequencing and/or gene expression array of cDNA libraries made from skin RNA of vector-experienced participants for each of the three vector groups at 4 hours and 48 hours after 4 feedings/exposures. Biopsies from seven participants within a group will be assigned for transcriptomic analysis.

3.2.2 Secondary endpoints

1. Flow cytometry analysis of PBMCs and cytokine analysis of plasma collected 24 hours after the one feeding in each vector group of vector-naïve participants in Cohort A.
2. Flow cytometry analysis of PBMCs and cytokine analysis of plasma collected 48 hours after the fourth and final feeding in each vector group of vector-experienced participants in Cohort B.

3.2.3 Exploratory endpoint

1. Comparison of gene expression of circulating white blood cells after the one feeding in each vector group of vector-naïve participants in Cohort A.
2. Comparison of gene expression of circulating white blood cells after the fourth and final feeding in each vector group of vector-experienced participants in Cohort B.

4 Study Population

4.1 Rationale for Participant Selection

Participants will be carefully screened using the inclusion and exclusion criteria described here to select the optimum participants for completing the study objectives

and minimize the risk of adverse events (AEs). We will enroll up to 140 healthy adults in this protocol in order to fill all cohorts/groups.

National Institutes of Health (NIH) employees and members of their immediate families may participate in this protocol. We will follow the Guidelines for the Inclusion of Employees in NIH Research Studies and will give each employee a copy of the "NIH Information Sheet on Employee Research Participation."

For NIH employees:

- NIH staff may be a vulnerable class of participants.
- Neither participation nor refusal to participate will have an effect, either beneficial or adverse, on the participant's employment or work situation.
- The NIH information sheet regarding NIH employee research participation will be distributed to all potential participants who are NIH employees.
- The employee participant's privacy and confidentiality will be preserved in accordance with NIH Clinical Center (CC) and National Institute of Allergy and Infectious Diseases (NIAID) policies, which define the scope and limitations of the protections.
- For NIH employee participants, consent will be obtained by an individual independent of the employee's team. Those in supervisory position to any employee and co-workers of the employee will not obtain consent.
- The importance of maintaining confidentiality when obtaining potentially sensitive and private information from co-workers or subordinates will be reviewed with the study staff at least annually and more often if warranted.

4.2 Recruitment Plan

Participants will be recruited through the screening study #11-I-0183 "Screening for LID Clinical Studies Unit Healthy Volunteer Protocols". Individuals will be carefully screened and evaluated, and those who meet the study eligibility criteria will be contacted and given the opportunity to be enrolled into the study.

If a potential participant has completed the screening study #11-I-0183 more than 90 calendar days prior to Day 0 of this study, they will be asked to come to the NIH CC for another visit under the screening study #11-I-0183 within 90 days prior to Day 0. Under that study, they will repeat HIV testing and complete any laboratory or other testing deemed necessary by the investigator to ensure that the individual is still eligible to take part in this study. All eligible participants will be consented and enrolled for this study only after completion of all necessary screening studies within 90 days prior to the first vector feeding (Day 0) unless they have completed participation in Cohort A and are participating in Cohort B, in which case rescreening is not required.

If a participant in Cohort A would like to also participate in Cohort B for the same vector group after they have completed Cohort A, they may be allowed to be reenrolled and counted as a new participant in Cohort B. The participant will be consented again prior to enrollment in Cohort B. In addition, any participant in Cohort A or B can also reenroll to participate in Cohort A or B of a different vector if eligible. In that case, they will participate in all feeding and other procedures for that cohort/vector group as per protocol. The participant will be consented again prior to enrolling in a different cohort.

4.3 Inclusion Criteria

Individuals must meet all of the following criteria to be eligible for study participation:

1. Healthy women and men who are ≥18 and ≤64 years of age.
2. Able to provide informed consent.
3. Willingness to complete all study visits and comply with all study requirements.
4. Willing to have samples stored for future research.
5. A female is eligible for this study if she meets 1 of the following:
    - Of non-childbearing potential (i.e., women who have had a hysterectomy or tubal ligation or are postmenopausal, as defined by no menses in ≥1 year).
    - Of childbearing potential but agrees to practice effective contraception or abstinence for 4 weeks prior to enrollment through the completion of the study. Acceptable methods of contraception include a male partner who is sterile and is the sole sexual partner of the female participant or a male partner who uses a condom with spermicide plus 1 or more of the following that is used by the female: 1) implants of levonorgestrel; 2) injectable progestogen; 3) an intrauterine device with a documented failure rate of <1%; 4) oral contraceptives; and 5) double barrier method including diaphragm.
6. Agrees to not use scented lotions, deodorants, or topical creams on each feeding day.
7. Agrees to not take aspirin or any other NSAID within 7 days of a biopsy.
8. Agrees to not use topical steroid creams or ointments throughout the study without prior permission of Principal Investigator (PI).
9. Vector-specific antibody enzyme-linked immunosorbent assay (ELISA) to one of the three vectors (the one to which the individual is assigned) is <2.5 standard deviations above the negative control for Cohort A only.

4.4 Exclusion Criteria

Individuals meeting any of the following criteria will be excluded from study participation:

1. Any underlying or current medical condition that, in the opinion of the investigator, would interfere with participation in the study.
2. Any participant that is HIV positive.
3. A clinically significant (as determined by the PI) baseline Grade 1 or greater toxicity by the toxicity table.
4. History of severe allergic reaction (including to mosquito or other insect bites) with generalized urticaria, angioedema, anaphylaxis, or anaphylactoid reaction.
5. Prone to allergic responses and/or significant history of allergies, including seasonal or specific allergies as determined by the PI.
6. Receipt of any investigational drug that is unlicensed within 3 months or 5.5 half-lives (whichever is greater) prior to enrollment.
7. Receipt of any unlicensed vaccine within 6 months prior to enrollment.
8. Self-reported or known history of alcoholism or drug abuse within 6 months prior to enrollment, or positive urine test for drugs of abuse at screening (excluding positive test for tetrahydrocannabinol [THC] or its metabolites if usage is less than 3 times per week).
9. Self-reported or known history of psychiatric or psychological issues that require treatment and are deemed by the PI to be a contraindication to protocol participation.
10. Any use of medications that affect blood clotting within 3 months, history of abnormal blood clotting, or result outside of the normal laboratory range for measurements of prothrombin time (PT), partial thromboplastin time (PTT), or international normalized ratio (INR) that may suggest a problem with blood clotting.
11. History of significant scarring after previous biopsies, lacerations, abrasions, surgeries, or other skin procedures (e.g., cosmetic piercings) that are deemed by the PI to be a contraindication to protocol participation.
12. Pregnant or breastfeeding.

Co-enrollment Guidelines: Co-enrollment in other trials is restricted, but may take place after consultation with the study staff and approval from the PI.

4.5 Justification for Exclusion of Special Populations

Exclusion of Pregnant Women: In this study, participants will have multiple exposures to mosquito or sand fly bites. Because pregnant women are known to be immunosuppressed to some degree and the primary objective of the study is to examine immune response to vectors, they will be excluded so as not to compromise the scientific validity of the study.

Exclusion of Children: This is a study of the adult immune response to insect bites. Therefore, children will be excluded from the study.

5 Study Intervention

5.1 Arthropod Feeding

At the Laboratory of Malaria and Vector Research (LMVR), established state of the art insectaries for both mosquitoes and sand flies have been inspected and certified by the USDA as ACL2 (arthropod containment level 2) insectaries. The sand fly colony of *Lutzomyia longipalpis* originates from Brazil and was initially established at the Laboratory of Parasitic Diseases and was brought to LMVR in 2004. The *Aedes aegypti* and *Anopheles gambiae* mosquitoes were first established in 1972 and 1973, respectively. The two colonies were brought to LMVR in 2004. The colonies have been raised in our insectaries since. The insectaries include temperature- and humidity-controlled rooms as well as incubators with temperature, humidity and light cycle controls. We also have experienced technicians that take care of the colonies on a daily basis. For our study, the mosquito pupae collected after emergence will be placed into clean new containers in incubators dedicated to mosquitoes for use in human studies, located in a separate room from the rest of the colony. Both sand flies and mosquitoes used in human studies never come into contact with human blood prior to their use.

Colony-reared, clean female arthropods from each of three species, *Aedes aegypti* mosquitoes, *Anopheles gambiae* mosquitoes, or *Lutzomyia longipalpis* sand flies, will be bred in the insectary at the LMVR at the Twinbrook campus. In Cohort A, participants will undergo one feeding. In Cohort B, participants will undergo 4 feedings by the same vector. Participants will be asked to not use scented lotions, deodorants, or topical creams on each feeding day. Post-feeding, insect bites will be counted. In individuals that lack a robust response to saliva, each bite site will be distinguished by a red pinpoint on the skin, visible to the eye with careful inspection of the skin. Other participants may be more reactive to component of saliva, such as vasodilators, and will exhibit clear redness and/or induration that denotes a clear site of bite.

Each feeding will be conducted as follows:

i. Assessment of the skin will be documented pre-feeding.
ii. Depending on the cohort, starved female *Aedes aegypti* mosquitoes, *Anopheles gambiae* mosquitoes, or *Lutzomyia longipalpis* sand flies will be selected from a colony approved for human feeding studies in LMVR, NIAID.
    a. 5-10 female insects will be aspirated or placed into a secured feeding device prior to feeding and brought to the CC from the LMVR Insectary.
iii. The feeding site will be wiped clean with mild unscented soap and water and the device will be placed on the participant's arm for up to 20 minutes. The insects will feed through a disposable mesh on the bottom of the feeding device. This device permits the evaluation of feeding on

the human participant at the end of exposure, where the abdomen of blood fed mosquitoes or sand flies will be distended and bright red, and clearly visibile through the mesh.

a. In the unlikely event of no feeding or poor feeding (only 0-2 insects fed or probed as noted by trained staff), the participant may undergo a repeat feed with 5-10 fresh insects once.
b. If a repeat feed is required, the post-feeding biopsies and blood draws will be done based on the timing of the repeat feed.
c. Post-feeding times will be calculated from the time the feeding device is removed.

iv. Post-feeding, redness, swelling and number of visible bites will be assessed and documented immediately (+15 minutes) and 30 minutes (+15 minutes) after the feeding device is removed. This may also include taking photographs of the bites.

v. Once the mosquitoes or sand flies have fed, they will be brought back to the lab for evaluation and then disposal.

After each feeding, participants may be offered standard treatment as needed to control reactions to vector bites. These treatments may include cold compresses, topical or oral antihistamines, or topical steroids. On days when biopsies will be performed, necessary treatment to control the reaction to the vector bite will be offered after the biopsy is completed. Any necessary treatment to control bleeding, discomfort, or reduce scarring from the biopsy will also be offered.

6 Study Schedule

This study will take place at the NIH CC. All aspects of the protocol will be carried out in accordance with NIH guidelines, and International Council on Harmonisation (ICH) Good Clinical Practice (GCP) involving human participant research. The study schedule is described below and in Appendix A: Schedule of Procedures/Evaluations.

6.1 Enrollment

At the time of enrollment, participants will be assigned to one of two cohorts and one of three vector groups based on their history of exposure and vector-specific ELISA results performed during screening (for Cohort A only, see Section 4.3). Vector assignment will also consider participant availability, since not all vectors may be available for feeding on the same feeding day. Participants may be assigned to any of the vectors or groups until a group is full. At that point, participants will only be assigned to vectors and groups that have not been completely enrolled. If a participant is only eligible for a vector or group that is already completely enrolled, they will not continue with consent and enrollment.

The research team will thoroughly discuss the informed consent form with each eligible individual and obtain consent prior to performing any study procedures. This process may take place within 90 days of or on Day 0 before any research procedures are performed.

6.2 Cohort A

Cohort A participants will undergo 1 vector feeding and 3 biopsy procedures, all on Day 0.

6.2.1 Vector feeding and biopsy (Day 0)

The vector feeding will take place in the NIH CC Special Clinical Studies Unit (SCSU) or other appropriate unit. The following procedures will be performed:

- Review of medical/medication history.
- Review of inclusion/exclusion criteria.
- Documentation of usual responses to mosquito or other insect bites as none (0), mild (1), moderate (2), or severe (3).
- Clinician exam/assessment.
- Vital signs.
- Urine pregnancy test (for women of childbearing potential; confirmed negative before feeding).
- Blood draw for research labs (section 7.2, Appendix A).

After these procedures, participants will undergo feeding with the assigned vector. Feeding and monitoring will be conducted as described in section 5.1.

After the feeding, participants will undergo the following procedures:

- 3 skin punch biopsy collections as follows:
    - 1 biopsy from bitten skin collected no earlier than 30 minutes and no later than 90 minutes after the vector feeding is completed.
    - 1 biopsy from bitten skin collected no earlier than 3.5 hours and no later than 4.5 hours after the vector feeding is completed.
    - 1 biopsy from unbitten skin on the opposite arm collected no earlier than 30 minutes and no later than 6 hours after the vector feeding is completed.
    - Biopsies will be placed immediately and no later than one minute after excision into pre-labeled Eppendorf tubes containing either a fixative (10% buffered formalin) or RNAlater for immunohistochemical or transcriptomic analyses, respectively.
- Any other clinical tests that are medically indicated or appropriate to ensure the safety of the individual participant as determined by the PI.

Following these study procedures, participants will be allowed to leave as long as there are no safety concerns. They will be provided with medications if needed before they leave, and they will be asked to notify the study team if they have any reactions or side effects.

6.2.2 24-Hour Post-Feeding Visit (Day 1 +1 day)

All Cohort A participants will return for a 24-hour post-feeding visit. At this visit, the following procedures will be performed.

- Vital Signs.
- Clinician assessment of bites and biopsy sites.
- Blood draw for research labs (section 7.2, Appendix A).

6.2.3 Final study contact (Day 7 ±3 days)

All participants will be contacted by telephone about 1 week after Day 0 to ensure there are no issues after the feedings or biopsies or adverse events. Any participant who experiences complications due to feeding or biopsies will be examined in the clinic and followed until complications have resolved and/or referral to the necessary medical care has been made. After this phone call, Cohort A participation will be complete.

6.3 Cohort B

Cohort B participants will undergo 4 vector feedings over about 8 weeks and 3 biopsy procedures after the fourth and final feeding.

6.3.1 Vector feeding and biopsy (Days 0, 14, 28, and 42, ±7 days)

All Cohort B participants will undergo feeding procedures on Days 0, 14 (±7 days), 28 (±7 days), and 42 (±7 days) for a total of 4 feedings. Feedings will be approximately 2 weeks apart with a minimum of 7 days in between feedings. These visits will occur in the SCSU, or other appropriate unit, and the following procedures will be performed:

- Review of medical/medication history.
- Review of inclusion/exclusion criteria.
- Documentation of usual responses to mosquito bites as none (0), mild (1), moderate (2), or severe (3). (only performed on Day 0).
- Clinician exam/assessment.
- Vital signs.
- Urine pregnancy test (for women of childbearing potential; confirmed negative before feeding).
- Blood draw for research labs (section 7.2, Appendix A).

After these procedures, participants will undergo feeding with the assigned vector. Feeding and monitoring will be conducted as described in section 5.1.

After each feeding on Day 0, 14, and 28, participants will be monitored for a minimum of 1 hour post feeding and the following procedures will be performed:

- Any clinical tests that are medically indicated or appropriate to ensure the safety of the individual participant as deemed by the PI.
- Participants will be asked to not apply any topical steroid treatments. Other topical treatments, such as antihistamines, may be applied as needed.

After the final feeding on Day 42, the following procedures will be performed:

- 2 skin punch biopsy collections as follows:
    - 1 biopsy from bitten skin collected no earlier than 3.5 hours and no later than 4.5 hours after the vector feeding is completed.
    - 1 biopsy from unbitten skin on the opposite arm collected no earlier than 30 minutes and no later than 6 hours after the vector feeding is completed.
- Any other clinical tests that are medically indicated or appropriate to ensure the safety of the individual participant as deemed by the PI.
- Participants will be asked to not apply any topical treatments after the fourth and final feeding. Other topical treatments may be applied after the Day 44 biopsy is completed.

Following these study procedures, participants will be allowed to leave as long as there are no safety concerns. They will be provided with medications if needed before they leave, and they will be asked to notify the study team if they have any reactions or side effects.

6.3.2 Follow-up and biopsy (Day 44 +3 days)

About 2 days after the final feeding procedure, all participants in Cohort B will return to the NIH CC for a follow-up visit. The following procedures will be performed:

- Vital signs.
- Review of medical/medication history.
- Clinician assessment of bite and biopsy sites.
- 1 skin punch biopsy from bitten skin at a minimum 40 hours after vector feeding is completed.
- Blood draw for research labs (section 7.2, Appendix A).

Following these study procedures, participants will be allowed to leave as long as there are no safety concerns. They will be provided with medications if needed before they leave, and they will be asked to notify the study team if they have any reactions or side effects.

6.3.3 Final study contact (Day 51 ±3 days)

All Cohort B participants will be contacted by telephone about 1 week after Day 44 to ensure there are no issues after the feedings or biopsies or adverse events. Any participant who experiences complications due to feeding or biopsies will be examined in the clinic and followed until complications have resolved and/or referral to the necessary medical care has been made. After this phone call, Cohort B participation will be complete.

6.3.4 Early termination visit

- Discussion to review reason for termination.
- Review of medical/medication history.
- Review of inclusion/exclusion criteria.
- Clinician exam/assessment.
- Vital signs.
- Blood draw for research labs (section 7.2, Appendix A) if participant is willing.

6.4. Unexpected or incidental medical conditions of the participant

If unexpected or incidental medical conditions are diagnosed during the medical evaluation in this protocol, the participant will be referred to an appropriate physician and/or hospital and encouraged to follow up for treatment of their condition. Standard of care treatment may be offered by the study team if necessary while the participant is being referred to appropriate outside medical care.

7 Study Procedures/Evaluations

See Appendix A for schedule of clinical and study procedures and laboratory evaluations. Blood volumes are provided in a tabular form in Appendix B: Blood Volumes for Specimen Collection.

7.1 Study Procedures

7.1.1 Vital signs

Participants must be sitting for a minimum of 5 minutes prior to these procedures being performed. Vital signs include blood pressure, mean arterial pressure, heart rate, respiratory rate, temperature, weight, and pulse oximetry. Height will be taken at the Day 0 visit only. On feeding days, vital signs will be performed pre-feeding.

7.1.2 Arthropod Feeding

Please see Section 5.1 (Arthropod Feeding)

7.1.3 Skin biopsies

From each participant, three 3-mm skin biopsies will be taken, two at the bite sites and one at an unbitten site on the opposite arm (control), using the following procedure:

1. Participant will be placed in a relaxed position with their arm out to expose the insect bites or control site on the ventral side of the appropriate forearm.
2. A time-out will take place where the participant's name and medical record number and the site of the biopsy will be confirmed. The time and date of the time-out will be documented.

3. Non-sterile gloves will be worn and pads will be laid out to protect clothing as needed.
4. Biopsy location will be marked with small circle.
5. 2% lidocaine with or without epinephrine will be drawn up in a 3-mL syringe.
6. A 30-gauge needle will be used to inject lidocaine: It will be inserted into the skin just outside the circle drawn around the biopsy site; 1-1.5 cc of lidocaine will be injected slowly.
7. Anesthesia will be tested using sharp forceps. More lidocaine will be injected as needed to achieve proper local anesthesia.
8. Chlorhexidine swabs will be used to disinfect the biopsy site by wiping concentrically from the center of the site to the outside of biopsy site.
9. A steri-drape may be applied around the biopsy site.
10. The biopsy will be performed by pulling skin taut with thumb and forefinger above and below the marked area. A punch biopsy tool will be used to apply pressure and turned back and forth 360 degrees. Forceps will be used to pull out the biopsy. Scissors may also be used if necessary to cut the specimen from the subcutaneous tissue.
11. Pressure will be held at the site using gauze, and then the site will be cleaned and dressed appropriately.
12. Sutures may be placed if deemed necessary by the clinical team. If sutures are placed, the participant will return to clinic approximately 10 days later for suture removal.

7.2 Laboratory Evaluations

7.2.1 Safety laboratory evaluations

For women of childbearing potential, a urine pregnancy test will be performed before each vector feeding procedure.

7.2.2 Laboratory testing of samples for study endpoints

Primary Endpoints:

- Immunohistochemistry of target proteins in vector-naïve participant skin biopsy samples for each of the three vector groups at 30 minutes and 4 hours after the one feeding.
- Transcriptomics via deep sequencing and/or gene expression array of cDNA libraries made from skin RNA of vector-naïve participants for each of the three vector groups at 30 minutes and 4 hours after the one feeding.

- Immunohistochemistry of target proteins in vector-experienced participant skin biopsy samples for each of the three vector groups at 4 hours and 48 hours after the fourth and final feeding.
- Transcriptomics via deep sequencing and/or gene expression array of cDNA libraries made from skin RNA of vector-experienced participants for each of the three vector groups at 4 hours and 48 hours after the fourth and final feeding.

Secondary Endpoints:

- Flow cytometry analysis of PBMCs and cytokine analysis of plasma collected 24 hours after the one feeding of in each vector group of vector-naïve participants.
- Flow cytometry analysis of PBMCs and cytokine analysis of plasma collected 48 hours after the fourth and final feeding in each vector group of vector-experienced participants.

Exploratory Endpoints:

- Comparison of gene expression of circulating white blood cells after the one vector feeding for each vector group of vector-naïve participants.
- Comparison of gene expression of circulating white blood cells after the fourth and final feeding in each vector group of vector-experienced participants.

8 Potential Risks and Benefits

8.1 Potential Risks

8.1.1 Risks of vector feeding

Vector feeding may result in itchiness, pain, swelling, and redness at the site of bites. These reactions resolve after a short period of time and pose little risk to the participant. Applying cold packs and administering over-the-counter pain medications or antihistamines (topical or oral) if necessary can generally treat these reactions. Vector feeding or scratching after vector feeding may result in scarring such as hyperpigmentation. In rare cases a more severe irritation could occur, such as anaphylactic reaction or secondary infection at the site of the bite. Signs of infection include pain, redness, swelling, and drainage at the site. Oral antibiotics or anti-inflammatory medications can be administered if necessary.

8.1.2 Risks of skin punch biopsy

Risks of biopsy include local pain, bleeding, redness, infection, a scar and possible keloid formation. Oral or topical antibiotics and oral analgesics will be used to manage pain and infection as needed. Any non-routine complications resulting from the

procedure will be addressed in consultation with the NIH CC Dermatology service. Injection of local anesthetic may cause a minimal burning discomfort or bruising at the site of the needle puncture.

8.1.3 Risks of blood draw

Risks of blood draw include pain, bruising, bleeding, local discomfort, lightheadedness, dizziness or possibly fainting and rarely infection or blood clot. The amount of blood drawn will be within the limits allowed for adult participants by the NIH CC (Medical Administrative Policy 95-9: Guidelines for Limits of Blood Drawn for Research Purposes in the Clinical Center: http://cc-internal.cc.nih.gov/policies/PDF/M95-9.pdf).

8.2 Potential Benefits

There is no direct benefit to the participant. The information collected from this study will allow a better understanding of the skin immune response to vector bites to inform future vaccine development.

9 Research Use of Stored Human Samples, Specimens, and Data

Intended Use: Samples, specimens and data collected under this protocol may be used to study aspects of leishmaniasis, Zika virus infection, and other vector-borne diseases.

Storage: All of the stored study research samples are labeled by a code that only the investigators can link to the participant. Samples are stored in a freezer within a secure laboratory with limited access. Data will be kept in password-protected computers. Only investigators or their designees will have access to the samples and data.

Tracking: Samples and data acquired under this protocol will be tracked using a database located on password-protected computers, which will be maintained by the investigators and their designees.

Disposition at the Completion of the Protocol:

- In the future, other investigators (both at NIH and outside) may wish to study these samples and/or data. Before any sharing of samples, data, or clinical information, either institutional review board (IRB) approval must be obtained or the NIH Office of Human Subjects Research Protections (OHSRP) must determine that the research is exempt from IRB oversight. OHSRP can make this determination for some research where the samples or data have no personal identifying information about the study participant and the researcher is not able to ascertain it.
- At the time of protocol termination, samples will either be destroyed, or after IRB approval, transferred to another existing protocol. Data will be archived by the study team in compliance with requirements for retention of research records; alternatively, after IRB approval, the data may be either destroyed or transferred to another repository.

Reporting the Loss or Destruction of Samples/Specimens/Data to the IRB:

- Any loss or unanticipated destruction of samples or data (for example, due to freezer malfunction) that meets the definition of protocol deviation or unanticipated problem (UP), and/or compromises the scientific integrity of the data collected for the study will be reported to the NIAID IRB.
- Additionally, participants may decide at any point not to have their samples stored. In this case, the PI will destroy all known remaining samples and report what was done to both the participant and to the IRB. This decision will not affect the individual's participation in this protocol or any other protocols at NIH.

10 Data Sharing Plan

Human data generated in this study will be shared for future research as follows:

- De-identified data in an NIH-funded or approved public repository.
- De-identified data in another public repository.
- Identified data in the Biomedical Translational Research Information System (BTRIS, automatic for activities in the CC).
- De-identified or identified data with approved outside collaborators under appropriate agreements.
- Data sharing may be complicated or limited in certain cases by contractual obligations or agreements with outside collaborators, such as cooperative research and development agreements (CRADA), clinical trial agreements (CTA), other restraints, etc.

Data will be shared through:

- BTRIS (automatic for activities in the Clinical Center).
- Approved outside collaborators under appropriate individual agreements.
- Publication and/or public presentations.

Data will be shared at the time of publication or shortly thereafter.

11 Remuneration Plan

Participants will be compensated according to Table 2 below. Study visits will be compensated according to the number of visits the participant completes. Participants will only be compensated for the protocol visits and interim visits requested by the investigators if the visits are medically necessary. Remuneration will be provided to the participants in Cohort A after the completion of the day 7 phone call. Remuneration will be provided to participants in Cohort B two times: after the completion of Day 28 and the remainder of the compensation will be provided after the day 51 phone call.

If a subject participates in Cohort A and Cohort B of the same vector, he/she will undergo Day 0 Feeding only once as part of Cohort A (which will also count for Day 0 Feeding for Cohort B). Therefore, these participants will not be compensated for Day 0 of Cohort B. They will receive compensation for the other protocol visits as described above and outlined below.

Table 2. Estimated participant remuneration.

Cohort A	**Compensation**
Day 0: Vector feeding and biopsy	$825
Day 1: 24-hour post-feeding blood draw	$100
Expected total for completion of Cohort A study visit	***$925***
Cohort B	
Day 0: Vector Feeding 1	$325
Day 14: Vector Feeding 2	$425
Day 28: Vector Feeding 3	$425
Day 42: Vector Feeding 4 and biopsy	$725
Day 44: 48-hour post-feeding biopsy and blood draw	$200
Expected total for completion of ALL Cohort B study visits	***$2100***
Investigator-requested interim visits:	$75

12 Assessment of Safety

12.1 Toxicity Scale

The investigator will grade the severity of each AE according to the U.S. Food and Drug Administration (FDA) "Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials" September 2007, which can be found at: https://www.fda.gov/downloads/BiologicsBloodVaccines/ucm091977.

Severity grading for clinical events that are not found in the FDA Healthy Volunteer Toxicity Table will be graded according to the following grading scale:

- Grade 1 (Mild)
  Events causing no or minimal interference with daily activity.
- Grade 2 (Moderate)
  Events causing greater than minimal interference with daily activity but not requiring medical intervention.
- Grade 3 (Severe)
  Events causing inability to perform daily activity and/or requiring medical intervention.
- Grade 4 (Potentially Life-Threatening)*
  Events causing inability to perform basic self-care functions OR medical or operative intervention indicated to prevent permanent impairment, persistent disability, or death.

***Note:** A severity assessment of "potentially life-threatening" is not necessarily the same as life-threatening as a "serious adverse event" (SAE) criterion. The latter means that the event is an immediate threat to life as opposed to a potential threat to life.

12.2 Recording/Documentation

At each contact with the participant, information regarding AEs will be elicited by appropriate questioning and examinations from consent to the last date on study. All events, both expected/unexpected and related/unrelated will be recorded on a source document. Source documents will include: progress notes, laboratory reports, consult notes, phone call summaries, survey tools, and data collection tools. Source documents will be reviewed in a timely manner by the research team. All reportable AEs that are identified will be recorded in the Clinical Research Information Management System of the NIAID (CRIMSON). The start date, the stop date, the severity of each reportable event, and the PI's judgment of the AE's relationship and expectedness to the study intervention will also be recorded in CRIMSON.

All vital sign, physical exam, and laboratory abnormalities found prior to the first arthropod feeding will be documented as a baseline finding (not an AE) and will be assessed for clinical significance. The participant will be reassessed for inclusion or exclusion to undergo each feeding. After the first arthropod feeding, all new gradable abnormalities not found at baseline will be reported as AEs.

Causality (likelihood that the event is caused by the study intervention) will be assessed considering the factors listed under the following categories:

Definitely Related

- Reasonable temporal relationship
- Follows a known response pattern
- Clear evidence to suggest a causal relationship
- There is no alternative etiology

Probably Related

- Reasonable temporal relationship
- Follows a suspected response pattern (based on similar products)
- No evidence of a more likely alternative etiology

Possibly Related

- Reasonable temporal relationship
- Little evidence for a more likely alternative etiology

Unlikely Related

- Does not have a reasonable temporal relationship

OR

- Good evidence for a more likely alternative etiology

Not Related

- Does not have a temporal relationship

OR

- Definitely due to an alternative etiology

Note: Other factors should also be considered for each causality category when appropriate. Causality assessment is based on available information at the time of the assessment of the AE. The investigator may revise the causality assessment as additional information becomes available.

12.3 Definitions

Expected Events

The following are mild (Grade 1) to moderate (Grade 2) signs or symptoms that are induced by or associated with vector feeding or skin biopsy. If deemed related to vector feeding or skin biopsy by the PI, they will be not be recorded as adverse events in CRIMSON per protocol, unless deemed by the PI to be abnormal or greater than Grade 2.

Feeding:

- Local swelling
- Pruritis
- Erythema

Biopsy:

- Local swelling
- Erythema
- Bleeding at biopsy site
- Scar at biopsy site

Reactions should be graded in the following manner:

Grade 1	Mild transient reaction; interruption not indicated; intervention not indicated.
Grade 2	Interruption indicated but responds promptly to symptomatic treatment (e.g., antihistamines, nonsteroidal anti-inflammatory drugs, narcotics, intravenous fluids); prophylactic medications indicated for less than 24 hours.
Grade 3	Prolonged (e.g., not rapidly responsive to symptomatic medication and/or brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for clinical sequelae.
Grade 4	Life-threatening consequences; urgent intervention indicated.
Grade 5	Death.

Adverse Event
Any untoward medical occurrence in a human subject, including any abnormal sign, symptom, or disease, temporally associated with the individual's participation in research, whether or not considered related to the individual's participation in the research.

Serious Adverse Event (SAE)
An SAE is an AE that results in 1 or more of the following outcomes:

- Death
- A life-threatening event (places the subject at immediate risk of death from the event as it occurred)
- An inpatient hospitalization or prolongation of an existing hospitalization
- A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
- A congenital anomaly/birth defect
- A medically important event*

*Medical and scientific judgment should be exercised in deciding events that may not be immediately life threatening or result in death or hospitalization but may jeopardize the participant or may require intervention to prevent one of the other outcomes listed above.

Protocol Deviation
Any change, divergence, or departure from the IRB-approved study procedures in a research protocol. Protocol deviations are designated as serious or non-serious and further characterized as:

- Those that occur because a member of the research team deviates from the protocol.
- Those that are identified before they occur, but cannot be prevented.
- Those that are discovered after they occur.

Serious Protocol Deviation
A deviation that meets the definition of an SAE or compromises the safety, welfare, or rights of subjects or others.

Non-compliance
The failure to comply with applicable NIH Human Research Protections Program (HRPP) policies, IRB requirements, or regulatory requirements for the protection of human subjects. Non-compliance is further characterized as:

1. Serious: Non-compliance that
    a. Increases risks, or causes harm, to participants.
    b. Decreases potential benefits to participants.
    c. Compromises the integrity of the NIH HRPP.
    d. Invalidates the study data.

2. Continuing: Non-compliance that is recurring.
3. Minor: Non-compliance that is neither serious nor continuing.

Unanticipated Problem (UP)
Any incident, experience, or outcome that meets all 3 of the following criteria:

1. Unexpected in terms of nature, severity, or frequency in relation to
   a. the research risks that are described in the IRB-approved research protocol and informed consent document, investigator's brochure, or other study documents; and
   b. the characteristics of the participant population being studied;
2. Related or possibly related to participation in the research;
3. Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

Serious UP

A UP that meets the definition of a SAE or compromises the safety, welfare, or rights of subjects or others.

UP that is not an AE (UPnonAE)
A UP that does not fit the definition of an AE, but which may, in the opinion of the investigator, involve risk to the subject, affect others in the research study, or significantly impact the integrity of research data. These events may involve a greater risk of social or economic harm to subjects or others rather than physical/psychological harm. Such events would be considered non-serious UPs. Examples of a UPnonAE include a breach of confidentiality, accidental destruction of study records, or unaccounted-for study drug.

12.4 Reporting Procedures to the NIAID IRB

12.4.1 Special reporting situations

Safety events of interest that may require expedited reporting and/or safety evaluation include, but are not limited to:

- Excessive reactions to study intervention.
- Inadvertent or accidental exposure to a study intervention.
- Medication error involving a product (with or without participant exposure to the study intervention, e.g., name confusion).

Special reporting situations will be recorded. Any special reporting situation that meets the criteria of an SAE should be reported as described in sections 12.4.2 and 12.4.3.

12.4.2 Expedited reporting to the NIAID IRB

Serious and non-serious UPs, deaths, serious deviations, and serious or continuing non-compliance will be reported within 7 calendar days of investigator awareness. SAEs that are possibly, probably, or definitely related to the research will be reported to the NIAID IRB within one calendar day of site awareness, regardless of expectedness.

12.4.3 Waiver of reporting anticipated protocol deviations, and expected UPnonAEs to the NIAID IRB

Anticipated deviations in the conduct of the protocol will not be reported to the IRB unless they occur at a rate greater than anticipated by the study team..

12.4.4 Annual reporting to the NIAID IRB

The following items will be reported to the NIAID IRB in summary at the time of continuing review:

- Serious and non-serious UPs
- SAEs that are possibly, probably, or definitely related to the research
- SAEs that are not related to the research
- All AEs, except expected AEs granted a waiver of reporting
- Serious and non-serious protocol deviations
- Serious, continuing, and minor non-compliance
- Any trends or events which in the opinion of the investigator should be reported
- A summary of accumulated safety data

12.5 Pregnancy

Although pregnancy itself is not an AE, events occurring during pregnancy, delivery, or in the neonate (e.g., congenital anomaly/birth defect) may be AEs or SAEs.

In the event of pregnancy, the following steps will be taken:

- Discontinue the study intervention and procedures but continue to follow-up for safety.
- Report to the IRB.
- Advise research participant to notify the obstetrician of study participation and study intervention exposure.

12.6 Type and Duration of the Follow-up of Participants after AEs

AEs that occur following enrollment of the participant (by signing the informed consent) will be followed until the final outcome is known or until the end of the study follow-up period. AEs that have not resolved by the end of the study follow-up period will be recorded as “not recovered/not resolved.” If a participant is lost to follow-up and AEs have not resolved, the outcome of these AEs will be recorded as “unknown.” Any participant who experiences complications due to feeding or biopsy will be followed until such complications have resolved or appropriate referral to the necessary medical care

has been made. For SAEs, if it is not possible to obtain a final outcome (e.g., the participant is lost to follow-up), the reason a final outcome could not be obtained will be recorded by the investigator in CRIMSON.

SAEs that have not resolved by the end of the follow-up period will be followed until final outcome is known. If it is not possible to obtain a final outcome for an SAE (e.g., the participant is lost to follow-up), the reason a final outcome could not be obtained will be recorded by the investigator in CRIMSON.

12.7 Pausing Rules for an Individual Subject

Pausing is the suspension of administration of study intervention to a single subject until a decision is made whether or not to resume administration of the study intervention.

The pausing criteria for a single subject in this study include any of the following:

- A subject experiences an SAE that is possibly, probably, or definitely related to a study intervention;
- A subject experiences two Grade 3 or greater AEs that are possibly, probably, or definitely related to a study intervention;
- Any safety issue that the investigator determines should pause administration of a study intervention to a single subject.

The PI will determine if study intervention administration to an individual participant should be paused. The study may also be paused for an entire group if a safety concern is identified.

12.7.1 Reporting a pause

If a pausing criterion is met, a description of the AE(s) or safety issue must be reported by the PI within 1 business day to the IRB by fax or email.

12.7.2 Resumption of a paused study

The PI will determine whether or not it is safe to resume administration of the study intervention to the participant. The PI will notify the IRB of the decision on resumption of the study intervention.

A subject who does not resume study interventions will continue to be followed for safety.

12.8 Halting Rules for the Protocol

Halting the study requires immediate discontinuation of study intervention administered for all participants and suspension of enrollment until a decision is made whether or not to continue enrollment and study intervention administration.

The halting rules are:

- 1 or more participants experience the same or similar SAEs that are possibly, probably, or definitely related to the study intervention;

OR

- 2 or more of the same or similar AE in different participants that are grade 3 or above and are possibly, probably, or definitely related to the study intervention;

OR

- any safety issue that the PI determines should halt the study.

The PI will determine if the study should be halted.

12.8.1 Reporting a study halt

If a halting rule is met, a description of the AE(s) or safety issue must be reported by the PI within 1 business day to the IRB by fax or email.

12.8.2 Resumption of a halted study

The PI will determine if it is safe to resume the study. The PI will notify the IRB of the decision on resumption of the study.

Participants who do not resume study intervention will continue to be followed for safety.

12.9 Premature Withdrawal of a Participant

An individual participant will be withdrawn for any of the following:

- An individual participant's decision. (The investigator should attempt to determine the reason for the participant's decision.)
- Non-compliance with study procedures to the extent that it is potentially harmful to the participant or to the integrity of the study data (e.g., participant misses a feeding visit beyond visit window).
- A change in the participant's baseline condition after enrollment so that the participant no longer meets one or more of the inclusion/exclusion criteria.
- The investigator determines that continued participation in the study would not be in the best interest of the participant.

12.10 Replacement of Withdrawn Participants or Participants Who Discontinue Study Intervention

Participants who withdraw at any time before all biopsies may be replaced. If a participant is removed prior to the first feeding, none of their data will be used in the analysis or publication of the study. If a participant completes at a minimum the first feeding, data will be included in the analysis and publication.

13 Clinical Monitoring Structure

13.1 Quality Management Plan

During the study, the PI and study team will be responsible for implementing a quality control plan. Additionally, the study team will be responsible for completing and submitting a summary report on the quality plan on a quarterly basis to the NIAID Clinical Director or designee. A courtesy copy will also be sent to Clinical Trials Management.

13.2 Safety Monitoring Plan

The data generated during participation in this protocol will be monitored by the PI and the study team for safety and compliance with protocol-specific requirements. The PI and the study team will closely monitor and analyze study data as it becomes available and will take any steps necessary to ensure that safety is maximized and risks are minimized and that subject protections are appropriate and comply with applicable regulations and guidance.

13.1 Monitoring

To help ensure that NIH/ ORSC Good Clinical Practices are being carried out, a Clinical Trials Management (CTM)/ RCHSPP designee on behalf of OCRPRO will conduct a Study Initiation Visit (SIV) prior to study enrollment beginning. The purpose of this meeting is to review with the Principal Investigator (PI) and study team their roles and responsibilities, as well as their commitment to adhere to the requirements of the protocol, especially in terms of AE, SAE and UP reporting requirements (NIH/OHSRP). In addition, the quality management and data management plans for the study will be reviewed.

14 Statistical Considerations

This study is highly exploratory as very little is known about skin immunity to bites of these vectors. The simple characterization of this skin immunity will be important, as even with a small samples size we expect to be able to identify important aspects of the innate and adaptive immune response after a vector bite by examining the transcriptomic responses of differentially expressed genes. Assessing the use of next generation transcriptomic and immunohistochemistry techniques, we have made estimates that will allow us to see useful differences between bittena dn unbitten skin.

14.1 Study Hypothesis

The study hypothesis is that there will be differential transcriptomic gene expression and immunihistochemical differences between the cases (bitten skin) and controls (unbitten skin from the opposite arm) with regard to innate immune markers at Day 0 after the first bite of an insect in a relatively naïve individual. We also exepect to see differential gene expression and immunohistochemical differences of adaptive immune markers between cases (bitten skin) biopsies collected on Day 42 and Day 44 (experienced cohort) as compared to controls (unbitten skin).

14.2 Sample Size Justification and Analysis Plan

Previous studies of the skin transcriptomes have estimated approximately 13,000 genes per biopsy of healthy individuals [18]. This large number of genes will be examined to find a smaller list of differentially expressed genes within cohorts. To analyze the data, non-normalized raw counts will be used with an analysis program (e.g., the EdgeR package) to perform differential gene expression analysis after quality control of samples. Our anticipated analysis program will utilize model-based scale normalization, estimate dispersions, and apply a negative binomial model for RNA transcriptomic data analysis to find differentially expressed genes.

Single comparisons will be made within cohorts of bitten skin at a particular timepoint to the unbitten skin biopsy. Because our study will analyze paired samples (bitten skin and unbitten skin biopsies from the same individual), we can employ two different approaches. First, we can apply general linear modelling (GLM) using a GLM likelihood ratio test for comparing bitten versus unbitten skin. Also, we can employ group-wise comparisons where negative binomial fitting is followed by exact test. FDR adjustment will be used for multiple testing correction. An FDR threshold of 0.1 for statistical significance is typically applied. Genes with larger differential expression will be defined with a log-fold-change threshold of 0.5 (i.e., 50% change between experimental conditions).

Currently it is unknown how uniform skin immune responses to bites of these vector are and how much variability will be observed. Therefore it is very difficult estimate an exact samples size. As this study is very exploratory in nature it is likely with the relative small number of individuals in each cohort the results will be very informative of future studies as it will offer more insight into the diversity or lack thereof in the skin responses. The cohort sizes of 15 will allow of us to have adequate numbers to perform these detailed and expensive immunohistochemical and transcriptomic techniques that could lead to future larger studies with more focused testing once the differential genes and important areas of immune response are identified.

15 Ethics/Protection of Human Participants

15.1 Informed Consent Process

Informed consent is a process where information is presented to enable persons to voluntarily decide whether or not to participate as a research participant. It is an ongoing conversation between the human research participant and the researchers, which begins before consent is given and continues until the end of the participant's involvement in the research. Discussions about the research will provide essential information about the study and include: purpose, duration, experimental procedures, alternatives, risks, and benefits. Participants will be given the opportunity to ask questions and have them answered.

The participants will sign the informed consent document prior to undergoing any research procedures. The participants may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the participants for their records. The researcher will document the signing of the consent form in the participant's medical record. The rights and welfare of the participant will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate or withdraw from this study.

15.1.1 Non-English–speaking participants

If a non-English–speaking participant is unexpectedly eligible for enrollment, the participant will be provided with the CC Short Written Consent Form for Non-English–Speaking Research Participants in the participant's native language and a verbal explanation of the purpose, procedures, and risks of the study as described in NIH HRPP standard operating procedure 12, and 45 Code of Federal Regulations (CFR) 46.117(b)(2). The IRB-approved English consent form will serve as basis for the verbal explanation of the study. The investigator will obtain an interpreter unless the investigator is fluent in the prospective participant's language. Preferably, the interpreter will be someone who is independent of the participant (i.e., not a family member). Interpreters provided by the CC will be used whenever possible. The interpreters will interpret all oral communications (English to target language and conversely) between the investigator and a limited English-proficient participant, facilitate discussions, and clarify information as necessary.

The IRB-approved English consent form will be signed by the investigator obtaining consent and a witness to the oral presentation. The CC Short Written Consent Form will be signed by the participant and a witness who observed the presentation of information. The interpreter may sign the consent document as the witness and, in this case, will note "Interpreter" under the signature line. A copy of both signed forms will be provided to the participant to take home.

The investigator obtaining consent will document the consent process in the participant's medical record (CRIMSON), including the name of the interpreter. Further, all instances of use of the CC Short Written Consent Form will be reported to the IRB at the time of annual review. If the CC Short Written Consent Form is used three times or more for the same language, this will be reported to the IRB immediately.

15.2 Participant Confidentiality

All records will be kept confidential to the extent provided by federal, state, and local law. The study monitors and other authorized individuals may inspect all documents and records required to be maintained by the investigator, including but not limited to medical records. Records will be kept locked and all computer entry and networking programs will be done with coded numbers only. Clinical information will not be released without written permission of the participant, except as necessary for monitoring by the IRB, NIAID, and Office for Human Research Protections.

16 Data Handling and Record Keeping

16.1 Data Capture and Management

Study data will be collected and maintained in CRIMSON and the Clinical Research Information System (CRIS) and collected directly from participants during study visits and telephone calls, or will be abstracted from participants' medical records. Source documents include all recordings of observations or notations of clinical activities and all reports and records necessary to confirm the data abstracted for this study. Data entry into these systems will be performed by authorized individuals. The investigator is responsible for assuring that the data collected are complete, accurate, and recorded in a timely manner.

16.2 Record Retention

The investigator is responsible for retaining all essential documents listed in the ICH GCP guidelines. Study records will be maintained by the PI according to the timelines specified in CFR 312.62 or a minimum of 5 to 7 years, and in compliance with institutional, IRB, state, and federal medical records retention requirements, whichever is longest. All stored records will be kept confidential to the extent required by federal, state, and local law.

SCIENTIFIC REFERENCES

[1] "WHO | A global brief on vector-borne diseases," *WHO*. [Online]. Available: http://www.who.int/campaigns/world-health-day/2014/global-brief/en/. [Accessed: 31-Mar-2017].
[2] A. S. Fauci and D. M. Morens, "Zika Virus in the Americas — Yet Another Arbovirus Threat," *N. Engl. J. Med.*, vol. 374, no. 7, pp. 601–604, Feb. 2016.
[3] F. J. Burt, M. S. Rolph, N. E. Rulli, S. Mahalingam, and M. T. Heise, "Chikungunya: a re-emerging virus," *Lancet Lond. Engl.*, vol. 379, no. 9816, pp. 662–671, Feb. 2012.
[4] R. S. Lanciotti *et al.*, "Origin of the West Nile Virus Responsible for an Outbreak of Encephalitis in the Northeastern United States," *Science*, vol. 286, no. 5448, pp. 2333–2337, Dec. 1999.
[5] M. Abdeladhim, S. Kamhawi, and J. G. Valenzuela, "What's behind a sand fly bite? The profound effect of sand fly saliva on host hemostasis, inflammation and immunity," *Infect. Genet. Evol.*, vol. 28, pp. 691–703, Dec. 2014.
[6] M. J. Conway, T. M. Colpitts, and E. Fikrig, "Role of the Vector in Arbovirus Transmission," *Annu. Rev. Virol.*, vol. 1, no. 1, pp. 71–88, Oct. 2014.
[7] S. Wichit, P. Ferraris, V. Choumet, and D. Missé, "The effects of mosquito saliva on dengue virus infectivity in humans," *Curr. Opin. Virol.*, vol. 21, pp. 139–145, Oct. 2016.
[8] N. C. Peters, N. Kimblin, N. Secundino, S. Kamhawi, P. Lawyer, and D. L. Sacks, "Vector Transmission of Leishmania Abrogates Vaccine-Induced Protective Immunity," *PLOS Pathog.*, vol. 5, no. 6, p. e1000484, Jun. 2009.
[9] J. M. Ribeiro, "Role of saliva in blood-feeding by arthropods," *Annu. Rev. Entomol.*, vol. 32, pp. 463–478, 1987.
[10] Z. Peng and F. E. R. Simons, "Mosquito allergy: immune mechanisms and recombinant salivary allergens," *Int. Arch. Allergy Immunol.*, vol. 133, no. 2, pp. 198–209, Feb. 2004.
[11] N. S. Zeidner, S. Higgs, C. M. Happ, B. J. Beaty, and B. R. Miller, "Mosquito feeding modulates Th1 and Th2 cytokines in flavivirus susceptible mice: an effect mimicked by injection of sialokinins, but not demonstrated in flavivirus resistant mice," *Parasite Immunol.*, vol. 21, no. 1, pp. 35–44, Jan. 1999.
[12] H. A. Wasserman, S. Singh, and D. E. Champagne, "Saliva of the Yellow Fever mosquito, Aedes aegypti, modulates murine lymphocyte function," *Parasite Immunol.*, vol. 26, no. 6–7, pp. 295–306, Jul. 2004.
[13] M. Pingen *et al.*, "Host Inflammatory Response to Mosquito Bites Enhances the Severity of Arbovirus Infection," *Immunity*, vol. 44, no. 6, pp. 1455–1469, Jun. 2016.
[14] N. Collin *et al.*, "Sand fly salivary proteins induce strong cellular immunity in a natural reservoir of visceral leishmaniasis with adverse consequences for Leishmania," *PLoS Pathog.*, vol. 5, no. 5, p. e1000441, May 2009.
[15] F. Oliveira *et al.*, "A sand fly salivary protein vaccine shows efficacy against vector-transmitted cutaneous leishmaniasis in nonhuman primates," *Sci. Transl. Med.*, vol. 7, no. 290, p. 290ra90, Jun. 2015.
[16] C. Teixeira *et al.*, "Characterization of the Early Inflammatory Infiltrate at the Feeding Site of Infected Sand Flies in Mice Protected from Vector-Transmitted Leishmania major by Exposure to Uninfected Bites," *PLoS Negl. Trop. Dis.*, vol. 8, no. 4, p. e2781, Apr. 2014.

[17] F. Oliveira *et al.*, “Delayed-Type Hypersensitivity to Sand Fly Saliva in Humans from a Leishmaniasis-Endemic Area of Mali Is TH1-Mediated and Persists to Midlife,” *J. Invest. Dermatol.*, vol. 133, no. 2, pp. 452–459, Feb. 2013.
[18] P.-H. D. Edqvist *et al.*, “Expression of Human Skin-Specific Genes Defined by Transcriptomics and Antibody-Based Profiling,” *J. Histochem. Cytochem.*, vol. 63, no. 2, pp. 129–141, Feb. 2015.
[19] Y. Guo, S. Zhao, C.-I. Li, Q. Sheng, and Y. Shyr, “RNAseqPS: A Web Tool for Estimating Sample Size and Power for RNAseq Experiment,” *Cancer Inform.*, vol. 13, no. Suppl 6, pp. 1–5, 2014.

Appendix A: Schedule of Procedures/Evaluations

Study Cohort	Cohort A			Cohort B					
Procedures/Evaluations	Study Days (window)								
	Day 0	Day 1 (+1 day)	Day 7 (±3 days)	Day 0	Day 14 (±7 days)	Day 28 (±7 days)	Day 42 (±7 days)	Day 44 (+3 days)	Day 51 (±3 days)
Informed Consent[1]	X			X					
Medical/Medication History	X			X	X	X	X	X	
Review of Eligibility Criteria	X			X	X	X	X	X	
Documentation of Usual Insect Bite Response	X			X					
Clinician Exam/Assessment	X	X		X	X	X	X	X	
Vital Signs[2]	X	X		X	X	X	X	X	
Urine Pregnancy Test[3]	X			X	X	X	X		
Blood Draw	X	X		X				X	
Vector Feeding	X			X	X	X	X		
Skin Punch Biopsy	X[4]						X[5]	X[6]	
Telephone Call to Assess Symptoms			X						X
Skin Biopsy Laboratory Evaluations									
Skin Immunohistochemistry	X						X	X	
Skin Transcriptomics	X						X	X	
Blood Laboratory Evaluations									
Post-feeding Labs (PBMC flow cytometry, plasma cytokine analysis, gene expression of circulating white blood cells)		X						X	

Abbreviations: RNA, Ribonucleic acid; PBMC, peripheral blood mononuclear cell

[1]Informed consent will be obtained within 90 days of or at Day 0 prior to any study procedures. Screening is performed under protocol 11-I-0183.

[2]Participants must be sitting for a minimum of 5 minutes prior to these procedures being performed; vital signs include blood pressure, mean arterial pressure, heart rate, respiratory rate, temperature, weight, and pulse oximetry; height will be taken at the Day 0 visit only. On days with feeding, vital signs will be performed pre-feeding.

[3]Females of childbearing potential only. Insect feeding will not be initiated until results are available.

[4]One biopsy from bitten skin will be collected no earlier than 30 minutes and no later than 90 minutes after the vector feeding is completed. One biopsy from bitten skin will be collected no earlier than 3.5 hours and no later than 4.5 hours after the vector feeding is completed. One biopsy will be collected from unbitten skin on the opposite arm no earlier than 30 minutes and no later than 6 hours after the vector feeding is completed.

[5] One biopsy from bitten skin will be collected no earlier than 3.5 hours and no later than 4.5 hours after the vector feeding is completed. One biopsy from unbitten skin on the opposite arm will be collected no earlier than 30 minutes and no later than 6 hours after the vector feeding is completed.

[6] One biopsy will be collected from bitten skin at a minimum 40 hours after vector feeding is completed.

Appendix B: Blood Volumes for Specimen Collection

Study Cohort	Cohort A		Cohort B				
Evaluations	Study Days (window)						
	Day 0	Day 1 (+1 day)	Day 0	Day 14 (±7 days)	Day 28 (±7 days)	Day 42 (±7 days)	Day 44 (+3 days)
	Blood Volume in mL						
Post-Feeding Labs							
Whole blood	(10 mL X 5)	(10 mL x 5)	(10 mL X 5)	0	0	0	(10 mL x 5)
PAXGene tube	(2.5 mL X 1)	(2.5 mL x 1)	(2.5 mL X 1)	0	0	0	(2.5 mL x 1)
Daily Volume	52.5mL	**52.5 mL**	52.5mL	0	0	0	**52.5 mL**
Cumulative Volume	**52.5 mL**	**105 mL**	**52.5 mL**	0	0	0	**105 mL**