Effect of influenza vaccination or infection on the development of protective immunity in children

DMID Protocol Number:
18-0009

Sponsored by:
National Institute of Allergy and Infectious Diseases (NIAID)

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DMID Clinical Project Manager:
Melinda Tibbals, RAC, CCRA

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04 February 2021

Statement of Compliance

The study trial will be carried out in accordance with Good Clinical Practice (GCP) and as required by the following:

- United States Code of Federal Regulations (CFR) 45 CFR Part 46: Protection of Human Subjects
- Food and Drug Administration (FDA) Regulations, as applicable: 21 CFR Part 50 (Protection of Human Subjects), 21 CFR Part 54 (Financial Disclosure by Clinical Investigators), 21 CFR Part 56 (Institutional Review Boards), 21 CFR Part 11, and 21 CFR Part 312 (Investigational New Drug Application), 21 CFR 812 (Investigational Device Exemptions)
- International Conference on Harmonisation: Good Clinical Practice (ICH E6); 62 Federal Register 25691 (1997); and future revisions
- Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research, Report of the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research
- National Institutes of Health (NIH) Office of Extramural Research, Research Involving Human Subjects, as applicable
- National Institute of Allergy and Infectious Diseases (NIAID) Clinical Terms of Award, as applicable
- Applicable Federal, State, and Local Regulations and Guidance

SIGNATURE PAGE

The signature below provides the necessary assurance that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH E6 Good Clinical Practice (GCP) guidelines.
I agree to conduct the study in compliance with GCP and applicable regulatory requirements.

I agree to conduct the study in accordance with the current protocol and will not make changes to the protocol without obtaining the sponsor's approval and IRB/IEC approval, except when necessary to protect the safety, rights, or welfare of subjects.

Clinical Site Investigator:

Signed: _____ Date: _____

_____

Principal Investigator (Administrative Site):

Signed: _____ Date: _____
Name

_____
Title, Site

Table of Contents

Table of Contents

Table of Contents

AE	Adverse Event/Adverse Experience
BCR	B-cell immunoglobulin receptor
CFR	Code of Federal Regulations
CIOMS	Council for International Organizations of Medical Sciences
CRF	Case Report Form
CoC	Certificate of Confidentiality
DMID	Division of Microbiology and Infectious Diseases, NIAID, NIH,
DHHS	Department of Health and Human Services
DSMB	Data and Safety Monitoring Board
FWA	Federal Wide Assurance
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	International Conference on Harmonization
IEC	Independent or Institutional Ethics Committee
IFNγ	Interferon Gamma
IL-2	Interleukin 2
IRB	Institutional Review Board
Kg	Kilogram
ILI	Influenza-like illness
LAR	Legally authorized representative
MOP	Manual of Procedures
N	Number (typically refers to participants)
NIAID	National Institute of Allergy and Infectious Diseases, NIH, DHHS
NIH	National Institutes of Health
OCRA	Office of Clinical Research Affairs, DMID, NIAID, NIH, DHHS
OHRP	Office for Human Research Protections
ORA	Office of Regulatory Affairs, DMID, NIAID, NIH, DHHS
PBMC	Peripheral blood mononuclear cells
PI	Principal Investigator
SAE	Serious Adverse Event/Serious Adverse Experience
SMC	Safety Monitoring Committee
SOP	Standard Operating Procedure
TNFα	Tumor Necrosis Factor Alpha
WHO	World Health Organization

PROTOCOL SUMMARY

Title: Effect of frequency and type of influenza vaccination on the development of the anti-influenza CD4 T cell and B cell response in children

Population: 220 children enrolled at between 6 months and 15 years of age vaccinated with the seasonal IIV (cohort A) or between 3 months and 15 years of age with an acute influenza infection (cohort B)

Number of Sites: One site

Study Duration: 3.5 years

Participant Duration: 2 influenza seasons in the main study plus 1 optional season

Objectives:

Primary Objective
To evaluate the relationship between influenza virus exposure through infection and vaccination and CD4 T cell reactivity in a cohort of children with well documented influenza exposures

Primary Outcome Measures:

- Assess the frequency and functional potential of CD4 T cell responses on a single cell level using full spectrum flow cytometry following either infection or inactivated influenza vaccine administration, with stratification by age and exposure history

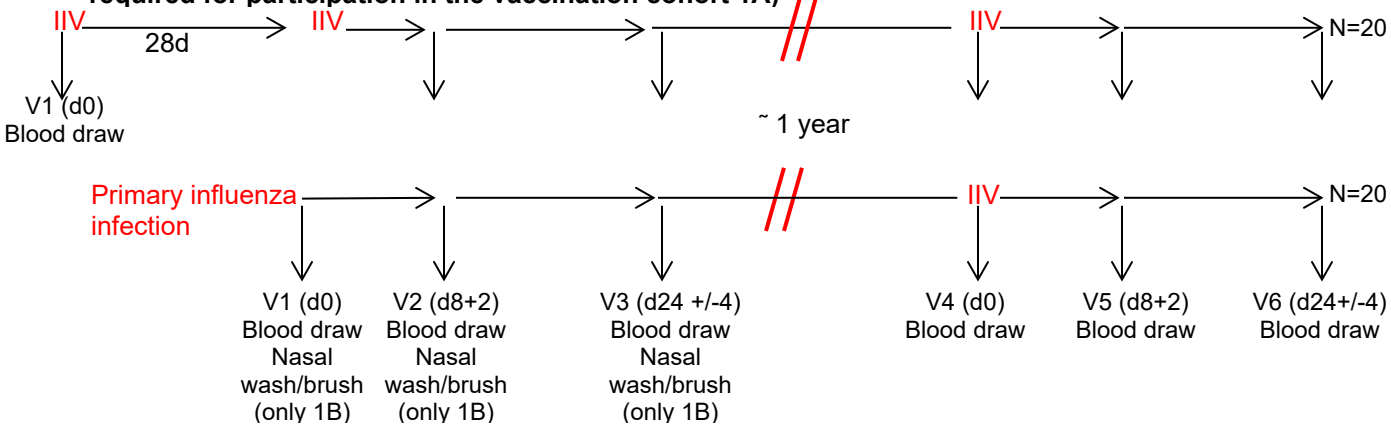
Exploratory Objectives
Determine the durability and breadth of the CD4 T cell and B cell/antibody responses and evaluate for relationships between response durability and factors such as age and exposure history.

Exploratory Outcome Measures:

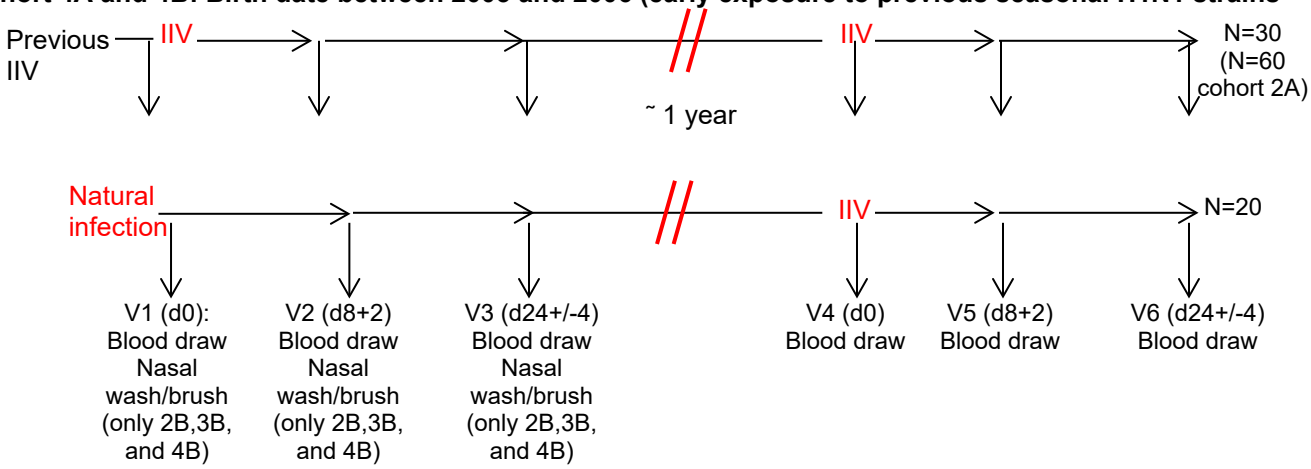
- Evaluate the humoral immune response that develops in response to infection or IIV vaccination using ELISA, microneutralization, and HAI assays with stratification by age and exposure history
- Determine the frequency of influenza-specific plasmablasts and memory B cells by ELISPOT and flow cytometry, with expression of monoclonal antibodies from cloned variable genes of single cells
- Determine the influence of the CD4 T cell response on the breadth and avidity of the post-vaccination antibody response
- Compare the cellular phenotype and functional potential observed at the day 24 post vaccination visit in study year one with that present at the prevaccination study visit in year 2 and year 3

Study Schematic

- **Cohort 1A and 1B: Children between 3 and 12 months of age (6 months of age or greater required for participation in the vaccination cohort 1A)**



- **Cohort 2A and 2B: Greater than 12 months of age, birth date after 2009 (early exposure to pH1N1)**
  - Cohort 2A will enroll 35 children <3 years of age and 25 children >=3 years of age
- **Cohort 3A and 3B: Birth date between 2006 and 2009**
- **Cohort 4A and 4B: Birth date between 2003 and 2006 (early exposure to previous seasonal H1N1 strains**



1. KEY ROLES

For questions regarding this protocol, contact Melinda Tibbals, Clinical Project Manager, or other appropriate DMID staff at NIAID/DMID (at the contact information below.

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2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

Current guidelines recommend yearly influenza vaccination for all children ≥6 months of age [1]; however the effect this has on the evolution of the anti-influenza immune response through childhood and into adulthood is poorly understood. Though far from idea, yearly vaccination is necessary at present due to the ongoing antigenic drift that occurs as viruses continually evolve to evade neutralizing antibody. There is currently much interest in the development of a universal influenza vaccination that would be able to provide protection against both seasonal and potentially pandemic strains of influenza [2]. However, a growing body of evidence suggests that early childhood influenza exposures result in imprinting that profoundly shapes lifelong CD4 T cell and B cell mediated immunity to this virus [3,4]. An improved understanding of immunity to influenza virus in early childhood is thus needed if a more broadly protective approach to influenza vaccination is to be successful.

Natural influenza infection stimulates vigorous inflammation with activation of multiple pattern recognition receptors. This leads to a robust innate immune response characterized by production of antiviral cytokines such as interferons that both limit early viral replication and instruct developing adaptive immunity [5-8]. As the infection progresses, viral replication results in abundant intracellular antigen that can be processed and presented to activate the adaptive immune response [5,6]. While neutralizing antibodies are thought of as the major correlate of protection [9-12], cellular immunity is increasingly recognized as important in protecting from severe disease [13-16], especially as T cells can recognize conserved epitopes and provide some degree of protection in years when antigenic drift leads to a poor vaccine match or a pandemic influenza strain emerges [17-21]. In contrast to the robust responses that develop following influenza infection, the immune response generated on vaccination with IIV is characterized by weak inflammatory signaling. IIV is produced by chemical inactivation of the H1N1, H3N2, and influenza B viral strains predicted to be circulating with disruption and purification of the surface glycoproteins (HA and NA) [22-24] This process results in production of an unadjuvanted vaccine that contains variable levels of NA, small amounts of internal virion proteins including NP, and only trace amounts of innate immune activators such as viral RNA [22,25-27].

Given the profound differences in antigenic context between natural infection and vaccination, this study will investigate how these different routes of early childhood exposure to influenza affect the functional potential of the anti-influenza immune response and determine the consequences this has on subsequent influenza vaccination. This improved knowledge of how early childhood influenza vaccination shapes the establishment of anti-influenza immunologic memory will enable both optimization of current influenza vaccination strategies and development of novel vaccines able to provide highly efficacious universal protection against both seasonal and potentially pandemic influenza strains.

2.2 Scientific Rationale

This protocol is a population-based study that will address the hypothesis that vaccination and infection differentially drive both the CD4 T cell and B cell immunity. Recent data in the literature [3,4,28], and our own preliminary data suggest that initial encounters with influenza have a long lasting effect on both CD4 T cell and B cell mediated immunity. This protocol will address how early immunity against influenza virus is primed by vaccination versus infection and whether this differentially poises subsequent immune responses on re-challenge with influenza vaccination. We will specifically test whether the more robust pro-inflammatory response initiated on first exposure to a natural influenza infection promotes imprinting of both CD4 T cell and B cell responses, influencing long term specificity and functionality. We also will examine the effects of age and exposure on the immune response that develops following influenza vaccination. To do this, we will first examine whether early exposure to a live viral infection compared to vaccination leads to a distinct phenotype and specificity in both the B cell and CD4 T cell compartments. Subsequently, we will determine whether the CD4 T cell repertoire is remodeled over time by examining CD4 T cell specificity, cytokine production, and expression of markers and transcriptional factors associated with a negative regulatory CD4 T cell phenotype in children of different ages versus adults.

The age range of the first cohort was chosen to enroll infants in their first influenza season with no history of prior exposure to influenza virus. Thus, this age cohort will allow examination of the true primary response to influenza infection or inactivated vaccination. Children between 6 and 12 months of age who have not previously had an influenza infection or vaccination will be eligible to participate in vaccination cohort 1A. During Visit 1, they will have an initial prevaccination blood draw and will then receive a dose of inactivated influenza vaccine. These children will then receive a booster dose of the inactivated influenza vaccine, with vaccine doses separated by 28 (+ 14) days as recommended by the AAP. A post vaccination blood draw will only be performed following the second vaccine dose [1]. To minimize blood draws, we will perform the next blood draw to interrogate the immune response following the second vaccine dose. Children between 3 and 12 months of age who present with an influenza-like illness will be eligible to participate in the infection cohort (1B). Study personnel will obtain nasal swabs from potential subjects for a rapid influenza antigen test on illness presentation (typically this occurs at day 2-4 post illness onset) if influenza testing was not completed by the clinical microbiology lab. In the event that influenza testing result is pending or completed by a medical provider, results of this test will be obtained through review of the medical record. If positive, subjects will be eligible for further study participation. Given the low sensitivity of rapid influenza testing, influenza real-time RT-PCR will be used to confirm all rapid antigen test results, with subjects recruited for further study participation if RT-PCR testing is positive. In addition, if the subject is eligible for enrollment based on a positive influenza test result by the clinical microbiology laboratory and symptomatic at the time of presentation, a nasal swab for additional influenza real time RT-PCR testing may be obtained during the first study visit (study visit 1, or visit 2 if visit 1 is bypassed) in order to obtain further information regarding the infecting influenza serotype if possible.

The age ranges of the subsequent cohorts were chosen to capture the likely exposure histories of enrolled children, specifically surrounding exposure to the 2009 pandemic H1N1 virus. All of

the children in cohorts 2 through 4 of the vaccination arm of the study will have previously received at least 2 doses of influenza vaccine, and thus will only require a single vaccine dose. However, children presenting with acute influenza infection may not yet have received 2 vaccine doses and thus may require both a priming and a booster dose of vaccine in year 2 of the study [29]. The second age cohort (> 12 months, birthdate after 2009) will likely have had exposure to the 2009 pandemic virus in early childhood. The third age cohort (birthdate between 2006 and 2009) may or may not have had early exposure to the 2009 pandemic virus, while the final cohort (birthdate between 2003 and 2006) will likely have had early childhood exposures to the previously seasonal H1N1 viral strains.

Blood draws will be obtained on study enrollment, day 8 (+2d), and day 24 (+/- 4d) post enrollment as depicted in Figure 1. The blood draw on study enrollment will evaluate influenza-specific immunity at the time of study entry. The blood draw on day 8 (+2d) post-vaccination will allow examination of each subject as CD4 T cell and plasmablast responses are peaking, while the day 24 (+/- 4d) time point will capture the peak of the neutralizing antibody response and allow us to examine CD4 T cell responses as they contract. All subjects will be followed through to the subsequent influenza season, at which point they will be rechallenged with IIV. Blood draws will again be obtained pre-vaccination and at days 8 (+2d) and 24 (+/- 4d) post vaccine administration, with the pre-vaccination blood draw providing a method to assess for undocumented influenza infection over the prior year. Upon completion of the planned follow up visits, subjects will be offered the opportunity to participate in the study for an addition influenza season to enable collection of longitudinal data on the development of early life anti-influenza immunity.

2.3 Potential Risks and Benefits

2.3.1 Potential Risks

The risks and discomforts of this study include risks associated with the vaccine, administration of the vaccine, the risks associated with study procedures (blood drawing and nasal and throat swabs) and possible loss of confidentiality.

In placebo-controlled trials in children, inactivated seasonal vaccines are associated with mild local pain at the site of administration and occasional systemic symptoms. According to the package insert, systemic symptoms like fever occur in 14.3% of infants and 7% of children. Likewise, some irritability (54.1%) was seen in infants, with abnormal crying occasionally reported. The most common adverse events reported remain local symptoms, with injection site pain (57-66%), tenderness (54.1%), erythema (37.3-34.1%), and swelling (21.6-24.8%) common. As influenza vaccination is considered the standard of care, these risks will be no greater than what children will be exposed to through current best practice recommendations [1].

During the swine influenza vaccine campaign of 1976, about 10 per 1,000,000 vaccine recipients in excess of the background rate developed the paralytic illness called Guillain-Barre Syndrome (GBS). In the subsequent decade, no association between seasonal influenza vaccine and GBS was found. More extensive investigations of this potential association occurring in the 1990s revealed that there was a small but detectable risk of GBS in the 6 weeks following seasonal

influenza immunization: an attributable risk of approximately 1 per 1,000,000, adjusted for potential confounders. In the period since the Vaccine Adverse Event Reporting System (VAERS) was established in 1990, the rates of GBS reports following influenza vaccination have declined substantially. The annual reporting rate in that period was highest in the 1993-1994 influenza season (1.7 per 1,000,000 vaccinees) and lowest in the last season analyzed in the report, 2002-2003 (0.4 per 1,000,000 vaccinees) [30]. GBS is thus considered highly unlikely after influenza vaccination and, as vaccination is considered the standard of care for all infants ≥6 months of age and children, is not a greater risk than children would be exposed to receiving standard pediatric care [1].

All blood will be drawn by experienced and trained pediatric nurses or physicians, with pain minimized through comforting and distraction techniques. Only the minimum amount of blood necessary to achieve the study objectives will be obtained, with the blood volume being within a level accepted as safe (Table 1). As a child's total blood volume is related to body weight, we have chosen a weight based approach to determine the volume of blood to be obtained. This will allow us to optimize our likelihood of being able to accomplish the study objectives while still prioritizing the safety of the child (see Scientific Rationale section). The proposed blood draws in this protocol impose a maximum upper limit of 1 mL/kg on any single draw (about 1.25% of estimated TBV), with a maximum of 3 mL/kg (about 3.75% of estimated TBV) over any 8 week period for all pediatric patients (Table 1). This volume remains well below the upper limit of 10% of total blood volume in an 8 week period and is consistent with the available evidence on what will present minimal risk to children [31] while still providing enough PBMCs to perform the in depth analysis of CD4 T and B cell specificity and functional capacity as well as antibody responses required for this study.

Drawing blood causes transient discomfort and may cause fainting. Bruising at the blood draw site may occur but can be prevented or mitigated by applying direct pressure to the draw site for several minutes. The use of alcohol swabbing and sterile equipment will make infection less likely at the site where blood will be drawn. The volume of blood per kg body weight being drawn is within a range considered safe. However, some older children may develop transient lightheadedness following blood drawing as a result of a vaso-vagal reflex. This will be discussed with parents/LARs at the time of the consent. If such a reaction occurs, the child will be laid down, have a blood pressure obtained, and be observed until the symptoms pass (but for at least 10 minutes).

Nasal swabs and washes can be uncomfortable and may cause gagging or occasionally minor brief irritation and rarely transient bleeding. Long term side effects from these procedures are considered extremely unlikely.

Personal health information of the subjects will be collected to determine eligibility and to evaluate outcomes throughout the study. Research personnel will make every effort to keep this information confidential. Still, a risk of participation is that the confidentiality of this information could be lost.

2.3.2 Known Potential Benefits

Although administration of influenza vaccines may result in the development of immunity that will prevent influenza infection, this intervention is considered the standard of care and presumably would occur regardless of study participation. Infants presenting with influenza-like illness during influenza season will receive influenza testing as part of their evaluation, which has the potential to diagnose and allow for the treatment of an active influenza infection. There are no other direct potential benefits to study participation.

3 OBJECTIVES AND OUTCOME MEASURES

3.1 Study Objectives

Primary Objective
To evaluate the relationship between influenza virus exposure, infection and vaccine history, and CD4 T cell reactivity in a cohort of children with well documented influenza exposures.

Exploratory Objectives
Determine the durability and breadth of the CD4 T cell and B cell/antibody responses and evaluate for relationships between response durability and factors such as age and exposure history.

3.2 Outcome Measures

3.2.1 Primary Outcome Measures

- Assess the frequency and functional potential of CD4 T cell responses on a single cell level using full spectrum flow cytometry following either infection or inactivated influenza vaccine administration, with stratification by age and exposure history

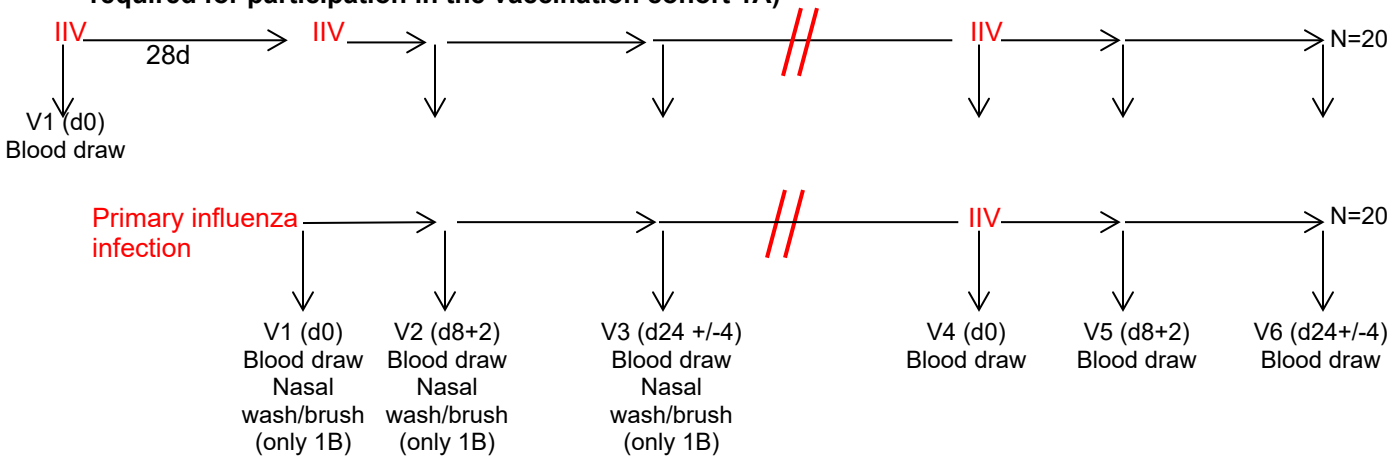
3.2.2 Exploratory Outcome Measures

- Evaluate the humoral immune response that develops in response to infection or IIV vaccination using ELISA, microneutralization, and HAI assays with stratification by age and exposure history
- Determine the frequency of influenza-specific plasmablasts and memory B cells by ELISPOT and flow cytometry, with expression of monoclonal antibodies from cloned variable genes of single cells
- Determine the influence of the CD4 T cell response on the breadth and avidity of the post-vaccination antibody response
- Compare the cellular phenotype and functional potential observed at the day 24 post vaccination visit in study year one with that present at the prevaccination study visit in year 2 and 3

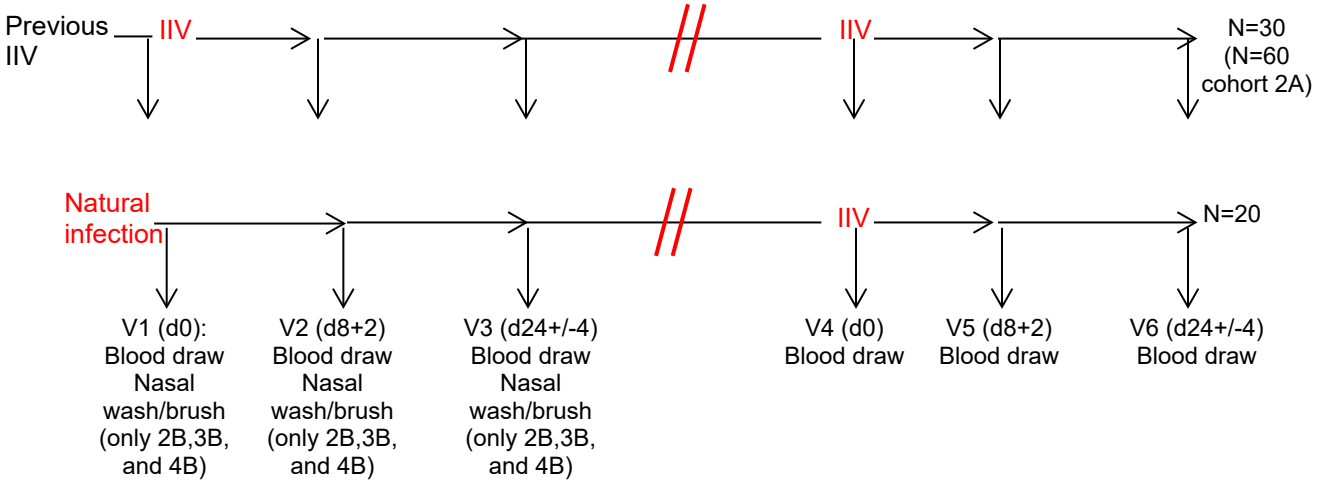
4 STUDY DESIGN

The study will be designed as a prospective surveillance of the immune response to seasonal vaccination in healthy children of varying ages. A schematic of the study design is shown below:

- **Cohort 1A and 1B: Children between 3 and 12 months of age (6 months of age or greater required for participation in the vaccination cohort 1A)**



- **Cohort 2A and 2B: Greater than 12 months of age, birth date after 2009 (early exposure to pH1N1)**
  - Cohort 2A will enroll 35 children <3 years of age and 25 children >=3 years of age
- **Cohort 3A and 3B: Birth date between 2006 and 2009**
- **Cohort 4A and 4B: Birth date between 2003 and 2006 (early exposure to previous seasonal H1N1 strains**



5 STUDY POPULATION

It is anticipated that this study will enroll a total of approximately 220 subjects. This includes 140 children in the vaccination cohort [20 children between 6-12 months, 60 children with a birth date after 2009 (35 children <3 years of age and 25 children >=3 years of age), 30 children with a birth date between 2006 and 2009, and 30 children with a birth date between 2003 and 2006]. We also will enroll up to 80 children total who present with acute influenza infection (20 children into each of the 4 age cohorts). We anticipate having to screen up to 500 children presenting with signs of ILI during the height of seasonal influenza circulation in order to identify and recruit these 80 children with natural influenza infection, as only a small number of children with symptoms possibly consistent with influenza will actually have respiratory infections as a result of influenza virus infection. It is anticipated approximately two-thirds of the children in all cohorts (both A and B) will enroll in the optional year of longitudinal follow-up.

The goal of this study is to understand the development of influenza-specific immunity in a population of children in the Rochester, NY area. Children will be excluded from participation in the study if they meet exclusion criteria of the protocol such as having a documented immunodeficiency or are on immunosuppressive medications expected to have a more than a minimal impact on the development of an immune response to vaccination or infection. Other conditions that may possibly have more than a minimal effect a child's immune system, such as allergies or other hypersensitivity syndromes, mild asthma, or obesity, will be able to enroll in the study. The presence or absence of concomitant medications and medical conditions will be documented and the effect of potential confounding variables will be examined in data analysis.

Children in the vaccination cohort will be recruited either by flyer, via a letter sent under authorship of his/her PCP, or in person at the Strong Pediatric Practice, Elmwood Pediatrics, or Lewis Pediatrics. Children recruited by letter may receive a follow-up phone call from study staff in 1 to 2 weeks to assess interest in participating when possible. We will also reach out to parents/LARs participating in RSRB protocol 58437 and 55570 who have consented for contact regarding future research studies to determine if the subject and parent/LAR are interested in study participation.

Acute influenza cases will be identified through the Strong Pediatric Practice, Elmwood Pediatrics, Lewis Pediatrics, the Pediatric Emergency Departments at Strong Memorial Hospital or Rochester Regional Health, or one of the UR-affiliated Urgent Care Centers by screening patients for ILI. Additionally, we will have flyers with contact information for subjects to call if they have a child with symptoms of ILI. Finally, records from influenza tests performed in the URMC Clinical Microbiology Laboratory will be reviewed and potentially eligible subjects with a documented acute influenza infection will be contacted to assess for study interest. Please see URMC Notice of Privacy Practices page 2 section on RESEARCH, which states "*We may use and disclose medical information about you for research purposes. In most cases we will ask for your written authorization. However, under some circumstances we may use and disclose your health information without your written authorization if doing so poses minimal risk to your privacy. We may also release your medical information without your written*

authorization to people who are preparing a research project, so long as any information identifying you does not leave our facility. The researchers may use this information to contact you to ask if you want to participate in such research." Subjects who present with an ILI will complete a brief consent for screening by rapid influenza test and future contact if influenza testing was not completed by the clinicial microbiology lab. In the event that influenza testing is pending or has been completed by a medical provider, results of this test will be obtained through review of the medical record. If positive, parents/LARs will be approached to participate in the full study. If rapid influenza testing is negative, a confirmatory PCR will be completed. Children with ILI that initially test positive by PCR performed by the clinical microbiology lab or test negative for influenza by rapid testing but later test positive by PCR will be contacted to determine if there is interest in full study participation. Every attempt will be made to have these children complete all study visits, but in the situation when the parent/LAR is unable to be reached prior to day 5 of illness onset, study visit 1 may be bypassed with enrollment completed at the time of study visit 2.

If funding is available, subjects will have the option to enroll for a additional 1 year follow-up upon reviewing and signing a "consent for longitudinal follow-up" to be presented to the subject and their parent/LAR at one of the scheduled year 2 follow up visits. If subjects sign this consent form and continues to meet all inclusion and exclusion criteria, they will be enrolled and followed under the same subject number, with an "R" appended at the end of the subject number so that they can be identified as a reenrollment.

5.1 Inclusion Criteria

- Age
  - Between 6 and 12 months at the time of enrollment to participate in the vaccination arm of age cohort 1A
  - Between 3 and 12 months at the time of enrollment to participate in the natural infection arm of age cohort 1B
  - > 12 months, birth date after 2009 for either the vaccination (A) or natural infection (B) arm of age cohort 2
  - Birth date between 2006 and 2009 for either the vaccination (A) or natural infection (B) arm of age cohort 3
  - Birth date between 2003 and 2006 for the vaccination (A) or natural infection (B) arm of age cohort 4
- Gestational age of ≥37 weeks at birth
- Parent/LAR can provide informed consent, with children ≥8 years of age providing informed assent
- Available for the duration of the study
- History of previous primary IIV vaccination (at least 2 previous doses for age <9 yrs, at least 1 previous dose for age 9 and older) **ONLY** for participation in the vaccination (A) arm of age cohorts 2, 3, or 4

- Acute illness documented by rapid influenza test, PCR testing, or testing done by either URMC Labs or RGH Clinical Microbiology Labs to be due to influenza virus **ONLY** for participation in the natural infection arms (B) of age cohorts 1-4
- Children enrolled in the Cohort A (vaccination cohort) are required to have an appropriate weight and vital signs as determined by a licensed medical provider. Children enrolled in the Cohort B (natural infection cohort) are required to have an appropriate weight and clinically stable vital signs as determined by a licensed medical provider.
    - Children will not qualify for study participation if their weight is more than 2.5 standard deviations below population norms. This will be determined through calculation of a Z score using the PediTools website (https://www.peditools.org/) utilizing the appropriate CDC growth calculators for age [32].

5.2 Exclusion Criteria

- Immunosuppression as a result of an underlying illness or condition (including HIV or a primary immunodeficiency syndrome)
- Active neoplastic disease
- Use of potentially immunosuppressive medications currently or within the past year (including chemotherapeutic agents) or chronic (>2 weeks) use of oral corticosteroid therapy
- A diagnosis of asthma requiring chronic inhaled corticosteroid use
- Participation in any clinical research study evaluating an investigational drug or therapy that is inconsistent with current standard of care within two (2) months of enrollment in this study.
- Previous administration of influenza vaccine in the current influenza season ONLY for subjects in the vaccination arm (A) of the study (subjects presenting with acute influenza infection with vaccine failure will be eligible to enroll in the B cohorts).
- Receipt of immunoglobulin or another blood product within the year prior to study enrollment
- An acute illness within the previous 3 days or temperature >38^{o}C on screening **EXCEPT** for participation in the natural infection (B) cohorts
- A contraindication to influenza vaccination EXCEPT infants between 3 and 5 months presenting with natural influenza infection whose only contraindication is their current age
- Anemia in the previous 6 months (children on iron supplementation with no documentation of abnormal hemoglobin and/or hematocrit for >6 months will be allowed to participate in the study)
- Recent (within 120 days) hospitalization, excluding hospitalization for delivery or subjects enrolled in the acute cohort who have been hospitalized for influenza-related reasons
- Any medical history or other condition that the study PI feels may have a more than a minimal impact on the immune response or may impact the safety of the subject.

5.3 Handling of Withdrawals

If the child and parent/LAR are unwilling to participate further, then they may withdraw consent for study participation without penalty. Participants who withdraw, or are withdrawn or terminated from the study, or are lost to follow-up after receiving the assigned vaccine will not be replaced. Participants who consented to study participation in the vaccination cohorts but did

not receive the influenza vaccine will be replaced. These subjects will be considered as screen failures.

Criteria for withdrawal from the study will include

- Study participants may withdraw their consent for study participation at any time during the study without penalty
- The subject is unable to comply with study procedures or visits
- The principal investigator determines that it is in the best interest of the participant to discontinue participation
- The participant is lost to follow-up
- The study is terminated

Whether to exclude a previously enrolled subject if exclusion criteria are met at a visit following enrollment will be decided on a case by case basis (a subject may be allowed to continue in the study in the case of initiation of controller asthma medication, a hospitalization with an apparently full recovery, a single short oral steroid treatment course to treat reactive airway disease exacerbation, or in other instances where the PI decides that study continuation will not compromise either subject safety or the integrity of the data). If a subject has a temperature >38.3^{o}C (101^{o}F) at the time of a follow up visit, more than one episode of vomiting or diarrhea in the previous 24 hours, or symptoms of illness (respiratory or otherwise) that are interfering with activities of daily living (such as resulting in school or daycare absence) the study visit will be rescheduled or cancelled until the child recovers.

5.4 Termination of Study

Although the study sponsor has every intention of completing the study, the sponsor reserves the right to terminate the study at any time for clinical or administrative reasons. Reasons for termination include, but are not limited to, study closure due to internal safety review and recommendation, or at the discretion of DMID.

6 STUDY INTERVENTION PRODUCT

6.1 Study Product Description

Influenza vaccines will be administered using age-appropriate guidelines in all years of the study. Inactivated vaccine will consist of Fluzone Quadrivalent vaccine (Sanofi Pasteur). This vaccine is approved for use in persons 6 month of age and older. It consists of a split and chemically inactivated influenza virus and is formulated to contain 7.5 mcg of each HA protein per 0.25 mL vaccine in sodium phosphate-buffered isotonic sodium chloride solution. This vaccine also may contain 12.5 mcg of thimerosal and trace amounts of formaldehyde in each 0.25 mL dose.

6.1.1 Acquisition

Vaccine will be ordered for each influenza season from the University of Rochester Medical Center hospital pharmacy, which will acquire the vaccine through distributors.

6.1.2 Formulation, Packaging, and Labeling

FLUZONE® Quadrivalent [Influenza Virus Vaccine Quadrivalent Types A and B (Split Virion)] for intramuscular use, is a sterile suspension containing four strains of influenza viruses propagated in embryonated chicken eggs, inactivated with formaldehyde, concentrated and purified by zonal centrifugation on a sucrose gradient, split with Triton® X-100, further purified and then suspended in sodium phosphate-buffered isotonic sodium chloride solution. The FLUZONE® Quadrivalent process uses an additional concentration factor after the ultrafiltration step in order to obtain a higher hemagglutinin (HA) antigen concentration.

The type and amount of viral antigens contained in FLUZONE® conform to the current requirements of the World Health Organization (WHO). FLUZONE® Quadrivalent is indicated for active immunization against influenza caused by the specific strains of influenza virus contained in the vaccine in adults and children 6 months of age and older.

6.1.3 Product Storage and Stability

Influenza vaccine will be stored according to manufacturer's directions in secure, limited-access temperature monitored refrigerator environment at 2°C to 8°C (36°F to 46°F) until needed. DO NOT FREEZE. The temperature of the storage unit will be monitored during the duration of the trial, and documentation of proper dedicated storage will be maintained. In the event of accidental deep-freezing or disruption of the cold chain, vaccines will not be administered; and the PI or the responsible person will contact the sponsor for further instructions

6.2 Dosage, Preparation and Administration of Study Intervention Product

All influenza vaccines will be administered at the recommended doses for age [33]:

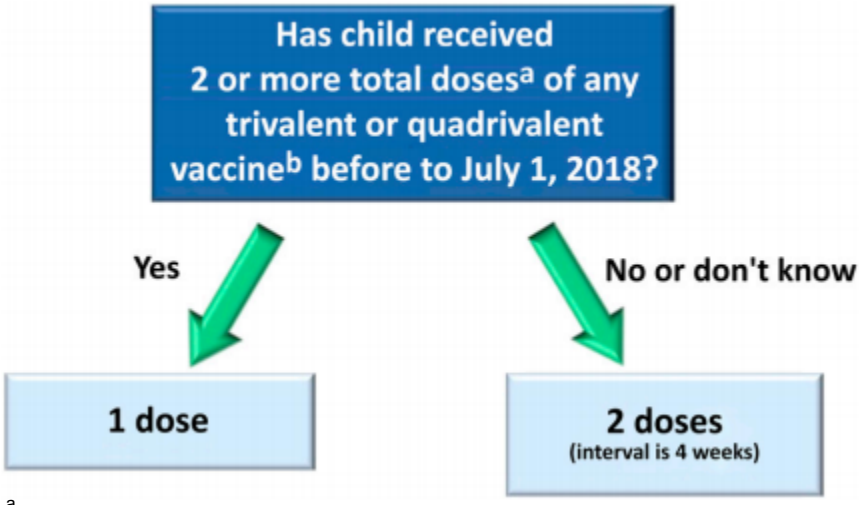
- IIV: Fluzone 0.25 mL administered intramuscularly to children between 6 and 35 months of age enrolled in the 2018-19 influenza season.

- IIV: Fluzone 0.25 mL or 0.5 mL per 2019 ACIP guidelines administered intramuscularly to children between 6 and 35 months of age enrolled during or after the 2019-20 season
- IIV: Fluzone 0.5 mL administered intramuscularly to children 36 months of age and older

Previously unvaccinated children 6 months to <9 years of age require 2 doses of seasonal influenza vaccine at an interval of at least 4 weeks. Eligible children <9 years of age who have properly received two or more doses of seasonal influenza vaccine in the past are recommended to receive one dose per season thereafter (see Figure 1).

Figure 1:

Number of seasonal influenza vaccine doses for children 6 months through 8 years of age.



[a] The 2 doses need not have been received during the same season or consecutive seasons. [b]Receipt of LAIV4 in the past is still expected to have primed a child's immune system, despite recent evidence for poor effectiveness. This is the protocol initiated for 2018-19 influenza season, which should be similar to recommendations released for future influenza seasons with the exception that the cut off year will shift to the current influenza season year.

6.3 Accountability Procedures for the Study Intervention Product(s)

Records of vaccine receipt and dispensation to the study subject as well as storage and destruction of the vaccine will be maintained according to existing standard operating procedures (SOPs)

6.4 Assessment of Participant Compliance with Study Intervention Product

Not applicable as vaccination is being given only at the VRU.

6.5 Concomitant Medications/Treatments

Administration of any medication, vaccines or therapies considered necessary for the subject's welfare will be recorded and documented in the subject's source documentation. Concomitant medications will include all medications taken within 30 days prior to enrollment through visit 3 of each season or early termination, whichever occurs first.

The following criteria will be reviewed with the subject's during each follow up visit. If these become applicable during the study, it will be noted in the subject's record.

1. Use of any investigational drug or investigational vaccine other than the study article.
2. Administration of chronic (defined as more than 14 days) immunosuppressants including inhaled corticosteroids, or other immune-modifying drugs (topical and nasal steroids are allowed).
3. Receipt of a licensed vaccine.
4. Receipt of immunoglobulins and/or any blood products.

7 STUDY SCHEDULE

Refer to Appendix A for the Schedule fo Events.

7.1 Screening

Potential subjects will be identified by providers in the Strong Pediatric Practice, Elmwood Pediatrics, Lewis Pediatrics, the Pediatric Emergency Departments at Strong Memorial Hospital, or one of the UR-affiliated Urgent Care Centers. The Emergency Department Research Associate (EDRA) Program is being utilized to recruit subjects seeking care for acute respiratory illness for the presence of signs and symptoms consistent with influenza as recorded in the medical record in the Strong Memorial Hospital Pediatric Emergency Department and UR-affiliated Urgent Care Centers. Records from influenza tests performed in the Clinical Microbiology Labs at URMC will be reviewed and potentially eligible subjects acutely infected with influenza will be contacted by phone to assess for study interest. Although University of Chicago is a scientific lead on this study, they will be sent coded samples with no recruitment or enrollment activities taking place at University of Chicago (MTA and LDUA in place).

We will also contact the parents/LARs of children previously participating in RSRB Protocol 58437 (Understanding how the initial encounter with influenza virus poises children for protective immunity) who have consented for contact regarding future research studies to assess interest in longitudinal participation in the vaccination arm (A) of the study.

On identification of a potential subject, the subject's care provider or study staff when there is a positive influenza test documented by clinical microbiology will ask if the parent/LAR is interested in learning more about this study, either in person at one of our participating practices, by phone, or via a letter sent to the parent/LAR. Parents/LARs that receive a letter describing the study may be contacted by phone approximately 7 to 10 days later to determine if there is any interest in study participation. If a parent/LAR expresses interest in the study and the subject is potentially eligible, screening and consent will take place either within the office/ED or the parent/LAR will be asked to schedule an appointment for screening and consent in the VRU. Screening records will be kept to document the reasons why an individual was consented for the study but failed trial entry criteria. Contact information, including e-mail addresses and phone numbers, will be collected from parents/LARs at the time of study enrollment. On obtaining consent, parents and/or the subject's LAR will be asked if they are willing to be contacted regarding future studies. Only those parents/LARs who consent to use of their information will be contacted for purposes outside of the present study. Remaining samples may be procured from clinical microbiology for additional viral phenotyping at a later timepoint.

A small cohort of subjects acutely infected with influenza may be referred from study protocol 14-0101 (RSRB # 55570), an acute influenza study protocol being run through the CEIRS New York Influenza Center of Excellence at the University of Rochester. Study nurses evaluating pediatric subjects enrolled under protocol 14-0101 will ask the parent/LAR of children acutely infected with influenza whether they are interested in also participating in

this study. If the parent/LAR is willing to participate, our study staff will ensure that the subjects meet our enrollment criteria and consent the parent/LAR during one of their visits in the VRU. These children will have weight-based blood samples obtained as per protocol 14-0101 in study year 1. Subjects will then be followed and vaccinated in season 2 as part of our study, with visits 4, 5 and 6 occurring as described in this protocol. Our study staff will have access to the blood samples obtained on days 0, 10, and 28 through collaboration as part of the CEIRS network.

Subjects will have the option to be screened and enroll if eligible for a additional 1 year follow-up.

To ensure appropriate safety precautions when conducting in-person study procedures, the process for conducting in-person visits outlined in the Guidance for Human Subject Research will be followed for all visits (https://www.urmc.rochester.edu/coronavirus/coronavirus-research/guidance-for-researchers/human-subjects-research.aspx).

7.2 Enrollment/Baseline (Visit 1)

Once subjects meet inclusion criteria and do not meet exclusion criteria and parents or a LAR provide consent for participation in the study, the subjects will be enrolled.

Subjects enrolled in the influenza vaccination cohorts will have a medical history obtained and demographic information collected, with particular attention to inclusion and exclusion criteria. If not already obtained, subject vital signs and weight will be measured. A targeted physical exam, focusing on the CV and respiratory systems, will then be performed as indicated by medical history. Following this, a sample of peripheral venous blood will be obtained if able. Subjects will then receive their initial influenza vaccination.

Subjects recruited with potential natural influenza infection will have a complete medical history obtained with particular attention to current symptoms of illness and inclusion and exclusion criteria. Demographic information will also be collected. If not already obtained, subject vital signs and weight will be measured. A targeted physical exam, with a focus on the respiratory and CV systems, may then be either performed by study staff or obtained from a review of the medical record. Following this, if influenza testing was not completed via the clinical microbiology lab, two nasal swabs will be obtained by placing UV-sterilized soft nylon flocked swabs into alternate nostrils of the subject and gently rotating them across the mucosa. One of these swabs will be used to perform a rapid influenza test, while the other will be placed into 1 mL of viral culture transport media for RT-PCR studies. All rapid influenza test results will be confirmed by influenza-specific RT-PCR. Subjects with negative influenza testing will not participate in the study further, although contact information will be collected and parents/LARs will be informed that we will contact them regarding possible enrollment in the full study if the influenza PCR testing comes back positive. Subjects that have a positive influenza test will have the opportunity to consent to full study participation. If this consent is obtained, a nasal wash, nasal brush, and a sample of peripheral venous blood will be collected if able. In addition, if the subject is eligible for enrollment based on a positive influenza test result performed by the clinical microbiology laboratory, a nasal swab for additional influenza real time RT-PCR testing will be obtained if

able during the first study visit in order to obtain further information regarding the infecting influenza serotype. Parents/LARs will then be given a memory aid to document the progression of their current illness and the symptoms of any future illnesses that occur, which we will collect at the time of their follow up visits. Children with ILI that initially test positive by PCR performed by the clinical microbiology lab or test negative for influenza by rapid testing but later test positive by PCR will be contacted to determine if there is interest in full study participation. Every attempt will be made to have these children complete all study visits. If parent/LAR is reached within 5 days post the onset of illness, subjects will be offered a home visit or asked to come to the VRU to complete the enrollment visit 1. In the situation when the parent/LAR is unable to be reached prior to day 5 of illness onset, the remaining study visit 1 procedures will be bypassed with enrollment completed at the time of study visit 2.

7.3 Follow-up and Final Visits

Visit 1.5: Vaccine booster visit (day 28 +14 days):

Subjects undergoing vaccination in cohort 1A will require an influenza vaccine booster dose. The subjects will be asked to return to VRU on day 28 (+14) following the initial influenza vaccination (Visit 1). At this time, the ICF, study requirements, any returned illness memory aids and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccinations, will be obtained and medications reviewed. Vital signs and subject's weight will be obtained and a targeted PE performed. Then a dose of seasonal IIV will be administered intramuscularly.

Visit 2 (day 8/36 +2 days):

Subjects may return to the VRU or have a home visit completed on day 8 (+2) following their presentation with influenza infection or their final influenza vaccination (this will be about day 36 following the initial vaccination for subjects requiring 2 doses of influenza vaccine). At this time, the ICF, study requirements, any returned illness memory aids that document recent symptoms of illness, and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccinations, will be obtained and medications reviewed. Vital signs and subject's weight will be obtained and a targeted PE performed. Following this, a sample of peripheral venous blood will be obtained, if able, for all cohorots. Those subjects enrolled in Cohorts 1B, 2B, 3B, and 4B will also have a nasal wash and nasal brush obtained if able. For subjects in the B cohorts enrolled based on a positive influenza test result by the clinical microbiology laboratory, a nasal swab for additional influenza real time RT-PCR testing will be obtained if able during the second study visit if the first study visit is bypassed in order to obtain further information regarding the infecting influenza serotype. This will only be obtained if the subject remains symptomatic at the time of presentation.

Visit 3 (day 24/52 +/- 4 days):

Subjects may return to the VRU or have a home visit completed on day 24 (+/-4) following their presentation with influenza infection or their final influenza vaccination (this will be about day 52 following the initial vaccination for subjects requiring 2 doses of influenza vaccine). At this time, the ICF, study requirements, any returned illness memory aids that document recent symptoms of illness and any adverse events or other illnesses will be

reviewed. An interim medical history, including vaccinations, will be obtained and medications reviewed. Vital signs and subject's weight will be obtained and a targeted PE performed. Following this, a sample of peripheral venous blood will be obtained, if able, for all cohorts. Those subjects enrolled in Cohorts 1B, 2B, 3B, and 4B will also have a nasal wash and nasal brush obtained if able.

Illness visits: Parents/LARs will contact study personal by phone if subjects develop symptoms consistent with influenza-like illness. These symptoms include temperature of 100.5 or more, cough, and/or runny or stuffy nose. If the reported symptoms are consistent with influenza infection, the parents/LARs will be offered the opportunity to complete an illness visit. At the illness visit, ICF, study requirements and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccines, will be obtained and medications reviewed. Vital signs will be obtained and a targeted PE performed. Following this, two nasal swabs will be obtained. At this time, a rapid antigen influenza test will be conducted. In the event the illness visit is completed as a home visit, the parent/LAR will be notified that the visit is being completed for research purposes only and does not take the place of contacting the subject's primary care provider for illness evaluation. The rapid influenza test will then be conducted in the home. If the rapid influenza test is positive, the parent/LAR will be notified at the time of the visit and study staff will recommend the subject's primary care practice be notified by the parent/LAR. All rapid influenza tests will be confirmed by influenza-specific RT-PCR. In the event a child is observed to be moderately or severely ill at an illness visit, study staff will recommend the parent or LAR contact their primary care practice for follow up or will follow local SOPs for emergency care procedures.

Visit 4 (Season 2 day 0): Subjects will be asked to return to the VRU the following fall to receive seasonal influenza vaccination. At this time, the ICF, study requirements and any AE or other illnesses will be reviewed. Subjects will have an interval medical history obtained, including vaccinations and a review of all medications. Any illness memory aids returned will be reviewed to determine if the parent/LAR recorded any symptoms consistent with influenza-like illness that were not reported to study personnel. Subject weight and vital signs will then be obtained and a targeted physical exam performed as indicated by medical history. Following this a sample of peripheral venous blood will be obtained if able. Subjects will then be vaccinated with seasonal IIV. Parents/LARs will use the memory aids to document the symptoms of any illnesses that occur and will be instructed to call the study coordinator if symptoms consistent with an influenza-like illness are present.

Visit 4.5: Vaccine booster visit (Season 2 day 28 +14 days):
Children <9 years of age acutely infected with influenza (cohort B) who have not appropriately received 2 or more total doses of influenza vaccine will present to the VRU to receive their influenza vaccine booster dose on day 28 (+14) following the initial influenza vaccination (Visit 4). At this time, ICF, study requirements, any returned illness memory aids, and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccinations, will be obtained and medications reviewed. Atargeted PE will be completed andvital signs and weight obtained. Then a dose of seasonal IIV will be administered intramuscularly.

Visit 5 (Season 2 day 8/36 + 2 days): Subjects may return to the VRU or have a home visit completed on day 8 (+2) following IIV administration (this will be about day 36 following the initial vaccination for children requiring 2 doses of influenza vaccine). At this time, the ICF, study requirements, any returned illness memory aids that document recent symptoms of illness and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccines, will be obtained and medications reviewed. Vital signs and subject's weight will be obtained and a targeted PE performed. Following this a sample of peripheral venous blood will be obtained if able.

Visit 6 (Season 2 day 24/52 +/- 4 days): Subjects may return to the VRU or have a home visit completed on day 24 (+/-4) following revaccination with IIV (this will be about day 52 following the initial vaccination for children requiring 2 doses of influenza vaccine). At this time, the ICF, study requirements, any returned illness memory aids that document recent symptoms of illness and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccines, will be obtained and medications reviewed. Vital signs and subject's weight will be obtained and a targeted PE performed. A sample of peripheral venous blood will be obtained if able.

Optional 1 year Follow-up Visit 1 (Season 3 day 0) -: Subjects will be asked to return to the VRU the following fall to receive seasonal influenza vaccination. At this time, the ICF, study requirements and any AE or other illnesses will be reviewed. Subjects will have an interval medical history obtained, including vaccinations, and a review of all medications. Any illness memory aids returned will be reviewed to determine if the parent/LAR recorded any symptoms consistent with influenza-like illness that were not reported to study personnel. Subject vital signs and a weight will be obtained and a targeted physical exam performed as indicated by medical history. Following this a sample of peripheral venous blood will be obtained if able. Subjects will then be vaccinated with seasonal IIV. Parents/LARs will use the memory aids to document the symptoms of any illnesses that occur and will be instructed to call the study coordinator if symptoms consistent with an influenza-like illness are present.

Optional 1 year Follow-up Visit 2 (Season 3 day 8+2 days) - Subjects may return to the VRU or have a home visit completed on day 8 (+2) following IIV administration. At this time, the ICF, study requirements, any returned illness memory aids that document recent symptoms of illness and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccines, will be obtained and medicatins reviewed. Vital signs and subject's weight will be obtained and a targeted PE performed. Following this a sample of peripheral venous blood will be obtained if able.

Optional 1 year Follow-up Visit 3 (Season 3 day 24 +/- 4 days) - Subjects may return to the VRU or have a home visit completed on day 24 (+/-4) following revaccination with IIV. At this time, the ICF, study requirements, any returned illness memory aids that document recent symptoms of illness and any adverse events or other illnesses will be reviewed. An interim medical history, including vaccines, will be obtained and medications reviewed. Vital

signs and subject's weight will be obtained and a targeted PE performed. A sample of peripheral venous blood will be obtained if able.

7.4 Home Visits

While our preference is for study visits to take place within the University of Rochester's Vaccine Research Unit, parent/LAR will be given the option to complete any visits that do not include the administration of vaccine as home visits if this is more convenient for them. Having home visits is optional and is not required to be part of the study. When a home visit is conducted, the following guidelines will be followed:

- The option of having certain study visits take place as home visits will be included in the consent form, and in all cases informed consent will be obtained prior to going into the subject's home.
- Study activities will be conducted using the same methodology regardless of the location of the visit. Study information will be collected on paper forms, which then will be transferred to our online study database on completion of the visit.
- The procedure to conduct blood draws and nasal wash specimens in the home setting will be the same as that used in outpatient setting (per local SOP). An experienced pediatric research nurse will go on all study visits to complete the blood draw. This nurse will be accompanied by either the study PI or a research assistant trained in how to hold subjects to optimize the success of blood collection. If blood is unable to be obtained, this will be documented on the Case Report Form. There will not be a requirement for the subject to present to the VRU in the event blood is unable to be collected.
- To ensure the safety of the researchers, study personnel will travel to the patient's residence in pairs. Members of the research team will be mandated reporters. In the event that potential child abuse or neglect is observed, the case will be reported to Child Protective Services as required by local SOP.

7.5 Early Termination Visit

If subjects discontinue from the study, they may be asked to make an early termination visit. At the time of the early termination visit, the reason for early termination will be recorded, current health status since the last visit will be reviewed, and all concomitant medications will be recorded. A targeted physical examination may be performed, as indicated, and information regarding AEs will be solicited. Any ongoing related AEs will be followed to resolution or until a stable chronic condition has been established. If subjects do not wish to present for an early termination visit, reasons for study termination will be solicited by phone when possible.

7.6 Unscheduled Visit

Unscheduled visits may occur at any time during the study. These visits may either be a clinic visit or a home visit. Depending on the nature of the visit, activities will include a review of the medical history including concomitant medications, assessment of adverse effects or other illnesses, vital signs, a targeted physical examination as necessary, and any additional testing deemed necessary.

7.7 Compensation for Participation

Subjects will be compensated for the time and effort of participation per local IRB requirements.

8 STUDY PROCEDURES AND EVALUATIONS

8.1 Clinical Evaluations

Medical History: Study personnel will take the medical history of all subjects. This history will include significant medical disorders of the head, eyes, ears, nose, throat, mouth, cardiovascular system, lungs, gastrointestinal tract, liver, pancreas, kidney, nervous system, blood, lymph glands, endocrine system, musculoskeletal system, skin, and genital/reproductive tract. It will also include a history of allergies, cancer, immunodeficiency, psychiatric illness, substance abuse, and autoimmune disease. Patients will also be queried regarding any previous influenza vaccinations, the date and source of vaccine, and the type of vaccine administered. Each subject's NYSIIS record will also be accessed to assess for vaccination history by study personnel.

Medication History: Study personnel will record all medications, including prescription and over-the-counter drugs (such as vitamins, minerals, supplements, homeopathic preparations and/or therapies), taken by the subject in the 30 days prior to enrollment.

Targeted Physical Examination: Licensed study clinicians (i.e., physician, physician's assistant, and study nurses) will obtain a subject's weight and vital signs (respiratory rate, pulse, and temperature) and a targeted physical examination will be conducted if necessary. This targeted physical examination will focus on the respiratory system and the cardiovascular system. In subjects enrolled through the ED, this information may be obtained through a review of the medical record.

8.2 Laboratory Evaluations/Assays

8.2.1 Specimen Collection, Preparation, Handling and Shipping

Blood draw: A weight-based sample of whole blood will be collected, if able, by venous, with the blood volume determined as described in Table 1. This sample will then be transferred to appropriate-sized tubes containing heparin or sodium citrate. If blood is available, a sample of fresh blood in sodium citrate tubes will be shipped to our collaborators at University of Chicago overnight using appropriate biosafety precautions to enable quantification, isolation, and determination of the BCR repertoire of plasmablasts. All other blood will be centrifuged and plasma and PBMCs will be isolated within several hours of the blood being obtained (blood is stable in heparinized tubes for at least 6 hours post draw). Plasma will be aliquoted and stored in an Ultra Low Temp Freezer at $\leq$ -70^{o}C for use in ELISA, antibody array, and microneutralization assays. PBMCs will be washed, counted, and frozen in liquid nitrogen for later use in intracellular cytokine staining, multiplex cytokine assays, RNA-seq, and CD4 T cell and memory B cell Elispot assays, with a subset of PBMCs sent to our collaborators NIAID for further analysis. If blood cannot be successfully obtained, this will be documented on the enrollment or case report form as "unable to be obtained." In the event that the child is unwilling to cooperate with research staff or attempts to draw blood are unsuccessful, the research staff may forego further intervention procedures for the given study visit. If the child and parent/LAR

are willing to participate further, then follow up visits will be scheduled and attempted as outlined in the timeline.

All blood will be drawn by experienced and trained pediatric nurses or physicians, with pain minimized through comforting and distraction techniques. Only the minimum amount of blood necessary to achieve the study objectives will be obtained, with the blood volume being within a level accepted as safe (Table 1). As a child's total blood volume is related to body weight, we have chosen a weight based approach to determine the maximum volume of blood to be obtained. This will allow us to optimize our likelihood of being able to accomplish the study objectives while still prioritizing the safety of the child. Current policy suggests that blood volume limits between 1% and 5% of the total blood volume on a single draw and up to 10% of total blood volume over an 8 week period will present minimal risk to children [31]. A child's total blood volume estimated at around 80 mL/kg. The proposed blood draws in this protocol impose a maximum upper limit of 1 mL/kg on any single draw (about 1.25% of estimated TBV), with a maximum of 3 mL/kg (about 3.75% of estimated TBV) over any 8 week period for pediatric patients. This volume remains well below the upper limit of 10% of total blood volume in an 8 week period and is consistent with the available evidence on what will present minimal risk to children while still providing enough PBMCs to perform the in depth analysis of CD4 T cell and B cell specificity and functional capacity and evaluation of antibody responses required for this study.

TABLE 1. BLOOD VOLUMES (mL) IN CHILDREN

Study Year 1					Study Year 2/3				Total	
Body Weight (kg)	Blood volume visit 1 (mL)	Blood volume visit 2 (mL)	Blood volume visit 3 (mL)	Max/8 weeks (mL)	Blood Volume Visit 4, 7 (mL)	Blood Volume Visit 5, 8 (mL)	Blood Volume Visit 6, 9 (mL)	Max/8 weeks (mL)	mL/kg per draw	Max % of TBV (over 8 weeks)
2-4	2	2	2	6	2	2	2	6	1	3.75%
≥5-10	4	4	4	12	4	4	4	12	0.8	3%
≥11-15	8	8	8	24	8	8	8	24	0.73	2.7%
≥16-20	12	12	12	36	12	12	12	36	0.75	2.8%
≥21-30	16	16	16	48	16	16	16	48	0.76	2.8%
≥31-40	20	20	20	60	20	20	20	60	0.64	2.4%
≥41-50	30	30	30	90	30	30	30	90	0.73	2.7%
≥51-60	40	40	40	120	40	40	40	120	0.78	2.9%
≥61	50	50	50	150	50	50	50	150	0.82	3%

Nasal swab: Nasal swabs will be obtained by placing two UV-sterilized soft nylon flocked swabs into alternate nostrils of the subject and gently rotating them across the mucosa to collect respiratory secretions. When appropriate, one of these swabs will be used to perform a rapid influenza test in the primary care office, URMC or RGH ED, the VRU, or the subject's home.

The other swab will be placed into 1 mL of viral culture transport media for RT-PCR studies and stored in an Ultra Low Temp Freezer at ≤ -70°C.

Nasal wash: Between 1.5 and 5 mL of sterile saline will be instilled and collected, if able, by gentle suction with a flexible catheter or bulb syringe inserted approximately 5 cm into the naris. Alternatively, we may opt to instill the entire volume into a single nostril, as this is considered safe and the anticipation of the 2nd nasal wash has been significantly distressing to some children [34]. In the event that the child is unwilling to cooperate with research staff or attempts to collect the nasal wash are unsuccessful, the research staff may forego further intervention procedures for the given study visit. If the child and parent/LAR are willing to participate further, then follow up visits will be scheduled and attempted as outlined in the timeline. Within several hours, the nasal wash fluid will be centrifuged to pellet the cells and the supernatant will be removed and stored in an Ultra Low Temp Freezer at ≤ -70°C . The cell pellet will be washed, after which any cells obtained will be counted and frozen in liquid nitrogen for later analysis.

Nasal Brush: A UV-sterilized pediatric flocked swab will be used to collect inferior turbinate nasal epithelial cells for genome-wide expression analysis. The brush specimen will be obtained, if able, immediately following the NW in order to obtain mucosal lining cells free of mucus and lymphocytes. The swab will be placed in the nostril and rotated with an up and down motion gently against the lining mucosa for ~ 5 seconds. The swab will then be placed into 2 mL of RNAprotect (Qiagen) and transported to the lab for RNA isolation, which will be completed within a 1 week time period. Recovered RNA will be stored in an an Ultra Low Temp Freezer at ≤ -70°C until use.

8.2.2 Laboratory Evaluations

Detection and quantification of influenza virus: Influenza will be detected from potentially infected subjects by (1) rapid antigen testing using a commercial assay (BinaxNOW, Alere, Waltham, MA or QuickVue Influenza A+B test, Quidel, San Diego, CA) and (2) real time RT-PCR using primers obtained from BEI Resources (Manassas, VA) or using the ABI 7500 real-time PCR platform. Two UV-sterilized soft nylon flocked swabs will be used to collect nasal swab samples from subjects potentially infected with influenza. One swab will be used for rapid antigen detection on site, while the other will be placed into 1 mL of viral culture transport media for use in real time RT-PCR to detect and quantify influenza viral load. This sample will be stored and may be used to sequence the infecting influenza virus at a later time point.

Multiparameter flow cytometry: PBMCs will be thawed, rested overnight, and, when appropriate, stimulated with influenza-specific peptide pools as experimental antigens, Staphylococcal enterotoxin B (a superantigen) as a positive control, or a pool of irrelevant peptides from the Sin Nombre virus glycoprotein precursor protein or media alone as a negative control for 10-20 hours, with Brefeldin A and monensin added to inhibit cytokine secretion for the final 8 hours of incubation. Cells will then be stained with a viability marker and a panel of antibodies against surface markers. When appropriate, they will then be fixed, permeabilized, and stained intracellularly using antibodies against cytokines as well as other markers. All events will be collected on a flow cytometer within the University of Rochester Medical Center Flow Core. We have the flexibility to adjust and modify these panels as necessary to optimize

our staining results.

Multiplex cytokine assay: Multiplex cytokine assays will be used to determine the antigen-specific cytokine production potential of the total CD4 T cell population. Cells will be thawed, rested overnight, and depleted of CD8 and NK cells. They then will be stimulated with HA and NP peptide pools or irrelevant peptides as a negative control. Following around 48 hours of coculture, supernatants will be assayed for levels of 30 common cytokines and chemokines with a human cytokine/chemokine magnetic bead panel (Bio-Rad Laboratories, Hercules, CA) and analyzed on a Bio-Plex200 system.

Peptide-specific CD4 T cell Elispot: IFNγ, IL-2, and granzyme B Elispot analysis of human PBMCs will be performed by established assays. Briefly, PBMC will be thawed, rested overnight, and depleted of CD8 and NK cells. They then will be cultured for 16-48 hours in Millipore 96-well PVDF plates coated with capture antibodies for either IFN Elispot analysis of human PBMCs will be performed by established assays. Briefly, PBMC will be thawed, rested overnight, and depleted

Detection of memory B cells: With collaborators at the University of Rochester, the method of Crotty and colleagues [35] will be adapted to determine frequencies of influenza-specific memory B cells. Cultured cells will be non-specifically stimulated by a mixture of mitogens, followed by HA, NP, and Ig Elispots for determination of (i) antigen-specific IgG ASC frequencies, and (ii) total IgG ASC frequencies. Antigen-specific MBC frequencies will be expressed as the percentage of antigen-specific IgG ASC/total IgG ASC.

RNA-seq: Total RNA will be extracted from PBMCs. This RNA will then be provided to the genomics core for construction of a cDNA library and high-throughput sequencing using an Illumina HiSeq 2500 System, with 20 million reads per sample to allow unbiased evaluation of whether the overall pattern of gene expression is influenced by a subject's prior influenza exposure.

Evaluation for cross reactive HA antibody by array: With collaborators at the University of Rochester, dilutions of serum will be applied to a chip coated with multiple HA proteins in microarray format [36] or evaluated using a Luminex-based technology [37] to rapidly quantify antibody levels against multiple HA proteins

Microneutralization assays: MDCK cells will be grown to confluence in a 96 well plate. Serial dilutions of serum will be preincubated with virus and transferred to MDCK cells to allow unneutralized virus to infect the cells. Free virus is then washed away and the plate is incubated to allow cytopathic effect to occur. The antibody titer is the highest serum dilution able to prevent MDCK cell infection.

ELISA: Plates will be coated with HA or NP protein and then incubated with dilutions of serum. After washing and blocking nonspecific binding, plates are incubated with a horseradish peroxidase-conjugated goat anti-human IgG antibody and developed with 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate with absorbance read at 450 nm. To determine antibody avidity, sera are incubated on a recombinant HA-coated plate in the presence of serial

dilutions of sodium thiocyanate. The serum avidity is defined as the concentration of sodium thiocyanate that induces 50% inhibition of antibody binding.

Surface Plasmon Resonance (SPR): SPR measures the off rate (rate at which antibodies dissociate from the antigen) of antibody-antigen binding. The off rates provide a measure of how well the antibodies bind, indicating their quality. In an ideal immune response to vaccine, the off rates should go down, indicating an improved recognition of antigen. For these studies, collaborators at the FDA will use recombinant influenza hemagglutinins that match that year's vaccine formulation, as well as HA head only and HA stalk only recombinant proteins to measure antibody off rates.

Isolation of Influenza-Specific Monoclonal Antibodies: We will send fresh blood samples to the University of Chicago at the baseline visit and days 8+2 and 24 +/- 4 days post-vaccination or infection in study years 1 and 2 and plasmablasts (day 8) and antigen-specific memory B cells (day 24) will be isolated by flow cytometry. Monoclonal antibodies will be expressed from the cloned variable genes of single cells and the antibodies and serum will be tested for binding, neutralization, and epitope specificity. In addition, BCR repertoire will be determined using the 10x genomics platform.

Systems Biology Approaches: We will be sending PBMCs to the NIAID Systems Genomics and Bioinformatics Unit to better understand immune development in childhood. Using a combination of approaches including flow cytometry, an analysis of cellular transcriptional activity in immune cell subset and single cells as analyzed by RNA-seq or microarrays, PBMC transcriptome analysis, and additional 'omics" assays for assessing immune receptor repertoires including TCR and BCR profiling, the state of the immune system both at baseline and following immune perturbations will be assessed.

Instructions for specimen preparation, handling, and storage are described in local SOPs.

8.3 Procedures to be Followed in the Event of Abnormal Laboratory Test Values or Abnormal Clinical Findings

Subjects will be informed of the results of their rapid influenza tests, together with a disclaimer that the sensitivity of this test is limited. Subjects will be notified that any repeat testing or treatment, if indicated, can be provided by their primary care physician.

If a subject tests positive for influenza by PCR testing they will be contacted to assess for interest in full study participation. This testing result will not be available real-time and will not be provided to primary care physicians as this test is not CLIA certified.

Subjects developing signs and symptoms of influenza during subsequent monitoring will be referred to an appropriate health care provider for evaluation and treatment, if indicated. If severe illness symptoms develop, subjects will be referred to their pediatrician or the pediatric emergency department per local SOP.

9 ASSESSMENT OF SAFETY

9.1 Specification of Safety Parameters

An FDA approved, licensed seasonal inactivated influenza vaccine will be administered in this protocol as per standard of care during influenza vaccine season following the manufacturers' instructions and safety precautions.

As **Quadrivalent Fluzone** is a licensed vaccine, NIAID does not expect that any new vaccine related safety signal will be detected in this trial. Therefore, the only adverse events reported to DMID will be adverse events that are reportable to Vaccine Adverse Event Reporting System (VAERS).

The National Childhood Vaccine Injury Act (NCVIA) **requires** healthcare providers to report to VAERS:

- Any adverse event listed by the vaccine manufacturer as a contraindication to further doses of the vaccine; and/or
- Any adverse event listed in the VAERS Table of Reportable Events Following Vaccination that occurs within the specified time period after vaccination (see below).
    - Anaphylaxis or anaphylactic shock (7 days)
    - Shoulder Injury Related to Vaccine Administration (7 days)
    - Vasovagal syncope (7 days), not related to blood draws
    - Guillain-Barré Syndrome (42 days)
    - Any acute complication or sequelae (including death) of above events (interval - not applicable)

Upon reporting to VAERS, any such report will also be provided to the DMID Medical Officer, DMID Clinical Project Manager, and local IRB as required. A paper copy of this information will be filed in the subject's chart and the information will be inputted into BLIS as an VAERS-reportable adverse event.

In addition, CDC encourages reporting any serious, unexpected (not listed in product label or Investigator's Brochure) or suspected adverse reaction to influenza vaccination to VAERS, with a copy of any such reports provided to the DMID Medical Officer, DMID Clinical Project Manager and local IRB as required.

Data on intermittent illnesses, hospitalizations, surgeries and other events that are unrelated to the study intervention but that may impact the health of the child will be collected from the first study visit to study conclusion, as these events may effect the immunologic endpoints of the study. This will include data on upper respiratory infections, febrile illnesses, common childhood procedures such as PE tube placement or tonsillectomy & adenoidectomy, and similar data. Collected information will include details on approximate date of onset, approximate date of resolution (if known), severity, whether the event was associated with respiratory symptoms or fever, and outcome; relatedness to vaccine will not be captured as these events are expected to

be unrelated to the vaccine. This information will be recorded on an illness/other event CRF and in Blis following adoption of Protocol v9. Although data on these illnesses/other events will be collected, this data will not be reported to DMID as Fluzone is a licensed vaccine and DMID does not expect any new vaccination safety signals to be detected as a result of this study.

10 CLINICAL MONITORING

The purpose of clinical monitoring is to protect the rights and well-being of human subjects in this study; to ensure that data are accurate, complete and verifiable from source documents; to ensure that conduct is in compliance with the currently approved protocol/amendments, with Good Clinical Practice, and with regulatory requirements.

10.1 Site Monitoring Plan

Site monitoring will be conducted using the DMID tools provided to ensure that human subject protection, study procedures, laboratory procedures, and data collection processes are of high quality and meet sponsor, GCP/ICH, and regulatory guidelines, and that the study is conducted in accordance with the protocol and sponsor standard operating procedures. DMID, the sponsoring agency, or its designee will conduct site-monitoring visits as defined in the site monitoring plan.

Site visits may be made at standard intervals as defined by DMID and may be made more frequently as directed by DMID. Monitoring visits will include, but are not limited to, review of regulatory files, sample tracking log, CRFs, informed consent forms, medical and laboratory reports, and protocol compliance. Study monitors will meet with investigators to discuss any problems and actions to be taken and document visit findings and discussions. The University of Rochester's IRB and other regulatory agencies may also conduct study monitoring visits.

11 STATISTICAL CONSIDERATIONS

11.1 Study Hypothesis

The primary hypothesis being tested in this study is that there will be differences in the specificity, magnitude and functionality of CD4 T cell and B cell reactivity in a cohort of young children depending on early childhood exposures.

11.2 Sample Size Considerations

Because the degree of variability in many of the measurements in this study are unknown, estimating with precision the correlation of patient characteristics (age and prior vaccination history) with outcomes (CD4 T cell response magnitude and function, antibody quality and quantity and B cell response) is not possible. The study will evaluate these outcomes and, if trends towards a relationship are discovered, subsequent studies will evaluate these in more detail.

11.3 Planned Interim Analyses (if applicable)

No formal interim analysis is planned. However, assay results will be reviewed as they become available

11.3.1 Safety Review

N/A, no interim analysis of safety is planned.

11.3.2 Immunogenicity or Efficacy Review

N/A, no interim analysis of safety is planned.

11.4 Final Analysis Plan

Data will be analyzed at the end of the study, with results available for publication at that time.

A cohort of n=20 to 60 healthy children that receive a prime-boost vaccination with IIV and n=20 children infected with influenza acutely will be recruited for each age cohort. Their T cell and antibody responses will be measured at three time points: baseline, day 8, and day 24 post-vaccination. We will apply Shapiro-Wilk's test to check the normality. If the data passes the normality test, two sample *t*-tests will be used to detect significant mean difference between the children of different ages and flu exposures at each time point. Otherwise, Wilcoxon rank-sum test will be used. For follow up visits, subjects will be followed longitudinally for one to two years with yearly revaccination with IIV. CD4 T cell responses (flow cytometry) and B cell and antibody responses will be measured at three time points: prior to IIV re-vaccination, 8 and 24 days post re-vaccination. We will apply one-way ANOVA *F*-test (or Kruskal-Wallis test, if the

normality assumption is rejected) to detect significant group differences at each time point. Because multiple response variables are used in this analysis, the *p*-values will be adjusted by the Benjamini-Hochberg multiple testing procedure [38] to control the false discovery rate (FDR) at the level of 0.05.

General linear models of difference or fold change in T/B cell response at following time points from baseline will be used to explore how childhood exposures affect immune responses over time, while controlling potentially confounding variables including age, sex, presence of asthma or allergies, or other acute or chronic illnesses and medications that may impact the immune response. In addition to time-specific comparisons, the following linear mixed effect model will be used to analyze data collected from all time points

$$y_{kit} = \alpha_k + \beta_{1k} G(i) + \beta_{2k} X_{kit} + \gamma_{kt} + \grave{o}_{kit}. \qquad (0.1)$$

Here y_{kit} represents the *k*th response variable measured from the *i*th subject at the *t*th time point; α_k is the overall mean value of y_{kit} ; *G*(*i*) is a binary variable which equals 0 if the *i*th subject is vaccinated with IIV and 1 otherwise; β_{1k} is a linear coefficient that quantifies the magnitude of the group-effect; β_{2k} are linear coefficients for potentially confounding clinical variables listed above; γ_{kt} is a random effect term that quantifies the time effects; and $\grave{o}_{kit}$ represents independent measurement errors. Likelihood ratio test will be used to test $H_0 : \beta_k = 0$ against $H_1 : \beta_k \neq 0$.

In case of high-dimensional data in different scales, standardization will be applied before model fitting and variable selection. Variable selection approaches using AIC or BIC criteria will be used to select the significant predictors. Penalized-based variable selection procedures such as LASSO [39] or SCAD [40,41] may be used to overcome the drawbacks of ad-hoc methods of stepwise, best subset selection, etc. post-hoc.

12 SOURCE DOCUMENTS AND ACCESS TO SOURCE DOCUMENTS

The site will maintain appropriate medical and research records for this study in compliance with ICH E6, Section 4.9 and regulatory and institutional requirements for the protection of confidentiality of subjects. The site will permit authorized representatives of the DMID, its designees, and appropriate regulatory agencies to examine (and when required by applicable law, to copy) clinical records for the purposes of quality assurance reviews, audits and evaluation of the study safety and progress. These representatives will be permitted access to all source data which include, but are not limited to, hospital records, clinical and office charts, laboratory notes, memoranda, pharmacy dispensing records, recorded data from automated instruments, x-rays, and subject files kept at the laboratories involved in the study. CRFs will consist of paper document collection forms which will then be entered into the database, and will serve as source documents. All electronic study documents will be secured by key and/or password protection.

13 QUALITY CONTROL AND QUALITY ASSURANCE

Following a written DMID-accepted site quality management plan, the sites are responsible for conducting routine quality assurance (QA) and quality control (QC) activities to internally monitor study progress and protocol compliance.

The Principal Investigators will provide direct access to all study-related sites, source documents, and reports for the purpose of monitoring and auditing by the sponsor, and inspection by local and regulatory authorities.

The Principal Investigators will ensure all study staff are appropriately trained and current documentations are maintained on site.

DMID-designated clinical monitors will verify that the clinical study is conducted and data generated, recorded, and reported in compliance with the protocol, GCP, and the applicable regulatory requirements. Clinical monitoring reports will be submitted to DMID.

The site staff will implement QC procedures with the data entry system and generate data QC checks that will be run on the database. Any missing data or data anomalies will be clarified and resolved by going back to the source documents and checking with the clinical team that collected the data.

14 ETHICS/PROTECTION OF HUMAN SUBJECTS

14.1 Ethical Standard

The investigator will ensure that this study is conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR Part 46 and/or the ICH E6; 62 Federal Regulations 25691 (1997).

14.2 Institutional Review Board

The University of Rochester Research Subjects Review Board (RSRB) will review this protocol and the associated informed consent documents. Any amendments to the protocol or consent materials must also be approved before they are placed into use unless it is in the best interest of the subjects' safety to implement changes prior to approval. The UR RSRB operates under U.S. Federal-Wide Assurance (FWA)

Prior to enrollment of subjects into this trial, the approved protocol and the informed consent form will be reviewed and approved by the appropriate IRB. Any amendments to the protocol or consent materials will also be reviewed and approved by the appropriate IRB and submitted to the sponsor. Notification of the IRB's composition, or the IRB's Federal-wide Assurance number, will be provided.

Should amendments to the protocol be required, the amendments will be written by the PI for submission to the IRB and also submitted to the sponsor. The site will submit to the sponsor a copy of the IRB letter of approval of the amendment.

14.3 Informed Consent

The parents/LARs of all subjects recruited into this study will provide written informed consent. There will be two separate consent forms: a briefer consent for a child presenting with influenza-like illness to be screened for influenza infection and contacted by the study team, and a full consent form for complete study participation. In addition after the 2 year follow up in the main study and if funding is available, subjects will have the option to enroll for a additional 1 year follow up upon reviewing and signing a "consent for longitudinal follow up." To obtain consent, a member of the investigative team will meet with potentially interested parents/LARs. The Emergency Department Research Associate (EDRA) Program will be utilized to recruit subjects acutely infected with influenza from both the Pediatric Emergency Department and UR-affiliated Urgent Care Centers. The parents or LARs of the subjects will be provided with a description of the study, including the purpose, risks, benefits, alternatives, and study procedures. The fact that there is no requirement for participation and that subjects are free to withdraw at any time will be stressed. All questions that arise during this discussion will be answered. Parents or LARs will then be provided with a copy of the consent form to read. If they agree to study participation, written informed consent will be obtained by a certified study team member or a member of the EDRA Program. A study assent form will also be reviewed with children ≥8 years of age and

signed. Once the consent and assent (as necessary) forms are signed, the subject will be considered enrolled in the study and subject information and samples will be obtained. A copy of the signed consent form will be provided to the parent/LAR.

Parents/LARs who initially sign the briefer consent form to have their children screened for influenza will be given the opportunity to consent to participation in the full study if their child screens positive for influenza. At that point, they will have the full study explained in detail and will again be given the opportunity to have any questions they have answered. There will not be a requirement to participate in the full study if consent for influenza testing is given and the child screens positive for infection.

14.4 Informed Consent Process

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continuing throughout the individual's study participation. As all participants in this study will be children, informed consent will be sought from the study participant's parent or LAR (although subjects will provide assent as appropriate for age). The risks and possible benefits of this study will be discussed extensively with parents/LARs and participants as appropriate. Consent forms describing in detail the study interventions/products, study procedures, and risks are given to the parent/LAR, with written documentation of informed consent required prior to starting intervention/administering study product. Consent forms will be IRB-approved and the participant/representative will be asked to read and review the document. Upon reviewing the document, the investigator will explain the research study to the participant's representative and the participant and answer any questions that may arise. The parent/LAR will sign the informed consent document prior to any procedures being done specifically for the study and assent from the study participant will be obtained if the subject is 8 years of age or older. The participants will have the opportunity to discuss the study with their parents/LARs or think about it prior to agreeing to participate. The parent/LAR may withdraw consent at any time throughout the course of the study. A copy of the informed consent document will be given to the participating parents/LARs for their records. The rights and welfare of the children will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

At the time the study staff seek informed consent, the study worker will ask the eligible participant's parent/LAR if he/she is literate. If the representative reports he or she is not literate, then the study staff will request that a witness be present while the study worker reads and explains the study and what participation will entail. If the eligible subject's representative agrees to study participation, he or she will make a mark on the signature line of the consent form. The witness will also sign and date the form, if the witness is confident that the participant has understood the explanation and is participating willingly. In addition, the witness will complete the date line for the participant.

14.4.1 Informed Consent/Assent Process (in Case of a Minor)

Appropriate consent will be obtained from all subjects prior to any study related procedures (see above). Parents/LARs of eligible subjects will sign informed consent. Children and adolescents

ages 8 years and older will also sign an assent document after consent is obtained from a parent or LAR.

14.5 Exclusion of Women, Minorities, and Children (Special Populations)

As this is a study of the development of immunity in children, all participants will be minors. There will be no exclusions based on race or ethnicity.

14.6 Participant Confidentiality

Participant confidentiality is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their agents. This confidentiality is extended to cover testing of biological specimens in addition to the clinical information relating to participants.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

The study monitor or other authorized representatives of the sponsor may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records. A representative from the University of Rochester IRB may also have access to the subject's record.

To protect privacy, the NIH has provided a Certificate of Confidentiality (CoC). With this CoC, the participating sites cannot be forced to release information that may identify the participant, even by a court subpoena, in any federal, state, or local civil, criminal, administrative, legislative, or other proceedings. The participating sites will use the CoC to resist any demands for information that would identify the participant, except as explained below.

The CoC cannot be used to resist a demand for information from personnel of the United States Government that is used for auditing or evaluation of federally funded projects, like this study, or local laws, such as for reporting of communicable diseases.

A CoC does not prevent the participant from voluntarily releasing information about themselves or their involvement in this research. If any person or agency obtains a written consent to receive study information, then the participating sites may not use the CoC to withhold that information.

The CoC does not prevent the participating sites from reporting without the participant's consent information that would identify the subject as a participant in the study regarding matters that must be legally reported including: child and elder abuse, sexual abuse, or wanting to harm themselves or others.

14.7 Study Discontinuation

If the study is discontinued, enrolled subjects will continue to be followed for safety assessments.

14.8 Future Use of Stored Specimens

Subjects will be asked to give permission to keep any remaining specimen for possible use in future research studies, such as further testing of innate or adaptive immune mediators, RNAseq analysis, or for antibodies against other viruses or bacteria. Samples will be stored at the local site and will not be sold or used directly for production of any commercial product. Each sample will be labeled only with a subject ID to protect subject's confidentiality. Such testing may be performed by collaborating laboratories located at other sites on deidentified samples or on samples labeled with the original sample ID when there is a limited data use agreement in place. All future research will need to be approved by the appropriate IRBs prior to research activities being undertaken.

There are no benefits to subjects in the collection, storage and subsequent research use of specimens. Reports about future research done with subject's samples will NOT be kept in their health records, but subject's samples may be kept with the study records or in other secure areas.

15 DATA HANDLING AND RECORD KEEPING

The site principal investigator is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported. All source documents will be completed in a neat, legible manner to ensure accurate interpretation of data. When making changes or corrections, the original entry will be crossed out with a single line, initialed and dated. The original text will not be erased, overwritten, or altered with correction fluid or tape on the original.

15.1 Data Management Responsibilities

All source documents and laboratory reports will be reviewed by the clinical team and data entry staff, who will ensure that they are accurate and complete.

Data collection is the responsibility of the clinical study staff at the site under the supervision of the clinical PIs. During the study, the investigators will maintain complete and accurate documentation for the study.

15.2 Data Capture Methods

Clinical data will be initially recorded on paper source documents, and then transferred to electronic case report forms (eCRF) within BLIS. Source documents will be retained for monitoring purposes in a secure location. Laboratory data will be directly entered into BLIS from laboratory notebooks or computer databases.

As detailed in the CEIRS contract, overall CEIRS data sharing will adhere to the following schedule:

- Sequence data: provided to Data Processing Coordinating Center within 45 days
- Surveillance data: provided to Data Processing Coordinating Center within 12 months
- Virus phenotypic data: provided to Data Processing Coordinating Center within 12 months
- Basic research data: provided to Data Processing Coordinating Center within 2 months post publication.

15.3 Types of Data

Data for this study will include subject demographics, clinical data, and research laboratory results including measures of CD4 T cell response magnitude, cytokine production, determination of antibody responses, and measurements of B cell and antibody responses. In addition, global gene expression profiling may be performed on RNA isolated from the peripheral blood mononuclear cells of a subset of subjects. RNA-seq will be used to determine in an unbiased way if the overall patterns of gene expression are influenced by influenza exposure history. The purpose of performing this testing is not to diagnose or determine susceptibility to any given genetic disease or condition, but instead to better understand how a

subject's previous history of influenza exposure influences future vaccine responses. Although we will not be testing for any specific diseases or conditions as part of this study, it is possible that genetic information regarding disease susceptibility will be obtained. As we will be examining expression levels of a multitude of mRNAs, there will be a low level of certainty that any obtained results are predictive of either the presence of or susceptibility to any disease. Thus, these results will be held in confidence and not released to the subject, placed in their medical record, or provided to any individual or organization.

15.4 Timing/Reports

The final report will include a comprehensive analysis of the data.

15.5 Study Records Retention

Records and documents pertaining to the conduct of this clinical study, including CRFs, source documents, and consent forms must be retained by the investigator for at least 2 years following the date of completion of the study. No study records will be destroyed without prior authorization by DMID. These documents should be retained for a longer period, however, if required by local regulations.

15.6 Protocol Deviations

A protocol deviation is any noncompliance with the study protocol, GCP, or protocol-specific MOP requirements or institution SOPs. The noncompliance may be either on the part of the subject, the site principal investigator, or other study personnel. As a result of deviations, corrective actions are to be developed by the site and implemented promptly.

These practices are consistent with ICH E6:

4.5 Compliance with Protocol, Sections 4.5.1, 4.5.2, and 4.5.3

5.1 Quality Assurance and Quality Control, Section 5.1.1

5.20 Noncompliance, Sections 5.20.1, and 5.20.2

It is the responsibility of the site principal investigator and other study personnel to use continuous vigilance to identify and report deviations within five working days of identification of the protocol deviation, or within five working days of the scheduled protocol-required activity. A line listing of deviations will be reported to DMID on a monthly basis.

All protocol deviations, as defined above, must be addressed in study subject data collection forms. A completed copy of the DMID Protocol Deviation Form must be maintained in the Regulatory File, as well as a copy kept in the subject's source document file. Protocol deviations must be sent to the local IRB/IEC per its guidelines. The site principal investigator and other study personnel are responsible for knowing and adhering to their IRB requirements.

16 PUBLICATION POLICY

Following completion of the study, the research investigators will share data as defined in the contract and as directed by the CO and COR.

17 LITERATURE REFERENCES

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APPENDIX A - SCHEDULE OF EVENTS

	Study Season 1					Study Season 2				Optional Study Season		
	Visit 1 (d0)	Visit 1.5 (d28)[b]	Visit 2 (d8/36)	Visit 3 (d24/52)	Illness visits	Visit 4 (d0)	Visit 4.5 (d28)[b]	Visit 5 (d8/36)	Visit 6 (d24/52)	Visit 1 (d0)	Visit 2 (d8/36)	Visit 3 (d24/52)
Review eligibility requirements	X									X		
Obtain informed consent and assent if ≥8 yo	X									X		
Review informed consent		X	X	X	X	X	X	X	X		X	X
Review study requirements	X	X	X	X	X	X	X	X	X	X	X	X
Medical history	X									X		
Interim history, including vaccines		X	X	X	X	X	X	X	X		X	X
Review medications	X	X	X	X	X	X	X	X	X	X	X	X
Vital signs	X	X	X	X	X	X	X	X	X	X	X	X
Targeted PE	X	X	X	X	X	X	X	X	X	X	X	X
Adverse event assessment and illness/other event assessment		X	X	X	X	X	X	X	X		X	X
Distribute illness memory aid	X					X				X		
Review illness memory aid if returned		X	X	X	X	X	X	X	X		X	X
Obtain blood for PBMC/plasma (if able)	X		X	X		X		X	X	X	X	X
Obtain nasal wash (if able)	X[c]		X[c]	X[c]								
Obtain nasal brush (if able)	X[c]		X[c]	X[c]								
Administer vaccine	X[d]	X[b]				X	X[b]					
Obtain nasal swabs for rapid antigen and influenza PCR testing[a]	X		X[e]		X							

[a] Natural infection and illness visits only
[b] Visit 1.5 will be required for subjects in Cohort 1A. Visit 4.5 will be required for subjects in the B cohorts who are <9 years and no history of 2 prior vaccine doses.
[c] Only for Cohorts 1B, 2B, 3B, and 4B
[d] Only for Cohorts 1A, 2A, 3A, and 4A
[e] This will only be obtained in subjects presenting with natural infection who only had testing completed by the clinical microbiology laboratory who have bypassed visit 1 AND who have ongoing symptoms at the time they are presenting for enrollment