Understanding how the initial encounter with influenza virus poises children for protective immunity

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List of Abbreviations:

aa:	Amino acid
Ab:	Antibody
Ag:	Antigen
AE:	Adverse Event
CD:	Cluster of Differentiation molecule
CPT:	BD Vacutainer Cell Preparation Tube with Sodium Citrate
ELISA:	Enzyme-linked immunosorbent assay
Elispot:	Enzyme-linked ImmunoSpot assay
ICS:	Intracellular cytokine staining
IFNγ	Interferon gamma protein
IIV:	Inactivated influenza vaccine
IL-2:	Interleukin 2 protein
HA:	Hemagglutinin protein
LAIV:	Live attenuated influenza vaccine
NP:	Nucleoprotein
M1:	Matrix protein 1
MHC	Major histocompatibility complex molecule
MN:	Microneutralization assay
NA:	Neuraminidase protein
NP:	Nucleoprotein
PBMC:	Peripheral blood mononuclear cells
RT-PCR:	Reverse transcription polymerase chain reaction
RNAseq	RNA sequence (gene expression)
TBV	Total blood volume
Th:	T helper cell
TNFα:	Tumor necrosis factor α protein
VRU	Vaccine research unit at the University of Rochester

1. PURPOSE OF THE STUDY AND BACKGROUND

1.1: Purpose of the Study:

Inactivated influenza vaccine ("IIV") is an intramuscular vaccine administered to children ≥6 months in age that is composed of highly purified HA and NA surface glycoproteins and contains trace, if any, activators of the immune system. In contrast, live attenuated vaccine ("LAIV") consists of intranasally delivered, attenuated viruses that stimulate the innate immune system while undergoing limited viral replication in the upper respiratory tract. Some children also continue to first encounter influenza through natural infection, where high levels of viral replication stimulate vigorous innate immunity. Although CD4 T cells play a key role in regulating the influenza-specific B cell, CD8 T cell, and innate immune responses, little is known about the immediate and long-term effects these different routes of early childhood priming have on CD4 T cell mediated immunity. The long-term goal of this research is to understand how a child's early exposure to influenza shapes immunity and poises the immune system to respond to subsequent influenza challenge. Our objective is to determine how the anti-influenza CD4 T cell and antibody responses are altered by the mode of primary influenza encounter and establish the consequences this has on subsequent influenza vaccination. To accomplish this objective, we will enroll two cohorts of young children: 1) a cohort of 24 through 35 month of children administered either LAIV or IIV and 2) a separate cohort of infants first exposed to influenza through prime-boost administration of IIV or natural infection during their initial influenza season. All enrolled subjects will be followed longitudinally to the subsequent influenza season and then rechallenged with IIV. These cohorts of children will allow us to test the following hypotheses:

1) Compared to intramuscular IIV, initial exposure to intranasal LAIV establishes a greater and more polyfunctional CD4 T cell response in children 24 through 35 months of age
2) Compared to a first exposure to influenza through infection, primary infant vaccination with IIV establishes a more limited CD4 T cell repertoire that consists predominately of IL-2 producing cells, and
3) The CD4 T cell responses established with this initial priming will differentially poise subjects to respond to revaccination with IIV the following influenza season

Completion of this research will determine the effect of different methods of priming on the specificity and functional potential of the anti-influenza CD4 T cell and antibody responses. These experiments will fill a critical void in our understanding of the primary human immune response to influenza and generate knowledge enabling the rational development of novel influenza vaccines.

The specific objectives for this study include the following:

- To comprehensively evaluate the specificity and functional potential of CD4 T cell responses using intracellular cytokine staining and Elispot assays following restimulation with pools of HA and NP peptides
- To assess the full spectrum of antigen-specific CD4 T cell cytokine and chemokine production using multiplex cytokine assays
- To evaluate the humoral immune response that develops in response to infection or administration of LAIV or IIV vaccines
- To evaluate the memory B cell response established by different routes of exposure to influenza
- To determine the influence of the CD4 T cell response on the breadth and avidity of the post-vaccination antibody response using arrayed imaging reflectometry to rapidly quantify antibody levels against multiple HA proteins, microneutralization assays to quantify neutralizing antibody titers against different viral strains, and enzyme linked immunosorbent assays to determine HA antibody avidity and quantify stalk reactive antibody
- To evaluate in an unbiased way the influence of previous influenza exposure on PBMC gene expression patterns using RNA-seq analysis
- To assess the mucosal immunity established by these modes of priming and determine whether any initially established differences in mucosal immunity are maintained on subsequent rechallenge with IIV

1.2: Background:

Influenza is a virus in the family *Orthomyxoviridae* that infects the host respiratory tract mucosa and causes a contagious acute respiratory infection (1). This virus contains a genome composed of eight strands of

negative-sense RNA that encode viral proteins (2). Vaccines against this virus have traditionally relied on inducing antibodies that protect against viral infection by binding to the HA surface glycoprotein and neutralizing virions. However, the rapid accumulation of point mutations in both the HA and NA proteins allow this virus to drift and escape neutralization, resulting in yearly epidemics despite the availability of influenza vaccines (1, 3). In addition, the segmented genome of influenza virus allows sporadic reassortment to occur, which can result in antigenic shift and creation of a strain against which the population has little preexisting immunity. Such a reassortment event occurred in 2009, when a novel swine-origin influenza virus emerged and spread globally, resulting in the first influenza pandemic of the 21st century (4, 5).

It is estimated that 20% of children become infected with influenza yearly, with increasing rates during severe epidemics (6, 7). In addition to providing a reservoir for community infection, childhood influenza infection results in a substantial number of outpatient and emergency room visits and can be associated with significant morbidity, including viral or secondary bacterial pneumonia (6-8). Hospitalization is highest in the very young (estimated at 4.5 per 1000 children 0 to 5 months old) (8), with overall childhood influenza-related mortality estimated at 0.21 deaths per 100,000 children (9). While annual influenza immunization is the most effective method to prevent infection, the influenza vaccine is suboptimal in children (10-12). Some evidence suggests that LAIV may induce broader, more protective immunity in the young (11, 13-16), however recently presented data demonstrated that LAIV failed to protect against H1N1 strains in the 2013-14 influenza season. Further, LAIV is not yet approved for use under the age of 2 due to concerns about the precipitation of wheezing (17, 18). As IIV is universally recommended in the USA beginning at 6 months of age (18, 19), increasing numbers of children are first exposed to influenza via IIV.

Major strides in understanding the protective immune response to influenza have been made in recent years. 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 (1, 20-23). As the infection progresses, viral replication results in abundant intracellular antigen that can be processed and presented to activate the adaptive immune response (1, 23). CD4 T cells have a multifaceted role in combating infection, providing help to promote B cell isotype switching and affinity maturation, CD8 T cell memory generation, and directly participating in viral clearance through the independent secretion of antiviral cytokines and direct, cell-mediated cytotoxicity (1, 3, 23-33). Additionally, CD8 T cells are activated and home to respiratory sites to eliminate infected cells (1, 3, 23, 28, 29) and antigen-specific B cells secrete high affinity, neutralizing antibodies that are able to provide strain-specific sterilizing immunity from future infection (1, 23, 34-37). While neutralizing antibody is thought of as the major correlate of protection (34-37), cellular immunity is increasingly recognized as important in protecting from severe disease (38-41). As T cells can recognize epitopes from conserved internal viral proteins (42-46), this protection can be very important in years when antigenic drift leads to a poor vaccine match or a pandemic influenza strain emerges.

In contrast to the robust response that develops following influenza infection, the immune response generated on vaccination with IIV is characterized by weak inflammatory signaling. These vaccines are 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) (47-49). Vaccine is solely quantified based on the content of the HA protein and generally 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 (47, 50-52). This vaccine is delivered intramuscularly without the addition of an adjuvant in the USA (50-52). While IIV stimulates antibody (53-59) and CD4 T cell responses (59-63), little CD8 T cell immunity

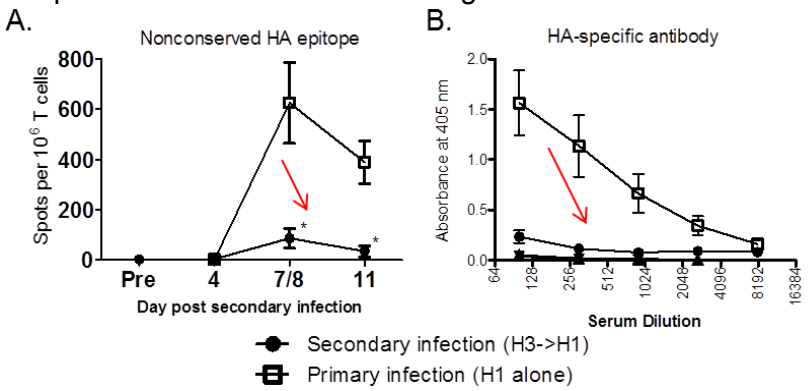

Figure 1: *Preexisting immunity can have a profound effect on both CD4 T cell and antibody responses.* Mice were initially infected with an H3 influenza virus, followed by challenge with an H1 strain 9 weeks later. (A) Splenocytes were enriched for CD4 T cells by MACS purification and incubated with known peptide-epitopes along with syngeneic splenocytes from uninfected animals as APC. Compared to following a primary infection (open squares), HA-specific CD4 T cell responses were suppressed following secondary infection (black circles). Asterisks denote a P-value <0.05 by Student's t test. (B) Sera obtained from mice following primary or secondary infection was tested for HA-specific antibody by ELISA. H1 HA-specific antibody was suppressed following secondary challenge with an H1 virus (black circles) compared to the primary infection (open squares). Triangles represent serum antibody from H3 infected animals (upwards) or naïve serum (downwards). Error bars represent the standard error of the mean.

is generated due to limited cross presentation of the extracellularly delivered antigen (54, 55, 62, 64, 65). Interestingly, LAIV is an intermediate between these two extremes. As an attenuated virus, LAIV results in activation of the innate immune system (56, 66-70) with stronger CD4 and CD8 T cell responses (55, 62, 71), but the more limited viral replication results in a decreased antigenic load.

Previous research has shown that the persistence, nature, and intensity of anti-influenza memory depend on the context of antigenic stimulation (72). Additionally, data we have generated in mice (Figure 1) and human subjects (Figure 2) suggests that previous influenza exposures shape the development of immunity on challenge with a drifted or shifted influenza strain. Given the profound differences in antigenic context between natural infection, IIV and LAIV, we 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.

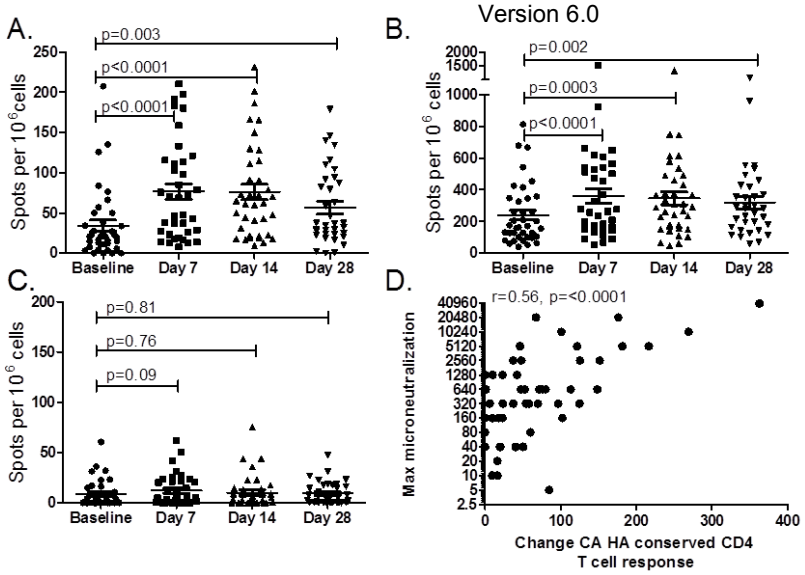

Figure 2: *Expansion of CD4 T cells directed against conserved peptides is seen following pandemic H1N1 vaccination, with a correlation between the development of an HA-specific CD4 T cell response and neutralizing antibody titer.* Subjects received the monovalent inactivated subvirion A/California/07/09 vaccine and CD4 T cell responses against conserved and nonconserved peptide pools were examined by IFNγ EliSpot assay using CD8 and CD56 depleted PBMCs. While a response to HA (A) and NP/M1 (B) peptide pools expected to be conserved compared to recently circulating H1N1 strains was detected, little response to peptides expected to be unique to this virus was seen in subjects without prior immunity (C). Error bars represent the standard error of the mean and P-values calculated using Wilcoxon signed rank test. Development of a CD4 T cell response was correlated with the maximum microneutralization titer achieved (D). R- and p-values determined using the Spearman correlation coefficient.

2. STUDY DESIGN:

2.1: Overview:

This study will be conducted as a single center, prospective, open-label evaluation of different methods of priming on the anti-influenza CD4 T cell and antibody response in young children. It is anticipated that this study will last 3 years, with approximately 1 year of follow up for each subject. The study objectives are to evaluate the specificity and functional potential of the CD4 T cell response and the quantity, breadth and avidity of the neutralizing antibody response following either influenza infection or receipt of different types of licensed influenza vaccine as follows:

- Primary objective: To comprehensively evaluate the specificity and functional potential of CD4 T cell responses on a single cell level using intracellular cytokine staining following restimulation with pools of HA and NP peptides

Secondary objectives will include:

- To assess the full spectrum of antigen-specific CD4 T cell cytokine and chemokine production using multiplex cytokine assays
- To perform in depth characterization of the specificity of the CD4 T cell response following influenza vaccination or infection by Elispot assay
- To evaluate the humoral immune response that develops in response to infection or administration of the LAIV or IIV vaccines
- To determine the frequency of HA-specific memory B cells established using these priming conditions
- To determine the influence of the CD4 T cell response on the breadth and avidity of the post-vaccination antibody response using arrayed imaging reflectometry to rapidly quantify antibody levels against multiple HA proteins, microneutralization assays to quantify neutralizing antibody titers against different viral strains, and enzyme linked immunosorbent assays to determine HA antibody avidity and quantify stalk reactive antibody

- To evaluate in an unbiased way the influence of previous influenza exposure on PBMC gene expression patterns using RNA-seq analysis
- To assess the mucosal immunity established by these modes of priming and determine whether any initially established differences in mucosal immunity are maintained on subsequent rechallenge with IIV

To accomplish these objectives, we plan to enroll a cohort of 60 children who have previously been vaccinated with at least 2 doses of IIV and who are between the ages of 24 and 35 months into cohort 1. These children will be block randomized to receive either LAIV (n=30) or IIV (n=30) in groups of 5 unless a history of isolated wheezing in the past year is present. As LAIV is not indicated in children with a history of recent wheezing, otherwise eligible children who have a history of wheezing will be defaulted to receive IIV. Children will have a blood draw and nasal wash on study entry and again at days 10 (+ 4 days/- 2 days) and 24 (+/- 4 days) post vaccination. They will then be followed through to the subsequent influenza season, when they will be rechallenged with IIV with blood draws and nasal washes pre-vaccination and again at days 10 (+ 4 days/- 2 days) and 24 (+/- 4 days) post vaccination (Figure 3).

The second cohort of children will be infants between 3 and 12 months of age who will be enrolled to investigate how the primary CD4 T cell response that develops following IIV administration differs from that established after natural influenza infection. Infants who receive vaccine in this cohort will be at least 6 months of age and will be enrolled in the fall before influenza begins to circulate in the community. Infants between 3 and 12 months of age who contract a natural influenza infection before any doses of vaccine are administered will be eligible for the natural infection arm of this cohort. Both of these infant groups will have blood drawn and a nasal wash obtained on study entry and at days 10 (+ 4d/- 2d) and 24 (+/- 4d) post-vaccination and then will be followed to the subsequent influenza season and rechallenged with IIV, with blood draws and nasal washes again completed pre-vaccination and at days 10 (+ 4d/- 2d) and 24 (+/- 4d) post-vaccination (Figure 3). Children who test positive for influenza will be allowed treatment with oseltamivir as recommended by the CDC (73). To accomplish this, the rapid influenza test result will be provided to the child's pediatrician, who will then coordinate treatment of the child.

Figure 3

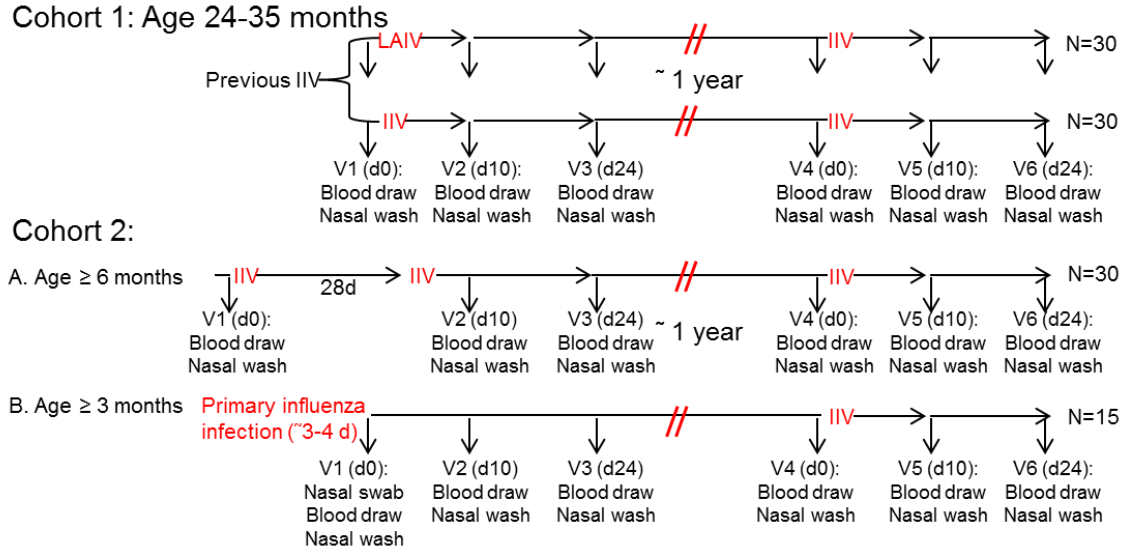

Figure 3: *Experimental schematic to determine whether the method of initial priming differentially poises subjects to respond to future influenza encounters.* Subjects that receive LAIV, IIV, or that are naturally infected with influenza will have CD4 T cell and antibody responses examined at enrollment and at days 10 and 24 post vaccination. Cohort 1 contains children approximately 24-36 months of age. Cohort 2A contains infants 6-12 months of age in their first influenza season, while cohort 2B contains infants 3-12 months of age. All enrolled subjects will be followed longitudinally for a year and then will be revaccinated with IIV. CD4 T cell responses, antibody response, and transcriptional profiles will be examined prior to revaccination and at days 10 and 24 post vaccination.

2.2: Rationale for Study Design:

The age range of the first cohort of children was chosen because children first become eligible to receive LAIV at the age of 24 months. Thus, the age range of 24 through 35 months will capture children receiving LAIV for the first time. A block randomization scheme will be used as there is a large population of children with a past history of isolated wheezing as a result of viral illnesses such as bronchiolitis that have been shown to respond

equivalently to influenza vaccine (59, 74, 75). We feel that including this population of children in the study will improve recruitment and allow for broader applicability of our study results, but as these subjects are not eligible to receive LAIV (17, 18), their inclusion also precludes a completely blinded randomization scheme. With block randomization, eligible subjects will be enrolled to receive either LAIV or IIV in groups of 5 unless a history of isolated wheezing in the past year is present, in which case they will be defaulted to receive IIV as long as no other exclusion criteria are present.

The age range of the second cohort was chosen to enroll infants in their first influenza season with no history of prior exposure to influenza virus. Thus, this 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 the vaccination group. All of these children will receive IIV, as LAIV is not approved for children this age. This cohort will receive a prime-boost vaccination series, with vaccine doses separated by 28 (+ 14) days as recommended by the AAP and ACIP (17, 18). To minimize blood draws, we will only interrogate the immune response following the second vaccine dose. Children between 3 and 12 months of age who present with an influenza-like illness and have no previous history of influenza vaccination will be eligible to participate in the infection group. Study personnel will obtain nasal swabs from potential subjects for a rapid influenza antigen test on illness presentation (typically this occurs at day 3-4 post illness onset). If positive, they will be eligible for further study participation. Influenza real-time RT-PCR will be used to confirm rapid antigen test results, however these results will not be available in time to influence subject enrollment.

Blood draws will be obtained on study enrollment, day 10 (+ 4d/- 2d), and day 24 (+/- 4d) post enrollment as depicted in Figure 3. The blood draw on study enrollment will evaluate influenza-specific immunity at the time of study entry. The blood draw on day 10 (+ 4d/- 2d) post-vaccination will allow examination of each subject as CD4 T cell responses are peaking, while the blood draw at day 24 (+/- 4d) 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 10 (+ 4d/- 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. Blood draw volume will be determined based on subject weight, with a maximum of 1 mL/kg of blood obtained on any single draw and 3 mL/kg of blood over a 3 month period (see Table 1). At a minimum, 4 million PBMCs will be needed for flow cytometry and 3.6 million PBMCs will be needed for multiplex immunoassays. Assuming blood cellularity allows for recovery of an average of 1.5 million PBMC/mL, obtaining 5 mL of blood per draw will provide approximately 7.5 million PBMCs, which is just under the minimum amount of blood needed for these two assays alone. When a child's weight allows, a greater blood volume will be obtained to a max of 16 mL of blood per draw to provide for the additional cells needed to examine CD4 T cell specificity by Elispot assay (approximately 12 million cells), memory B cell responses (approximately 4 million cells), completion of gene expression studies, and allow for additional restimulation conditions to be tested using ICS and multiplex immunoassay as possible.

TABLE 1. BLOOD VOLUMES (mL) IN CHILDREN

Study Year 1					Study Year 2					
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 (mL)	Blood Volume Visit 5 (mL)	Blood Volume Visit 6 (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%
>20	16	16	16	48	16	16	16	48	0.76	2.8%

2.3: Rationale for Dosage:

All influenza vaccines will be administered at the age-appropriate recommended dosages as follows:

- IIV: Fluzone (Sanofi Pasteur, Swiftwater, PA) 0.25 mL administered intramuscularly to children between 6 and 35 months of age

- Children in cohort 2 (6-12 months of age) who are receiving influenza vaccine for the first time will receive 2 doses separated by 28 (+14) days

- LAIV: Flumist Quadrivalent (MedImmune) 0.2 mL administered intranasally

3. CHARACTERISTICS OF THE RESEARCH POPULATION

3.1: Subject Characteristics

a. Number of Subjects: It is anticipated that this study will enroll a total of approximately 180 subjects. This includes 60 children between the ages of 24 and 35 months in the first cohort. These children will be divided equally into two groups of 30, with one group receiving IIV and the other receiving LAIV as described above. We expect to screen up to 120 subjects into the second cohort, of who 45 will be enrolled in the full study and evaluable. This will include 30 children between the ages of 6 month and 1 year that will receive IIV and 15 children between the ages of 3 months and 1 year who become naturally infected with influenza virus. However, in order to enroll these 15 naturally infected children, we anticipate having to screen up to 90 children with signs of influenza like illness during the height of seasonal influenza circulation, as only a small number of these children will have respiratory infections as a result of influenza virus infection.

b. Gender and Age of Subjects: It is anticipated that there will be an approximately equal distribution of males and females enrolled. This will include 60 children between the ages of 24 and 35 months in the first cohort and up to 120 children between the ages of 3 months and 1 year in the second cohort, of whom 45 will be evaluable.

c. Racial and Ethnic Origin: It is anticipated that the demographic distribution of enrollment will approximately resemble that of the City of Rochester, which is about 40% Caucasian, 40% African-American, 15% Hispanic, and 5% other. No subject will be excluded because of their racial or ethnic origin.

d. Vulnerable Subjects: As the goal of this study is the study of the early immune response to childhood influenza infection and vaccination, by definition it needs to include children, who are a vulnerable population. To safeguard rights and welfare of these children, the parents or legal guardians of all subjects recruited into this study will provide written informed consent. Since the subjects involved will all be less than 3 years of age, it will not be possible to obtain subject assent, although study procedures will be explained to all subjects as developmentally appropriate based on the subject's age. To maintain subject privacy, paper copies of subject PHI will be stored in a locked file cabinet within a locked office, with an electronic extraction of this data maintained on a password protected and secured university computer.

All blood will be drawn by experienced and trained pediatric nurses, 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. 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 (76). 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 all 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 specificity and functional capacity and antibody responses required for this study.

3.2: Inclusion and Exclusion Criteria

a. Inclusion Criteria:

- Age

  - Between 24 and 35 months for cohort 1
  - Between 6 and 12 months to participate in the vaccination arm of cohort 2 (cohort 2A)
  - Between 3 and 12 months to participate in the natural infection arm of cohort 2 (cohort 2B)
- Gestational age of ≥37 weeks at birth
- Parent/guardian can provide informed consent
- Available for the duration of the study
- History of previous IIV administration **ONLY** for participation in cohort 1
- Acute illness documented to be due to influenza virus **ONLY** for participation in the natural infection arm of cohort 2 (cohort 2B)

b. 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 or inhaled steroid therapy
- A diagnosis of asthma requiring chronic controller medication
- Previous administration of influenza vaccine in the current influenza season
- 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} on screening **EXCEPT** for participation in in the natural infection arm of cohort 2 (cohort 2B)
- A contraindication to influenza vaccination **EXCEPT** infants between 3 and 5 months presenting with natural influenza infection whose only contraindication is their current age

3.3: Discussion of Subject Population:

The main objective of this Study is to evaluate the specificity and functional potential of the CD4 T cell response and the quantity, breadth and avidity of the neutralizing antibody response following influenza infection or vaccination. In order to eliminate potential confounding factors, we plan to exclude any subject who has an acute or chronic medical condition that may result in immunosuppression. Also excluded will be potential subjects that have recently received immunosuppressive medications or blood products that could potentially interfere with vaccine immunogenicity. This exclusion will include children with a history of asthma who require chronic controller medicines, including inhaled steroids. Children with only a history isolated wheezing not on chronic controller therapy will be allowed to participate, although they will not be administered LAIV.

4. SUBJECT IDENTIFICATION, RECRUITMENT AND CONSENT

4.1: Method of Subject Identification and Recruitment

Potential subjects will be identified by providers in the Strong Pediatric Practice or Elmwood Pediatrics. On identification of a potential subject, the subject's primary care provider will ask if the family is interested in learning more about this study, either in person or via a letter sent to the family. When possible, families receiving a letter describing the study will receive a phone call from study personnel after 7-10 days to determine if there is any interest in study participation. If a family expresses interest in the study and the subject is determined to be eligible, the consent process will be undertaken. 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 at the time of study enrollment. On obtaining consent, parents will be asked if they are willing to be contacted regarding future studies. Only those parents who consent to this future use of their information will be contacted for purposes outside of the present study.

Our division has extensive experience in recruiting children to studies that examine the immunology of respiratory viruses. Dr. Mary Caserta and Dr. Edward Walsh recruited infants between the ages of 0 and 9 months into an immunologic study of RSV infection (12-004). Currently this study has enrolled 226 infants into a cohort that was recruited at birth and followed prospectively for 1 year. In addition, this study has enrolled 82 infants hospitalized with RSV infection and 149 outpatients with respiratory illness, of whom 43

were subsequently documented to have infection due to RSV and participated in the complete study. Similarly, Dr. John Treanor recently recruited children into a longitudinal family study of influenza (07-0046) and was able to recruit 132 children under the age of 4. Similar to the proposed study, both of these studies involved enrolling and obtaining blood samples from young children. This supports the feasibility of our proposed enrollment goals in this young population.

4.2: Process of Consent:

The parents or legal guardians of all subjects recruited into this study will provide written informed consent. There will be two separate consent forms: a briefer consent to have a child presenting with influenza-like illness screened for influenza infection and a full consent form for complete study participation. To obtain consent, a member of the investigative team will meet with potentially interested families at the Strong Pediatric Practice, Elmwood Pediatrics, or in the Vaccine Research Unit (VRU) at the University of Rochester. The parents or guardians 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 guardians 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. Once the consent form is 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.

Parents 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 on rapid antigen testing. 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. All rapid influenza test results will be confirmed by influenza-specific RT-PCR, however these results will not be available in time to be used for the purpose of subject enrollment.

5. METHODS AND STUDY PROCEDURES

While our preference is for study visits to take place within the University of Rochester's Vaccine Research Unit, families will be given the option to complete visits 2, 3, 5, and 6, as well as any illness visits as home visits if this is more convenient for them. For a full description of the home visit protocol, please see the home visit addendum (section 5.11).

5.1: Study Schedule

Enrollment visit (visit 1, d0)

Subjects in the influenza vaccination groups will have a complete 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 directed physical exam will then be performed as indicated by medical history. Following this, a nasal wash and a sample of peripheral venous blood will be obtained. Subjects will then receive their initial influenza vaccination. Post vaccination, parents will be given memory aids to document adverse events following vaccination and the symptoms of any future illnesses that occur. They also will be instructed to call study personnel if symptoms consistent with an influenza-like illness develop.

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. If not already obtained, subject vital signs and weight will be measured. A directed physical exam will then be performed as indicated by the medical history. Following this, two nasal swabs will be obtained by placing UV-sterilized soft nylon flocked swabs into alternate nostrils of the infant 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. Subjects with a negative rapid influenza test will not participate in the study further. Subjects that have a positive rapid antigen test will have the opportunity to consent to full study participation. If this consent is obtained, a nasal wash and a sample of

peripheral venous blood will be obtained. Parents will then be given a memory aid to document the progression of their current illness and the symptoms of any future illnesses that occur.

While some subjects will have their initial enrollment completed in the office of their primary care physician, this may not be possible on days when clinic space is limited. In this situation or when the family is recruited by letter, the subject will have their enrollment completed and study vaccine administered (when appropriate) in the University of Rochester VRU. Only staff experienced in the collection of samples from and the vaccination of children will perform study procedures.

Vaccine booster visit:
Infants undergoing vaccination in cohort 2A will have the option of either presenting to the VRU or their primary pediatric practice to receive their influenza vaccine booster dose. If parents opt to present to the VRU, the subjects will have an interval history and targeted PE completed, vital signs and weight obtained, and then will be administered a dose of IIV intramuscularly. If parents opt to obtain the booster dose of this vaccination in their primary pediatric clinic, follow up will be scheduled prior to exiting the clinic and this date recorded so that parents can be reminded of this appointment the day prior by phone.

Visit 2 (day 10/38 + 4 days/- 2 days):
Subjects will return to the VRU or have a home visit completed on day 10 following their presentation with influenza infection or their final influenza vaccination (this will be day 38 following the initial vaccination for infants requiring 2 doses of influenza vaccine). At this point, an interim history will be obtained with a targeted PE as appropriate. The memory aids will also be reviewed to document recent symptoms of illness or adverse events following vaccination. Following this, a nasal wash and a sample of peripheral venous blood will be obtained.

Visit 3 (day 24/52 +/- 4 days): Subjects will return to the VRU or have a home visit completed on day 24 following their presentation with influenza infection or their final influenza vaccination (this will be day 52 following the initial vaccination for infants requiring 2 doses of influenza vaccine). At this point, an interim history and targeted PE will be obtained and the memory aids will be reviewed. A nasal wash and a sample of peripheral venous blood will then be obtained.

Table 2: Study Schedule

	Study year 1				Study year 2		
	Visit 1 (d0)	Visit 2 (d10/38)	Visit 3 (d24/52)	Illness visits	Visit 4 (d0)	Visit 5 (d7)	Visit 6 (d21)
Medical history	X						
Vital signs	X	X	X	X	X	X	X
Complete PE	X				X		
Review medications	X	X	X	X	X	X	X
Review eligibility requirements	X						
Review study requirements	X	X	X	X	X	X	X
Obtain informed consent	X						
Adverse event assessment		X	X	X	X	X	X
Review interim vaccine Hx		X	X	X	X	X	X
Administer vaccine	X				X		
Distribute memory aid	X						
Review memory aid		X	X	X	X	X	X
Obtain blood for PBMC/plasma	X	X	X		X	X	X
Obtain nasal wash	X	X	X		X	X	X
Interim history		X	X	X	X	X	X
Targeted PE		X	X	X		X	X
Review informed consent		X	X		X	X	X
Obtain nasal swabs for rapid antigen and influenza PCR testing[a]	X			X			

[a] Group 4 and illness visits only

Illness visits: Parents will contact study personal by phone if subjects develop symptoms consistent with influenza-like illness. If the reported symptoms are consistent with influenza infection, the parents will be offered the opportunity to complete an illness visit. At the illness visit, an interval medical history, vital signs, and targeted PE will be obtained. Following this, two nasal swabs will be obtained by placing UV-sterilized soft nylon flocked swabs into the nostrils of the infant and gently rotating them across the mucosa to collect respiratory secretions. 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 future RT-PCR studies.

In the event the illness visit is completed as a home visit, the family 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 family will be notified at the time of the visit and the subject's primary care practice will be notified by phone that same day. No results from the RT-PCR studies will be available for clinical use regardless of where the swabs are collected, as this test will be run in a batched manner days to weeks following the illness event. In the event a child is observed to be moderately or severely ill at an illness visit, their PCP will be contacted and the child will be sent to their PCPs office or transported to the ED as appropriate regardless of the visit location.

Visit 4 (year 2 d0): Subjects will return to the VRU the following fall to receive seasonal influenza vaccination. Subjects will have an interval medical history obtained and subject vital signs and weight will be measured. The illness memory aid will be reviewed to determine if the family recorded any symptoms consistent with influenza-like illness that were not reported to study personnel. A directed physical exam will then be performed as indicated by medical history. Following this, a nasal wash and a sample of peripheral venous blood will be obtained. Subjects will then be vaccinated with seasonal IIV. Parents will use the memory aids to document adverse events following vaccination or the symptoms of any illnesses that occur. They also will be instructed to call the study coordinator if symptoms consistent with an influenza-like illness are present.

Visit 5 (year 2 d10 + 4 days/- 2 days): Subjects will return to the VRU or have a home visit completed on day 10 following IIV administration. At this point, an interim history will be obtained with a targeted PE as appropriate. The memory aids will also be reviewed to document recent symptoms of illness or adverse events following vaccination. Following this, a nasal wash and a sample of peripheral venous blood will be obtained.

Visit 6 (year 2 d24 +/- 4 days): Subjects will return to the VRU or have a home visit completed on day 24 following revaccination with IIV. An interim history and targeted PE will be obtained and the memory aids will be reviewed. A nasal wash and a sample of peripheral venous blood will be obtained.

5.2: Study Procedures:

Blood draw: A weight-based sample of whole blood will be collected by venous puncture using CPT tubes, with the blood volume determined as described in Table 1. While not required, a lidocaine 2.5% and prilocaine 2.5% anesthetic cream (EMLA cream) may be applied at the start of the study visit in order to provide local anesthetic affect. This cream will be applied in the following circumstances: 1) on parental request, 2) in the situation of high parental or child anxiety regarding the blood draw, or 3) when there is a history of a prior difficult blood draw. On collection of blood, the specimen will be centrifuged and plasma and PBMCs will be isolated within several hours of the blood being obtained (per the manufacturer's instructions, blood is stable in CPT tubes for at least 2 hours post draw). Plasma will be aliquoted into equal volumes and stored at -80^{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. If blood cannot be successfully obtained, this will be documented on the case report form as "unable to be obtained."

Nasal swab: Nasal swabs will be obtained by placing two UV-sterilized soft nylon flocked swabs into alternate nostrils of the infant and gently rotating them across the mucosa to collect respiratory secretions. One of these swabs will be used to perform a rapid influenza test in the primary care office, the VRU, or the

subject's home. The other swab will be placed into 1 mL of viral culture transport media for future RT-PCR studies.

Nasal wash: Between 1.5 and 5 mL of sterile saline will be instilled into each naris and collected by gentle suction with a flexible catheter or bulb syringe inserted approximately 5 cm into the naris, after which the catheter will be washed with media to remove any residual fluid or cells. Alternatively, in the 24-35 month age group, we may opt to instill 5 mL into a single nostril, as this volume is considered safe and the anticipation of the 2nd nasal wash has been significantly distressing to some children (77). Within several hours, the nasal wash fluid will be centrifuged to pellet the cells and the supernatant will be removed and stored at -80°C. The cell pellet will be washed until no visible clumps of mucous remain, after which the cells will be counted and frozen in liquid nitrogen for later analysis.

5.3: Laboratory Evaluations:

Detection and quantification of influenza virus: Influenza will be detected from potentially infected infants by (1) rapid antigen testing using a commercial assay (BinaxNOW, Alere, Waltham, MA) and (2) real time RT-PCR using primers obtained from BEI Resources (Manassas, VA) or TLDA methodology. Two UV-sterilized soft nylon flocked swabs will be used to collect nasal swab samples from infants 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.

Intracellular cytokine staining: PBMCs will be thawed, rested overnight, and stimulated with HA and NP 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 CD4, CD8, CD14, CD19, CXCR5, CD27, CD57, ICOS, and CD45RA. They then will be fixed, permeabilized, and stained intracellularly using antibodies against CD3, CD69, IL-2, IFNγ, TNFα, and granzyme B . All events will be collected on an 18 color LSR II 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 and IL-2 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 IFNγ or IL-2. Pools of overlapping peptides will be used for restimulation to allow detection of antigen-specific CD4 T cell responses.

Detection of memory B cells: The method of Crotty and colleagues (78) 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: In collaboration with Dr. Benjamin Miller, dilutions of serum will be applied to a chip coated with multiple HA proteins in microarray format. After incubation, chips are washed, dried, and imaged using a prototype reflectometer to rapidly quantify antibody levels against multiple HA proteins (79).

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.

5.4: Safety Assessments:

As IIV and LAIV are well characterized childhood vaccinations currently recommended by ACIP (18) and the AAP (17), we do not expect any serious adverse events to occur. However, we will provide the parents with a post-vaccination memory aid and instruct them to record any adverse events that develop over the course of the study. An interval history will be obtained, including the development of any adverse events, and the memory aid will be reviewed at each subsequent study visit. Solicited systemic AEs will include feverishness, fussiness, vomiting, and headache. Solicited local AEs will include URI symptoms or injection site pain, redness, or swelling. The investigator will be responsible for monitoring and recording all AEs that are observed or reported during the study, regardless of their relationship to study product.

5.5: Assessment of Subject Compliance:

The only at-home study activity required by the parents is to record any adverse events or symptoms of illness in the memory aids and contact study personnel if symptoms consistent with an influenza-like illness develop. It is possible that parents will not comply with this request. However, we will also obtain a sample of blood for measurement of baseline CD4 T cell and antibody responses at each subject's presentation for seasonal IIV administration in study year 2. If CD4 T cell and antibody responses increase between the final visit in study year 1 and day 0 in study year 2, it will provide strong evidence of that subject having had an undocumented influenza infection during the previous influenza season. These subjects will be noted and data will be analyzed both including and excluding these subjects to determine the effect they have on the results obtained.

5.6: Data and Specimen Banking for Future Research Use:

Subjects will be asked for permission to keep any remaining specimens for possible use in future research studies. All biological specimens will be assigned a unique identifying number which will identify subject and visit information, allowing confidentiality to be maintained. The specimens will be stored in secure facilities within the University of Rochester. Use of specimens remaining for additional studies not described in the original protocol must be approved by the IRB and will only be considered for those subjects who have consented to future research use of their specimens. There are no benefits to subjects in the collection, storage and subsequent research use of specimens. Information from additional testing will not be provided to the subject or in any way entered into their medical record.

5.7: Genetic/Genomic Research Activities:

Global gene expression profiling will be performed on RNA isolated from the peripheral blood mononuclear cells of a subset of subjects in study year 2. RNA-seq will be used to determine in an unbiased way if the overall patterns of gene expression are influenced by influenza exposure history. To perform this assay, total RNA will be extracted from PBMCs and 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. 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. Submission of RNA-seq data to public data repositories supported by the National Institutes of Health for potential future analyses is often required for study publication. While all such data is submitted deidentified, there is a very small risk that this information could be reconstructed to identify study subjects in the future. This potential will be discussed with parents during the consent process.

5.8: Costs to the Subject:
Costs to the subject will include the cost of transportation to and from the study site. This should be compensated for by the monetary incentive given for each study visit; however in certain circumstances it may be necessary for the study to find an alternative method of transportation for the subject. In this situation, either livery service or bus tokens may be provided to the subject free of charge. The cost of parking will be covered as part of this study. There will be no charges made against the subject's insurance as a direct result of participation in this study, although any vaccines, including influenza vaccines, that are administered through the patient's primary care physician during this time will be charged to insurance as usual

5.9: Payment for Participation:
Subjects will be compensated for the time and effort of participation at a rate of $25.00 per completed study visit. This cost will be covered as part of the Doris Duke Charitable Foundation Clinical Scientist Development Award.

5.10: Return of Individual Research Results:
Subjects will be informed of the results of the testing for influenza. Subjects will be notified that any repeat testing or treatment, if indicated, can be provided by their primary care physician. No other individual research results will be provided to study subjects or their parents.

5.11: Home Visits:
While our preference is for study visits to take place within the University of Rochester's Vaccine Research Unit, families will be given the option to complete visits 2, 3, 5, and 6, as well as any illness visits 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 (see home visit 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. All compensation payments will be made either by Bank of America VISA Prepaid card or by check mailed to the subject after the study visit. When a Bank of America VISA card is used, the card will carry no balance on being handed to the patient. The $25 will be added to the card by study personel on return to the hospital following completion of the study visit. 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. The consent form will

include mandatory reporting language to inform parents that the members of the research team are required to report any observed or suspected child abuse or neglect.

6. CONCOMITANT AND DISALLOWED MEDICATIONS:

There are no restrictions on the medications that can be provided during the course of the study. All medications necessary for the health and well-being of the child will be allowed. All medications used during the study will be recorded on the data collection form and updated at each study visit. No medications other than yearly seasonal influenza vaccine will be administered as part of this study.

7. SUBJECT WITHDRAWLS:

Subjects may withdraw consent for study participation at any time during the study without penalty. The subject will also be considered a study withdrawal if they are lost to follow up or are unable to comply with study procedures or visits.

8. STUDY DRUG/DEVICE/BIOLOGIC ADMINISTRATION/ASSIGNMENT

8.1: Study Drug/Device/Biologic:

Influenza vaccines will be administered using age-appropriate guidelines in years 1 and 2 of the study. In year 2, all subjects will be administered IIV during a fall study visit in the VRU.

- Live vaccine will consist of Flumist Quadrivalent (MedImmune Vaccine), supplied in a pre-filled, single-dose intranasal sprayer. Each 0.2 mL dose contains $10^{6.5\text{-}7.5}$ fluorescent focus units of live attenuated influenza virus reassortants of each of the 4 strains (A/H1N1, A/H3N2, B/Yamagata and B/Victoria). Each dose is formulated in a sucrose phosphate buffer and may contain residual amounts of ovalbumin, gentamicin, and EDTA. Flumist contains no preservatives.
- Inactivated vaccine will consist of Fluzone Trivalent or Quadrivalent vaccine. 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.

8.2: Dosage of Study Drug/Biologic

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

- LAIV: Flumist Quadrivalent (MedImmune) 0.2 mL administered intranasally
- IIV: Fluzone 0.25 mL administered intramuscularly to children between 6 and 35 months of age
    - Children in cohort 2 (6-12 months of age) who are receiving influenza vaccine for the first time will receive 2 doses separated by 28 days +14 days

8.3: Study Enrollment/Randomization:

We plan to enroll a cohort of 60 children who have previously been vaccinated with at least 2 doses of IIV and who are between the ages of 24 and 35 months into vaccination cohort 1. These children will be block randomized to receive either LAIV (n=30) or IIV (n=30) in groups of 5 unless a history of isolated wheezing in the past year is present. As LAIV is not indicated in children with a history of recent wheezing, eligible children who give a history of isolated wheezing will be defaulted to receive IIV.

Infants between the ages of 6 months and 1 year of age receiving IIV before influenza reaches epidemic levels in the community will be eligible to enroll in the vaccination arm of cohort 2. These infants will not be randomized as they can only receive IIV. Two doses of IIV separated by 28 days will be administered to these children as they will be influenza-vaccine naïve. Infants between 3 and 12 months of age who are unvaccinated and present with influenza-like illness will be eligible to enroll in the infection arm of cohort 2.

8.4: Accountability of Investigational Supplies:

Some children will receive their first dose of influenza vaccine at the time of study enrollment in the office of their primary care physician. The clinic will be responsible for storing and maintaining these vaccines as recommended by the manufacturer. A supply of both vaccines will also be stored in a secure, limited-access, temperature monitored refrigerator at 2°C to 8°C in the VRU until needed. The temperature of the storage unit will be monitored throughout the duration of the study and documentation of proper storage will

be maintained. In the event of accidental deep-freezing or disruption of the cold chain, the vaccines will not be administered.

8.5: Subject Withdrawal of Study Drug

No medications will be chronically administered as part of this study.

8.6: Emergency Drug Disclosure:

Vaccine administration will not be blinded.

9. SAFETY AND REPORTABLE EVENTS

9.1: Adverse Event Definition:

An adverse event is any symptom, sign, illness, or experience which develops or worsens during the course of the study, whether or not the event is considered related to study drug. It does not necessarily have a causal relationship with the vaccination. This can include exacerbations of underlying diseases or the development of an intercurrent illness. All subjects will be followed for the development of any adverse events throughout the course of the study.

9.2: Serious Adverse Event:

A serious adverse event is defined as any adverse medical experience that results in any of the following outcomes:

- Death;
- Is life-threatening;
- Requires inpatient hospitalization;
- Results in persistent or significant disability/incapacity;
- Requires medical or surgical intervention to prevent permanent impairment or damage.

9.3: Recording Adverse Events:

Study staff will assess adverse events by recording all voluntary complaints of the subject and by assessing the subject's clinical appearance. The subject will be questioned directly regarding the occurrence of any adverse experience since his/her last visit at each study visit. Solicited systemic AEs will include feverishness, malaise, fussiness, vomiting, and headache. Solicited local AEs will include URI symptoms or arm pain, tenderness, redness, or swelling.

All adverse events, whether observed by the Investigator or elicited from or volunteered by the subject, will be documented. Each adverse event description will include a brief description of the experience, the date of onset, the date of resolution, the duration and type of experience, the severity, likely relationship to study vaccine, any contributing factors, and any action taken.

9.4: Responsibilities for Reporting Serious Adverse Events:

The investigator will record all serious adverse experiences that occur during the study period in the case report forms and in an adverse event log. All unanticipated and serious events deemed possibly related to the study will be reported promptly to the RSRB.

10. RISK/BENEFIT ASSESSMENT

10.1: Potential Risks and Protection against Risks:

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

While there are possible risks associated with administration of either LAIV or IIV (commonly including the development of a low grade fever or fussiness, the development a runny nose, headache, or sore throat from LAIV, or pain, redness, or swelling at the injection site from IIV), the administration of influenza vaccine to children is considered the standard of care. Thus, these risks are no greater than what would be experienced by children receiving the standard of care.

Blood draws may cause transient discomfort, which will be minimized using sucrose water in infants or distraction in older children. Bruising at the blood draw site also may occur, but can be prevented or

mitigated by applying direct pressure to the draw site for several minutes post blood draw. Development of infection is also possible, although unlikely, and will be prevented through the use of sterile technique (alcohol swabbing and the use of sterile equipment). The volume of blood per kg body weight being drawn is within a range considered safe. Nasal swabs are very safe and carry no significant risk in infants or children. Risks occasionally associated with nasal swabs include brief, minor irritation, and, rarely, transient bleeding. Nasal washes are also considered minimal risk, with the main risk being mild transient irritation, or, possibly transient bleeding. Long term side effects from any of these procedures are considered extremely unlikely.

Personal information on study subjects and their parents will also be collected to determine study eligibility and to account for possible confounding factors when performing data analysis. Research personnel will make every effort to keep this information confidential; however, a risk of participation is that the confidentiality of this information could be lost.

10.2: Potential Benefits to Subjects:

Although administration of influenza vaccines may result in the development of immunity that will prevent future 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 potentially allow for the treatment of an active influenza infection. There are no other direct potential benefits to study participation.

10.3: Alternatives to Participation:

Subjects have the option to not participate in this study without any negative consequences.

11. CONFIDENTIALIATY OF DATA AND INFORMATION STORAGE:

All data collected will be maintained in strict confidentiality according to accepted procedures and relevant HIPAA regulations. All demographic data, medical history, and identifiers will be stored in a coded manner in secured password protected files on computers, with hard-copy source documents secured in a locked area of the University of Rochester Medical Center. All subject samples will be identifiable only by a unique sample id and study number without any identifiers (i.e., name, date of birth, social security number or phone number). Identifying data that links the clinical and demographic data to clinical samples will be maintained by the investigators and kept in a secure location.

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. However, the clinical study site will permit access to all documents and records if required for inspection by regulatory authorities, including but not limited to the records for study subjects. Information that appears in publications will not include any data that can be directly linked to a specific individual.

All data will be stored until analyzed. It is anticipated that analysis of such complex data may continue beyond the proposed duration of the study. All aspects of confidentiality will continue to be maintained during this period, and all additional analyses will be covered by the original IRB approval, or a new IRB approval will be obtained. The data will be maintained indefinitely by one of the investigators, or an approved designee.

12. RESEARCH INFORMATION IN MEDICAL RECORDS:

Information or data generated as a result of the study will not be deposited into the subject's medical record. However, a flag will be added to the medical record for the duration of study participation to indicate that the subject is a study participant and will receive the subsequent year's influenza vaccine per the study protocol.

13. DATA ANALYSIS AND MONITORING

13.1: Sample Size Determination:

	d=0.3	d=0.35	d=0.4
n1=25, n2=25	0.738	0.858	0.934
n1=30, n2=30	0.815	0.915	0.968
n1=35, n2=35	0.871	0.95	0.985
n1=30, n2=10	0.517	0.646	0.761
n1=30, n2=15	0.64	0.772	0.871
n1=30, n2=20	0.721	0.844	0.924

The primary endpoint of this study is CD4 T cell responses. Based on a study of immune response in children and adults (62), the overall mean percentage of IFNγ+ CD4 T cells in 5-9 year old children vaccinated with IIV or LAIV is around 0.1%, or -3 in log10 scale. The standard deviation (STD) in log10 scale is around 0.4. The geometric mean differences induced by vaccinations or between the two types of vaccination is around 0.3 in log10 scale. The above table summarizes statistical power based on Student's two sample *t*-test. Assuming our responses are similar, recruiting n=30 subjects in each group will give us adequate power (0.815) for Aim 1. Because LAIV is an attenuated virus, it is reasonable to assume that the difference in CD4 T cell responses induced by IIV and natural influenza infection is larger (d=0.4), therefore recruiting n=30 subjects in the IIV group and n=15 subjects in the infection group can achieve adequate power (0.871) for Aim 2. For Aim 3, assuming that within-group STD is 0.4 and between-group STD is just 0.2, the power is 0.851 if we recruit n=105 subjects divided them into four subgroups (n1=30, n2=30, n3=30, n4=15).

13.2: Planned Statistical Analysis:

We outline the statistical analysis strategies for the specific aims as follows. **Aim 1:** A cohort of n=60 healthy children will be recruited and divided into two groups with equal sample size (n=30); one group will be vaccinated with LAIV and the other with IIV. Their T cell and antibody responses will be measured at three time points: Baseline, day 10, 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 two groups at each time point. Otherwise, Wilcoxon rank-sum test will be used. Because multiple response variables are used in this analysis, the *p*-values will be adjusted by the Benjamini-Hochberg multiple testing procedure (80) to control the false discovery rate (FDR) at the level of 0.05. In addition to pairwise comparisons, the following linear mixed effect model will be used to analyze data collected from all time points:

$$y_{kit} = \alpha_k + \beta_k G(i) + \gamma_{kt} + \grave{o}_{kit}. \qquad (0.1)$$

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 LAIV and 1 otherwise; β_k is a linear coefficient that quantifies the magnitude of the group-effect; 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 $H_1 : \beta_k \neq 0$. **Aim 2:** Two groups of infants will be recruited for this aim. The first group (n=30) will receive a prime-boost IIV series; the other group (n=15) consists of infants who are infected by influenza naturally. Multiple clinical endpoints will be recorded at 3 time points for both groups. We will apply two sample *t*-test (or Wilcoxon rank-sum test, if Shapiro-Wilks test rejects the assumption of normality) at each time point to detect significant group differences. In addition, we will apply a linear mixed effect model similar to Equation (1.1) to detect the overall group effect. The resulting *p*-values will be properly adjusted to control for FDR at a 0.05 level. **Aim 3:** All subjects (four subgroups; n=105) will be followed longitudinally for a year and then be revaccinated with IIV. CD4 T cell responses, antibody responses, and transcriptional profile (high-throughput RNA-seq) will be measured at three time points: prior to IIV re-vaccination, d10 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. Post-hoc analysis based on Tukey's adjustment will be used to test for significant differences between groups. We will also apply a linear mixed effect model similar to Equation (1.1) to detect the overall group effect. For high-throughput transcriptional data, first we will apply suitable pre-processing techniques such as non-specific filtering (81), normalization (82) and batch-effect removing

(83). We then select differentially expressed genes at each time point by specialized procedures such as DEseq (84) and DEGseq (85). Benjamini-Hochberg procedure will be used to control FDR at a 0.05 level for all analyses.

13.3: Data and Safety Monitoring:

The PI is responsible for the accuracy and completeness of all study data. As this is an open label, single site clinical study in which the intervention being examined is considered the standard of care, safety monitoring will be completed by the PI. All adverse events will be documented on the case report form along with a narrative about the event. Any serious, unexpected adverse events deemed related to the study will be reported promptly to the RSRB.

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