Influenza Immunity: Protective Mechanisms against a Pandemic Respiratory Virus

Fall 2009 Seasonal Influenza Vaccine Protocol

Project 2: T-cell and General Immune Responses to Influenza
Project 3: Single-cell Phospho-protein Signaling Analysis of the Response to Influenza Vaccination
Technical Development Project 1: Measuring the Immunome: Genomic Approaches to B-cell Repertoire

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STATEMENT OF COMPLIANCE

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

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46; 21 CFR Part 50, 21 CFR Part 56, and 21 CFR Part 312)
- ICH E6; 62 Federal Register 25691 (1997)
- NIH Clinical Terms of Award

All key personnel (all individuals responsible for the design and conduct of this study) have completed Human Subjects Protection Training.

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances 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 guidelines.

Site Investigator:

Signed: _____ Date: _____

Name: Cornelia L. Dekker, M.D.
Title: Professor of Pediatrics, Principal Investigator

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LIST OF ABBREVIATIONS

AE	Adverse Event/Adverse Experience
CFR	Code of Federal Regulations
CIOMS	Council for International Organizations of Medical Sciences
CONSORT	Consolidated Standards of Reporting Trials
CFR	Code of Federal Regulations
CRF	Case Report Form
CRO	Contract Research Organization
DAIT	Division of Allergy, Immunology and Transplantation
DCC	Data Coordinating Center
DHHS	Department of Health and Human Services
DMID	Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS
DSMB	Data and Safety Monitoring Board
eCRF	Electronic Case Report Form
FDA	Food and Drug Administration
FWA	Federalwide Assurance
GCP	Good Clinical Practice
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IDE	Investigational Device Exemption
IEC	Independent or Institutional Ethics Committee
IND	Investigational New Drug Application
IRB	Institutional Review Board
ISM	Independent Safety Monitor
JAMA	Journal of the American Medical Association
LAIV	Live attenuated influenza vaccine
MedDRA ®	Medical Dictionary for Regulatory Activities
MOP	Manual of Procedures
N	Number (typically refers to subjects)
NCI	National Cancer Institute, NIH, DHHS
NDA	New Drug Application
NEJM	New England Journal of Medicine
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

OHSR	Office for Human Subjects Research
ORA	Office of Regulatory Affairs, DMID, NIAID, NIH, DHHS
PHI	Protected Health Information
PI	Principal Investigator
PK	Pharmacokinetics
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event/Serious Adverse Experience
SMC	Safety Monitoring Committee
SOP	Standard Operating Procedure
TIV	Trivalent inactivated influenza vaccine
US	United States
WHO	World Health Organization

PROTOCOL SUMMARY

Title: Fall 2009 Seasonal Influenza Vaccine Protocol, Project 2: T-cell and General Immune Responses to Influenza. Project 3: Single-cell Phospho-protein Signaling Analysis of the Response to Influenza Vaccination. Technical Development Project 1: Measuring the Immunome: Genomic Approaches to B-cell Repertoire

Phase: IV

Population: Group A (suppl.): 8-17 yo Identical Twin Pairs Given TIV/LAIV (n=16)

Group B: 18-30 yo Identical Twin Pairs Both Given LAIV (n=20)

Group C: 18-30 yo Fraternal Twin Pairs Both Given LAIV (n=20)

Group D: 40-49 yo Identical Twin Pairs Both Given LAIV (n=20)

Group E: 40-49 yo Fraternal Twin Pairs Both Given LAIV (n=20)

Group F (suppl.): 70-100 yo Elderly Adult Twin Pairs Given TIV (n=14)

Group G (suppl.): 70-100 yo Elderly Adults Given TIV (n=30)

Number of Sites: 1

Study Duration: 12 months for enrollment, vaccination, analysis

Subject Participation Duration: 1 month

Description of Agent or Intervention: Live, attenuated influenza vaccine, Trivalent, inactivated influenza vaccine

Objectives:

PrimaryPrimary:

- Compare lymphocyte responses at Days 9-14 and the lymphocyte and serology responses at Day 28 post-immunization following annual administration of the influenza vaccines
- Evaluate changes in cytokine profile in the immune response (CD4, CD8, $\gamma\delta$ cells, B cell, responses) from Day 0 to Day 9-14 for T cells and antibody-secreting cells (ASCs)
- Evaluate changes in cytokine profile in the immune response from Day 0 to Day 28 for HAI responses to the vaccine antigens
- Evaluate any potential gene expression signatures either at Day 0 or during the early response period Day 9-14 that are associatedwith immune senescence, potentially providing insight into mechanism
- Compare lymphocyte and monocyte reactivity to activating stimuli at Days 0 and 9-14 with the lymphocyte and serology responses at Day 28 post-immunization following administration of seasonal live attenuated influenza vaccine and trivalent inactivated influenza vaccine (TIV).
- Identify age-specific biomarkers or clusters of markers in general and especially those that are predictive of immune sufficiency or insufficiency
- Quantify the frequency of influenza specific T-cells pre and post vaccination
- Determine the effective breadth of T cell repertoire to an influenza vaccine within an individual.

Secondary:

- Compare differences in response between live attenuated vaccine (LAIV) and killed vaccine (TIV)
- Develop comprehensive signaling maps downstream of immune stimulation in response to immune perturbations using single cell phosphor-signaling and cell interrogation approaches.
- Determine signaling-based correlates of influenza A immunity in children, adults and elderly subjects
- Evaluate the variation in immune response (as measured by all study assays) between individuals and assess whether it changes

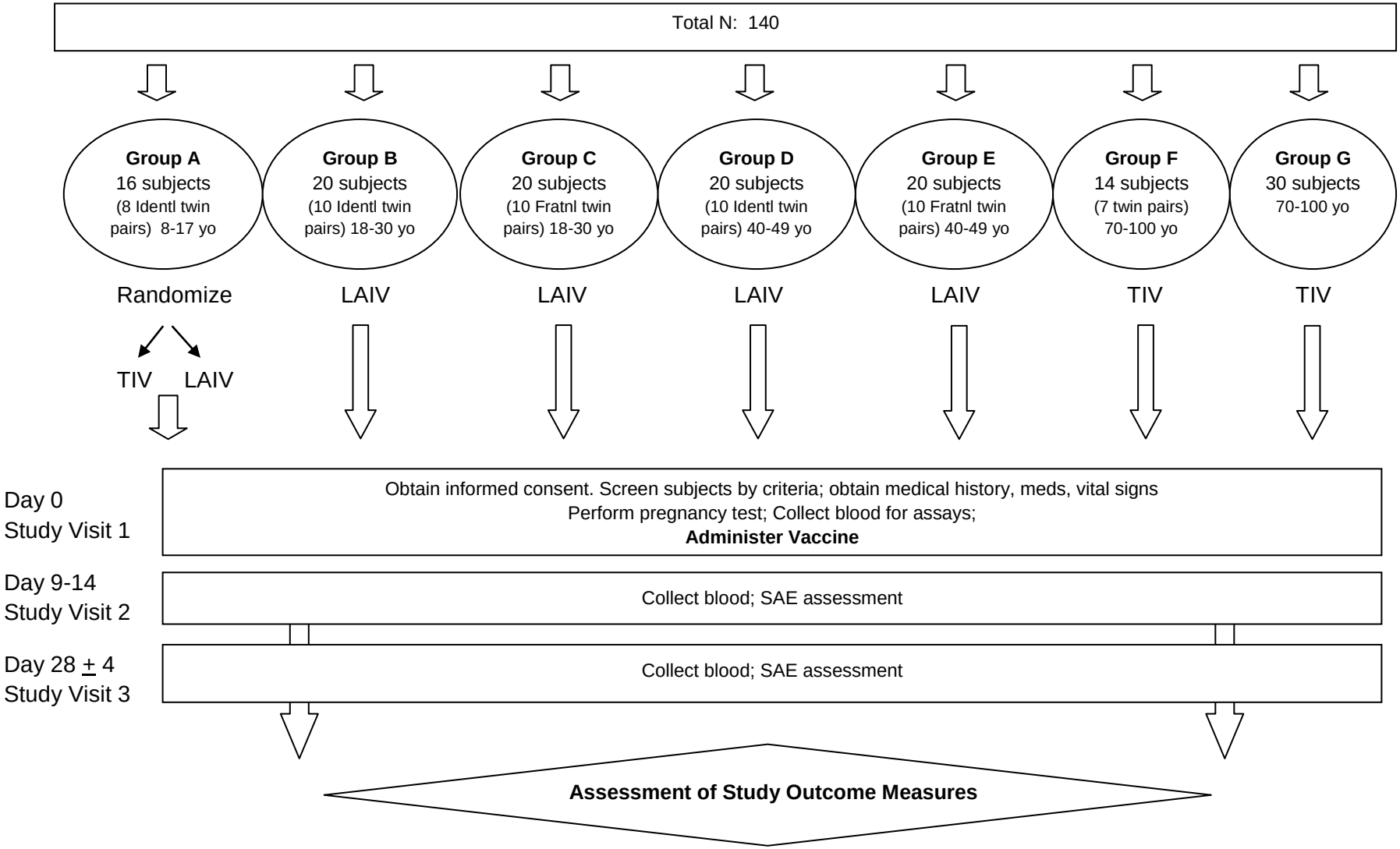
as a function of age and similarity in genetic and environmental background (by comparing differences between monozygotic and dizygotic twin pairs of different ages) for determining follow-up study design.

- Analyze the antibody and B-cell receptor repertoire in the same study subjects

Description of Study Design: This study will be conducted in healthy male and female volunteers. Group A of identical twins will be randomly assigned to receive a single administration of the 2009-2010 formulation of either TIV or LAIV (twins in a pair will receive different vaccines), Groups B-E twins all will receive LAIV, Groups F and G, elderly subjects, will all receive a single TIV immunization. Follow-up post-immunization will be for serious adverse events. Blood samples to conduct the assays described will be taken at pre-immunization, Days 9-14 and 28 post-immunization.

Estimated Time to Complete Enrollment: 3 months (September 1-November 30)

Schematic of Study Design:



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2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

The fundamental objective of the Stanford Cooperative Center for Translational Research on Human Immunology and Biodefense is to use the analysis of vaccine-induced and naturally acquired influenza A immunity as a model for defining adaptive and innate immune mechanisms and antimicrobial protection of the respiratory tract in children and younger and older adults. Influenza A was chosen as a model system relevant to biodefense because influenza A has significant potential to be modified genetically to produce a microbial agent of bioterrorism. Further, influenza A causes natural pandemics, which can incapacitate a large fraction of the population simultaneously, even in the absence of human modification, and may endanger defense preparedness. In either circumstance, influenza A has many characteristics of microbial pathogens that could become agents of civilian bioterrorism. Among these are its capacity to cause illness with high morbidity and mortality rates, potential for highly efficient person-to-person transmission, high infectivity by aerosol, resulting in the capacity to cause large outbreaks, potential to cause anxiety in the public and in health care workers, and potential to be weaponized.

For purposes of conveying the potential impact of introducing a novel influenza virus into the population, the table below illustrates the impact of the four influenza pandemics that occurred during the 20th century in the United States (1, 2). The 1918 influenza pandemic caused more than 500,000 deaths in the United States and an estimated 20 million or more deaths worldwide. Mortality rates were unusually high among young, otherwise healthy adults. The attack rate of 29% is the most severe pandemic ever recorded.

Year	Subtype	Excess mortality per 100,000	% Excess deaths in persons >65 years old
1918	H1N1 Spanish flu	529	99
1957	H2N2 Asian flu	39	36
1968	H1N1 Hong Kong flu	8.1	48
1977	H1N1 Russian flu	3.8	14

While influenza vaccines exist, the immunologic mechanisms by which protection is induced in the respiratory tract are poorly understood in the human host. Genetically altered influenza A viruses that express antigenically unique hemagglutinin (HA) and neuraminidase (NA) proteins have the capacity to infect the population in all age groups, regardless of antiviral immunity that was elicited by prior infection. While generally successful in healthy adults, influenza vaccination is often not successful in the very young or especially in older people, for reasons that are not well understood. This results in significant morbidity and mortality, especially in the elderly, where influenza infection is the 10th leading cause of death (3) , with as many as 36,000 fatalities a year in the US alone (4, 5). There is also the very real prospect of the H1N1 strain of flu mutating to a variant that could be spread between humans where the consequences could be catastrophic and in which young adults could be at risk, as they were in the 1918 pandemic (6), or that a recombinant influenza strain might be created and used for bioterrorism. In addition, the repeated failure of potential HIV vaccines and those against other serious diseases shows that vaccine development in general might be improved significantly if we understood more about how “successful” vaccines, like those for Influenza modulate the human immune system. Thus there is a vital need to understand how our standard influenza vaccines work and where they fail, why they do so.

During an influenza virus infection both innate and adaptive immune defenses are activated to eliminate the virus. Both arms of the adaptive immune system, the production of virus-specific antibodies and the induction of cytotoxic T cell responses contribute to virus elimination and protective immunity against influenza (7), (8). However, it is only by antiviral CD8^{+} cytotoxic T lymphocytes (CTL) that the immune system can terminate intracellular virus replication (9). Moreover, in a recent work, McElhaney et al. showed provocative evidence that, in the elderly, certain T-cell responses may be better correlates of vaccine protection than antibody titers (10). Thus, T-cell immunosenescence may be an important factor in the failure to make an adequate response to influenza infection in the elderly (11), (12).

Multiple alterations have also been observed in different components of the innate immune system such as neutrophils, macrophages, NK cells, and NKT cells (13), (14). However, the most dramatic changes of immunosenescence affect the adaptive arm of immunity (15), (16). A decrease in the proliferative capacity of B cells and diminished antibody potency have been observed in the elderly (17). Furthermore, decreased antibody production and a shortened period of protective immunity following immunization are also characteristic (18), (19).

One likely root cause of the T and B cell defects is likely the impaired production of new T cells due to the involution of the thymus (20). This leads to a decrease in circulating $CD95^-$ naïve T cells (21), (22) that are critical for the response to primary infection.

Concomitant with such reduction in naïve T cells, a reduced CTL activity has been observed in the elderly which seems to be linked to a shift from Th1 to Th2 cytokines including IL-10 (23). It is assumed that a decline in influenza-specific CTL activity with aging contributes to the decline in vaccine efficacy (24-26) and diminished protection against the influenza virus (27).

Other changes in T cells that are associated with age include reduced influenza-specific CTL proliferation *in vitro* (24, 26, 28), a reduced frequency of IFN-γ^+ influenza-specific memory T cells (26) and the accumulation of oligoclonal $CD28^-$ T cells (25) characterized by shortening telomeres and reduced proliferative capacity (29), which could be a consequence of chronic antigenic stimulation with CMV virus throughout life (30-32). Many of these deficiencies in older people may be the result of signaling defects and, in this regard, multiple alterations have been found in the early activation downstream pathways of T lymphocytes. These include defects in protein kinase pathways in freshly isolated $CD4^+$ T cells from older mice (33), (34), (35), decreases in MAP kinase activity in T cells in both mice (36) and humans (37) following activation. Declines in phosphorylation of the TCR-associated CD3ζ chains (38) as well as in calcium influx (39), (40) have also been reported. More recently, age-related changes have been described in the formation of immunological synapses involving T cells (41), including early cytoskeletal modifications that appear to impair the ability to discriminate agonist from antagonist peptides (42). There are also reports of changes in cell physiology with age such as modified glycosylation patterns of the CD43 and CD45 cell surface molecules (43). Changes in membrane lipid composition in the elderly have also been associated to a decline in T cell activity (44), (45). It has also been reported that the sequence diversity of T cell receptors in both young and elderly cohorts is impaired compared to healthy adults (46), (47), (48). In young children, this is likely the result of a T cell compartment that is still being populated whereas in older people it may be the long term result of thymic involution.

Thus, as discussed above there has been a great deal of work exploring markers and mechanisms which correlate with immune deficiencies associated with age, especially in the elderly. Many questions still remain, in particular, it is unclear how these different findings on immune impairment relate to one another and the definitive nature of T cell impairment with age is not yet clear. Currently, we have conducted two surveys of healthy adults receiving the standard TIV (killed vaccine) over the last two years in subjects aged 20-96 years. Volunteers had blood drawn prior to immunization, were vaccinated, and then had blood drawn on day 5-7 (the peak of T-cell responses) and on day 25-28 (for serum titers). Data collected include details of white blood cell subsets (16+) by flow cytometry, up to 42 serum cytokines, whole blood gene expression analysis, signaling characteristics using phosphoflow and proliferation assays. Further assays of T-cell specificity are planned on banked PBMCs. Our analysis reveals many changes between different age groups including phosphoflow, cell subset variations, gene expression and serum cytokines. Particularly striking is a significant drop in response to stimuli amongst the elderly, in particular for IL-6, IL-21, INFα and INFγ, as well as parabolic patterns in the percentage of many different cell subsets, including $\gamma\delta$ T cells, with a low point in ages 40-65 approximately. Also interesting and potentially significant is a link we have found between serum HAI titers to influenza and both a serum chemokine MDC/CCL22 and the expression of the AID gene in B cells, which is important in the affinity maturation of antibodies.

Previous work on influenza specific T cell responses using peptide-MHC tetramers has demonstrated a number of interesting effects, including differences in phenotype evoked in CD8+ cells with the TIV versus the LAIV vaccine (49) and an apparent ceiling in the number of CD4+ T cells (49). But these studies and those with the related ELISpot technology suffer from a number of limitations, namely that the number of specificities analyzed is usually quite limited; and yet, it is likely that many different T cells specificities are mobilized, as shown by the work of Kwok and colleagues with CD4+ T cells (50). In order to broaden the range of epitopes that can be surveyed, we have devised a simple method that involves staining cells with a set of tetramers each labeled with a different combination of dyes to distinguish one specificity from another. The number of specificities that can be measured depends on the number of different fluorescent dyes being used. As a proof of principal we have used four tetramer colors to discriminate between fifteen different specificities at once and we believe that it would be possible to use at least 100 (using seven colors and their combinations). Thus we should be able to look at many specificities as well as many functional attributes (e.g. patterns of cytokine expression, signaling pathways utilized) in a standard (10cc) blood sample.

The work of Jenkins and colleagues (51) showed that, for the first time, it was possible to at least visualize primary CD4+ T cells from mice that were specific to a foreign antigen. In that work, three different specificities were found to vary from 1 in 100,000 to 1 in 1,000,000 CD4+ cells. The most abundant species gave a proportionally larger

proliferative response and the depletion of a particular population in reconstitution experiments abrogated the T-cell response to that antigen. If this is true in humans, it has substantial implications for vaccine development, as it would mean that an important benchmark for a vaccine to stimulate a good T-cell response would be that a sizeable majority of the potential recipients should have pre-existing T-cell pools with the appropriate specificity, and the more there were, the better the response would be. Preliminary work in our lab analyzing naïve populations of $CD8^+$ cells specific for epitopes in HLA-A0201 individuals that had most likely never encountered those organisms. This initially has involved an HIV epitope (SL9) in HIV seronegative individuals and an avian flu epitope (52). A modified version of the Moon et al. approach succeeded in the enumeration of naïve $CD8^+$ T-cell cohorts to both peptide-MHC complexes in four individuals with frequencies ranging from 1 in 100,000 to 1 in 1,000,000. This is similar to the mouse results with $CD4^+$ epitopes but differs significantly from recent work by LeFrancois and colleagues who have examined the naive $CD8^+$ T cell repertoire in mice and found them to be significantly (~5-10X) more abundant (53). Thus, humans may have a somewhat larger $CD8^+$ T-cell repertoire than mice. In most cases the vast majority of these T cells in our study had a naïve cell surface marker phenotype, ($CD45RA\text{-}CD62L^{hi}$) indicating that they are truly naïve.

Current technologies to measure antibody or T cell receptor diversity are limited in their throughput and provide only a small piece of the true picture for B or T cell specialization development. As a result, the conclusion drawn on such a small data set may not reflect the true underlying mechanism (54-58). It is undoubtedly advantageous to analyze antibody genes and T-cell receptor genes in all B- and T-cell subpopulations from each donor so that one can assess the "clonality" of antigen specific cells, responses of different populations of B and T cells to a particular antigen, and the effects of overall B- and T-cell repertoire on a specific immune response in a much more comprehensive manner. Despite the tremendous success of whole genome sequencing and expression analysis, these technologies have not yet made significant inroads into problems of antigen receptor diversity and B- and T-cell repertoires. It will be extremely valuable to our understanding of these problems if we can develop technologies to measure the changes in the human "immunome" as human subjects are challenged with influenza vaccine.

Feasibility of recruiting and retaining twin volunteer subjects

These studies will enroll healthy male and female subjects in various age groups according to the Schematic of Study Design shown above. Except for Group G, these subjects will be largely drawn from the SRI Northern California Twin Registry (NCTR). The NCTR was created by SRI in 1995 to fill the need for a pool of twins for genetic studies of pharmacokinetics and pharmacodynamics. In 1996, an extensive advertising campaign was conducted and included advertising in 19 newspapers, San Francisco Bay Area-wide movie theaters, and AM/FM radio stations. Within 2.5 years, this

campaign resulted in the enrollment of a total of 1,054 twins. In addition to the advertising campaign, a five-year NCTR anniversary celebration party was held in July 2000, which increased enrollment to 1,765 individual twins. Ongoing recruitment efforts include print advertisements and outreach to local twin organizations. Referrals to the NCTR by registered twins or friends and family is encouraged and to date has resulted in a 4% increase in membership. SRI International is now specifically targeting dizygotic (DZ) twin pairs as these are somewhat underrepresented in the overall registry. Contact is maintained with twins in the NCTR via annual newsletters and birthday cards. SRI maintains a NCTR website (http://www.sri.com/policy/twin/), email address (sri.twin@sri.com), and a toll-free twin line (1-800-SRI-TWIN [1-800-774-8946]). Currently, there are well over 2000 local and non-local adult twins registered with the NCTR. The Table below provides a description of the demographic characteristics of Registry twins living within 50 miles of Stanford University.

Demographic characteristics of twins in the SRI NCTR.

Age	Zygosity		Gender			Race/Ethnicity				
	Fraternal	Identical	Male	Female	Mixed	Caucasian	African-American	Asian	Hispanic	Other
8-17	57%	43%	29%	54%	17%	73%	9%	3%	9%	6%
18-70	33%	67%	30%	60%	10%	79%	4%	5%	7%	5%

In parallel to ongoing recruitment of twins to the NCTR, SRI's Center for Health Sciences will develop a multi-media advertising campaign that will be specific to the proposed study. The advertisements will focus on increasing the pool of children 8-17 years, local fraternal twins, twins of diverse race/ethnicity, and male twins. The SRI research team will utilize the experience and knowledge of the SRI corporate communications group to develop a campaign to further develop a sample of twins representative of the SF Bay area. In addition to advertisements, SRI will develop an outreach program to identify desired twins. This will involve working with community colleges, medical clinics, church groups, local twin organizations, and racially/ethnically diverse communities. An example of SRI's outreach efforts includes a recently formed alliance with seven local community colleges for the recruitment of volunteers to an ongoing NIH-funded grant (DA016427, G. Swan, PI). College staff members actively recruit volunteers and post study flyers and brochures throughout campus. Based on previous recruitment success, we expect the Registry to contain >3000 twins in the near future.

2.2 Rationale

This is an exploratory study to investigate markers and mechanisms and define general predictor correlates with immunological health. This goal is analogous to what has been achieved in cardiovascular medicine where the levels of different forms of cholesterol have provided useful benchmarks for cardiovascular health. In this context vaccination with approved influenza strain formulations represents a safe and accessible opportunity to gauge the immune response in a particular individual as a function of age and genetics and then to try to find predictive biomarkers.

Our particular focus here is on the difference in immune responsiveness between young healthy adults and older cohorts where one can expect a high frequency of inadequate responsiveness. There is already a large body of work on the effects of aging on the immune system, the most severe manifestation being known as immunosenescence, which impairs responses to infectious diseases. Influenza, Respiratory Syncytial Virus, and pneumonia are all major killers of older people because of their generally weaker immune systems.

The experiments here follow-up on our current study of aging and responsiveness to the TIV vaccine with a similar effort largely centered around the LAIV vaccine. As the peak of T-cell response post vaccination varies for the two vaccines (5-7 days post vaccination for TIV, 10 or more for LAIV) we have chosen to schedule Visit 2 (the first post vaccination point) at an interimediate time of 9-14 days post vaccination. Because the LAIV vaccine is only approved for subjects from 2-49 years of age, we cannot assess the responses of the most elderly subjects, but since there may already be some decline in immunity starting around age 40, we will compare adults of 18--30 years old (Group B-C) with those that are 40-49 years of age (Group D-E). By drawing on the large cohorts of monozygotic and dizygotic twins, we will be able to address a number of important questions simultaneously:

1. **How do white blood cell subsets, serum cytokines, whole blood gene expression, and T cell signaling responses change with age and to what degree is this genetically determined?** Does this change between early adulthood and late middle age, reflecting divergent environmental and/or infectious disease histories? Unique features of this study are that: (1) we are able to survey a very large number of traits/biomarkers for each subject across multiple visits, (2) with the twin pairs determining (to a first approximation) which traits are genetically determined versus those that are a function of the environment.

2. **Is the size of the precursor pool an accurate prediction of the subsequent response?** Here, by enumerating the pool of strain specific peptide-MHC staining cells before and after immunization, we can gauge the degree of

amplification. If this hypothesis is true, it could be a considerable aid to vaccine design in many situations including avian flu and other infectious diseases.

3. **Are there changes in the effective T cell repertoire of an influenza vaccine response with age?** Indirect (TCR sequence) evidence suggests that younger and older people generally have a reduced TCR repertoire (51), but this has not been shown at a functional level. With the ability to sample large numbers of epitopes at once in different age groups receiving the same vaccine it should be very apparent whether there is such a difference.

4. **Is there a correlation between the repertoire used and a robust/efficacious response?** It has been suggested in other infectious disease systems that a broader T-cell response to a pathogen is more protective. Here, we will correlate the breadth of the T-cell response to serological and other measures of responsiveness to the vaccines.

5. **Is there a correlation between the cytokine expression pattern or the signaling pathways activated and a robust/efficacious response?** It has been suggested that a broad cytokine response correlates with good vaccine efficacy. Here, we will look at the 25 different cytokines that T cells can make in the context of an antigen-specific T-cell response. We will also couple our peptide-MHC tetramer capabilities with analysis of signaling pathways by phosphflow to determine whether there are variations in the signaling capacities of influenza epitope specific T cells as a function of age, genetics, or the robustness or lack thereof of the response.

The five year nature of our subject accrual plan and its focus on each flu season means that we can use each succeeding year as an opportunity to test fewer and fewer potential markers and thus achieve significance, despite the large number of measurements made. All study numbers we list here as well as the statistical analysis made is based on the number of subjects for the first year. We have also powered the study of the young and older adult twin cohorts (ages 20-30 years, and 40-49 years) fairly robustly (50 monozygotic twin pairs, 50 dizygotic pairs for each age group) to give at least a preliminary determination about whether or not a given trait is genetically determined. With the twin cohorts particularly, this approach will allow us to ask the following important questions:

1. **What are the effects of genetic identity on the T cell repertoire?** By immunizing monozygotic (Group A, B, D) versus dizygotic twin pairs (Group C,E), we can assess the effect of genotype on the resulting T-cell response.

2. **How does exposure to different vaccine modalities influence the evolution of a particular response in monozygotic versus dizygotic twins?** It would be important to know whether or not the two vaccine types (LAIV or TIV) are distinguishable in their ability to trigger naïve, strain-specific T-cell cohorts and that, once stimulated, there was any difference in what cytokines or signaling pathways were induced. Particularly in the case of CD8+ T cells, there could be marked differences between activation through "cross-priming" (TIV) versus activation through direct contact with virally infected cells (LAIV).

3. **How do different vaccine types (TIV vs LAIV) influence the repertoire of responding B cells?** By immunizing the different individuals in monozygotic twin pairs (Group A) with a different vaccine type (LAIV or TIV), we can assess the effect of the vaccine type on the B-cell repertoire independently of genetics.

4. **How does genetic identity influence the size, TCR composition and evolution of a particular B-cell response?** Here, we will analyze identical twin pairs immunized with the same influenza vaccine (TIV or LAIV) and gauge the effects on strain-specific epitopes that are naïve in both twins but present in the vaccine, for their proliferative ability in vivo, their ability to make different cytokines and their TCR diversity (by single cell PCR of TCR V regions).

2.3 Potential Risks and Benefits

2.3.1 Potential Risks

This protocol will immunize children and adult subjects with either TIV or LAIV that is licensed for use in the age groups studied. The discomforts of this study are those of having blood drawn from an arm vein, IM injection or intranasal application of the vaccine, and possible reactions to the vaccine. Drawing blood causes transient discomfort and may cause fainting. Infection at the site where blood will be drawn or where the vaccination is given is extremely unlikely, but is a potential risk. Bruising at the site of blood drawing may occur, but can be prevented or lessened by applying pressure for several minutes immediately after the blood draw. Intramuscular injection may cause injection site pain, swelling, and redness. Intranasal administration may be associated with nasal congestion. Immediate allergic reactions to vaccine, including anaphylaxis, are in general extremely rare (approximately 1 person in 4,000,000), and might occur as a skin rash such as hives, difficulty breathing, fainting, drop in the blood pressure and death. Such reactions can usually be stopped by emergency medications administered by study personnel. Vaccine recipients may develop systemic reactions such as fever, headaches, body aches, and fatigue. These reactions are usually greatest within the

first 24 to 72 hours after vaccination and last 1 to 2 days. Analgesics (*e.g.*, aspirin or Tylenol®) and rest will generally relieve or moderate these symptoms. Other hypersensitivity reactions, including Arthus reactions resulting in large local swelling reactions, are also possible. Although Guillain-Barré syndrome may have been associated with the 1976-77 inactivated swine influenza vaccine and TIV vaccines used in early 1990's, subsequent inactivated vaccines have not been associated with an increased risk of this condition. The attenuated live virus vaccine has the potential for transmission of influenza to close contacts with severely weakened immune systems (requiring care in a protected environment, such as a bone marrow transplant unit). People in close contact with those whose immune systems are less severely weakened (including those with HIV) may receive LAIV.

2.3.2 Known Potential Benefits

Subjects given influenza vaccines are likely to experience decreased frequency and severity of subsequent influenza infection. The beneficial role of influenza vaccination in the elderly and healthy young adults has been recognized increasingly over the past several years as more information has become available about the high rate of morbidity and mortality from this respiratory pathogen.

3 OBJECTIVES

3.1 Study Objectives

Primary:

- Compare lymphocyte responses at Days 9-14 and the lymphocyte and serology responses at Day 28 post-immunization following annual administration of the influenza vaccines
- Evaluate changes in cytokine profile in the immune response (CD4, CD8, $\gamma\delta$ cells, B cell, responses) from Day 0 to Day 9-14 for T cells and antibody-secreting cells (ASCs)
- Evaluate changes in cytokine profile in the immune response from Day 0 to Day 28 for HAI responses to the vaccine antigens
- Evaluate any potential gene expression signatures either at Day 0 or during the early response period Day 9-14 that are associated with immune senescence, potentially providing insight into mechanism
- Compare lymphocyte and monocyte reactivity to activating stimuli at Days 0 and 9-14 with the lymphocyte and serology responses at Day 28 post-immunization following administration of seasonal trivalent inactivated influenza vaccine
- Identify age-specific biomarkers or clusters of markers in general and especially those that are predictive of immune sufficiency or insufficiency

Secondary:

- Compare differences in response between live attenuated vaccine (LAIV) and killed vaccine (TIV)
- Develop comprehensive signaling maps downstream of immune stimulation in response to immune perturbations using single cell phospho-signaling and cell interrogation approaches
- Determine signaling-based correlates of influenza A immunity in children, adults and elderly subjects
- Evaluate the variation in immune response (as measured by all study assays) between individuals and assess whether it changes as a function of age and similarity in genetic and

environmental background (by comparing differences between monozygotic and dizygotic twin pairs of different ages) for determining follow up study design

- To analyze the antibody and B-cell receptor repertoire in the same study subjects

3.2 Study Outcome Measures

3.2.1 Primary Outcome Measures

1. Serum antibody assay: HAI titer
2. Abundance levels of cell subsets, serum cytokines, and mRNA transcripts in blood.
3. Single cell phosphoprotein abundance changes in response to immune perturbations.
4. Quantitation of the size of the antigen specific T cell response by flow cytometry.
5. Affinity of recombinant monoclonal antibodies to various influenza strains.
6. T-cell receptor repertoire analysis: gene segment usage preference, sequence abundance distribution and possible polymorphism on T cell receptor genes.
7. T cell receptor repertoire to various influenza strains.

3.2.2 Secondary Outcome Measures

1. Antibody repertoire analysis: gene segment usage preference, sequence abundance distribution and possible polymorphism on B cell receptor genes.

4 STUDY DESIGN

This is a Phase IV study of healthy children and adults who are given either TIV or LAIV. There are no exclusions for gender, ethnicity or race. Following confirmation of written informed consent, baseline blood samples will be drawn from all study subjects prior to immunization. Group A of identical twins will be randomly assigned to receive a single administration of the 2009-2010 formulation of either TIV or LAIV (twins in a pair will receive different vaccines), Groups B-E twins all will receive LAIV, Groups F and G, elderly subjects, will all receive a single TIV immunization. All subjects will receive a single dose of their assigned influenza vaccine, either by IM injection (TIV) or intranasal application (LAIV). Two subsequent study visits at 9-14 days and at 24-32 days will consist of monitoring for SAEs and drawing blood for requested assays.

5 STUDY ENROLLMENT AND WITHDRAWAL

5.1 Subject Inclusion Criteria

1. Otherwise healthy, ambulatory children or adults, ages 8-17 years (identical twin pairs), 18-30 years (identical or fraternal twin pairs), 40-49 years (identical or fraternal twin pairs) or 70-100 years (twin or non-twin adults).
2. Willing to complete the informed consent process.
3. Availability for follow-up for the planned duration of the study at least 28 days after immunization.
4. Acceptable medical history and vital signs.
5. Negative urine pregnancy test for women of childbearing potential
6. If the subject is female and of childbearing potential, she must use an acceptable method of contraception and not become pregnant for the duration of the study. (Acceptable contraception includes implants, injectables, combined oral contraceptives, effective intrauterine devices (IUDs), sexual abstinence, or a vasectomized partner).

5.2 Subject Exclusion Criteria

1. Prior vaccination with TIV or LAIV in Fall 2009
2. Allergy to egg or egg products, or to vaccine components, including gentamicin, gelatin, arginine or MSG (for LAIV only), or thimerosal (TIV multidose vials only).
3. Life-threatening reactions to previous influenza vaccinations
4. Asthma (LAIV groups only)
5. Active systemic or serious concurrent illness, including febrile illness on the day of vaccination
6. History of immunodeficiency
7. Known or suspected impairment of immunologic function, including, but not limited to, clinically significant liver disease, diabetes mellitus treated with insulin, moderate to severe renal disease, blood pressure >150/95 at screening, or any other chronic disorder which, in the opinion of the investigator, might jeopardize volunteer safety or compliance with the protocol.
8. Hospitalization in the past year for congestive heart failure or emphysema.
9. Chronic Hepatitis B or C.

10. Recent or current use of immunosuppressive medication, including glucocorticoids (corticosteroid nasal sprays are permissible).
11. Subjects in close contact with anyone who has a severely weakened immune system should not receive LAIV.
12. Malignancy, other than squamous cell or basal cell skin cancer (includes solid tumors such as breast cancer or prostate cancer with recurrence in the past year, and any hematologic cancer such as leukemia).
13. Autoimmune disease (including rheumatoid arthritis treated with immunosuppressive medication such as Plaquenil, methotrexate, prednisone, Enbrel) which, in the opinion of the investigator, might jeopardize volunteer safety or compliance with the protocol.
14. History of blood dyscrasias, renal disease, or hemoglobinopathies requiring regular medical follow up or hospitalization during the preceding year
15. Use of any anti-coagulation medication such as Coumadin or Lovenox, or anti-platelet agents such as aspirin, Plavix, Aggrenox may be acceptable after review by investigator
16. Receipt of blood or blood products within the past 6 months
17. Medical or psychiatric condition or occupational responsibilities that preclude subject compliance with the protocol
18. Inactivated vaccine 14 days prior to vaccination
19. Live, attenuated vaccine within 60 days of vaccination
20. History of Guillain–Barré Syndrome
21. Pregnant or lactating woman
22. Use of investigational agents within 30 days prior to enrollment
23. Donation of the equivalent of a unit of blood within 6 weeks prior to enrollment
24. Any condition which, in the opinion of the investigator, might interfere with volunteer safety, study objectives or the ability of the participant to understand or comply with the study protocol.

5.3 Treatment Assignment Procedures

5.3.1 Randomization Procedures

Individuals within each pair of identical twins will be randomized such that one receives TIV and the other LAIV. Randomization will follow a permuted block design, where each

twin pair is a block. Random assignment within each pair will be done by computer. Using these computer assignments, a set of envelopes will be created, one for each twin pair. Each envelope will contain two numbered slips of paper, labeled either 1. TIV and 2. LAIV or 1. LAIV and 2. TIV. The member of each twin pair of earlier birth time will be receive the number 1 vaccine type within a block and the other member will receive the number 2 vaccine type.

5.3.2 Reasons for Withdrawal

A study subject will not receive the study product if a clinical intercurrent illness, or other medical condition or situation occurs that meets the exclusion criteria. Subject will be withdrawn from the study if participation in the study would not be in the best interest of the subject, or if doing so would harm the subject in the opinion of the investigator. Subject may withdraw from the study voluntarily.

5.3.3 Handling of Withdrawals

If, for safety reasons a subject is deemed by the investigators to not be eligible to receive the study product as per protocol, he/she will be terminated from the study. If a subject voluntarily withdraws from the study or is withdrawn by the investigator after immunization, the subject will be followed for safety until Day 28 post-immunization or until any serious adverse events are resolved.

5.3.4 Termination of Study

The study may be terminated for administrative reasons or other unanticipated circumstances.

6 STUDY INTERVENTION/INVESTIGATIONAL PRODUCT

6.1 Study Product Description

6.1.1 Acquisition

The study product will be shipped from the manufacturer to the investigational pharmacy at the study site. The TIV will be supplied by Sanofi-Aventis as Fluzone (2009-2010 formulation). The LAIV will be supplied by MedImmune as FluMist (2009-2010 formulation).

6.1.2 Formulation, Packaging, and Labeling

TIV. Fluzone® (Influenza Virus Vaccine) Suspension for Intramuscular Injection 2009-2010 Formula: Each 0.5 mL dose will contains a total of 45 µg of influenza virus hemagglutinin of each of the 3 strains selected for the 2009-2010 formulation in a prefilled, single dose syringe, 0.5 mL, no preservative.

LAIV. FluMist® Influenza Virus Vaccine Live, Intranasal Spray 2009-2010 Formula: Each 0.2 mL dose contains 106.5-7.5 FFU (fluorescent focus units) of live attenuated influenza virus reassortants of each of the three strains for the 2009-2010 season in a 0.2 mL pre-filled, single-use intranasal spray (0.1 mL per nostril.).

The 2009–2010 trivalent influenza vaccines will contain A/Brisbane/59/2007 (H1N1)-like, A/Brisbane/10/2007 (H3N2)-like, and B/Brisbane/60/2008-like antigens. Compared to the 2008-09 Northern Hemisphere influenza vaccines, only the B strain has changed.

6.1.3 Product Storage and Stability

Fluzone vaccine presentations should be refrigerated at 2° to 8°C (35° to 46°F).. Vaccine that has been frozen will be discarded. Between uses, multi-dose vials will be returned to the recommended storage conditions at 2° to 8°C (35° to 46°F).

FluMist should be stored in a refrigerator between 2-8°C (35-46°F) upon receipt and until use. The product must be used before the expiration date on the sprayer label and must not be frozen. The cold chain (2 to 8°C) must be maintained when transporting FluMist. Once FluMist has been administered, the sprayer should be disposed of according to the standard procedures for medical waste (e.g., sharps container or biohazard container).

6.2 Dosage, Preparation and Administration of Study Intervention/Investigational Product

Previously unvaccinated children through 8 years of age should receive two doses of either influenza vaccine, one on day 1 followed by another dose at least one month later.

TIV: Dosage, 36 months-adults, 0.5 mL. Vaccine will be administered as a 0.5 mL dose, with a sterile, disposable syringe and needle by IM injection into the deltoid muscle. The subject will choose whether the injection will be administered into the right or left deltoid.

LAIV: dosage, 2-49 years of age, is 0.2 mL. Vaccine will be administered as an intranasal spray. Each sprayer contains a single dose of FluMist; approximately one-half of the contents should be administered into each nostril. 0.1 mL (i.e., half of the dose from a single FluMist sprayer) is administered into each nostril while the recipient is in an upright position. Insert the tip of the sprayer just inside the nose and rapidly depress the plunger until the dose-divider clip stops the plunger. The dose divider clip is removed from the sprayer to administer the second half of the dose (0.1 mL) into the other nostril.

6.3 Accountability Procedures for the Study Intervention/Investigational Product(s)

The study article will be shipped by the manufacturer to the investigational pharmacy at the study site. Use and disposition of each unit of vaccine will be appropriately documented in accordance with ICH GCP. Unused products will be returned to the investigational pharmacy.

6.4 Concomitant Medications

At each study visit, the subject will be questioned about any concomitant medication use and the information will be recorded. Medication history will include currently taken prescription and over-the-counter medications. Subject will be ineligible if taking insulin or glucocorticoids other than corticosteroid nasal sprays, or have received an inactivated vaccine within 14 days or live, attenuated vaccine within 60 days. The investigator should be consulted regarding eligibility if the subject is taking medications for treatment of autoimmune disease (such as Plaquenil, methotrexate, prednisone, Enbrel), anti-coagulation medications such as Coumadin, Lovenox, aspirin, Plavix, Aggrenox, or any antiviral or psychiatric medications which might indicate a condition that precludes subject compliance with the protocol.

7 STUDY SCHEDULE

7.1 Screening

Subjects will be contacted by phone prior to the first study visit. Study staff will briefly review the eligibility criteria, subject availability and the study procedures. If the subject is eligible and interested, study visits will be scheduled.

7.2 Study Visit 1 (Day 0) Enrollment/Baseline

- Potential subjects will be provided with a verbal description of the study (purpose and study schedule and procedures). They then will be asked for any questions and to read/sign the consent form. The consent form will be signed prior to the performance of any study procedures.
- The study staff will discuss with the subject his/her medical history, study eligibility criteria and concomitant medication use.
- A urine pregnancy test will be performed if indicated and negative result confirmed prior to vaccination.
- Obtain vital signs (oral temperature, blood pressure, pulse, respiratory rate), height and weight.
- The following blood volume samples will be collected:
    - From adults ages 18-49 years,obtain a 4.5 mL blood sample from an arm vein into a serum separator (gold top) tube, a 2.5 mL sample into a PaxGene tube, a 1-2 mL sample into a microtainer for CBC with differential and a 90 mL sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 100 mL.
    - From children weighing more than 100 lbs and from elderly adults ages 70-100, obtain a 7 mL blood sample from an arm vein into a serum separator tube (gold top) , a 2.5 mL sample into a PaxGene tube, a 1-2 mL sample into a microtainer for CBC with differential and a 54 mL blood sample in heparin (green top) tubes prior to vaccination. Total volume not to exceed 65 mL.
    - From children weighing 66-100 lbs, obtain a 3 mL blood sample from an arm vein into a serum separator (gold top) tube, a 2.5 mL sample into a PaxGene tube, a 1 mL sample into a microtainer for CBC with differential, and a 20 mL sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 30 mL.

- From children weighing 61-65 lbs, obtain a 3 mL blood sample from an arm vein into a serum separator (gold top) tube, a 2.5 mL sample into a PaxGene tube, a 1 mL sample into a microtainer for CBC with differential, and an 18 mL sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 25 mL.
- From children weighing 41-60 lbs, obtain a blood sample from an arm vein for a 3 mL sample into a serum separator (gold top) tube, a 2.5 mL sample into a PaxGene tube, 1 mL sample into a microtainer for CBC with differential, and a 12 mL sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 20 mL.

- Subjects will be vaccinated in the deltoid muscle with TIV or by intranasal spray of LAIV and observed in clinic for 30 minutes for any immediate serious adverse events. Study questionnaires will be completed during the observation period.
- Subsequent study visits will be scheduled.
- Memory aids will be given with written and oral instructions for collection of information for serious adverse events. The memory aid is a simple diary for the volunteer to record a change in health status between study visits.

7.3 Follow-up Study Visit 2 (Day 9-14 after immunization)

- Review medications and memory aid for serious adverse events
- The following blood volume samples will be collected:
  - From adults ages18-49 years, obtain a 4.5 mL blood sample from an arm vein into a serum separator (gold top) tube, a 2.5 mL sample into a PaxGene tube, a 1-2 mL sample into a microtainer for CBC with differential and a 90 mL sample into heparin (green top) tubes. Total volume not to exceed 100 mL.
  - From children weighing more than 100 lbs and from elderly adults ages 70-100, obtain a 7 mL blood sample from an arm vein into a serum separator tube (gold top) , a 2.5 mL sample into a PaxGene tube, a 1-2 mL sample into a microtainer for CBC with differential and a 54 mL blood sample in heparin (green top) tubes. Total volume not to exceed 65 mL.
  - From children weighing 66-100 lbs, obtain a 3 mL blood sample from an arm vein into a serum separator (gold top) tube, a 2.5 mL sample into a PaxGene tube, a 1 mL sample for CBC with differential, and a 20 mL sample into heparin (green top) tubes. Total volume not to exceed 30 mL.
  - From children weighing 61-65 lbs, obtain obtain a 3 mL blood sample from an arm vein into a serum separator (gold top) tube, a 2.5 mL sample into a

PaxGene tube, a 1 mL sample for CBC with differential, and a 18 mL sample into heparin (green top) tubes. Total volume not to exceed 25 mL.

- From children weighing 41-60 lbs, obtain a blood sample from an arm vein for a 3 mL sample into a serum separator (gold top) tube, a 2.5 mL sample into a PaxGene tube, a 1 mL sample for CBC with differential, and a 12 mL sample into heparin (green top) tubes. Total volume not to exceed 20 mL.

7.4 Final Study Visit 3 (Day 24-32 after immunization)

- Review medications and memory aid for serious adverse events
- Children 8 years of age who have not previously been immunized against seasonal influenza prior to enrollment in the study will receive a second dose of vaccine (the same vaccine given at Visit 1) at least 28 days after the initial immunization.
- The following blood volume samples will be collected:
  - From adults ages 18-49, obtain a 4.5 mL blood sample from an arm vein into a serum separator (gold top) tube, a 1-2 mL sample into a microtainer for CBC with differential and 90 mL sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 100 mL.
    - From children weighing more than 100 lbs andfrom elderly adults ages 70-100, obtain a 7 mL blood sample from an arm vein into a serum separator tube (gold top) , a 1-2 mL sample into a microtainer for CBC with differential and a 54 mL blood sample in heparin (green top) tubes prior to vaccination. Total volume 65 mL.
  - From children weighing 66-100 lbs, obtain a 3 mL blood sample from an arm vein into a serum separator (gold top) tube, a 1 mL sample into a microtainer for CBC with differential and a 24 mL sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 30 mL.
  - From children weighing 61-65 lbs, obtain a 3 mL blood sample from an arm vein into a serum separator (gold top) tube, a 1 mL sample into a microtainer for CBC with differential, and a 18 mL sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 25 mL.
  - From children weighing 41-60 lbs, obtain a blood sample from an arm vein for a 3 mL sample into a serum separator (gold top) tube, a 1 mL sample into a microtainer for CBC with differential, and a 16 mL blood sample into heparin (green top) tubes prior to vaccination. Total volume not to exceed 20 mL.
- Children 8 years of age who have not previously been immunized against seasonal influenza prior to enrollment in the study will receive a second dose of vaccine (the

same vaccine given at Visit 1) at least 28 days after the initial immunization. Blood draw will be obtained prior to vaccination.

7.5 Early Termination Visit

If a volunteer is terminated from the study early, every effort should be made to perform the following procedures:

- Review current health status and note any changes since the last visit. Solicit information regarding SAEs and record all concomitant medications. Any ongoing related AEs will be followed to resolution or until a stable chronic condition has been established. Volunteers will be encouraged to permit continued follow-up of AEs if necessary

- Obtain remaining blood samples, if possible

8 STUDY PROCEDURES/EVALUATIONS

8.1 Clinical Evaluations

Clinical Evaluation will be obtained by interview with a research nurse, and will include:

- Medical history including influenza immunization history, medical problems, hospitalizations and allergies.
- Medication history of currently taken prescription and over-the-counter medications. Subject will be ineligible if they are taking insulin or glucocorticoids other than corticosteroid nasal sprays, or have received an inactivated vaccine within 14 days or live, attenuated vaccine within 60 days. The investigator should be consulted regarding eligibility if the subject is taking medications for treatment of autoimmune disease (such as Plaquenil, methotrexate, prednisone, Enbrel), anti-coagulation medications such as Coumadin, Lovenox, aspirin, Plavix, Aggrenox, or any antiviral or psychiatric medications which might indicate a condition the precludes subject compliance with the protocol.
- Review of memory aid for serious adverse events occurring during the 28 day study period.

8.2 Laboratory Evaluations

8.2.1 Clinical Laboratory Evaluations

- Hematology: Complete blood count (CBC) with differential count (1-2 mL blood).

8.2.2 Special Assays or Procedures

1. **Subject Analysis** – We will use fluorescence activated cell sorting to determine the precise number of each white blood cell type in a given patient sample and their activation state (B cells, T cells, NK cells, $\gamma\delta$ T cells, monocytes, dendritic cells).

2. **Gene Expression Analysis of Whole Blood** – We will collect part of the blood sample in a PAX gene tube and prepare probes from the mRNA for gene expression analysis using Agilent microarrays that survey global gene expression. This will tell us if there is a particular “gene expression signature” that correlates with immune senescence.

3. **Serum Cytokine Analysis** – As there have been reports of changes in the serum cytokine repertoire in older people, we will survey serum samples for 26 different cytokines using the Panomics/Luminex system now running the Human Immune Monitoring Center at Stanford.

4. **Responses to Cytokine Stimulation** – We will stimulate PBMCs with various cytokines and will analyze the phosphorylation status of different STATs and other key mediators of cellular activation in B cells, T cells and monocytes by flow cytometry.

5. **Specific Responses to the Influenza vaccine** – To study specific immune responses, T cells will be stimulated ex vivo with live virus, and GrzB and other cytokine activites will be measured. This sensitive method, can detect slight differences in the immune response to the Influenza virus in older people vs. their younger counterparts. Proliferation assays will also be conducted to address differences in the proliferative potential of multiple cell subsets from the different age groups after stimulation with TIV.

6. **HAI Antibody Titer** – The standard HAI antibody assay will be run on samples at baseline and at Day 28 to evaluate response to vaccination.

7. **Gene expression analysis of PBMCs** – We will process blood to obtain PBMC, from which we will prepare probes from the mRNA for gene expression analysis using Illumina microarrays that survey global gene expression. This will provide a finer resolution of data for adaptive immune response gene expression different from what we will obtain from whole blood (#2). Coupled with phenotyping data, we will be able to obtain cell specific gene expression signatures for each cell type we analyze.

8. **T cell isolation:** We will analyze total T-cell populations, as well as subpopulations of naïve T-cells (on day 0 and day 24-32), memory T-cells (day 0 and day 24-32) and activated T cells (day 9-14). Using cell surface markers and multiplex peptide MHC tetramer binding (Newell EW, Klein OL, Wong Y, and Davis MM. Nature Methods, in press) the differentiation state and antigen specificity of individual T cells will be determined. This will allow for the collection of different populations of T cells by fluorescence activated cell sorting.

9. **Combinatorial tetramer analysis of various influenza T cell specificities**. CD8+ and/or CD4+ T cells will be enriched using Rosette-Sep (Stem Cell Technologies) and stained with prepared combinatorially labeled pMHC tetramer reagents (made in house). After staining with these tetramers, the cells will be stained with additional phenotypic cell surface on intracellular markers before analysis using a flow-cytometer.

10. **High-throughput dynamic array chip assay for VJC combination and 454 sequencing of antibody and T cell receptor repertoire**. In preparation for deep sequencing of antibody heavy chain and B cell receptor genes, mRNA will be isolated and reverse transcripted and amplified. This amplified material will serve as the input for 454 GS sequencer to do the high-throughput sequencing as well as Fluidigm dynamic array chip for coarse analysis of VJC combination. The fraction of cell lysate that contains DNA will be banked in -80°C for future genetic comparison if needed.

    B cell-containing rosettes in the T-cell isolation step will be collected for deep sequencing of antibody repertoire VJC gene segment combinatorial usage using dynamic array chips from the same subject. The same library preparation procedure will be applied to the B cells.

11. Genotyping for Zygosity: To verify zygosity, we will use an Applied Biosystems AmpFLSTR Identifiler PCR kit for 15 tetranucleotide repeat markers and a gender-determining marker. Two laboratory personnel will independently determine the final zygosity calls. Any discrepancy will be reconciled by a blind retest conducted by two additional laboratory personnel.

8.2.3 Specimen Preparation, Handling, and Shipping

Serum: Gold-top tube for HAI antibody determination and Luminex assays per weight guidelines in section 7.

Procedural recommendations:

- Slowly invert tubes 8-10 times immediately after collection
- Store upright at room temperature until centrifugation
- Centrifuge within 2 hrs of collection at 1100 RCF for 15 minutes at room temperature
- Aliquot and freeze at -20C within 2 hrs of collection, and transfer to -80C within 2 weeks

PBMCs: Heparinized tubes (see section 7 for lower pediatric blood volume instructions) for flow cytometric analysis of PBMC subsets and phospho-proteins, stimulation assays (cytokine production and gene expression), MHC tetramer arrays. Tilt tubes immediately after filling and deliver to Davis lab. At the time of T-cell purification in the Davis lab, B-cells will be made available to Quake lab for repertoire analysis studies and deep sequencing.

T cell isolation: Total T-cell population, as well as naïve T-cell (day 0 and day 24-32), memory T-cell (day 0 and day 24-32) and activated T cell (day 9-14) populations sorted by flow cytometry, will be used for T-cell specificity and repertoire analysis as well as deep sequencing of immunoglobulin genes.

RNA from whole blood: One 2.5 ml PaxGene Blood RNA Tube for transcriptional (mRNA) profiling using microarrays, MicroRNA analysis, quantitative PCR (qPCR) analysis of mRNA expressed in peripheral blood

Recommendations:

- Should be drawn as 2nd or later tube (not first)
- Invert 3 – 5 times immediately following collection
- Freeze within 1 hour at -20°C or -70°C, long term storage at -70°C prior to processing
- Do not freeze PAXgene Blood RNA Tubes standing upright, instead place the tubes horizontally in a plastic bag or tray for freezing

9 ASSESSMENT OF SAFETY

9.1 Specification of Safety Parameters

All SAEs occurring while the volunteer is on study will be collected and appropriately reported.

9.2 Methods and Timing for Assessing, Recording, and Analyzing Safety Parameters

9.2.1 Serious Adverse Events

Serious Adverse Event (SAE): An SAE is defined as an adverse event (AE) that meets one of the following conditions:

- Death during the period of protocol defined surveillance
- Life-threatening event (defined as a subject at immediate risk of death at the time of the event)
- An event requiring inpatient hospitalization or prolongation of existing hospitalization during the period of protocol defined surveillance
- Results in congenital anomaly or birth defect
- Results in a persistent or significant disability/incapacity
- Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgment, the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

All SAEs will be:

- reviewed and evaluated by a study clinician
- recorded on the appropriate VAERS AE reporting form
- reported to the Stanford IRB as required
- followed through resolution by a study clinician

9.3 Reporting Procedures

9.3.1 Regulatory Reporting for Studies Not Conducted Under DAIT-Sponsored IND

For those events meeting the previously described definition of Serious Adverse Events, the completion of a VAERS report form is required. The VAERS form will be filled out and submitted to the Vaccine Adverse Event Reporting System (VAERS) per federal regulations. The VAERS form will simultaneously be sent to DAIT. SAEs that are unexpected and related to vaccine and harmful to the subject will be reported directly to the Stanford IRB within 10 working days from when the investigator learns of the event or new information. Unexpected deaths or life-threatening experiences related to the research must be reported to the IRB within 5 working days from when the investigator learns of event.

9.4 Type and Duration of Follow-up of Subjects after Adverse Events

All SAEs will be followed until satisfactory resolution or until the PI or Subinvestigator deems the event to be chronic or the patient to be stable.

9.5 Safety Oversight

The Principal Investigator will oversee compliance with the protocol, the subject's safety and any unanticipated problems involving risks to subjects and will report these events as described above. Unanticipated problems and serious adverse events will be reported to the Stanford IRB as required.

10 CLINICAL MONITORING

This study will not be monitored by the sponsor. This is a Phase 4 study using a licensed product. The investigator is responsible to ensure compliance with the protocol, and the accuracy, completeness, legibility, and timeliness of the data reported.

11 STATISTICAL CONSIDERATIONS

11.1 Study Hypotheses

This is an exploratory discovery based study using a strategy that has not been employed previously to investigate the effect of age, genetic and epigenetic background on responses to influenza vaccines, as well as the differential immune response to TIV versus LAIV. Our goal will be to identify age-specific biomarkers or clusters of markers in general and especially those that are predictive of immune sufficiency or insufficiency. The questions we will address are:

1. What traits correlate with specific age groups?
2. What traits correlate with a robust immune response or a poor one?
3. What traits change with age and presumed environmental influence?
4. Does the frequency of antigen specific T-cells change with age? And is pre-vaccination antigen specific T-cell frequency a predictor of subsequent immune response.
5. What is the T cell receptor repertoire to various influenza strains ?

At a secondary degree, our study is aimed at providing initial estimates for subsequent studies design for several intriguing questions, namely:

6. What traits of immune response change between vaccination with TIV vs. LAIV?
7. What traits are genetically determined entirely or partially?
8. Does the variation in immune biomarkers grows as a function of age and genetic dissimilarity?
9. What is the B-cell immune repertoire? Systemic analysis of immune repertoire at detailed sequence level has not been documented before. Based on our analysis of zebrafish repertoire sequencing (49), we expect to see the sequence abundance exhibits different distributions in different B cell populations. Gene segment usage preference will likely be observed between two different vaccines. We will also discover possible polymorphism on immunoglobulin genes.

11.2 Sample Size Considerations

Paired twins make up 6 of the 7 groups in this study. As indicated in the synopsis, enrollment of some groups is contingent on funding of the administrative supplement for this contract. At present, it is unknown what the correlation between individuals within twin pairs is for many of the assays we will be performing. Thus we are unable to assess how their genetic similarity and the growing differences in their environment with age, will affect sample size. To address this, we analyze and provide power calculations for two extreme cases: first assuming twins are fully correlated to one another and second when assuming that they are fully independent of one another. We expect the analysis from this study design to provide crucial information for subsequent study designs.

The hypotheses we are testing in this study can be divided in terms of sample size considerations to five categories:

(1) Between group analyses of differences associated with age: Assuming twins are independent a total of 140 individuals 16 8-17 yo, 40 18-30 yo, 40 40-49 yo, 44 70-100 yo. This total drops to 86 individuals 8 8-17 yo, 20 18-30 yo, 20 40-49 yo, 37 70-100 yo assuming twins are in full correlation.
(2) Between group analyses of differences in gene expression, cytokines, cell subsets and cellular signaling between vaccine responders to non-responders as measured by HAI titers: Typically the young adults display immunity (either seroprotection or seroconvertion) whereas 40-60% of the elderly do not. We can thus expect roughly 120 responders and 20 non-responders in the case of independent twins and 14 non-responders. From an ongoing study we are conducting in the normal healthy individuals of different age groups, we will be able to augment the non-responder group with an additional 20 people, to a total of 40 (34 if fully correlated twins), and responders to a total of 200 (140 if fully correlated twins).
(3) Within group analysis of differences in gene expression, cytokines, cell subsets and cellular signaling to vaccine type response (LAIV, TIV): 8 pairs of twins in which one twin is given LAIV and the other TIV.
(4) Exploratory studies for which no prior estimates of variation are known.

Sample size for cell subset, serum cytokine and cytokine stimulation studies:

Preliminary analysis of an ongoing study (comparison of 30 young, middle age and elderly adults, 10 per group) has shown that we have approximately five candidate serum cytokine markers, five cell subset differences and ten cytokine stimulation differences between young individuals and the older cohorts. Gene expression, cytokine, cell subset and cell signaling (cytokine stimulation) assays will be regressed by age (Category 1). For a moderate value of R^2 (.1) and a 1/100 Type I error our regression model is powered at 89% assuming no dependence between twins and at 85% with a 1/20 Type I error assuming full correlation between twins. Thus, this study can provide an independent validation of our ongoing study. For vaccine response comparisons (Category 2) at a p-value of 0.05, the power is only 0.53. To increase the power of our analysis we will thus pool these studies samples with those for our ongoing study

which will increase our power to 82% (73% in the case of full correlation between twins). For assessing differences between vaccine types (Category 3), one individual within each twin pair will be randomized to TIV and the other to LAIV. This should enhance statistical power for detecting differences between the two vaccines by blocking on genetic factors. Analysis will be via permutation testing (59). With a sample size of 8 twins (n = 16), two-tailed permutation analysis can reject the null hypothesis of no difference in means between groups with attained significance levels as small as $2/2^8 = 1/128$, which is comfortably below the standard threshold on Type I error of 1/20.

Sample size for gene expression studies:

Out of 45015 probes, assuming 2.6% of probes are active (differentially expressed), yields 1170 active probes and 43845 inactive probes. A per-probe Type I error rate (α) of 0.3% and a standard deviation = 0.56 per probe on a $\log_2$ scale of the normalized data, 35 participants per each age class (Category 1) provide at least 90% power to detect at least 1.3-fold over-expression/under-expression, and a 95% power to detect 2-fold change differences between Group A (n=16) and the other age groups. Similarly, vaccine response comparisons (Category 2) analysis at a 1.5-fold change will be powered at 95%. 90% of 1170 active probes gives an expected 1053 true positives while 0.3% of 43845 gives an expected 132 false positives, for a marginal false discovery rate (mFDR) of ~11.1%.

Sample size B cell repertoire and T cell epitope usage studies:

We plan to enroll 10 subjects in each group for deep-sequencing immunoglobulin and T cell receptor genes. The projected sample size is comparable to published study of B cell repertoire by deep sequencing (60). The T cell epitopes analysis study is exploratory and it is unknown at this time what breadth of influenza epitopes will be detectable in one individual.

11.3 Final Analysis Plan

Cell subset, HAI titer, serum cytokine and cytokine stimulation studies:

Cell subset responses responses (absolute counts and percentages) will be summarized as sample means and sample standard deviations by age group and sampling period (0, 9-14 or 24-32 days); and serological responses (HAI titer) will be summarized as sample geometric means by age group and sampling period (0 and 24-32 days). For serum cytokines, the change in cytokine response from baseline to days 9-14 will be summarized as sample means and sample standard deviations by age group. We will estimate the Spearman correlation coefficient between levels of each measured cytokine and HAI titer. Spearman correlation will be employed because titer data are interval censored. For cytokine stimulation assays for each cell type,

cytokine stimulant, phosphoprotein combination we will summarize the data in two ways: (a) group differences in mean baseline unstimulated cells for each of the five hypotheses type categories above. (b) fold-change between cytokine stimulated and unstimulated cells. Both median and 90th percentile will be used for descriptive summaries of gated cell distributions. The centroids (multivariate means) of each age groups and responder group (seroconverted and non-seroconverted) will be compared at baseline and post-vaccination using multivariate analysis of variance (MANOVA) performed separately for cell subset, serum cytokine and cytokine stimulation assay.

Permutation testing will be employed to compare mean response between TIV and LAIV recipients in group A and to construct confidence intervals (61) on mean response within groups B through G. Permutation testing was selected because results are exact and unbiased in small samples (62). In addition, for group A, permutation testing is motivated by the randomized design (63). For Group A, permutation will be of individuals within each twin pair (matched-pairs design). For the remaining groups, permutation will be of twin pairs, not of individuals.

Gene expression studies:

We will use SAM (64) to identify differentially expressed genes between groups for each of the Categories defined above. We will also consider clustering our variables or narrowing our testing list a priori by restricting ourselves to immune system related genes (65) or by performing our analysis at the pathway level to decrease the number of tested hypotheses. Identified differentially expressed genes will be analyzed for functional enrichment (66). In addition, we will compile a restricted list of genes previously associated with flu or aging genes and test for their differential expression in our data at a more limited multi-hypothesis correction than could be performed over the entire array. In addition to the above discussed hypotheses, we will specifically test for the association between measured serum cytokines, cell frequency abundance of cells secreting those cytokines and the measured gene expression of the transcripts coding for those cytokines.

Analysis of T cell epitope usage using combinatorial pMHC tetramer staining: We recently described and validated a method for assessing large numbers of T cell peptide specificities using pMHC tetramers coded by combinations of fluorescent labels (70). Here we will apply this system to assess the usage and phenotypes of T cells responding to various Influenza epitopes before and after vaccination. Individual responses will be compared between age groups.

B cell receptor repertoire analysis: Similar to our published analysis on fish antibody repertoire (60), rarefaction of both VDJ combinations and receptor sequences will verify complete sampling of sequences. Correlations of VDJ-isotype profiles and sequence-level convergence among subjects will be used to determine the effect of age or a given vaccine on the global antibody and as well as B cell receptor repertoire. Our high-throughput sequencing measurement will then be used to characterize global antibody and B cell receptor repertoire

responses across different control groups of subjects. Specifically, this measurement will allow us to detect more subtle effects of the vaccine among those subpopulations of B -cells that would otherwise not be observed in small samples of Sanger-sequenced cells.

12 SOURCE DOCUMENTS AND ACCESS TO SOURCE DATA/DOCUMENTS

All subject information will be obtained by the investigators and their support staff and will remain confidential. Specimens for laboratory testing will be coded by subject number, and data for individual subjects will be coded for data analysis. A database containing a code key will be kept on a computer that is password secured and available only to study staff. Participants will not be identified in any reports or publications that may result from the study. Personal identifiers will be removed for all analyses and any publications. Upon completion of the study, data will be stored for 25 years.

Subject confidentiality is held strictly in trust by the participating investigators, their staff, and their agents. This confidentiality extends to genetic and biological sample tests, in addition to the clinical information relating to participating subjects.

The study protocol, documentation, data, and all other information generated will be held in strict confidence.

The clinical study site will permit access to all documents and records that may require inspection by the sponsor or its authorized representatives, including but not limited to, medical records (office, clinic or hospital) and pharmacy records for the subjects in this study.

13 QUALITY CONTROL AND QUALITY ASSURANCE

Quality Control and Quality Assurance activities will generally be completed as outlined in the current version of the Stanford Quality Management Plan. Chart audits for the trial will be conducted for research subject records on a monthly basis utilizing the DMID-approved Chart Audit Tool. Audits will be conducted on a random sampling of subject charts in accordance with the low risk to volunteers participating in the trial. Charts will be randomly selected from among those not previously audited. Results of these audits will be summarized monthly using the DMID QM_QA Summary Report tool, and shared with research staff at staff meetings, or more often as necessary.

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

Prior to enrollment of subjects into this trial, the protocol and the informed consent form will be reviewed and approved by the appropriate IRB.

The responsible official for the IRB will sign the IRB letter of approval of the protocol prior to the start of this trial and a copy will be provided to DAIT. Notification of the IRB's composition and the institutions Federal Wide Assurance number will be provided to DAIT as needed.

Should amendments to the protocol be required, the amendments will be reviewed by the sponsor and submitted to the IRB.

Subjects will be compensated for their participation in this study. Compensation will be in accordance with the local IRB’s policies and procedures, and requires IRB approval.

14.3 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. Extensive discussion of risks and possible benefits of participation in this study will be provided to the subjects and their families. Consent forms describing in detail the study procedures and risks are given to the subject or parent of a child and written documentation of informed consent is required prior to enrolling in the study. Consent forms will be IRB-approved and the subject or parent/ legally authorized representative (LAR) will be asked to read and review the document. After reviewing the document, the investigator or authorized study staff will explain the research study to the subject or

parent/LAR and answer any questions that may arise. The subject or parent/LAR will sign the informed consent document prior to being enrolled in the study. The subject or parent/LAR should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The subjects or 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 subjects for their records. The rights and welfare of the subjects 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.

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

An assent form approved by the IRB will be discussed with minors and signed by participants who are 7-17 years of age after the parent/ LARprovides consent.

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

Group A consists of children 8-17 years of age. There will be no exclusions based on gender, race or ethnicity.

14.5 Subject Confidentiality

All subject information will be obtained by the investigators and their support staff and will remain confidential. Specimens for laboratory testing will be coded by subject number, and data for individual subjects will be coded for data analysis. A database containing a code key will be kept on a computer that is password secured and available only to study staff. Participants will not be identified in any reports or publications that may result from the study. Personal identifiers will be removed for all analyses and any publications. Upon completion of the study, data will be stored for 25 years.

Subject confidentiality is held strictly in trust by the participating investigators, their staff, and their agents. This confidentiality extends to genetic and biological sample tests, in addition to the clinical information relating to participating subjects.

The study protocol, documentation, data, and all other information generated will be held in strict confidence.

The clinical study site will permit access to all documents and records that may require inspection by the sponsor or its authorized representatives, including but not limited to, medical records (office, clinic or hospital) and pharmacy records for the subjects in this study.

14.6 Study Discontinuation

The investigators have the right to terminate this study at any time. Reasons for terminating the study may include, but are not limited to, the following:

- Incidence or severity of adverse events indicates a potential health hazard
- Data recording is inaccurate or incomplete
- Study staff does not adhere to the protocol or applicable regulatory guidelines in conducting the study.

A subject may withdraw or may be withdrawn from the study for the following reasons:

- Subject withdraws consent
- Development of serious adverse event warranting withdrawal
- Trial termination
- Any reason that, in the opinion of the investigator, precludes the subject's participation in the study.

14.7 Future Use of Stored Specimens

After the study is complete, residual specimens will be stored for future research. As new scientific discoveries identify technologies or mediators that might be useful in studying the immune response, stored samples may be used to explore new information that could be made available with these advanced methods. Volunteer specimens will be stored under a unique identifier. The volunteer's name or other identifiers will not be available in any data shared with other investigators. New studies using stored samples will be reviewed by the IRB.

15 DATA HANDLING AND RECORD KEEPING

The investigator is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported. All source documents and data collection forms should be completed in a neat, legible manner to ensure accurate interpretation of data. Black or dark blue ink is required to ensure clarity of reproduced copies. When making changes or corrections, the original entry will be crossed out with a single line, and initialed and dated to indicate the change. DO NOT ERASE, OVERWRITE, OR USE CORRECTION FLUID OR TAPE ON THE ORIGINAL.

Data entered into the data entry system that are derived from source documents or data collection forms should be consistent with the source documents and data collection forms or the discrepancies should be explained.

15.1 Data Management Responsibilities

All source documents will be reviewed by the clinical team and data entry staff, who will ensure that they are accurate and complete. Serious adverse events will be assessed for causality, and reviewed by the site PI or designee.

Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site PI. During the study, the investigator must maintain complete and accurate documentation for the study.

The PI (or designee) will be responsible for data management, quality review, analysis, and reporting of the study data.

15.2 Data Capture Methods

Clinical data (including SAEs) will be entered into a 21 CFR Part 11-compliant data entry system. The data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents or data collection forms.

15.3 Types of Data

Data for this study will include clinical, safety and outcome measures.

15.4 Timing/Reports

Data will be reviewed on an ongoing basis according to the site Quality Management Plan. Data analysis will begin once all clinical data have been collected and verified for accuracy. Preliminary analyses of outcome measures will begin as soon as laboratory data are available. Subjects will receive a unique study ID at enrollment. All data for study analysis will be de-identified (coded by study ID number) and password-protected. De-identified protected health information will be provided only as needed for data analysis of study outcome measures.

Protected health information may be disclosed as requested by The Office for Human Research Protections in the U.S. Department of Health and Human Services, the sponsor, or Stanford University Administrative Panel on Human Subjects in Medical Research and any other unit of Stanford University as necessary.

15.5 Study Records Retention

Records and documents pertaining to the conduct of this study, including CRFs, source documents, data collection forms, consent forms, and medication inventory records, must be retained by the investigator for at least 2 years or until DAIT authorizes transfer or destruction of study records.

15.6 Protocol Deviations

A protocol deviation is any noncompliance with the clinical trial protocol or Good Clinical Practice (GCP). The noncompliance may be either on the part of the subject, the investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly.

These practices are consistent with Good Clinical Practice:

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 to use continuous vigilance to identify and report deviations. All deviations from the Protocol will be addressed in a study subject data collection form. Protocol deviations will be sent to the local IRB/IEC per their guidelines. The site PI/study staff are responsible for knowing and adhering to their IRB requirements.

16 PUBLICATION POLICY

Following completion of the study, the investigator may publish the results of this research in a scientific journal. The International Committee of Medical Journal Editors (ICMJE) member journals have adopted a trials-registration policy as a condition for publication. This policy requires that all clinical trials be registered in a public trials registry such as ClinicalTrials.gov, which is sponsored by the National Library of Medicine (NLM). Other biomedical journals are considering adopting similar policies. Any clinical trial starting enrollment after 01 July 2005 must be registered either on or before the onset of subject enrollment.

The ICMJE defines a clinical trial as any research project that prospectively assigns human subjects to intervention or comparison groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Studies designed for other purposes, such as to study pharmacokinetics or major toxicity (*e.g.*, Phase 1 trials), would be exempt from this policy.

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SUPPLEMENTS/APPENDICES

Appendix A. Schedule of Events

Appendix B. Fluzone 2009-2010 Package Insert (to be added when available)

Appendix C. FluMist 2009-2010 Package Insert (to be added when available)

APPENDIX A: SCHEDULE OF EVENTS

Procedures	Baseline	D. 9-14	D. 24-32	Premature discontinuation
Obtain Informed Consent	X			
Medical History	X			
Concomitant Meds	X	X	X	X
Vital Signs[1]	X			
Draw Blood for T-cell and B-cell Studies	X	X	X	X
Immunization	X		X[2]	
Review SAEs		X	X	X

[1] Vital Signs: height, weight, temperature, pulse, blood pressure

[2] For vaccine-naïve 8 yo participants only- to occur at least 28 days after the initial immunization.