Immune Senescence in the Elderly

Comparison of Immune Responses to Influenza Vaccine in Adults of Different Ages_Year 3 Amendment

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8215

SLVP Protocol Number:
SLVP015

Principal Investigator:
Cornelia L. Dekker, MD

Co-Investigator:
Mark M. Davis, PhD

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Statement of Compliance

This trial will be conducted in compliance with the protocol, International Conference on Harmonisation guideline E6: Good Clinical Practice (ICH E6): Consolidated Guideline, and the applicable regulatory requirements from United States (US) Code of Federal Regulations (CFR) (Title 45 CFR Parts 46 and Title 21 CFR including Parts 50 and 56) concerning informed consent and Institutional Review Board (IRB) regulations.

SIGNATURE PAGE

The signatures below document the approval of this protocol and the attachments, and provide the necessary assurances that this clinical study will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality and according to local legal and regulatory requirements and to the principles outlined in applicable U.S. federal regulations and ICH guidelines.

Principal Investigator

Signed: Date:

Cornelia L. Dekker, M.D.
Professor, Pediatrics

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AE	Adverse Event
CMI	Cell-Mediated Immunity
CMV	Cytomegalovirus
CFR	Code of Federal Regulations
GCRC	General Clinical Research Center
HA	Hemagglutinin
HAI	Hemagglutination Inhibition
HIV	Human Immunodeficiency Virus
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
IEC	Independent or Institutional Ethics Committee
IFN	Interferon
IL	Interleukin
IRB	Institutional Review Board
MHC	Major Histocompatibility Complex
MOP	Manual of Procedures
mRNA	Messenger RNA
N	Number (typically refers to subjects)
NA	Neuraminidase
NIAID	National Institute of Allergy and Infectious Diseases, NIH, DHHS
NIH	National Institutes of Health
NLME	Nonlinear Mixed Effects Model
PBMC	Peripheral Blood Mononuclear Cell
PI	Principal Investigator
SAE	Serious Adverse Event
SOP	Standard Operating Procedure
TNF	Tumor Necrosis Factor

Protocol Summary

Title: Comparison of Immune Response to Influenza Vaccine in Adults of Different Ages

Population: <u>Year 1:</u>
A maximum of 30 healthy ambulatory male and female volunteers 18 to 30 years inclusive (n=10), 60 to 79 years inclusive (n=10) and 80 to 100 years inclusive (n=10), enrolled at Stanford University Hospital

<u>Years 2 and 3:</u>

A maximum of 90 healthy ambulatory male and female volunteers 18 to 30 years inclusive (n=30), 60 to 79 years inclusive (n=30) and 80 to 100 years inclusive (n=30), enrolled at Stanford University Hospital

Number of Sites: Stanford University School of Medicine
Department of Pediatrics
300 Pasteur Drive, Room G312
Stanford, CA 95305

Subject Duration: Enrolled Year 1: Approximately 3 ½ years (annually through the 2009-2010 influenza season)

Enrolled Year 2: Approximately 2 ½ years (annually through the 2009-2010 influenza season)

Enrolled Year 3: approximately 6-8 months depending upon date of initial enrollment (through the 2009-2010 influenza season)

Primary Objectives:

- Compare lymphocyte responses at Days 5-7 and the lymphocyte and serology responses at Day 28 post-immunization following administration of seasonal trivalent inactivated influenza vaccine
- Evaluate changes in cytokine profile in the immune response (CD4, CD8, γδ cells, B cell responses) from Day 0 to Day 5-7 for T cells and antibody-secreting cells (ASCs)
- Evaluate changes in cytokine profile in the immune response (CD4, CD8, γδ cells, B cell, and serology responses) from Day 0 to Day 28 for HAI responses
- Evaluate any potential gene expression signatures either at Day 0 or during the early response period Day 5-7 that are associated with immune senescence, potentially providing insight into mechanism
- Compare monocyte reactivity to activating stimuli at Days 0 and 5-7 with the lymphocyte and serology responses at Day 28 post-immunization following administration of seasonal trivalent inactivated influenza vaccine (Year 1 only)

Secondary objectives:

- Determine whether any of the immune responses correlate with protection from reported influenza-like illness at the end of the flu season
- Determine the effect of level of physical activity on the generation of immune response to influenza vaccine
- Determine the effect of habitual caffeine consumption on the generation of immune response to influenza vaccine (Years 2 and 3 only)

- Compare cell specific gene expression differences between age groups for both whole blood and whole PBMC
- Determine the effect of cell subset frequency variation on serum cytokine abundance

Study Design

Total N: Obtain informed consent from up to 30 subjects enrolled at Stanford in Year 1, and 90 subjects in Years 2 and 3.

Screen subjects by criteria; obtain history document [1]

Sample Collection [2]

- Day 0 (Before 1st dose)
- Day 5-7 (after 1st dose)
- Day 28 (after 1st dose)

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Sample Processing [3]

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Sample Testing [4]

- B cells, T cells, NK cells, γδ T cells, monocytes, dendritic cells
- Gene Expression Analysis
- Serum Cytokine Analysis
- HAI Antibody

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Data Analysis

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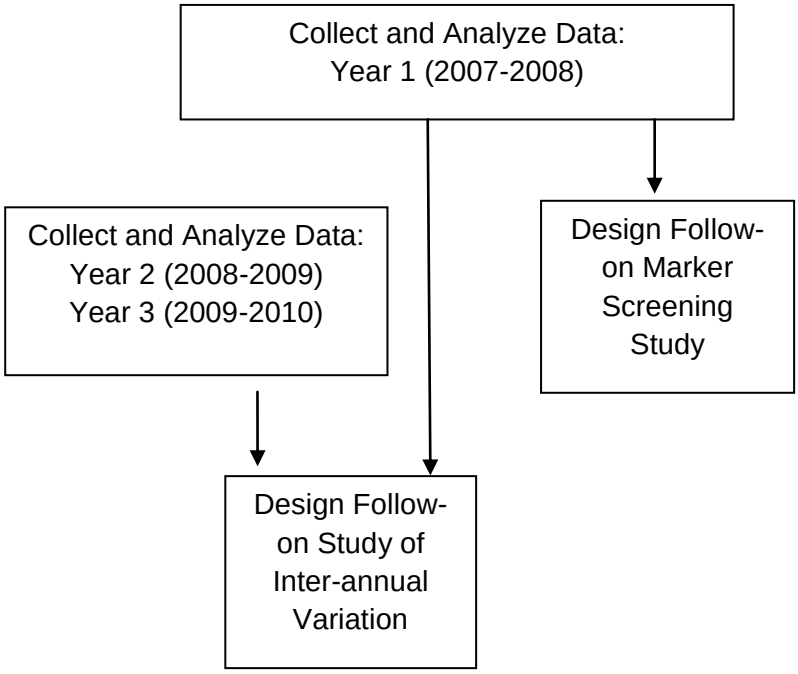
Repeat Annually Through the 2009-2010 Influenza Season

Program Design



1 KEY ROLES

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2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

We plan to use the analysis of vaccine-induced and naturally acquired influenza A immunity as a model for defining adaptive immune responses in older adults. Influenza A was chosen as a model system because there is significant morbidity and mortality in the elderly due to variants of this virus and we wish to understand how immune function declines in the elderly. Further, influenza A causes natural pandemics that can incapacitate a large fraction of the population simultaneously, regardless of age. Biomarkers that correlate with a poor immune response to flu in elderly people may also predict younger people who are at greater risk.

Because elderly people are recommended for annual influenza vaccination, this represents a large population of subjects in which to examine the variability of an immune response to a defined pathogen. B-cell and T-cell immune mechanisms are used by the host to control infections of the respiratory system. Influenza A and B are primary causes of human infection. Influenza A viruses are classified into subtypes based on two surface antigens, hemagglutinin and neuraminidase. Immunity to these antigens reduces the likelihood of infection and lessens the severity of the disease during usual annual influenza epidemics. However, because influenza strains undergo antigenic drift, prior infection or vaccination may provide little or no protection if the vaccine is not well-matched to circulating strains. The frequent emergence of antigen variants through antigenic drift is the virologic basis for seasonal epidemics. However, when major antigenic variation occurs, with the incorporation of new genes encoding novel HA and/or NA proteins, a pandemic situation can develop (1-3). Influenza pandemics are characterized by (1) the introduction of a genetically new influenza virus, (2) the ability of the new virus to infect, cause disease, and be transmitted efficiently in humans throughout the world, and (3) the presence of a large susceptible population lacking immunity to the new virus. Influenza virus is spread primarily by inhalation of airborne particles produced by coughs and sneezes.

2.2 Rationale

Previous work has shown that older adults can exhibit a number of immune deficiencies, such as reduced lymphocyte proliferation to new antigens (4-7) or failure to produce neutralizing antibodies (4-7) that may explain the increased morbidity and mortality to infectious diseases. There are also a number of other indicators of immune dysfunction that may or may not be a

factor, such as increased numbers of $CD8^+$ T cells lacking the co-stimulatory molecule CD28 (8-10), lower numbers of $\gamma\delta$ T cells (11) or decreased telomere length. What is lacking is a rapid diagnostic tool for immune senescence that correlates strongly with the lack of an effective response to infectious diseases. For this reason, we have obtained funding from the Ellison Foundation to use our newly developed functional cellular array technology (12, 13), and from the Bill and Melinda Gates Foundationto analyze flu-specific T-cell responses in elderly patients receiving a seasonal flu vaccination. This technology consists of arraying particular antigenic peptide-HLA complexes onto coated slides using protocols that we have developed. Purified $CD4^+$ and $CD8^+$ T-cell populations are then incubated with these slides briefly and specific T cells bind to the appropriate peptide-HLA combination. The cells are then incubated for six hours at 37ºC, which results in T-cell activation and cytokine secretion. Specific cytokines (up to 25 different ones) are then assayed using anticytokine antibodies that have been "spotted" onto the slides at the same time as the peptide-HLA complexes, and then the amount of cytokine is "read out" by using a second step antibody coupled to a fluorophore (13). This allows us to simultaneously assess both the number of T cells that are present in a sample and the cytokine profile of these cells. It has become increasingly clear that the ability of different T cells to make different cytokines is an important part of any given type of T-cell response (or the lack of one). We are particularly interested in any differences in the cytokine profile of responding T cells between older people and younger controls.

While we can expect to see a diminished number of T cells responding to vaccination in at least some older adults based on previous work, no one has been able to analyze the cytokine profiles of responding cells so extensively and, thus, we hope with this new approach to find evidence of cytokine disruption. This could provide both potential new diagnostics for immune senescence in the elderly and perhaps therapeutic options as well, in the provision of or suppression of specific disregulated cytokines.

Thus, our main effort here will be to survey elderly and younger control subject responses to influenza vaccination for up to three influenza vaccine seasons using our functional cellular array technology. We will compare these results with serum antibody responses from day 28 blood samples measuring hemagglutination inhibition (HAI) titers against the vaccination strains. This is the accepted standard for determining whether there is an effective antibody response to influenza vaccine antigens. In addition, we will follow-up subjects yearly by phone to ask whether they have experienced any influenza-like illness episodes following immunization. In this way, we will be able to correlate the accepted standard assay for an appropriate antibody response to influenza vaccination with our new assay, and also begin what we hope will be a valuable longitudinal study of immune responsiveness in the elderly. We expect that some of our elderly subjects will exhibit signs of immune senescence by the HAI assay and that will also be associated with an increased susceptibility to influenza which would emerge in the follow-up surveys.

We also think that there will be a T-cell array "signature" of immune senescence both in terms of the number of responding cells and their cytokine profile that will both allow a more rapid read-out of this condition (day 5-7 after vaccination) and give some mechanistic clues regarding this phenomenon. In addition, we want to obtain as much information as possible with these samples and thus propose to do the following assays:

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). We are particularly interested in whether the reports of increased $CD8^{+}CD28^{-}$ T cells (9, 10) or decreased $\gamma\delta$ T cells (11, 14) correlate with immunosenescence. This subset analysis also will be important for normalizing the gene expression data.

2. **Gene Expression Analysis** -- – 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. **Monocyte Activation Potential** – As antigen presentation capacity may also be a variable influencing the robustness of the adaptive immune response, in Year 1 we examined circulating monocytes as a source of antigen presenting cells. We examined the potential of these cells to respond to in vitro activating stimuli (cytokines and LPS) as measured by expression of cell surface activation markers (DR, CD86, CD40) and intracellular phosphoproteins (eg. pp38, pSTATs). Expression of these markers was measured by FACS analysis. No striking changes were seen in monocyte signaling between the younger and older groups in an extensive signaling analysis done in collaboration with Garry Nolan and colleagues, therefore the monocyte assay will not continue Years 2 and 3.

The first year of this study was completed in 2007-2008. The second and third years will continue through the 2009-2010 influenza season. The primary objective for Years 2-3 is to compare lymphocyte responses at Days 5-7 and the lymphocyte and serology responses at Day 28 post-immunization following administration of the influenza vaccines. We will evaluate any changes in cytokine profile in the immune response (CD4, CD8, $\gamma\delta$ cells, B cell, and serology responses) from Day 0 to Day 5-7 for T cells and antibody-secreting cells (ASCs), and from Day 0 to Day 28-for HAI antibody responses.

2.3 Potential Risks and Benefits

2.3.1 Potential Risks

The discomforts of this study are those of having blood drawn from an arm vein, IM injection 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 two minutes immediately after the blood draw. Intramuscular injection may cause injection site pain, swelling, and redness. 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.*, Ibuprofen 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.

2.3.2 Known Potential Benefits

There are no benefits to the subject for participating in this study other than receiving the seasonal influenza vaccine, which is available publicly. Subjects given the influenza vaccines are likely to experience decreased frequency and severity of subsequent influenza infection. The beneficial role of influenza vaccination in the elderly 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. Participation will help investigators understand the cellular immune response of persons to influenza vaccine.

3 OBJECTIVES

The primary objectives of this study are to:

- Compare lymphocyte responses at Days 5-7 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 5-7 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 5-7 that are associated with immune senescence, potentially providing insight into mechanism
- Compare monocyte reactivity to activating stimuli at Days 0 and 5-7 with the lymphocyte and serology responses at Day 28 post-immunization following administration of seasonal trivalent inactivated influenza vaccine (Year 1 only)

The secondary objectives are to:

- Determine whether any of the immune responses correlate with protection from reported influenza-like illness at the end of the flu season
- Determine the effect of level of physical activity on the generation of immune response to influenza vaccine
- Determine the effect of level of habitual caffeine consumption on the generation of immune response to influenza vaccine (Years 2 and 3 only)
- Compare cell specific gene expression differences between age groups for both whole blood and whole PBMC.
- Determine the effect of cell subset frequency variation on serum cytokine abundance

Study 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 STUDY DESIGN

Healthy ambulatory adult subjects are eligible for enrollment into three age categories: 18-30 years, 60-79 years and 80-100 years, inclusive. There are no exclusions for gender, ethnicity or race. Up to 30 volunteers will be recruited for enrollment in the Year 1 exploratory study conducted at Stanford. Following the analysis of Year 1 data, new subjects will be recruited for Years 2. Enrollment will be increased in Years 2 and 3 to a study total of up to 90 subjects in each year by increasing the number enrolled to approximately 30 participants in each age category. Participants enrolled in Years 1 and 2 are encouraged to return in Years 2 and 3. The enrollment period for Years 2 and 3 will be from September to December. Individual study participation will continue annually for two and a half more years through the 2009-2010 influenza season. Each study year will include three study clinic visits at Day 0, 5-7 and 21-35, and one phone-call at Month 6 to report the occurrence of influenza-like illness during the past flu season.

All studies, data analysis and manuscript preparation will be performed at Stanford University.

5 Study Population

5.1 Selection of Study Population

Approximately 30 healthy male and female subjects were enrolled at Stanford in Year 1. Enrollment will be increased in Years 2 and 3 to a total of up to 90 volunteers in each year by increasing the number enrolled in each age category to approximately 30 volunteers. Participants enrolled in Years 1 and 2 are encouraged to return for Years 2 and 3. Enrollment will be stratified by age: up to 30 volunteers will be age 18-30 years, up to 30 will be age 60-79 years, and up to 30 will be age 80-100 years, inclusive.

5.2 Inclusion/Exclusion Criteria

Inclusion Criteria

- Age 18-30, 60-79, or 80-100 years, inclusive at time of initial enrollment
- General good health and ambulatory at time of enrollment
- No acute illness at time of vaccination
- Willing and able to sign Informed Consent
- Available for follow-up for the planned duration of the study
- Acceptable medical history by screening evaluation and brief clinical assessment

Exclusion Criteria

- Prior vaccination with TIV or LAIV in Fall 2009
- Adult ages <18, 31-59 or >100 years at time of initial enrollment
- Allergy to egg or egg products
- Allergy to vaccine components, including thimerosal
- Active systemic or serious concurrent illness, including febrile illness on the day of vaccination
- History of immunodeficiency
- 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.

- Hospitalization in the past year for congestive heart failure or emphysema.
- Chronic Hepatitis B or C.
- Recent or current use of immunosuppressive medication, including glucocorticoids (corticosteroid nasal sprays are permissible).
- 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).
- 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.
- History of blood dyscrasias, renal disease, or hemoglobinopathies requiring regular medical follow up or hospitalization during the preceding year
- 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.
- Receipt of blood or blood products within the past 6 months
- Medical or psychiatric condition or occupational responsibilities that preclude subject compliance with the protocol
- Inactivated vaccine 14 days prior to vaccination
- Live, attenuated vaccine within 60 days of vaccination
- History of Guillain–Barré Syndrome
- Pregnant or lactating woman
- Use of investigational agents within 30 days prior to enrollment
- Donation of the equivalent of a unit of blood within 6 weeks prior to enrollment
- 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.

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.

Subject Withdrawal/Discontinuation

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.

6 STUDY PROCEDURES/EVALUATIONS

6.1 Test Agent

Test Agent Administration:

Subjects will receive Fluzone 0.5 mL given intramuscularly into the non-dominant deltoid muscle. Vaccine will be administered by licensed clinicians.

Drug Formulation:

Subjects will receive Fluzone®, Influenza Virus Vaccine, (Zonal Purified, Subvirion) manufactured by Sanofi Pasteur for intramuscular use. Fluzone®, Influenza Virus Vaccine, (Zonal Purified, Subvirion) for intramuscular use, is a sterile suspension prepared from influenza viruses propagated in embryonated chicken eggs. The virus-containing fluids are harvested and inactivated with formaldehyde. Influenza virus is concentrated and purified in a linear sucrose density gradient solution using a continuous flow centrifuge. The virus is then chemically disrupted using a nonionic surfactant, octoxinol-9, (Triton® X-100 – A registered trademark of Union Carbide, Co.) producing a "split virus." The split virus is then further purified by chemical means and suspended in sodium phosphate-buffered isotonic sodium chloride solution. The package insert for the 2008-2009 and 2009-2010 Fluzone® vaccine are not yet available. Fluzone vaccine was standardized according to USPHS requirements for the 2007–2008 influenza season and is formulated to contain 45 micrograms (µg) hemagglutinin (HA) per 0.5 mL dose, in the recommended ratio of 15 µg HA each, representative of the following three prototype strains: A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. Gelatin 0.05% is added as a stabilizer. Fluzone® vaccine is supplied in four different presentations: a 5 mL vial of vaccine which contains the preservative thimerosal [(mercury derivative), (25 µg mercury/dose)]; a 0.25 mL prefilled syringe (No Preservative: Pediatric Dose, for 6–35 months of age) distinguished by a pink syringe plunger rod; a 0.5 mL prefilled syringe (No Preservative, for 36 months of age and older); and a single dose vial (No Preservative, for 36 months of age and older). There is no thimerosal used in the manufacturing process of the No Preservative unit dose presentations of Fluzone® vaccine. Fluzone® vaccine, after shaking syringe/vial well, is essentially clear and slightly opalescent in color.

New formulations of Fluzone will be developed for the 2008-2009, 2009-2010 influenza seasons based upon the predominant strains for each season.

Preparation

Vaccine will be supplied in multi-dose vials and drawn up individually at the time of each immunization visit. Vaccine will be stored according to guidelines in the package insert.

6.2 Study Procedures

Clinic Visit 1 (Day 0)

- Review informed consent, and answer questions. Obtain signed informed consent before beginning screening procedures
- Vital signs: height, weight, temperature, pulse, respirations, and blood pressure
- Urine pregnancy test for females of childbearing potential
- Medical history
- Brief clinical assessment including a physical exam focused on cardiovascular, respiratory, and HEENT systems
- A blood sample will be collected (up to 54 mL in heparinized green top tubes, 5 mL in a red top tube, 2.5 mL in a PaxGene Blood RNA Tube, and 2 mL into a lavender top tube for a CBC with differential)
- Immunize with TIV
- Observe in clinic 30 minutes for immediate AEs
- Administer physical activity and habitual caffeine intake questionnaires
- Dispense memory aid and give instructions to record any AEs

Clinic Visit 2 (Day 5-7)

- The Memory Aid will be reviewed with volunteer for safety assessment and adverse events
- A blood sample will be collected (up to 54 mL in heparinized green top tubes, 5 mL in a red top tube, 2.5 mL in a PaxGene Blood RNA Tube, and 2 mL into a lavender top tube for a CBC with differential)
-
- Concomitant medications will be reviewed and updated if applicable.
- A targeted physical exam and vital signs will be completed if indicated.

Clinic Visit 3 (Day 21-35)

- The Memory Aid will be reviewed with volunteer for safety assessment and adverse events

- A blood sample will be collected (up to 54 mL in heparinized green top tubes, 5 mL in a red top tube, 2.5 mL in a PaxGene Blood RNA Tube, and 2 mL into a lavender top tube for a CBC with differential)
- Concomitant medications will be reviewed and updated if applicable.
- A targeted physical exam and vital signs will be completed if indicated.
- Volunteers will be instruct on the influenza-like illness form in preparation for the phone call at Month 6

Phone Call Follow-up (Month 6 +/- 4 weeks)

- Call volunteer to ask about influenza-like illness during the flu season, assess for SAEs, and complete final visit case report form.

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 AEs 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 up to 64 mL of blood for cell-mediated immune assays and microarray assay if possible.

6.3 Laboratory Evaluations

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

5. **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.

6. **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.

6.4 Specimen Collection, Preparation, Handling and Shipping

6.4.1 Specimen Preparation, Handling, and Storage

Serum: 5 mL in a red top tube for HAI antibody determination

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: Up to 54 mL in heparinized green top tubes 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, non-T cells (monocyte-rich) will be made available to Mellins lab for monocyte activation potential studies.

RNA from whole blood: 2.5 ml in a 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

Hematology: 2 mL blood in a lavender top tube sent to Clinical Laboratory for complete blood count (CBC) with differential to assess lymphocytes.

7 STATISTICAL CONSIDERATIONS

7.1 Analyses of Data from Year 1

This study is exploratory, using a strategy that has not been employed previously to investigate cellular responses to viral vaccines. The data from Year 1 allowed us to set sample size for a follow-on study in a separate sample of participants. As an amendment to this pilot study, the study will screen six panels, a) CD4/CD8 abundance, b) lymphocyte composition, c) cytokine composition (functional cellular assay), d) cytokine composition (plasma assay), e) gene-expression profiles and f) monocyte activation potential, to identify markers that differ in mean response among the three age groups, between seroconverted and non-seroconverted and between seroprotected and non-seroprotected. Year 1 allowed us to collect preliminary data on these six panels.

Sample size for Year 1: For Year 1, we propose a total sample size of 10 per group ($\times$ 3 groups). We allowed for the possibility that standard deviations may be unequal among groups and plan on estimating the standard deviation separately by group for each outcome which required at least 10 participants per each age group to achieve minimally sufficient relative precision for an estimate of the each mean (15). The estimate of each standard deviation was adjusted for small-sample bias per (16).

Descriptive summaries of immune response: For Objective 1, lymphocyte responses (absolute counts and percentages) will be summarized as sample means and bias-adjusted sample standard deviations by age group and sampling period (0, 5-7 or 28 days); and serological responses (HAI titer) will be summarized as sample geometric means by age group and sampling period (0 and 28 days). For objective 2, the change in cytokine response from baseline to days 5-7 will be summarized as sample means and bias-adjusted sample standard deviations by age group. For objective 3, 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 objective 4, monocyte phenotypes will be descriptively summarized in two ways: a) univariately as mean MFI and mean % positive cells for each of the four phosphoproteins by age group; and b) multivariately via a plot of the data from all three age groups on the first two components of a principal components analysis on the eight outcomes—2 measures (MFI and % positive cells) $\times$ 4 phosphoproteins.

Preliminary hypothesis testing: Three sets of hypothesis tests will be conducted to inform design of the Year 1 study. Details on how these results will be used to design the follow-on study are detailed in the next paragraph. a) The centroids (multivariate means) of three age groups will be compared at baseline using multivariate analysis of variance (MANOVA). This will be performed separately for CD4/CD8 abundance, lymphocyte composition, each cytokine

panel (functional cellular and plasma) and the set of eight monocyte outcomes. b) MANOVA will be used to compare centroids separately for these same three panels between i) seroconverted and non-seroconverted and ii) seroprotected and non-seroprotected (defined in next paragraph). For monocytes, these MANOVA will be performed separately for data from Day 0 and from Days 5-7. Unless the Year 1 results from Day 0 and Days 5-7 are strikingly different, we plan on dropping monocyte assays for Days 5-7 in the Year-1 follow-on study.

Sample-size calculations for follow-on screening study: We will use the data obtained in Year 1 to set the size of a new sample of participants for a follow-on study. Our goal will be to identify up to six respective subsets of markers whose means differ a) among the three age groups, b) between seroconverted and non-seroconverted, and c) between seroprotected and non-seroprotected. Here we define seroconverted as $\geq$ 4-fold increase in HAI titer and seroprotection as HAI titer $\geq$ 1:32, each after vaccination. For our high-dimensional panel, gene expression at Days 5-7, we will test these three hypotheses in the follow-on study using significance analysis of microarrays (SAM; 17). The Year 1 data will be used to set sample size for the follow-on study using procedures available for a 1-way ANOVA ("oneclass") in the SAM v3 software (18). For each of our five lower-dimensional panels (CD4/CD8, lymphocyte, cellular and plasma cytokine, and monocyte), in the follow-on study the three hypotheses will first be tested using 1-way MANOVA, and only if this omnibus test is significant will separate F tests will be performed to assess which markers differ significantly among groups (19, p 134). Sample size from the Year 1 data will be calculated as per Rencher (19, pp 141-142) for each of the three sets of comparisons.

Preliminary analysis of Year 1 has shown that we have approximately five candidate markers that differ between young individuals and the older cohorts. More will undoubtedly emerge from the gene expression data which is still being analyzed. But for five informative variables and a moderate value of R2 superscript (.15) one can achieve a P-value of .05 with 84 subjects, 6 less than the number proposed here. This will be adequate for a first pass at the subset and cytokine data and with the large number of indicators that are expected to emerge from the gene expression data we will employ multiple hypotheses correction (FDR) to correct for this. We will also consider clustering our variables or narrowing our testing list a priori (focusing only on immune system related genes, for example) to decrease the number of tested hypotheses.

7.2 Analyses of Data from Years 1-3

At the end of Year 3, we will have data across inter-annual variation in immune response to vaccination on up to 90 participants across the three age groups. In addition to the follow-on study that will be designed from the Year 1 data, an additional follow-on study (with separate amendment) across inter-annual variation in immune response will be designed from the combined data for Years 1-3. The two goals of the combined Years 1-3 data will be a) to use

these as a preliminary data set for assessing if immune response (HAI titer at Day 28) can be predicted from factors including level of physical activity prior to each vaccination and b) testing for association between HAI titer at Day 28 and whether or not the participants develops influenza during that season.

Regression modeling: The analytic goal here is to develop a prognostic model for immune response. Potential prognostic factors of interest are age, pre-vaccination level of physical activity and pre-vaccination level of HAI titer. To develop this prognostic model, we will use the data from Years 1, 2 and 3. Fold-change in HAI titer (Day 0 to Day 28) will be regressed on age, pre-vaccination level of physical activity and pre-vaccination level of HAI titer using nonlinear mixed-effects models (NLME, 20). We chose NLME for two reasons. a) Their nonlinear structure permits wide latitude in choice of likelihood formulation, which is desirable when modeling a complex outcome such as fold change of an interval censored variable (HAI titer). b) Examination of the distribution of subject-specific effects will shed light on whether or not unidentified prognostic factors are missing from the model. If this analysis suggests that important prognostic factors remain unidentified, the investigators will brainstorm to develop an exhaustive list of additional candidate prognostic factors for evaluation in the follow-on study to the Years 1-3 analysis. This list along with the estimates from the Years 1-3 regression analysis will be used to set a sample size for appropriate variable selection methods (e.g., lasso) in the follow-on study. In the event that analysis of the Years 1-3 data do not suggest the need for a more complex model, the model estimates of Years 1-3 will be used to set sample size for model validation in the Years 1-3 follow-on study.

Association of HAI titer and illness: Using the Years 1-3 data, we will test for association between HAI titer (Day 28 post-vaccination) and the presence/absence of influenza during the remainder of the year using NLME for a binary outcome. Degrees of freedom for hypothesis testing will be adjusted for small sample size per (21) The mixed-effects aspect of NLME will allow us to account for the repeated-measures structure of the sampling (multiple years observed within a participant.) We will employ Akaike's Information Criterion with adjustment for small sample size (22) to choose between logistic versus complementary log-log response functions.

8 SUBJECT CONFIDENTIALITY

Participant confidentiality is held strictly in trust by the participating investigators, their staff, the sponsor(s), and their agents. This confidentiality extends to biological sample tests, in addition to the clinical information relating to participating subjects. Volunteers will be assigned a unique ID code number and will not be identified by name except as required by the GCRC for clinical purposes.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor. The results of the research study may be published, but participants' names or identities will not be revealed. Records will remain confidential. To maintain confidentiality, the principal investigators at the sites will keep records in locked cabinets and results of tests will be coded to prevent association with participants' names. Volunteers' records will be available to the National Institutes of Health (NIH), investigators, and their Institutional Review Board (IRB) as required.

9 INFORMED CONSENT PROCESS

The study staff will obtain documented informed consent from each potential volunteer. Consent must be documented by the volunteer's dated signature on a Consent Form along with the dated signature of the person conducting the consent discussion. A copy of the signed and dated consent form should be given to the volunteer before participation in the trial. The original signed consent form must remain in each volunteer's study file and must be available for review at any time. The initial informed consent form and any subsequent revised written informed consent form, and written information must receive the IRB's approval in advance of use. The volunteer should be informed in a timely manner if new information becomes available that may be relevant to the volunteer's willingness to continue participation in the trial. The communication of this information should be documented.

This protocol and informed consent documents and all types of volunteer recruitment or advertisement information will be submitted to the IRB for review and must be approved before the study is initiated. Any amendments to the protocol must also be approved by the IRB prior to implementing any changes in the study. The investigator is responsible for keeping the IRB apprised of the progress of the study and of any changes made to the protocol as deemed appropriate, but in any case at least once a year. The investigator must also keep the IRB informed of any significant adverse events. The trial may be discontinued for administrative reasons or if new data about the investigational product resulting from this or any other trials become available, and/or on advice of the Investigator and/or IRB. If a trial is prematurely terminated or suspended, the Investigator shall promptly inform the IRB and the regulatory authorities of the reason for termination or suspension. If for any reason the trial is prematurely terminated, the Investigator should promptly inform the participants and ensure appropriate therapy and follow-up for participants.

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