Transcriptomic Signatures of Influenza Vaccine Responses

NCT03603509

May 19, 2019

MAYO CLINIC

IRB Minimal Risk Protocol Template

General Study Information

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Study Title: Transcriptomic Signatures of Influenza Vaccine Responses

Protocol version number and date: Version 7, 05/19/2019

IRB #17-010601

Research Question and Aims

Specific aims and Hypotheses:

Specific Aim 1: Identify Innate Cell Transcriptomic Signatures Associated with Sex and Immune Response to Influenza Vaccines in the Elderly. To do this we will:
• **Aim 1.1** Test the hypotheses that vaccine type (MF59Flu vs HDFlu) and/or sex are associated with variations in innate cell immune outcomes (e.g., APC activation phenotype and cytokine/chemokine secretion).
• **Aim 1.2** Test the hypothesis that the increased Ag dose in HDFlu results in greater activation/suppression of the innate genes/genesets previously associated with immune responses to SDFlu. We will also test the hypothesis that the MF59 adjuvant results in activation/suppression of additional specific innate genes/genesets compared to HDFlu (see Overview and Rationale of Specific Aim 1).
• **Aim 1.3** Test the hypothesis that innate immune cell transcriptomic signatures mediate the association of vaccine type (or sex) with innate cell immune outcomes.
• **Aim 1.4** Test the hypothesis that the innate cell immune outcomes and transcriptomic signatures from Aim 1.3 will predict markers of humoral immunity (HAI Ab and B cell ELISPOT response).

Specific Aim 2: Identify CD4+ T Helper (Th) Cell Transcriptomic Signatures Associated with Sex and Immune Response to Influenza Vaccines in the Elderly. To do this we will:
• **Aim 2.1** Test the hypotheses that vaccine type (MF59Flu vs HDFlu) and/or sex are associated with variations in Th cell immune outcomes (e.g., Th phenotype and cytokine production).
• **Aim 2.2** Test the hypothesis that the increased Ag dose in HDFlu results in greater activation/suppression of the Th cell genes/genesets previously associated with immune responses to SDFlu. We will also test the hypothesis that the MF59 adjuvant results in activation/suppression of additional specific genes/genesets in CD4+ Th cells compared to HDFlu (see Overview and Rationale of Specific Aim 2).
• **Aim 2.3** Test the hypothesis that Th cell transcriptomic signatures mediate the association of vaccine type (or sex) with Th cell immune outcomes.
• **Aim 2.4** Test the hypothesis that the Th cell immune outcomes and transcriptomic signatures from Aim 2.3 will predict markers of humoral immunity (HAI Ab and B cell ELISPOT response).

Background:

Scientific Premise of the Proposed Research. While older populations are at the highest health risk from seasonal influenza, these populations have poor immune responses to standard-dose seasonal influenza vaccine (SDFlu).1, 2 Our study is focused on two influenza vaccines for use in subjects ≥65 years of age: **MF59Flu**, an adjuvanted subunit vaccine (licensed in late 2015);3 and **HDFlu**, a split-virus vaccine containing 4x the hemagglutinin protein (licensed in 2009).4, 5 These vaccines have demonstrated greater immunogenicity than SDFlu in older populations, but little is known about the underlying mechanisms.5-16 **For these two vaccines, systems biology studies designed to identify the mechanisms for improved immune response have never been conducted in older individuals, nor have sex-based differences been adequately studied.** The goals of this proposed project are to examine inter-individual variations in innate and T helper (Th) responses, and identify *transcriptomic signatures* associated with immune responses (including correlates of protective immunity) to influenza vaccines in older individuals.

This is important for the following reasons:
A. Public Health Importance. Seasonal influenza A is believed to kill between 3,000–49,000 annually,17 resulting in over 250,000 excess hospitalizations and annual costs of >$90 billion in the US.18 Influenza morbidity and mortality increase significantly with age.19, 20 **More than 90% of influenza-associated deaths occur in individuals ≥65 years of age, and are predominantly associated with influenza A/H3N2.**21 During the 2014–2015 influenza season, adults age ≥65 years had an influenza-related hospitalization rate of 314 per 100,000 people—a record high since surveillance began in 2005.19, 20 The efficacy of influenza vaccines is diminished in older adults and varies widely (15% to 75%), averaging <50%.1, 2, 22, 23 Considering the unprecedented population growth in persons ≥65 years of age and the significant public health impact of influenza, **it is imperative that influenza vaccine-induced immunity in older adults be better understood**.24-26 Due to in-creased mortality in the elderly and cognizant of budgetary limitations, we will focus on influenza A/H3N2.

B. Knowledge Gap—Immune Response Differences to Adjuvanted vs. High-Dose Influenza Vaccines. HDFlu contains 60ug HA per influenza strain, and has been the focus of high-quality studies, including a study of 31,989 older individuals (≥ 65 years of age) that demonstrated higher immunogenicity (ratio of GMT 1.8) and 24% greater protection against influenza illness than SDFlu (15ug HA/strain/dose).5, 13, 14 MF59Flu also induces higher immunogenicity and longer persistence of Ab titers than non-adjuvanted SDFlu.6-12, 15, 27 A recent large study in 7,082 individuals (≥ 65 years of age) demonstrated significantly higher immunogenicity ($p<0.001$, seroconversion and HAI GMT) of MF59Flu vaccine compared to SDFlu (particularly against A/H3N2).15 A single small systems biology study comparing MF59Flu and SDFlu in immunoimmature children (14- to 24-months-old, n=90) identified significantly higher transcriptional responses to MF59Flu and identified early innate response signatures correlated with Day 28 Ab titers.28 These include M16 (a module associated with TLR and inflammatory signaling); M11 (a module regulating monocyte function); M75 (a module controlling IFN-induced antiviral response); M156 (a module associated with Ab secreting cells); and S3 (a module with genes involved in immunoglobulin production). These genesets and those identified in other systems biology studies of influenza vaccine response will be evaluated for their influence on immune responses to MF59Flu and HDFlu in this proposal.

The underlying immunologic mechanisms for the improved immunogenicity of MF59Flu and HDFlu are largely unknown for older adults. Our proposal will answer key questions (as illustrated in Figure 1) regarding innate and Th immune responses to these vaccines, the underlying transcriptomic signatures, and their impact on humoral immunity in older adults. At a minimum, this proposal will fill the knowledge

gap by examining whether the higher immunogenicity of these vaccines is a result of greater activation/suppression of genes/genesets (mRNA and miRNA) previously associated with immune responses to SDFlu, or additional unsuspected genes/genesets controlling immune responses.

C. Knowledge Gap—Sex-Based Differences in Immune Responses to Influenza Vaccines. Studies of Multiple vaccines (influenza, yellow fever, MMR, hepatitis A and B, herpes simplex [HSV] 2, rabies, smallpox, and dengue) demonstrate significantly higher Ab responses in females than males, as reported in a series of high-quality papers.16, 29-38 Sex-based differences in humoral immune responses are observed prior to puberty, during the reproductive years, and after reproductive senescence,29-38 suggesting that sex hormones are not the necessary—or sole—mediators of sex differences in humoral immune responses to vaccines.39, 40 **Despite significant evidence of immune response differences between the sexes, most vaccine studies do not analyze immune response outcome data by sex.**41, 42
Across a cohort of 556 older (ages 50–64) and 558 younger (ages 18–49) subjects, the SDFlu vaccine induced >1.5-fold higher A/H3N2-specific HAI Ab titers in women than men.29 Similarly, a study of SDFlu and HDFlu vaccine responses in 414 elderly subjects (ages 65–95) demonstrated higher rates of seroconversion in females than in males ($p<0.05$).31 However, no significant differences in Ab measures were found in cohorts of 494 and 158 older adults after receiving SDFlu,43, 44 and sex was also not reliably associated with seroprotection and/or Ab titers in children and young adults receiving MF59Flu.45 **These publications demonstrate inconsistent—and sometimes conflicting—findings regarding sex-based effects on immune responses to influenza vaccine.** Further research is needed, which is a stated priority for NIH research.
Additionally, new vaccines containing the MF59 adjuvant are likely to alter the transcriptomic signatures associated with vaccine responses in elderly populations, and potentially alter the influence of sex on those responses. The current lack of knowledge is a critical barrier to understanding poor vaccine responses in the elderly, and such knowledge is foundational to the future development of new influenza vaccines.

D. Impact of this Work. Given the substantially diminished efficacy of influenza vaccines with age and the importance of developing improved influenza vaccines,46 data from our studies could be used to inform directed and rational development of next-generation influenza vaccines and/or therapeutics47-57—although the development of such a vaccine is not the purpose of this proposal. Age-related immune dysfunction (immunosenescence) might be overcome by adjuvant stimulation of innate and/or Th cell-specific genes, which may be different in males and females (see Expected Results). For example, a TLR4 agonist GLA-SE has been shown to enhance Th1 responses to influenza vaccine in older adults.58 The identification of critical chemokine pathways involving CCR5 and its ligands (CCL5) (see Preliminary Data #7), predictive of influenza vaccine-induced humoral immunity, may support the usage of CCR5 small molecule agonists/antagonists approaches in vaccines to modulate inflammatory response and T cell chemotaxis/activation for optimal Ab response.44, 59-62

Study Design and Methods

Methods:

Subject Recruitment, Enrollment and Screening

We will screen and recruit 400 generally healthy males and females ages 65 and older who meet all inclusion and exclusion criteria and are willing to receive a flu vaccine.

We will also screen and recruit 20 generally healthy males and females ages 18-40 as a control group. These subjects will only have a baseline blood draw (no flu vaccine administration and no follow-study visits).

Eligible subjects who consent and enroll into the study will undergo a baseline blood draw (up to 108 mL) and then be randomly assigned to receive either the MF59 (Fluad) flu vaccine (N=200) or the High Dose (Fluzone) flu vaccine (N=200). Subjects in the control group will undergo the baseline blood draw only (up to 108 mL). They will NOT receive a flu vaccine. The following sections describe the remaining study visits for the subjects who are ages 65 and older.

Subjects will then be asked to come back for three additional blood draws after their flu vaccination: Day 1, Day 8, and Day 28 after vaccination. Each blood draw will be up to 105mL of blood.

Flu vaccination can occur up to 2 weeks after the baseline blood draw. The Day 1 blood draw will occur the following day after vaccination. The Day 8 blood draw will occur 7-10 days after the vaccination. The Day 28 blood draw will occur 25-31 days after vaccination.

During the Baseline visit we will collect patient demographic information, height, weight, BMI, medication history, medical information, and information on alcohol and smoking use. Patients will also be offered the CDC's influenza vaccine-specific Vaccine Information Sheet that is routinely provided to Mayo patients.

During the 3 follow-up visits we will collect weight, medication history and medical information.

Subjects will be remunerated $40 for each blood draw visit they complete.

We will also mail subjects, roughly a year after participation, 3 different sleep questionnaires as a way to assess sleep hygiene and immune response.

Resources: Potential participants will be identified through routine clinical appointments, advertising with flyers, brochures, an EMR search to identify mail-merge candidates, and existing lists/databases of individuals interested in being contacted about participation in research studies. Subject recruitment and advertisement will occur on Mayo campus and at various sites off campus (e.g., retirement centers). Permission will be obtained from each location before recruitment begins. We will also use newspaper and social media advertisements.

Based on a 2016 census by the United States Government, there were between 30,447 - 54,748 people 65 years of age or older in Olmsted County.

This project has been funded through the NIH through an R01 award.

The majority of the laboratory assays will be performed in the Vaccine Research Group Laboratory which occupies approximately 3,500 square feet on the sixth floor of the Mayo Clinic Guggenheim Research Building. Equipment not in this laboratory is readily available in institutional core laboratories and is freely shared.

☐ (1a) This is a multisite study involving Mayo Clinic and non Mayo Clinic sites. *When checked, describe in detail the research procedures or activities that will be conducted by Mayo Clinic study staff.*

☐ (1b) Mayo Clinic study staff will be engaged in research activity at a non Mayo Clinic site. *When checked, provide a detailed description of the activity that will be conducted by Mayo Clinic study staff.*

Subject Information

Target accrual: 420. This includes 400 subjects ages 65 and older (over-recruiting by 10% to account for subject drop-out and insufficient cell recovery from biospecimens) and 20 younger subjects (18-40 years of age) as a control group.

Subject population: Potential participants will be identified through routine clinical appointments, advertising with flyers, brochures, an EMR search to identify mail-merge candidates, and existing lists/databases of individuals interested in being contacted about participation in research studies. Subject recruitment and advertisement will occur on Mayo campus and at various sites off campus (e.g., retirement centers). Permission will be obtained from each location before recruitment begins.
Inclusion Criteria:

- Male or female adults ages 18-40 or 65 and older at the time of enrollment
- Eligible to receive Fluad® (MF59Flu) or Fluzone® (HDFlu) if age 65 or older
- No history of anaphylactic reaction to gelatin, neomycin, or other vaccine component
- Not pregnant
- No immunosuppression or immunodeficiency
- No acute illness at time of vaccination
- Determined by medical history and clinical judgment to be eligible for the study, by being generally healthy, with no autoimmune or immunosuppressive conditions and having stable current medical conditions (subjects with preexisting stable disease, defined as disease not requiring significant change in therapy or hospitalization for worsening disease 12 weeks before receipt of study vaccine, will be eligible. A change in dose or therapy within a category (e.g., change from one nonsteroidal anti-inflammatory drug to another) is allowed. A change to a new therapy category (e.g., surgery or addition of a new pharmacological class) is only allowed if it is not caused by worsening disease. A change to a new therapy category caused by worsening disease is considered significant and therefore ineligible for enrollment.

- Patients with diabetes mellitus are eligible for inclusion if they have had a hemoglobin A1c measurement of <8.0 within the past 6 months prior to enrollment. These hemoglobin A1c measurements are recommended at least twice yearly by the American Diabetes Association (ADA), and the target levels here are representative of the goals of the ADA. These hemoglobin A1c levels will ensure that these participants have good glycemic control. (*American Diabetes Association. American*

Diabetes Association Position Statement: Standards of Medical Care in Diabetes— 2015. Diabetes Care 2015;38(Suppl. 1): S1–S94)

- Able to follow study procedures in the opinion of the investigator
- Expected to be available for the duration of the study
- Weighs ≥110 lbs

Exclusion Criteria:

- Known or suspected immunodeficiency or receiving treatment with immunosuppressive therapy including cytotoxic agents (e.g., for cancer, HIV, or autoimmune disease).
- Subjects on corticosteroids will be excluded if ≥ 20mg of Prednisone (or equivalent drug) has been (or will be) administered daily for 2 weeks or more. Subjects will be eligible if corticosteroid therapy has been discontinued for at least 30 days.
- Serious chronic medical conditions including metastatic malignancy, severe chronic obstructive pulmonary disease requiring supplemental oxygen, end-stage renal disease with or without dialysis, clinically unstable cardiac disease, or any other disorder that, in the investigator's opinion, precludes the subject from participating in the study. Diabetic patients will be excluded if they do not have a hemoglobin A1c measurement within the past 6 months or if they had a hemoglobin A1c measurement of an A1c >8.0
- Receipt of any blood products, including immunoglobulin, within 6 months of study enrollment.
- Current anticoagulant therapy or a history of bleeding diathesis that would contraindicate intramuscular (IM) injection. (Note: antiplatelet drugs such as aspirin and clopidogrel are permitted.)
- Receipt of any vaccines within the past 30 days prior to enrollment
- Receipt of the current seasonal influenza vaccine other than in this study
- Acute illness within the last 30 days
- Blood donation within the last 56 days prior to study enrollment and within 56 days following the last study visit
- Pregnancy, Nursing or trying to conceive at the time of the study or for 28 days following the baseline visit
- Any condition (e.g. allergic reaction, Guillain-Barre Syndrome) that precludes their receipt of the influenza vaccine
- Currently taking antibiotics to treat a serious infection. Preventative use of antibiotics (i.e. oral surgery) is not an exclusion criterion.
- Diagnosis of a cognitive disorder (e.g. Alzheimer's, Dementia)
- Anemia
- Any medical condition that would, in the opinion of the investigator, interfere with the evaluation of the study objectives

Visit Schedule for subjects 18-40

	Baseline Visit - Vaccination
Review of Eligibility/Ineligibility	X

Demographics	X
Study Questionnaire	X
Medication Review	X
Height/Weight	X
Informed Consent	X
Blood Draw – 108ml	X[b,]

[b]Following standard practice, subjects will be provided juice, water and cookies after providing their blood sample

Visit Schedule for subjects 65 and older

	Baseline Visit - Vaccination	**Post-Vaccination Visit**		
		Day 1	**Day 8 (-1 day/+2 days)**	**Day 28(±3 days)**
Review of Eligibility/Ineligibility	X			
Demographics	X			
Study Questionnaire	X			
Medication Review	X	X	X	X
Height	X			
Weight	X	X	X	X
Informed Consent	X			
Blood Draw – 108ml	X[b,]			
Blood Draw – 105ml		X[b]	X[b]	X[b]
Flu vaccine	X			
Adverse Events	X	X	X	X

[b]Following standard practice, subjects will be provided juice, water and cookies after providing their blood sample

Blood will either be collected using a blood collection bag or with tubes. The blood bag collection is the preferred method.

Blood bag collection

- Baseline visit: 108 mL collected in the blood bag. 5 mL will be separated into a redtop tube x1 and 3 mL into an EDTA tube x1
- Day 1 visit: 105 mL collected in the blood bag. 5 mL will be separated into a redtop tube x1
- Day 8 visit: 105 mL collected in the blood bag. 5 mL will be separated into a redtop tube x1
- Day 28 visit: 105 mL collected in the blood bag. 5 mL will be separated into a redtop tube x1

Biospecimen tube collection

- Baseline visit: 5 mL redtop tube x1; 10 mL green top sodium heparin tubes x10; 3 mL EDTA tube x1
- Day 1 visit: 5 mL redtop tube x1; 10 mL green top sodium heparin tubes x10
- Day 8 visit: 5 mL redtop tube x1; 10 mL green top sodium heparin tubes x10
- Day 28 visit: 5 mL redtop tube x1; 10 mL green top sodium heparin tubes x10

Experimental Design:

Below, we briefly describe the assays we will use to monitor/characterize transcriptional changes and specific innate immune response variables at three important time points as in-formed by other studies and our preliminary data: Baseline (Day 0) and Days 1 and 8 after vaccination. The baseline blood draw allows us to ascertain each subject's pre-existing immune status and provides a baseline upon which to evaluate immunologic changes caused by the vaccine. The Day 1 blood draw allows us to examine early, innate immune responses. The Day 8 sample will be used to characterize plasmablast responses which are directly responsible for humoral immunity and the development of protective Ab titers. The Day 28 blood draw will allow us to examine the peak adaptive immune response.
The assays will be performed either on T- and B-cell depleted PBMCs isolated at each timepoint (for APC immunophenotyping, mRNA, and miRNA) or on T- and B-cell depleted PBMCs stimulated *in vitro* with influenza A/H3N2 (for cytokine/chemokine secretion). This will allow us to capture the *in vivo* and *in vitro* effects of these two vaccines.

The clinical characterization of our study subjects will include demographic information, height, weight, medications, and medical conditions that do not meet exclusion criteria. We will also run a CBC to quantify blood leukocyte populations.

Secreted Cytokine/Chemokine Mediators. Meso Scale Discovery kits will be used to detect cytokines and chemokines in culture supernatants before and 24 hours after *in vitro* stimulation with influenza A/H3N2.
Detection of Functional Innate Cell Subsets by Flow Cytometry. Cellular phenotypes of cells (before and 24 hours after *in vitro* stimulation with influenza virus) will be characterized in Baseline, Day 1, and Day 8 PBMCs by flow cytometry.259 We will examine the frequency of immune cell types, and characterize their activation by detecting the presence of cell surface markers, co-stimulatory molecules, and activation markers.
Influenza A/H3N2 Ab Assay. The HAI Ab assay will be performed using standard protocols.262, 359, 369-373 Given the known issues with some A/H3N2 strains, we will utilize influenza antigen matching the vaccine strain and test the antigen for neuraminidase binding to RBCs.374, 375
CMV Serostatus. CMV Ab titers (IgG) will be measured by multiplex flow-based immunoassay (Bio-Rad).
B Cell ELISPOT. Influenza-specific Ab-secreting cells/plasmablasts and memory IgG-like B cells will be quantified in PBMCs using ELISPOTPLUS for Human IgG kits (Mabtech) with (for memory B cells) or without (plasmablasts) R848/IL-2 pre-stimulation.262, 361
mRNA Transcriptomics.141 mRNASeq will be performed in Mayo Clinic's Medical Genome Facility on RNA samples extracted from PBMCs harvested at Baseline, Day 1 and Day 8. RNA libraries will be multiplexed with six samples per lane. Flow cells will be sequenced as 51x2 paired-end reads on an Illumina HiSeq. Base-calling will be performed using Illumina's RTA version 1.17.21.3. We will use StringTie376, 377 to process the resulting BAM files and sQTLseekeR378 to evaluate alternately spliced transcripts.
miRNA Sequencing. RNA libraries will be prepared with RNA extracted from Baseline, Day 1, and Day 8-harvested PBMCs (NEBNext Multiplex Small RNA Kit, New England Biolabs). Following multiplex adapter ligation (24 samples/lane) and library enrichment by PCR, equimolar amounts of each library will be pooled, purified, and loaded onto flow cells for sequencing (Illumina HiSeq 2000, Illumina cBot and cBot Paired end cluster kit version 3). Bioinformatics analysis will use adapter trimmed reads and miRDeep2.379

Research Activity

Check all that apply and complete the appropriate sections as instructed.

1. ☐ **Drug & Device**: Drugs for which an investigational new drug application is not required. Device for which (i) an investigational device exemption application is not required; or the medical device is cleared/approved for marketing and being used in accordance with its cleared/approved labeling. (Specify in the Methods section)

2. ☒ **Blood**: Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture.

3. ☐ **Biological specimens other than blood**: Prospective collection of human biological specimens by noninvasive means that may include: urine, sweat, saliva, buccal scraping, oral/anal/vaginal swab, sputum, hair and nail clippings, etc.

4. ☐ **Tests & Procedures:** Collection of data through noninvasive tests and procedures routinely employed in clinical practice that may include: MRI, surface EEG, echo, ultrasound, moderate exercise, muscular strength & flexibility testing, biometrics, cognition testing, eye exam, etc. (Specify in the Methods section)

5. ☒ **Data** (medical record, images, or specimens): Research involving use of existing and/or prospectively collected data.

6. ☐ **Digital Record**: Collection of electronic data from voice, video, digital, or image recording. (Specify in the Methods section)

7. ☒ **Survey, Interview, Focus Group:** Research on individual or group characteristics or behavior, survey, interview, oral history, focus group, program evaluation, etc. (Specify in the Methods section)

☐ NIH has issued a *Certificate of Confidentiality* (COC). *When checked, provide the institution and investigator named on the COC and explain why one was requested*. _____

Biospecimens – Categories 2 and 3

(2) Collection of blood samples.

a. **From healthy, non-pregnant, adult subjects who weigh at least 110 pounds**. For a minimal risk application, the amount of blood drawn from these subjects may not exceed 550ml in an 8 week period and collection may not occur more frequently than 2 times per week.

Volume per blood draw (Baseline only): ___108__ml
Volume per blood draw (Follow-up visits): ___105__ml

Frequency of blood draw: Four blood draws total. Blood draw #1 can occur the day of or up to 2 weeks prior to receiving the flu vaccination. Blood draw #2 will occur the following day after vaccination. Blood draw #3 will occur 7-9 days after the vaccination. Blood draw #4 will occur 25-31 days after vaccination.

b. **From other adults and children considering age, weight, and health of subject.** For a minimal risk application, the amount of blood drawn from these subjects may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period, and collection may not occur more frequently than 2 times per week.
Volume per blood draw: _____ml
Frequency of blood draw (e.g. single draw, time(s) per week, per year, etc.) _____

(3) Prospective collection of biological specimens other than blood: _____

Review of medical records, images, specimens – Category 5

For review of existing data: provide a date range or an end date for when the data was generated. The end date can be the date this application was submitted to the IRB.
Date Range:

Check all that apply (data includes medical records, images, specimens).

☐ (5a) Only data that exists before the IRB submission date will be collected.

☐ (5b) The study involves data that exist at the time of IRB submission **and** data that will be generated after IRB submission. Include this activity in the Methods section.
Examples
- The study plans to conduct a retrospective chart review and ask subjects to complete a questionnaire.
- The study plans to include subjects previously diagnosed with a specific disease and add newly diagnosed subjects in the future.

☐ (5c) The study will use data that have been collected under another IRB protocol. Include in the Methods section and enter the IRB number from which the research material will be obtained. *When appropriate, note when subjects have provided consent for future use of their data and/or specimens as described in this protocol*.

Enter one IRB number per line, add more lines as needed

☐ Data ☐ Specimens ☐ Data & Specimens _____

☐ Data ☐ Specimens ☐ Data & Specimens _____

☐ Data ☐ Specimens ☐ Data & Specimens _____

☐ (5d) This study will obtain data generated from other sources. Examples may include receiving data from participating sites or an external collaborator, accessing an external database or registry, etc. Explain the source and how the data will be used in the Methods section.

☐ (6) Video audio recording:

HIPAA Identifiers and Protected Health Information (PHI)

Protected health information is medical data that can be linked to the subject directly or through a combination of indirect identifiers.

Recording identifiers (including a code) during the conduct of the study allows you to return to the medical record or data source to delete duplicate subjects, check a missing or questionable entry, add new data points, etc. De-identified data is medical information that has been stripped of all HIPAA identifiers so that it cannot be linked back to the subject. De-identified data is **rarely** used in the conduct of a research study involving a chart review.

Review the list of subject identifiers below and, if applicable, check the box next to each HIPAA identifier being recorded at the time of data collection or abstraction. Identifiers apply to any subject enrolled in the study including Mayo Clinic staff, patients and their relatives and household members.

Internal refers to the subject's identifier that will be recorded at Mayo Clinic by the study staff.
External refers to the subject's identifier that will be shared outside of Mayo Clinic.

Check all that apply:	INTERNAL	EXTERNAL
Name	X	
Mayo Clinic medical record or patient registration number, lab accession, specimen or radiologic image number	X	
Subject ID, subject code or any other person-specific unique identifying number, characteristic or code that can link the subject to their medical data	X	
Dates: All elements of dates [month, day, and year] directly related to an individual, their birth date, date of death, date of diagnosis, etc. **Note:** Recording a year only is not a unique identifier.	X	
Social Security number	X	
Medical device identifiers and serial numbers		
Biometric identifiers, including finger and voice prints, full face photographic images and any comparable images		
Web Universal Resource Locators (URLs), Internet Protocol (IP) address numbers, email address		
Street address, city, county, precinct, zip code, and their equivalent geocodes	X	
Phone or fax numbers	X	
Account, member, certificate or professional license numbers, health beneficiary numbers		
Vehicle identifiers and serial numbers, including license plate numbers		
Check 'None' when none of the identifiers listed above will be recorded, maintained, or shared during the conduct of this study. (exempt category 4)	☐ None	☒ None
Data Analysis		

Data Analysis Plan:

The planned analyses for Aims 1 and 2 will follow the same strategies, so for Aim 2 analyses we point out the differences needed to adapt the analyses for Aim 2.

Aim 2.1 Test the hypotheses that vaccine type (MF59Flu vs HDFlu) or sex is associated with variations in Th cell immune outcomes: The analysis plans for Aim 1.1 will be followed, but using the Th phenotype and cytokine production outcomes, evaluating change from Baseline to Days 8 and 28.

Aim 2.2 Test the hypothesis that the expression of specific genes is associated with differences in Th cell immune outcomes: Analyses will parallel those for Aim 1.2, but use differential Th cell-specific gene and miRNA expression (change from Baseline to Days 8 and 28) as independent variables to evaluate for their association with change in Th cell immune responses.

Aim 2.3 Test the hypothesis that Th cell transcriptomic signatures mediate the association of vaccine type (or sex) with Th cell immune outcomes: Analyses will parallel those described in Specific Aim 1.3.

Aim 2.4 Test the hypothesis that Th cell immune outcomes and transcriptomic signatures from Aim 2.3 will predict markers of humoral immunity (HAI Ab and B cell ELISPOT response): The analysis plans closely follow the mediation analyses described in Aim 1.4. We will test the hypothesis that Th immune response and their associated genes (Aim 2.3), predict humoral immunity (HAI Ab titer and B cell ELISPOT response) at Day 28. We will also test the hypothesis that additional genes mediate humoral immunity independently of Th responses. To achieve this, we will treat Th responses as adjusting covariates, using the regression model: By adjusting for Th response, we seek genes/genesets that are only associated with humoral immune response.

Power: See Specific Aim 1. We anticipate stronger signals in the transcriptomic data from purified CD4+ T cells compared to PBMCs. The increased signal to noise ratio will allow for detection of smaller effects.

Potential Limitations and Alternative Strategies for Aims 1 and 2.

False-positive associations will be controlled by our clearly defined analysis plan, the use of false-discovery rates to assess significance, and geneset/pathway analysis to maximize power and minimize false discovery. Statistical power to detect effects is driven by our sample size of 400 subjects, which is larger than most systems biology studies, including our prior study of SDFlu in older subjects where we and others were able to successfully identify transcriptomic signatures of vaccine response.16, 44, 59-61, 260, 261, 263, 264, 275, 280, 437 Accumulation of information across multiple genes increases the percent of variability explained by the model and hence increases the likelihood of detecting effects. Our analysis approach will also test the association of each innate and Th cell immune response with the expression of all genes in a specified geneset using kernel methods.438-441 Advantages of kernel methods are the following: 1) they allow for correlations among the different genes in a geneset; 2) they are robust to when some genes are positively associated with a response, others are negatively associated with a response, and other genes in the geneset are not associated; 3) and they allow adjustment for covariates. Finally, we will evaluate data-driven genesets using WGCNA to group genes into biological modules,118 which is an approach we have successfully used (see Preliminary Data).

Separate mediation analyses will be performed for mRNA and miRNA. If we find both mRNA and miRNA to be separate mediators in our initial analyses, we will evaluate their joint effects as a pathway of mediators using techniques developed by VanderWeele.106 Other confounding factors (e.g., immunosenescence, underlying medical conditions, obesity, and CMV serostatus) will be evaluated in our analyses as potential confounders.

Expected Results and Impact of Findings for Specific Aims 1 and 2. Our publications44, 59, 258, 259, 263-265, 442, 443 and preliminary data indicate a high likelihood of identifying not only significant vaccine type-

and sex-specific differences in immune responses, but also transcriptomic signatures associated with those differences. Furthermore, the preliminary data from our mediation analysis support our hypothesis that changes in gene expression influence the effect of sex on immune response. We therefore anticipate identifying comparable sex-specific differences along with the genes/genesets mediating those responses (i.e., transcriptomic signatures), following receipt of MF59Flu and HDFlu. Pinpointing the innate and/or Th cell responses associated with humoral immunity to these vaccines facilitates our understanding of the critical pathways vaccines must trigger in order to elicit protective antibody titers.
Our data will also provide new insights into why men and women respond differently to influenza vaccines, and highlight the specific genetic pathways associated with MF59- and sex-based immune response differences. Aims 1.1 and 2.1 may provide additional correlates of protection that can be used in conjunction with HAI titer to more accurately assess immune status. Understanding what genes and genesets are associated with the increased immunogenicity of MF59Flu and HDFlu (Aims 1.2 and 2.2) will inform functional studies verifying the mechanistic relationship between gene expression and immune function. For example, if TLR4 and TLR8 activation are strongly associated with increased APC activation and HAI titers, it suggests the use of a combination of GLA-SE (a TLR4 agonist) and CpG (a TLR8/9 agonist) might make an effective adjuvant. As another example, MF59 has been shown to enhance APC recruitment to the Ag site and transport of Ag to the draining lymph nodes. Due to reasons that are currently poorly understood, those processes are followed by enhanced Th cell responses and more robust (in terms of both titer and breadth of targets) Ab responses. Evaluating the transcriptomic changes occurring in innate and Th cells after MF59Flu vaccination is likely to identify gene expression patterns occurring in the APCs and responding T cells, providing critical clues regarding the nature of the interactions between these cell types, and may highlight the receptor-ligand pairs and downstream signaling cascades responsible for enhanced T cell responses. Such information allows for directed and informed engineering of novel vaccine candidates and adjuvants. In the unlikely event that we do not identify vaccine type or sex-dependent differences, this finding will be interesting its own right, suggesting that further studies be conducted to find additional variables that do impact innate and/or Th responses to influenza A/H3N2.

Endpoints:
Secreted cytokine levels
Detection of Functional T_h Cell Subsets
mRNA and miRNA Sequencing

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