Single Cell Molecular Analysis of Influenza Vaccine Induced T Cell Responses in Adults 65 Years of Age or Older

NCT04077424

November 11, 2019

VERSION: 4.0
DATE: November 11, 2019

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1.0 BACKGROUND AND RATIONAL FOR THE STUDY

Each year influenza virus is associated with approximately 5,546 deaths in adults ≥65 years in the United States.[1] The reasons for the disproportionate morbidity and mortality of influenza in older individuals are not well defined, and they increase with age.[2] In fact, persons aged 85 years and older are 32 times more likely to die of influenza and associated pneumonia than those only 65-69 years of age.[2] Annual influenza vaccination has been recommended for adults ≥65 years[3] of age since 1960 to reduce excess morbidity and mortality in older adults. However, a steady and systemic declined in the immune system with increasing age, termed Immunosenescence,[4] appears to reduce vaccine effectiveness (VE) in older adults.[5]

The reasons for decreasing response to vaccination with age are multifactorial and may be related to changes in adaptive immune responses, including decreased T cell [6-8] and B cell [9,10] function. T cell phenotypic changes associated with aging include a gradual loss of CD4+ and CD8+ naïve cells [11], and an accumulation of late-stage differentiated CD4+ and CD8+ memory cells with loss of CD28 expression[6,12-14]. CD28 is a costimulatory molecule required for activation, proliferation, and differentiation of T cells [15].

Immune senescence is defined as phenotypic and functional changes in T cell populations associated with aging. These include skewing of the phenotype of CD4+ and CD8+ T cells toward a “late stage differentiated” phenotype, characterized by lack of expression of the costimulatory molecules CD27 and CD28 [16]. Expansion of this T cell phenotype has been associated with cytomegalovirus (CMV) seropositivity and CMV antigen recognition [17], suggesting an antigen driven expansion of these cell populations. Despite their decreased proliferative ability, these cells are functional, as they are still able to produce cytokines and maintain lytic capability (expressing granzyme and perforin) [17-22]. Poor vaccine responses in older persons have been linked to expansion of memory T cell populations with decreased CD28 expression, elevated levels of exhaustion markers (increased PD-1, CTLA-4 and LAG-3), and an accumulation of T cells with decreased proliferative potential (increased CD57 and KLRG1) [23-26].

Herpesvirus infection is ubiquitous in humans, and is relevant for immune senescence and aging. It is estimated that >90% of the adult population globally is infected with at least one HHV.[27] These include herpes simplex virus (HSV), CMV, varicella zoster virus, and other HHV. In immunocompetent persons, most of these are latent infections that do not contribute to overt clinical disease over the course of a lifetime. There is mounting evidence, however, that maintaining HHV latency comes at a cost to the immune system, even in the immunocompetent host [27]. CMV is a beta herpesvirus, and like the other viruses in this family, causes chronic latent infection [28,29]. The incidence of CMV seropositivity increases with age [28,30-32], and has been associated with poor responses to influenza vaccination in elderly individuals [12,33], and with reports of negative [34] and beneficial [35] effects in young individuals. The links between CMV infection and influenza vaccine response therefore are not straightforward. A recent study evaluated antibody responses to a new antigen strain (H1N1pdm in 2009) in adults 18-52 years and found diminished antibody responses associated with age, but not with CMV infection [36]. Poor immune responses in the setting of chronic CMV infection have been linked to accumulation of late-stage differentiated senescent T cells with diminished helper function[12,22,33,37 38].

The mechanism by which CMV infection may alter the host’s response to new infections or vaccination is not clear. However, the chronic nature of the infection leads to a feature of the immune response termed “memory inflation”. Classical CD4+ and CD8+ memory T cell responses against viruses expand during primary infection and contract to low magnitudes during post infection resolution [18]. However, CD8+ T cell responses to select epitopes of human CMV (HCMV) [39,40], rhesus CMV [41], and murine CMV (MCMV) [42,43] persist for decades at very high magnitudes after primary infection or during latency. This phenomenon is termed “memory inflation” and has

been best characterized among CMV- specific CD8+ T cells that consist primarily of CD45RO+ CCR7- CD27- T cells (effector memory T cells [T_{EM}]) and their CD45RA+ revertants, CD45RO- CCR7- CD27- T cells (effector memory RA+ [T_{EMRA}]) [21,44-47]. We have recently identified this same feature of CMV-specific CD4+ T cells [19]. We will evaluate CD4+ CMV-specific T cell memory inflation in this cohort of elderly influenza participants to determine whether it contributes to poor vaccine responses.

The term “older adults” represents a diverse population of individuals over 65 years of age ranging from physically and mentally fit to physically and/or cognitively-impaired with very different requirements for daily care ranging from those who are entirely independent to those who must rely on others for their complete care. Hence when performing studies in older adults, it is essential to describe the population being studied in explicit detail to interpret study results and for generalizability of findings. One key characteristic of older adults that may differ greatly is the degree of frailty. Frailty is the conceptualization of a phenotype of poor physiologic reserve and poor resistance to stressors and hence is associated with a high risk of morbidity and death from diseases.[48] One of the most accepted methods to measure frailty is one described by Fried et al,[49] that is operationalized by declines in lean body mass, strength, endurance, balance, walking performance, and low activity. Frailty as a syndrome in the geriatric population encompasses a person’s chronic medical conditions, functional status, and risk of mortality.[50] Frailty may be a better predictor of the immune response in older adults than chronologic age.[51] In previous studies, frailty scales have predicted vaccine response to polysaccharide pneumococcal vaccine better than age [52] and to be correlated with poor antibody response to influenza vaccination.[53]

Immune responses to both influenza and influenza vaccines are moderated by prior exposure to influenza and influenza vaccines. The original concept was called antigenic sin[54] but recently has been termed imprinting.[55] Influenza A viruses can be divided into two phylogenetic groups. The first influenza A group that a person is exposed to will provide better protection for the remaining influenza viruses within the group but not in the other group.[55] The birth cohort ranging from 1918 to 1957 is the majority of the population ≥65 years of age was first exposed to an H1N1 virus leaving this birth cohort at increased morbidity and mortality to H3N2 group viruses.

The current circulating H3N2 has adapted well to humans and evades antibody binding by glycosylating common antigenic sites. These same glycosylations make growth for vaccine production in eggs difficult. Egg adaption can change the vaccine strain less well-matched to the circulating strain.

As a strategy to increase immunogenicity of influenza vaccines, FDA approved high-dose (HD) influenza vaccine (Fluzone® High-Dose, IIV-HD) containing 4-times more hemagglutinin (HA) (60µg HA/strain) than the standard-dose (SD) vaccine (15µg HA/strain) for people aged ≥ 65 years in 2009.[56] In clinical trials, the high dose vaccine demonstrated significantly higher HAI antibody (Ab) responses (seroconversion and seroprotection rates) without an increase in clinically relevant systemic adverse reactions, but a slight increase in local reactions.[57] The large phase IIIb/IV study of HD, led by Dr. Talbot, showed improved clinical efficacy with a relative VE of 24%. In 2015, the FDA approved an MF-59 adjuvanted inactivated influenza vaccine (FLUAD™, aIIV) for the same population of older adults. The adjuvanted vaccine, FLUAD, has been in use in Europe for over 25 years with an excellent safety and tolerability experience.[58] The effectiveness of the MF-59 adjuvanted vaccine was compared with standard influenza vaccine and was found to be 63% more effective.[59] A recombinant inactivated influenza vaccine (rIIV)comprised of 45mcg hemagglutinin per strain (total of 180mcg) was licensed for use in the United States in 2013. Likely due to the higher dose of antigen, the rIIV was shown to have higher efficacy in adults ≥50 years of age in a trial during the 2014-2015

season.[60] At this time, the, the Advisory Committee on Immunization Practices recommends all adults ≥65 years of age receive an influenza vaccine but does not preferentially recommend any vaccine for this population.

2.0 Specific Aims:

Studies of immune function in elderly populations have been limited. We propose to advance multiple high-priority fields simultaneously: T cell immunology, immunologic aging and immune senescence, precision medicine and vaccine responses, and the relationship between cellular immune responses to chronic CMV infection and response to influenza vaccination in immunocompetent older populations.

Specific Aim 1. To perform HAI and microneutralization assay individuals who receiving influenza vaccine.

Specific aim 2. Determine whether profile of immune senescence is predictive of vaccine responses in elderly individuals receiving IIV-HD, aIIV3 or rIIV.

Specific aim 3. Determine whether inflated CMV-specific immune responses in elderly populations are responsible for the immune senescence profile of elderly individuals and limits their ability to mount immune responses to vaccines

Specific aim 4. Determine whether measures of frailty are associated with immune senescence and decreased ability to respond to vaccines.

STUDY PROCEDURES

3.0 Eligibility for enrollment.

Community dwelling adults ≥65 years of age without contraindication for influenza vaccination will be eligible. Participants will not be excluded based on comorbid conditions in order to comprehensively capture the spectrum of the aging population.

Accural Goal

Our goal is to enroll 120 people each year.

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria

1. Age ≥65 years
2. Independent Living (including Assisted Living)

Exclusion Criteria

1. Unable to understand the consent or the study.
2. Allergic to any vaccine components, excluding eggs.
3. History of Guillain-Barre.
4. Anaphylaxis egg allergy
5. Residing in a long-term care facility such as a nursing home.

5.0 Study procedures:

Data Collection: During the first year of the study, the following forms will be designed to complete the objectives of this study.

1. ***REDCap Case Report Form:*** Administered by the enrollers, and includes: demographics; height and weight; past medical history; history of recent falls and hospitalizations; active medications; alcohol use; use of home oxygen; questions regarding ability to perform activities of daily activity; frailty measures (see section on Frailty Metrics); sarcopenia assessment; depression symptom screen; cognitive assessment; and contact information for the participant.
2. ***Frailty Metrics:*** As mentioned earlier, the evaluation of frailty is very important in the assessment of vaccine responses. Frailty can be measured by multiple methods. One of the most accepted tools is the Fried Frailty Evaluation.[49] This tool evaluates shrinkage (loss of mean body mass), weakness, endurance, slowness, and physical activity on a scale of 0-5. This tool is predictive of falls, worsening mobility or disability in the activities of daily living, and in predicting hospitalization and death over a three-year period. This measure of frailty is not synonymous with comorbidities or functional status. A composite frailty measure will be calculated using each of the evaluation scales and utilized to compare the safety profile and ultimately the immune responses.[61] This measure will be based on the five parameters of weight loss, exhaustion, low activity level, slowness, and weak strength, described below.

1. Weight Loss	*Meets criteria for frailty if answers yes to:* Self-report of loss of >10 pounds unintentionally over the past year
2. Exhaustion	*Meets criteria for frailty if answers:* Self-report of "moderate" or "most of the time" for either: 1. I felt that everything I did was an effort in the last week: a. Rarely or none of the time (<1 day) b. Some or little of the time (1-2 days) c. Moderate amount of the time (3-4 days) d. Most of the time 2. I could not get going in the last week: a. Rarely or none of the time (<1 day) b. Some or little of the time (1-2 days) c. Moderate amount of the time (3-4 days) d. Most of the time
3. Low Activity Level	*Meets criteria for frailty if answers:* Self-reported "limited a lot" for: Does your health limit vigorous activities such as running, lifting heavy objects, or participating in strenuous sports? a. No, not at all b. Yes, limited a little c. Yes, limited a lot
4. Slowness	*Meets criteria for frailty if:* Time to walk 15 feet (4.57 meters) at usual pace (averaged over 2 trials) Men: ≥ 7sec for height ≤ 173cm; ≥ 6sec for height > 173cm Women: ≥ 7sec for height ≤ 159cm; ≥ 6sec for height > 159cm

5. Weakness	*Meets criteria for frailty if:* Grip strength measured by hand dynamometer (averaged over 3 trials of dominant hand): Men / Women ≤ 29kg for BMI ≤ 24 / ≤ 17kg for BMI ≤ 23 ≤ 30kg for BMI 24.1-26 / ≤ 17.3kg for BMI 23.1-26 ≤ 30kg for BMI 26.1-28 / ≤ 18kg for BMI 26.1-29 ≤ 32kg for BMI >28 / ≤ 21kg for BMI >29 (BMI: body mass index)

Participants with total scores of 3 or greater meet criteria for frailty. Those participants with a higher composite score (and therefore a greater degree of frailty) will be expected to have lower reactogenicity to vaccine and likely a lower serologic response, but this hypothesis will be tested.

3. ***Five-times Sit-to-Stand (5STS) Test for Sarcopenia.*** All participants will be undergo two trials of timed duration to go from sitting position to standing position and back to sitting five times. The average of the 2 trials will be used for the result. The 5STS is a routinely-used geriatric assessment for sarcopenia and fall risk. An average 5STS time of >12 seconds is indicative of fall risk [62].
4. ***The Montreal Cognitive Assessment (MoCA)****:* The MoCA is a validated, performance-based tool used to detect cognitive impairment with score ranges from 0 to 30 points. It will be administered at enrollment. The following ranges may be used to grade severity: 18-26 = mild cognitive impairment, 10-17 = moderate cognitive impairment and < 10 = severe cognitive impairment. This tool will be included as many geriatricians feel cognitive impairment is often associated with frailty. As presence of depressive symptoms may affect cognition and memory, we will also include a depression symptom screen as part of the assessment. All participants will complete the Patient Health Questionnaire as part of routine care. The results of the Patient Health Questionnaire performed during routine medical care will be included in the chart review.
5. ***Baseline clinical laboratory assessment***

   At the first visit, blood specimen collection will also include evalution of two clinical tests to be conducted by the Vanderbilt Laboratory: CMV serology and CD4/CD8 lymphocyte ratio calculation. Detection of serum CMV IgM and IgG antibodies will be conducted on all participants. Calculation of absolute CD4 and absolute CD8 lymphocytes will also be performed using the clinical lab for calculation of CD4/CD8 ratio. Inversion of the CD4/CD8 in older adults has been found to correlate with measures of adaptive immune senescence, CMV seropositive, as well as frailty and falls [63-65].

	Visit 1 (D0)	Visit 2 (D7[+/2days])	Visit 3 (D28 [-4/±14 days])	Visit 4 (Month 6/ D180[+/-14 days]) Optional
Baseline Data Collection: - Case report form - Frailty Metrics - 5STS - MoCA	X			

- Clinical laboratory tests				
Blood Draw	X	X	X	X
Vaccination	X			

Recruitment and Retention: Although most industry-sponsored vaccine studies in older adults seek to recruit a very healthy, mobile population, in this study we will pay special attention to the enrollment of older adults ≥80 years of age and those who are frail so we can get a more comprehensive assessment of the safety and immunogenicity of these two vaccines in a larger spectrum of older adults. The population in Davidson County is 61% white and 27% black with 37,787 woman and 25,574 men ≥65 years of age who reside in the County.

Informed Consent in Older adults: Previous research conducted by our group to identify viral respiratory disease in hospitalized patients has led our team to work closely with our Institutional Review Board to improve the consenting process for older adults. Consents have been simplified to include only necessary information and they are printed in larger fonts. Each enroller also utilizes magnifiers and hearing amplifiers to assist the participants. In addition, we also have utilized a standardized assessment to determine a person's ability to self-consent wherein they are asked specific questions to determine their understanding of the consent form. These practices will be used for the proposed study.

Table : Enrollment Plan for 2019-2020

Samples (100 subjects)	**Tests**	**Amount of Blood/Participant**
Prevaccination (Day 0)	PBMC/HLA + Ab + CBC w/diff	45 ml +10 ml + 10ml = 65 ml
Day 7 (+/- 2 days)	PBMC + Ab + CBC w/diff	45 ml +10 ml + 10 ml= 65 ml
Day 28 (-4/+14 days)	PBMC + Ab + CBC w/diff	45 ml +10 ml + 10 ml= 65 ml
Month 6/Day 180 (+/- 14 days)	PBMC + Ab + CBC w/diff	45 ml +10 ml + 10 ml= 65 ml
Total		270 ml

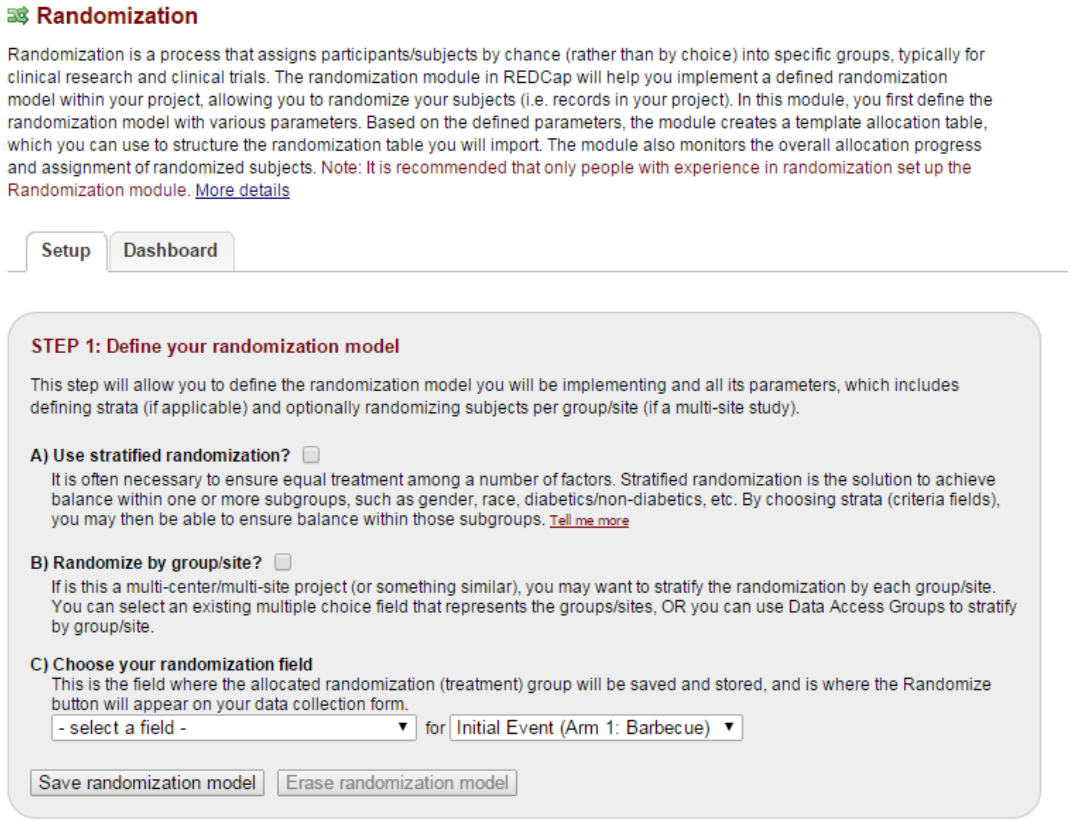
PBMC = peripheral blood mononuclear cells
Ab= antibody

Approximately 100 adults ≥65 years of age will be enrolled. After consent, blood and serum will be collected and then subjects will be randomized to either high dose influenza vaccine or adjuvanted influenza vaccine (both FDA approved vaccines). Vaccination will be done by an unblinded nurse. All further evaluations and analysis will be done by a blinded staff member. Medical history, relevant medications, and frailty status will be assessed at the first visit. Subjects will return (Table 3) for blood and serum draws on days 7 (+/- 2 days), 28 (-4/+14 days) and for an optional visit at 6 months/180 days (+/- 14 days) (after influenza season).

Influenza vaccination

The ACIP recommends influenza vaccine for all adults ≥65 years of age. This includes the egg-based vaccines, the cell-based vaccines and the recombinant vaccines. This study will use the egg-based high-dose inactivated influenza (IIV-HD), the egg-based adjuvanted influenza vaccine (aIIV), and the recombinant influenza vaccine (rIIV). Each of these vaccines are recommended and licensed in adults ≥65 years of age and will be used in this study according to licensure.

Subjects Randomized: Subjects will be randomized 1:1:1 using the REDCap System, hosted at Vanderbilt. Since this is a web-based system, Randomization in REDCap allows users to create their custom allocation list, which will serve as a lookup table for deciding how to randomize subjects. First, users will set up their randomization model and all its parameters, after which REDCap will provide some examples of how to set up the allocation table. The randomization table must be generated outside of REDCap using other software (e.g., SAS, Stata, R) before users will finally upload it. By letting users create their own allocation table outside of REDCap, they can structure their allocations and assignments (e.g., block sizes, permutations, stratification balancing). The figure below demonstrates the options to set up the randomization model.



6.0 Laboratory Methods

Specimen processing for peripheral blood mononuclear cells (PBMCs):

Blood will be collected from a vein. Study personnel will collect the blood samples, label without personal identifiers, and deliver to the VUMC Cell Processing Core (Directed by Dr. Kalams) for preparation of serum and and isolation of peripheral blood mononuclear cells (PBMCs). Serum will be frozen until further testing at Vanderbilt University Medical Center for HAI, MN testing, B and T cell influenza-specific responses. Processing, cryopreservation, storage, and maintenance of sample logs will be done according to standard operating procedures that have been in place and maintained for over 15 years All clinical labs will be sent to the local lab.

Specimen processing for serum:

Whole blood drawn in a tube without anticoagulant will be left at room temperature for a minimum of 30 minutes and maximum of 2 hours. The samples will then be placed at 4ºC.

Samples will be processed within 24 hours of collection. Tubes will be placed in a table top centrifuge and serum will be clarified by centrifugation at 3000 rpm for 10 minutes at 4ºC. Serum will be aliquotted into labeled cryovials with an adjustable pipette. Aliquot size will be determined based on the testing to be performed. Serum will then be stored at –80ºC in labeled fiberboard boxes until shipping or testing is performed.

HAI Assay:

The hemagglutination inhibition assay (HAI) assay is adapted from the CDC laboratory-based influenza surveillance manual and we have extensive experience in its use. Briefly, three parts of receptor destroying enzyme (RDE) will be added to one part sera and incubated overnight in a 37°C water bath. Then the RDE will be inactivated by incubation in a 56°C water bath for 30 minutes. Following inactivation of RDE, PBS will be added to the sample for a final serum concentration of 1:10. Turkey red blood cells (RBC) will be washed twice and diluted in PBS to a final concentration of 5% RBC. The cells are kept at 4°C and expire in one week. Twenty-five microliters of RDE-treated sera are then serially diluted across the plate (2-fold). Twenty-five microliters of prepared flu antigen tittered to 4HAU is added to the diluted sera. The plates are then incubated at room temperature for 30 minutes followed by the addition of 50 µl of 5% turkey RBC. Then, the plates are mixed and the RBCS are allowed to settle for 1 hour at room temperature. The test will be read by observing a lack of agglutination in the button RBC that is settled in the bottom of the well. The HAI titer is determined by the reciprocal dilution of the last well with lack of agglutination. Every plate will have a virus and a serum control. The virus control will consist of PBS + 4HAU of antigen + turkey RBC. The cell control will consist of PBS + RBC. A negative titer is reported as a <10. All samples are tested in duplicate and results are expected to be +/- one well. If duplicate results are more than one well different the sample will be repeated. The lower of the two results within one well will be recorded as the titer.

7.0 Statistical Analysis:

This is an exploratory study. Antibody titers will be compared pre- and post-vaccination on the samples collected. Paired measures of cell mediated immunity will be compared before and after vaccination. In addition, post-vaccination measures of cell mediated immunity will be compared across age strata and correlated with serologic response to vaccination. Analyses will be done accounting for age, frailty, and underlying medical conditions.

Data Management: All data will be collected and stored under the REDCap database system. This system is a secure web application for building and managing online surveys and databases. While REDCap can be used to collect virtually any type of data, it is specifically geared to support data capture for research studies. The REDCap Consortium is composed of 1,920 active institutional partners in 103 countries who utilize and support REDCap in various ways including the CDC. The REDCap application allows users to build and manage online surveys and databases quickly and securely, and is currently in production use or development build-status for more than 273,000 projects with over 372,000 users spanning numerous research focus areas across the consortium. This system will allow each site to access the database and will provide a secure means to send a study dataset without personal identifiers upon request to the CDC.

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