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# Statistical modeling using early markers of innate immunity to explain variation in humoral responses to influenza vaccine in older adults

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ABSTRACT

Greater understanding of the factors associated with a protective response to influenza vaccine in older adults could have tremendous public health benefits. We studied 158 participants age 50–74 years vaccinated with 2010–2011 inactivated influenza vaccine and performed innate immunity and humoral immunity assays directed against influenza A/California/2009 (H1N1) as measured through hemagglutination inhibition (HAI), microneutralization, and B cell ELISPOT at days 0, 3, and 28 postvaccination. We report the results of statistical modeling using Day 3 cytokines, chemokines, and innate cell populations to model Day 0 to Day 28 HAI seroconversion, viral neutralization seroconversion, and B cell ELISPOT results.



## 1. Background

The elderly population has the highest risk of morbidity and mortality from influenza infection, and is the population least likely to respond to inactivated influenza vaccine [1,2]. In generating protective immunity, antigens introduced through vaccination activate innate immune pathways that trigger adaptive responses leading to the production of humoral immunity [3]. Identifying early innate immune markers that are associated with humoral immune response to influenza vaccine may help distinguish between those who are likely to generate protective immunity shortly after vaccination from those who are not. This is of particular importance in older individuals whose immune systems are less capable of responding to vaccines and infections. This immunosenescence, or age-related decline in immune function, has a significant impact on health and longevity in older individuals. In the long term, early biomarkers may also inform development of novel influenza vaccines to generate protective immunity more reliably in the elderly.

The hemagglutination inhibition assay (HAI) has been used as the correlate of protection for influenza vaccine response since the latter half of the 20th century [1,4]. Studies in healthy adults and children have found that an HAI titer of 1:40 corresponds with a 50% reduction in influenza infection and is considered the benchmark for seroprotection; a four-fold rise in HAI titer has been conventionally used to indicate immunologic response to vaccination (i.e., seroconversion) [1,4–7]. At this time, influenza vaccines must demonstrate adequate HAI response for licensure by the Food and Drug Administration (FDA); however, HAI alone is insufficient to characterize humoral response to influenza vaccination, especially in older adults [6–8]. Newer assays such as viral neutralization antibody (VNA) and influenza B cell ELISPOT offer complementary assessment of protective antibody responses through analysis of inactivation of influenza infectivity, and influenza-specific IgG secreting B cells, respectively [7,9,10]. Further validation is needed to evaluate the use of these assays as correlates of protective immunity from influenza vaccination with regard to vaccine efficacy and licensure.

In this study, we describe a cohort of older adults who received 2010–2011 inactivated influenza vaccine and present the results

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of statistical modeling to identify early innate immune markers that are associated with humoral immune responses to influenza A/California/2009 (H1N1), as measured through HAI, microneutralization, and B cell ELISPOT.

## 2. Methods

### 2.1. Study participants

The following methods are similar to or identical to previously published studies using this cohort [9,11,12]. We recruited 200 generally healthy adult volunteers age who were age 50–74 years prior to the 2010–2011 influenza season. Volunteers were excluded from the study if they had already received a dose of 2010–2011 influenza vaccine at the time of enrollment, had a history of severe allergic reaction to influenza vaccine, were allergic to egg or chicken proteins, had a history of Guillain-Barré Syndrome, had any immunocompromising conditions, had any serious chronic medical conditions, had any new medical diagnoses or medications in the preceding three months, received any blood products or immunoglobulin within six months prior to enrollment, were on chronic anticoagulation, or had received (or intended to receive) any investigational agents during the course of the study. Blood was drawn from each participant prior to vaccination (Day 0) with 2010–2011 seasonal influenza vaccine (Fluarix [GlaxoSmithKline], containing A/Christchurch/16/2010 NIB-74XP [H1N1] [an A/California/7/2009-like virus], A/Texas/50/2012 NYMC X-223A [H3N2] [an A/Victoria/361/2011-like virus], and B/Brisbane/60/2008 strains), as well as Days 3, and 28 following vaccination. The assays described below were run on the 159 subjects who had blood drawn at all timepoints. One subject was excluded because of extremely high cytokine/chemokine values and clinical features of possible immune deficiency; hence, 158 subjects were included in subsequent analyses. Mayo Clinic's institutional review board approved this study.

### 2.2. Assays of innate immunity

Meso-Scale Discovery (MSD) electrochemiluminescence was used to quantify cytokine and chemokine levels from sera for each participant at Day 3 following vaccination and have been described previously [11]. Cytokines and chemokines investigated were INFγ, IL-2, IL-4, IL-10, IL-7, IFNα-2a, IL-8, IL-1b, GM-CSF, IL-6, TNFα, Eotaxin, Eotaxin-3, MIP-1b, TARC, IP-10, MCP-1, MDC, MCP-4, RANTES, and MIP-1a. The relative frequency of innate and antigen presenting cells (B cells, NK cells, NK T cells, dendritic cells, classical monocytes, intermediate monocytes, and non-classical monocytes) was assessed through polychromatic flow cytometry to simultaneously identify surface markers on peripheral blood mononuclear cells (PBMC) from each participant at Day 3 following vaccination.

### 2.3. Assays of humoral immunity

Influenza A/California/2009 (H1N1) was grown in embryonated chicken eggs, as previously described [13]. Virus was quantified using HAI and $TCID_{50}$ following infection of MDCK cells; a single viral stock was used for all assays of humoral immunity.

The HAI assay was performed as previously described [13]. Sera from each participant at Day 28 were supplemented with receptor destroying enzyme and was diluted 1:10 and then serially diluted. Each dilution was incubated with 8 HA units/50 μL of influenza virus for 15 min followed by addition of 0.65% guinea pig erythrocytes; agglutination was recorded after 1 h of incubation. Pooled high titer antiserum was used as a positive control and pooled serum from previously unvaccinated subjects was used as a negative control.

The VNA assay was performed as previously described [10]. Sera from each participant at Day 28 were heat-inactivated, serially diluted, and incubated with 8 HA units/50 μL of influenza virus for 2 h. MDCK cells were added and incubated for 24 h followed by acetone fixation. Influenza A nucleoprotein (NP) was detected in infected cells through ELISA. The Reed-Muench method was used to determine the 50% inhibiting titer [14]. Pooled high titer antiserum was used as a positive control and pooled serum from previously unvaccinated subjects was used as a negative control.

The B cell ELISPOT was performed using ELISPOT$^{PLUS}$ for Human IgG (Mabtech), as previously described, after coating plates with 50,000 $TCID_{50}$/well of influenza virus [9]. ELISPOT plates were analyzed using ImmunoSpot S4 Pro Analyzer and ImmunoSpot version 4.0 software (Cellular Technology Ltd., Cleveland, Ohio, USA) [9].

### 2.4. Statistical analyses

Spearman correlations were computed to assess the association between Day 3 innate markers and Day 28 humoral immunity (HAI, VNA, and B cell ELISPOT), as well as the association between Day 3 cytokines/chemokines with Day 0 immunosenescence markers. Multivariable models were developed for Day 28 humoral response from Day 3 innate markers using elastic net penalized regression with tuning parameter $\alpha = 0.9$ [15]. First, redundancy analysis was used to reduce the number of independent variables[16]. Specifically, regression models were built for each Day 3 marker as a function of the other Day 3 markers, and Day 3 markers with an $R^2 \geq 0.75$ were eliminated. Next, multivariable models were developed separately for each humoral response. HAI and VNA were modeled with logistic regression, with the dependent variable defined as a positive four-fold change between Day 0 and Day 28; subjects who had a titer of 1:640 or more were excluded because they deemed not able to achieve a positive four-fold change [15]. Model fit was assessed for the logistic regression models by comparing the Brier's Score [17,18] for the model with the minimum cross-validated misclassification error rate to the intercept only model 9 (i.e., a non-informative model). A model with perfect accuracy would have a Brier's score of 0. B cell ELISPOT ($\log_2$) at Day 28 was modeled with linear regression, with Day 0 B cell ELISPOT ($\log_2$) included as a covariate. Model fit for the linear model was assessed by comparing the $R^2$ value from the final model to the model with only the Day 0 B cell value as a covariate. Penalized regression models were fit using the "glmnet" function in R [15]. The R statistical software version 3.0.1 was used for all analyses (www.r-project.org).

## 3. Results

One hundred fifty-eight participants were included in the analyses: 60 (38.0%) were male, 156 (98.7%) were Caucasian, and the median age was 59.6 years (IQR 55.3–66.4). Regarding HAI, 157 (99.4%) had titers ≥ 1:40 at Day 28; 58 (36.7%) had a four-fold increase in titer from Day 0 to Day 28; and 20 (12.7%) had a Day 0 titer ≥ 1:640, for which a four-fold increase was not observed in any participants (i.e., antibody ceiling). The median B cell ELISPOT (median stimulated–unstimulated) for Day 28 was 34.8 Spotforming units (SPUs) per 200,000 cells (IQR 15.1–56.5). For VNA, 157 (99.4%) had titers ≥ 1:40 at Day 28; 67 (42.4%) had a four-fold increase in titer from Day 0 to Day 28; and 20 (12.7%) had a Day 0 titer ≥ 1:640, for which a four-fold increase in titer at Day 28 was unlikely to be physiologically feasible. Day 3 serum levels of several cytokines and chemokines were determined using a multiplex ELISA-based assay, and the distributions of the innate cell populations are reported in Table 1.