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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.

Keywords

Influenza Vaccines; Immunity; Humoral; Models; Statistical; Aged; Adult; Age Factors

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Conflicts of Interest:

Dr. Poland is the chair of a Safety Evaluation Committee for novel non-rubella investigational vaccine trials being conducted by Merck Research Laboratories. Dr. Poland offers consultative advice on vaccine development to Merck & Co. Inc., CSL Biotherapies, Avianax, Sanofi Pasteur, Dynavax, Novartis Vaccines and Therapeutics, PAXVAX Inc, Emergent Biosolutions, Vaxess and Adjuvance. Dr. Poland holds two patents related to measles and vaccinia peptide research. These activities have been reviewed by the Mayo Clinic Conflict of Interest Review Board and are conducted in compliance with Mayo Clinic Conflict of Interest policies. None of the other authors have any relevant conflicts of interest to disclose. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo Clinic Conflict of Interest policies.

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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 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.

Methods

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.

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 $\text{INF}\gamma$, IL-2, IL-4, IL-10, IL-7, $\text{INF}\alpha$ -2a, IL-8, IL-1b, GM-CSF, IL-6, $\text{TNF}\alpha$, 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.

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 minutes followed by addition of 0.65% guinea pig erythrocytes; agglutination was recorded after 1 hour 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 hours. MDCK cells were added and incubated for 24 hours 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 TCID₅₀/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].

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 $R^2 \leq 0.75$ 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 fourfold 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).

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 $\geq 1:40$ 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 $\geq 1:640$, 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 $\geq 1:40$ 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 $\geq 1:640$, 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.

We assessed several markers of immunosenescence in our cohort (e.g., chronological age, T-cell receptor excision circles [TREC], telomerase expression, and the percent of CD4⁺ or

CD8⁺ T cells that were CD28^{neg}). The serum levels of several of the cytokines and chemokines that were assayed in this study correlated with these markers of immunosenescence. Age was positively correlated with both CCL26 (Eotaxin 3: $r=0.18$, $p=0.02$) and CXCL10 (IP-10: $r=0.20$, $p=0.01$). The percent of CD28-negative T helper cells was negatively correlated with serum levels of IL-10 ($r=-0.17$, $p=0.03$), while the percent of CD28-negative cytotoxic T lymphocytes was positively correlated with CCL11 (Eotaxin; $r=0.16$, $p=0.04$), and negatively correlated with IL-4 ($r=-0.17$, $p=0.03$) expression. Telomerase activity correlated with IL-4 levels ($r=0.22$, $p=0.005$). TREC levels positively correlated with four cytokines (IL-10: $r=0.18$, $p=0.02$; IL-6: $r=0.23$, $p=0.005$; IL-7: $r=0.17$, $p=0.04$; and TNF α : $r=0.27$, $p=0.001$) and one chemokine (MDC: $r=0.20$, $p=0.01$). In addition, there were several significant correlations identified between body mass index (BMI) and Day 3 markers including IL-10 ($r=0.26$, $p=0.002$), IL-7 ($r=0.20$, $p=0.01$), IL-8 ($r=0.24$, $p=0.005$), IL-6 ($r=0.32$, $p<0.001$), TNF α ($r=0.24$, $p=0.005$), IP-10 ($r=0.18$, $p=0.03$), MCP-1 ($r=0.23$, $p=0.005$), MDC ($r=0.28$, $p<0.001$), MCP-4 ($r=0.23$, $p=0.006$), MIP-1a ($r=0.17$, $p=0.04$), and percent plasmacytoid DCs ($r=-0.17$, $p=0.04$).

The univariate correlation analysis of Day 3 innate immune markers and Day 28 humoral immune response is summarized in Table 2. GM-CSF was significantly correlated with HAI titer ($p=0.007$) and VNA titer ($p=0.010$) and negatively correlated with B cell ELISPOT ($p=0.032$). IL-6 and the level of HLA class II expression on classical monocytes were significantly correlated with HAI titer ($p=0.003$ and 0.042 , respectively) and VNA titer ($p=0.010$ and 0.022 , respectively) but not B cell ELISPOT. INF- γ ($p=0.027$) and IL-8 ($p=0.042$) were significantly correlated with HAI titer. CD86 expression on B cells ($p=0.018$), the percent of B cells present in the PBMC sample ($p=0.0001$), and CD86 expression on classical monocytes ($p=0.020$) were correlated with B cell ELISPOT, and MIP-1b ($p=0.012$) and TARC ($p=0.040$) were significantly negatively correlated with B cell ELISPOT. No other early markers of innate immunity were significantly correlated with humoral immune response at Day 28.

The penalized standardized coefficients from these regression models utilizing Day 3 innate markers to model a four-fold rise in HAI and VNA and B cell ELISPOT at Day 28 are presented in Figure 1. The Day 3 innate markers with the largest positive standardized effects in the HAI seroconversion model were IL-2, IFN α -2a, the percent of intermediate monocytes, MIP-1b, IL-8, and NK T-cells (standardized coefficients 0.39, 0.27, 0.15, 0.14, 0.13, and 0.08 respectively); the Day 3 innate markers with the largest negative standardized effects in the HAI seroconversion model were percent non-classical monocytes, MIP-1a, IL-4, -IL-1b, Eotaxin-3, and IL-7 (standardized coefficients -0.31 , -0.23 , -0.22 , -0.14 , -0.11 , and -0.07 , respectively). In the VNA seroconversion model Day 3 IL-6 had the largest positive standardized effect (standardized coefficient 0.05) while the percent non-classical monocytes and IL-1b had the largest negative standardized effects (standardized coefficients -0.14 , and -0.11 , respectively). The statistical modeling found B cells to have the largest positive association with Day 28 B cell ELISPOT (standardized coefficient 0.07). Assessing model fit, the penalized logistic models for HAI had the best improvement when adding covariates, and Brier's Scores for the HAI model improved to 0.18 compared to 0.24 for the null model. VNA had a Brier' Score of 0.23 compared to 0.25 for the null model.

The increase in R^2 for the B cell model was <0.01 , which indicates there was negligible gain over just modeling the baseline value.

Discussion

In this study of generally healthy older volunteers aged 50–74 years receiving influenza vaccination prior to the 2010–2011 influenza season, we investigated early innate immune markers associated with humoral immune response to vaccination. As expected, the median pre-vaccination HAI titers reflected a past history of vaccination and/or infection in our subjects. Not all subjects responded in the same manner; in fact, subjects could be grouped together into several distinct response profiles, which we have previously reported [19]. There was a correlation between BMI and acute inflammatory markers, as described previously [20].

Immunosenescence markers were, by and large, positively correlated with serum levels of pro-inflammatory cytokines and chemokines. Note that none of the correlations were extremely large, likely due to the relatively narrow age range of our cohort (50–74 years of age). Interestingly, TREC was correlated with both pro-inflammatory markers IL-6 and TNF α , and MDC, as well as with IL-7[21, 22]. We also identified classical Th2 markers (IL-4 and IL-10) associated with telomerase activity and TREC, respectively. While it is tempting to assume that this reflects an age-related Th2 shift, we also identified negative correlations between other immunosenescence markers. This likely reflects the fact that each of these markers that are typically linked to immunosenescence involve discrete and separate immunologic pathways. Given the complex nature of immunosenescence and the myriad ways in which immune function is altered as we age, further investigation in this area is needed.

In univariate analyses, Day 28 HAI titer was significantly correlated with Day 3 INF- γ , IL-8, GM-CSF, IL-6, and HLA class II expression levels on classical monocytes. Not surprisingly, Day 28 VNA titer was significantly associated with similar innate markers. These include Day 3 GM-CSF, IL-6, and HLA class II expression on classical monocytes. These are primarily proinflammatory cytokines and may reflect a more robust innate response to the vaccine which, in turn, stimulates a stronger humoral response. The correlation between HLA class II expression (indicative of maturation) on monocytes further supports this hypothesis. Production of GM-CSF has been shown to amplify marginal zone B cell antibody production [23] through enhanced secretion of cytokines (BAFF, APRIL, IL-12) by B cell-helper neutrophils[24]. For the immune outcome associations, please note that the cytokine levels in most subjects were only detectable at very low levels and the correlation coefficients were small. For the correlations discussed above, several may have limited impact biologically. Day 28 B cell ELISPOT was significantly correlated with the number of B cells present at Day 3. This is not surprising, and may simply reflect having a greater number of B cells in the PBMCs tested by the ELISPOT assay. Total B cell numbers actually dropped from 3.7% of PBMCs on Day 0 to 3.1% of PBMCs on Day 3 ($p=2.7 \times 10^{-5}$) and then remained at a similar level at Day 28. We also identified a positive correlation between B cell ELISPOT response and the expression of CD86 on B cells, which may reflect early stages of B cell activation [25], and CD86 expression on classical

monocytes. In contrast, memory B cell ELISPOT responses were negatively correlated with expression levels of GM-CSF, MIP-1b, and TARC. The correlation between total B cells and antigen-specific B cells is not surprising; the correlations between costimulation/activation markers on both B cells and monocytes likely reflect the increased innate activation state in the higher responders. The inverse correlation between antibody response and MIP-1a levels is similar to the effect observed in an HIV plasmid vaccine study [26], where administration of plasmids encoding MIP-1a and the Gag protein resulted in lower levels of IgG, IgG1, and IgG2a. TARC elicits chemotactic responses in T cells through CCR4 binding [27] and may influence humoral responses indirectly through its effect on CD4⁺ T helper lymphocytes.

Recently, a number of reports have been published using systems biology approaches to evaluate how early events following vaccination correlate with downstream immune outcomes. Most of these reports focus on gene expression profiles shortly after vaccination. Bucasas, et al. identified an upregulated pattern of genes involved in IFN and IL-6 signaling as well as antigen processing and presentation pathways that occurred within 24 hours of vaccination[28–31]. Our study presented in this manuscript focused on serum cytokine/chemokine measurements and cellular subsets and phenotypes. By day 3, we found very low levels of IL-6 and IFN- α 2a in the sera of vaccine recipients (Table 1), nevertheless we did identify significant, positive correlations between IL-6 levels and both HAI and VNA (Table 2). Thus, our results support the prior report despite different timepoints (day 1 versus day 3), different cohorts (ages 18–40 compared to 50–74), and the transcriptomic versus proteomic focus of the respective studies. A number of other reports have identified early gene signature in antibody secreting cells that control the unfolded protein response [30], baseline signatures of vaccine responses that might serve as predictive biomarkers [31], and another study focused on sex-based differences in influenza vaccine response [29]. This last study identified a greater inflammatory cytokine profile and elevated antibody responses in female vaccine recipients. We have also reported on enhanced humoral vaccine responses in females [11, 32, 33].

In this study, statistical modeling was able to identify early innate markers that were associated with HAI seroconversion, VNA seroconversion, and B cell ELISPOT results. The percent B cells at Day 3 had the largest impact (largest standardized coefficient) on influenza-specific B cell ELISPOT, a quantitative measure of antigen specific B cells. Interestingly, the percent myeloid dendritic cells and their surface expression of CD86 were also associated with B cell ELISPOT responses. CD14⁺ DCs have been shown to promote plasma cell development through the secretion of cytokines such as IL-12 and IL-6 [34]. Similarly, IL-6 levels and the percent B cells at Day 3 were also identified as markers associated with a positive VNA response, while IL-1b and the percent of non-classical monocytes were negatively associated. With regard to the accepted measure of vaccine response, HAI titer, we found that the percent of B cells at Day 3 was associated with a positive response. While this finding was not surprising, it was interesting to note that the response model included other cell types such as intermediate monocytes, NK T cells, and plasmacytoid DCs (all positive), while the percent of non-classical monocytes had a negative association. It is entirely possible that differential cytokine secretion or other survival signals (BAFF) by these cells may exert differential effects on the developing B cell

responses [35–37]. We are intrigued by the differential effect of MIP-1 α and MIP-1 β in our model. Perhaps this differential effect relates to the proinflammatory cytokine production caused by MIP-1a, but not MIP-1b. Alternatively, it might reflect the different cell subsets upon which these chemokines exert their influence.

In this study, we more broadly examined the humoral immune response to influenza vaccination rather than HAI alone. Although HAI response is required for influenza vaccine licensure in the United States, it is insufficient as a stand-alone correlate of protection against influenza infection, especially in older adults [7, 8]. Analysis of inactivation of influenza infectivity through VNA and influenza-specific, IgG-secreting B cells (by ELISPOT assay) provides a more complete characterization of humoral immune response to influenza. VNA has a potential advantage over HAI by being able to demonstrate a reduction in infectivity; however, further research is needed to identify VNA titers that correlate with protection from virologic challenge, as well as an increase in VNA titer that indicates a successful immunologic response to vaccine. B cell ELISPOT can quantify the number of influenza-specific B cells present; however, further assays are needed to assess functional antibody secreted by these B cells. The effect of pre-existing immunity as measured by HAI, VNA, and B cell ELISPOT on response to influenza vaccination in this study is further discussed in a previously published manuscript [19].

Understanding humoral response in the elderly based on early biomarkers may have practical ramifications, as well as utility, in influenza vaccine development. We were able to identify chemokines and cytokines that were associated with HAI, VNA, and B cell ELISPOT after vaccination, with the HAI model having the best performance. Due to large inter-individual variability, the accuracy of these results can still be improved. The utility of data from these modeling results may inform further analysis and studies targeting specific genes and proteins collected with high dimensional omics data as well as further refinement (requiring larger sample sizes or additional cohorts). However, the current data may be useful in identifying specific biological pathways for more in-depth, targeted examination. As older adults have the highest burden of influenza disease and the weakest response to vaccination, it would be ideal to quickly distinguish between those who are likely to be protected from infection by the vaccine and those who are not. This may lead to new strategies such as repeat vaccination, development and use of novel more immunogenic vaccines, antiviral prophylaxis, or early initiation of antiviral therapy to protect patients who are predicted to be unprotected from the vaccine. This would be especially useful for optimal resource allocation in the setting of pandemics with novel influenza strains where two-dose regimens may be required to achieve adequate protection in some people and vaccine antigens may be in limited supply. In the long-term, understanding the innate immune factors that contribute to protection through humoral immunity will help to inform vaccine development to create more efficacious influenza vaccines, especially for the elderly. For example, our data indicate that increased levels of certain cytokines/chemokines (IL-12, IFN α 2a, IL-8, MIP-1b) are associated with stronger HAI response; while other cytokines/chemokines (IL-1b, IL-4, IL-7, MIP-1a) are negatively correlated with humoral response. This suggests that cytokines/chemokines may serve as useful adjuvants in influenza vaccines for the elderly.

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a) HAI

b) VNA

c) B cell ELISPOT

Figure 1. Barplots of standardized regression coefficients using Day 3 innate immune markers from adult volunteers age 50–74 years to model a) hemagglutination inhibition (HAI) seroconversion, b) viral neutralization antibody (VNA) seroconversion, and c) B cell ELISPOT results at Day 28

Standardized regression coefficients are presented in order to facilitate comparison of effect sizes between markers and models. For logistic regression models, these can be interpreted as the increase in log odds of response for every 1 standard deviation increase in the marker. For linear regression models, these are interpreted as the amount the response increases for every one standard deviation increase in the marker.

Table 1

Distribution of Day 3 Serum Cytokines, Chemokines and Innate Cell Populations.

Cytokines (pg/ml)	N	Median	IQR
IFN γ	158	0.50	0.25–0.91
IL-2	158	0.11	0.05–0.16
IL-4	158	0.01	0.00–0.06
IL-10	158	0.91	0.72–1.43
IL-7	158	4.13	2.77–5.80
IFN α -2a	148	0.13	0.04–0.23
IL-8	148	5.38	3.67–9.29
IL-1b	148	0.06	0.00–0.25
GM-CSF	148	0.18	0.04–0.42
IL-6	148	0.50	0.31–0.83
TNF α	148	5.30	4.13–6.80
Chemokines (pg/ml)			
Eotaxin	158	220.08	171.47–299.53
Eotaxin-3	158	22.98	12.55–43.98
MIP-1b	158	125.53	89.07–169.03
TARC	158	406.15	249.92–627.37
IP-10	158	151.21	109.37–228.57
MCP-1	158	418.69	327.61–545.13
MDC	158	250.39	179.54–341.24
MCP-4	158	694.84	521.14–1035.97
RANTES	158	101187.00	81321.19–151880.75
MIP-1a	158	1.15	0.63–2.39
Innate Cell Populations (percentage or mean fluorescence intensity)			
% B Cell	158	3.13	2.33–4.11
HLA Expression on B cells	158	3287.30	2789.76–3940.86
CD86 Expression on B cells	158	62.38	55.53–68.37
% NK Cells	158	11.97	7.82–16.91
% NK T Cells	158	3.02	1.86–6.15
% mDC	158	1.82	1.01–3.62
HLA Expression on mDC	158	1843.60	1403.04–2308.10
CD86 Expression on mDC	158	366.00	326.49–412.37
% pDC	158	0.14	0.10–0.19
HLA Expression on pDC	158	3490.70	2681.16–4718.87
CD86 Expression on pDC	158	139.22	125.19–151.36
% Classical Monocytes	158	3.77	2.49–5.45
HLA Expression on Classical Monocytes	158	2355.60	1784.33–2895.41
CD86 Expression on Classical Monocytes	158	558.91	512.98–620.34

Cytokines (pg/ml)	N	Median	IQR
% Intermediate Monocytes	158	0.15	0.07–0.26
HLA Expression on Intermediate Monocytes	157	7393.40	5912.88–9401.89
CD86 Expression on Intermediate Monocytes	157	738.72	650.03–825.46
% Non-classical Monocytes	158	0.63	0.39–0.94
HLA Expression on Non-classical Monocytes	157	3253.90	2558.76–4011.82
CD86 Expression on Non-classical Monocytes	157	598.31	547.23–690.53

Table 2

Spearman's Correlations between innate immune markers three days after inactivated influenza vaccination in adult volunteers from Mayo Clinic, Minnesota age 50–74 with humoral immune responses 28 days after vaccination

Day 3 innate immune marker	Day 28 Hemagglutination inhibition (HAI) titer		Day 28 Viral neutralization antibody (VNA) titer		Day 28 B cell ELISPOT	
	Spearman Correlation Coefficient	P-value	Spearman Correlation Coefficient	P-value	Spearman Correlation Coefficient	P-value
<i>cytokines</i>						
IFN γ	0.18	0.027	0.12	0.142	0.03	0.681
IL-2	0.06	0.464	-0.01	0.923	0.01	0.873
IL-4	-0.02	0.801	-0.02	0.795	0.07	0.377
IL-10	0.13	0.107	0.06	0.467	0.00	0.977
IL-7	0.15	0.056	0.12	0.123	0.02	0.817
IFN α -2a	0.12	0.154	0.05	0.568	0.12	0.137
IL-8	0.17	0.042	0.14	0.086	0.01	0.930
IL-1b	-0.02	0.829	-0.05	0.531	-0.09	0.286
GM-CSF	0.22	0.007	0.21	0.010	-0.18	0.032
IL-6	0.24	0.003	0.21	0.010	-0.03	0.716
TNF α	0.15	0.068	0.14	0.088	-0.07	0.427
<i>chemokines</i>						
Eotaxin	-0.05	0.497	-0.03	0.706	-0.08	0.289
Eotaxin-3	-0.01	0.866	0.03	0.675	-0.14	0.072
MIP-1b	0.12	0.123	0.10	0.234	-0.20	0.012
TARC	-0.02	0.847	0.06	0.455	-0.16	0.040
IP-10	0.12	0.121	0.06	0.451	-0.07	0.380
MCP-1	0.03	0.696	0.04	0.623	-0.09	0.275
MDC	0.14	0.085	0.13	0.098	-0.10	0.214
MCP-4	0.02	0.796	0.07	0.353	-0.07	0.412
RANTES	0.05	0.533	0.09	0.255	0.06	0.492
MIP-1a	-0.02	0.835	-0.03	0.677	0.03	0.709
<i>Innate and antigen presenting cell populations</i>						
% B Cells	0.14	0.079	0.14	0.089	0.31	0.0001
HLA Expression on B cells	-0.10	0.196	-0.12	0.121	-0.06	0.47

Day 3 innate immune marker	Day 28 Hemagglutination inhibition (HAI) titer			Day 28 Viral neutralization antibody (VNA) titer			Day 28 B cell ELISPOT		
	Spearman Correlation Coefficient	P-value		Spearman Correlation Coefficient	P-value		Spearman Correlation Coefficient	P-value	
CD86 Expression on B cells	-0.001	0.985		-0.03	0.706		0.19	0.018	
% NK Cells	0.02	0.845		0.01	0.874		-0.01	0.934	
% NK T Cells	-0.02	0.765		0.03	0.682		-0.05	0.505	
% mDC	-0.01	0.922		0.02	0.803		0.12	0.149	
HLA Expression on mDC	0.12	0.129		0.10	0.191		-0.01	0.914	
CD86 Expression on mDC	0.15	0.066		0.12	0.124		0.15	0.061	
% pDC	-0.13	0.102		-0.11	0.169		-0.04	0.625	
HLA Expression on pDC	-0.05	0.569		-0.03	0.719		-0.04	0.591	
CD86 Expression on pDC	-0.01	0.881		-0.06	0.487		0.08	0.318	
% Classical Monocytes	-0.12	0.127		-0.09	0.242		0.15	0.055	
HLA Expression on Classical Monocytes	0.16	0.042		0.18	0.022		0.01	0.861	
CD86 Expression on Classical Monocytes	0.04	0.632		-0.01	0.932		0.19	0.020	
% Intermediate Monocytes	-0.02	0.838		0.03	0.664		0.03	0.746	
HLA Expression on Intermediate Monocytes	0.03	0.741		0.06	0.435		-0.10	0.195	
CD86 Expression on Intermediate Monocytes	0.07	0.363		0.03	0.722		0.10	0.221	
% Non-classical Monocytes	-0.11	0.177		-0.06	0.460		0.01	0.888	
HLA Expression on Non-classical Monocytes: HLA	0.02	0.782		0.03	0.751		-0.01	0.852	
CD28 Expression on Non-classical Monocytes	0.07	0.402		0.01	0.880		0.14	0.075	