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Longitudinal and Integrative Biomodeling of Effector and Memory Immune Compartments after Inactivated Influenza Vaccination

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Most vaccines, including those against influenza, were developed by focusing solely on humoral response for protection. However, vaccination activates different adaptive compartments that might play a role in protection. We took advantage of the pandemic 2009 A (H1N1) influenza vaccination to conduct a longitudinal integrative multiparametric analysis of seven immune parameters in vaccinated subjects. A global analysis underlined the predominance of induction of humoral and CD4 T cell responses, whereas pandemic 2009 A (H1N1)–specific CD8 responses did not improve after vaccination. A principal component analysis and hierarchical clustering of individuals showed a differential upregulation of influenza vaccine–specific immunity including hemagglutination inhibition titers, IgA+ and IgG+ Ab-secreting cells, effector CD4 or CD8 T cell frequencies at day 21 among individuals, suggesting a fine-tuning of the immune parameters after vaccination. This is related to individual factors including the magnitude and quality of influenza-specific immune responses before vaccination. We propose a graphical delineation of immune determinants that would be essential for a better understanding of vaccine-induced immunity in vaccination strategies. The Journal of Immunology, 2013, 191: 000–000.

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Abbreviations used in this article: A(H1N1)pdm09, pandemic 2009 A(H1N1); ASC, Ab-secreting B cell; D0, day 0; GMT, geometric mean titer; HA, hemagglutinin; HI, hemagglutination inhibition; M4, month 4; MN, microneutralization; PCA, principal component analysis; SP, single-positive.

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immune responses to vaccine, and to define immune behavior and longitudinal equilibrium in a target population. In this study, we explored the plexus of immune parameters that could be used as hallmarks of vaccine efficacy and the relation between pre-existing immunity and immune response to influenza vaccination. We propose a novel model of evaluation of vaccine immunogenicity that takes into account heterogeneity of individual immune responses to influenza vaccination.

Materials and Methods

Study design

One dose of an adjuvanted A(H1N1)pdm09 influenza vaccine (Pandemrix; GlaxoSmithKline, Marly-le-Roi, France) was given i.m. to 147 hospital health care staff members enrolled in a Phase IV clinical trial from October 21, 2009, through December 16, 2009, in two university hospitals located in Paris (France); 10 subjects discontinued their participation in the study. Eligibility criteria were the following: age $\geq$ 18 y, clinical examination and interview for medical history, documented history of influenza vaccinations, and written informed consent. Further exclusion criteria were any acute or chronic illness, local or systemic immunosuppressive treatments, and pregnancy that might interfere with the study protocol. Supplemental Table I summarizes the subjects’ demographic characteristics. Information on the total number of previous influenza vaccinations was recorded for 127 individuals with an average of 2.59 serum dilutions equivalent to $2^{10^{3}}$ TCID$_{50}$ A/California/07/2009 (H1N1) influenza virus and incubated at 37°C for 2 h before being transferred onto 96-well microtiter plates containing sodium thiocyanate (Sigma). HRP-conjugated mouse anti-human IgG (Panenza; Sanofi Pasteur) were measured by a microtiter HI assay modified and analyzed for Boolean combination gating with the FlowJo software (Tree Star).

Statistical analyses

In univariate analyses, we used Wilcoxon matched-pairs tests for kinetic immune responses and Mann–Whitney tests for continuous variables. A Bonferroni correction was applied to compare groups. Statistical significance was set at $p < 0.05$. All statistical analyses were performed with SPSS statistical software 17.0 (SPSS, Chicago, IL) and SAS 9.2 (SAS Institute, Cary, NC), and Prism 5.0 or Microsoft Excel for Mac OS X for data handling and graphic representation.

Radar chart, principal component analysis

The radar charts were designed with R, a free software environment for statistical computing and graphics (http://www.r-project.org/). The analysis by principal component analysis (PCA) and hierarchical clustering is based on the fold increase of immune responses between D0 and D21. Data on all 7 parameters were available for 79 subjects. Log10 fold increases were normalized using MeV 4.7.4 software. To stratify the population and visualize the clusters, we used a part of the TM4 software suite, the Multi Experiment Viewer, MeV (22). The best partition for the initial population was obtained for five clusters (inflection point of the Figure of Merit) (23). To cluster the data, for example, to stratify the initial population, we ran a K-Mean Clustering for five clusters and performed Hierarchical Clustering on the elements in each cluster created (24). The hierarchical clustering was done using complete linkage and Pearson correlation. Each cluster was then colored separately. A PCA was run and used to attribute the overall variability in the data to a reduced set of variables, for example, the principal components. We used the three first principal components to map each element into a three-dimensional viewer.

Results

A(H1N1)pdm09 influenza vaccine induced immunity of highly variable intensity and quality in healthy individuals

We performed a longitudinal analysis of humoral, T cell, and B cell immunological responses at D0, D21, and M4 in the FLUHOP cohort vaccinated with adjuvanted inactivated influenza A(H1N1) pdm09 vaccine (Supplemental Table I). We chose the immunological responses that are involved in protection against influenza infection or in reducing severity of illness postinfection. These parameters included HI titers (Fig. 1A), MN titers (Fig. 1B), serum Ab avidity (Fig. 1C), A(H1N1)pdm09-specific IgA- and IgG-secreting memory B cells (Fig. 1D, IE, respectively), A(H1N1)pdm09-specific IFN-γ/IL-2/TNF-α-secreting CD4 (Fig. 1F) and CD8 (Fig. 1G) T cells, and A(H1N1)pdm09-specific CD107a+ CD8 T cells (Fig. 1H).
As expected, HI titers increased significantly between D0 (geometric mean titer [GMT] = 11.45, 17.52% HI titers $\geq 40$) and D21 (GMT = 133.5, 94.81% HI titers $\geq 40$) and then decreased at M4 (GMT = 72.44, 81.75% HI titers $\geq 40$; $p$, 0.0001 compared with D21; Fig. 1A, Supplemental Table II). Trends in GMTs were similar when seroprotection was defined as an HI titer $\geq 80$ postvaccination (Supplemental Table II). In addition, Fig. 1B and 1C show that the neutralization activity and serum avidity of A(H1N1)pdm09-specific Abs also increased at D21 compared with D0 ($p$, 0.0001) and decreased at M4 (compared with D21, $p < 0.0001$). These results, which are consistent with the literature (25–28), present the conventional way to measure vaccination immunogenicity.

In view of the prime role of B cells in the generation of Abs, we measured A(H1N1)pdm09-specific IgA–Ab-secreting memory B cells (Fig. 1D) and IgG ASCs (Fig. 1E). We found that both types of ASCs were significantly amplified at D21 (IgA-ASC and IgG-ASC: $p$, 0.0001) and decreased at M4 (compared with D21, IgA-ASC: $p = 0.0046$ and IgG-ASC: $p = 0.0018$; Fig. 1D, 1E). One hallmark of the efficacy of T cell responses against viral infection is the production of multiple cytokines by CD4 and CD8 cells, most specifically IFN-$\gamma$. Accordingly, we analyzed the longitudinal frequency of single-positive (SP; IFN-$\gamma$ IL-2$^+$TNF-$\alpha$), double-positive (IFN-$\gamma$ IL-2$^+$TNF-$\alpha$, or IFN-$\gamma$ IL-2$^+$TNF-$\alpha$, or IFN-$\gamma$ IL-2$^+$TNF-$\alpha$, or IFN-$\gamma$ IL-2$^+$TNF-$\alpha$), and triple-positive (IFN-$\gamma$ IL-2$^+$TNF-$\alpha$)
cytokine-secreting T cells. In addition, the expression of CD107a, a molecule defining degranulation capacity, was evaluated for CD8 T cell response. Fig. 1F and 1G showed the percentages of A(H1N1)pdm09-specific total cytokine (IFN-γ, IL-2, and/or TNF-α)-secreting CD4 and CD8 T cells, respectively (after subtraction of background nonstimulated cells). A(H1N1)pdm09-specific CD4 T cells increase significantly between D0 and D21 (p = 0.0005) and do not change between D21 and M4 (Fig. 1F). Interestingly, the A(H1N1)pdm09-specific CD8 responses (either cytokine- or CD107a+ cells) did not change significantly (Fig. 1G, 1H).

The polyfunctionality of T cells was represented in pie-chart analyses (Fig. 2). A significant difference was observed for the amplification of IFN-γ–IL-2–TNF-α–producing CD4 T cells between D0 and D21 (p = 0.0004) and its subsequent decrease at M4 (compared with D21, p = 0.0001; Fig. 2A). The CD8 cytokine profile, however, did not change significantly over time (Fig. 2B). Furthermore, the increase in A(H1N1)pdm09-specific CD4 T cells observed from D0 to D21 was correlated with increased HI titers (p = 0.0048), attesting of potential helper function of CD4 T cells.

A radar chart summarizing the view of overall adaptive immunity showed that influenza vaccination shaped immune responses toward major humoral responses, including amplification of effector CD4 T cells (Fig. 1I). However, we noted the absence of A(H1N1)pdm09-specific CD8 T cell amplification in the population and the extreme heterogeneity of humoral and cellular immune responses both at baseline and postvaccination.

**Heterogeneity of magnitude and quality of influenza-specific immune compartments**

According to the maximum of fold changes after vaccination at D21 and at M4, we classified individuals as responders and nonresponders (i.e., no change from baseline; Fig. 3). Subjects with a fold increase ≥4 in HI titers (85.4% of the cohort) were considered responders to the A(H1N1)pdm09 vaccination (Fig. 3A). Similarly, a high proportion of our subjects (66.7%) had MN titers that increased by ≥4-fold (Fig. 3B). We also found that 53.3 and 63.6% of subjects had amplified ≥2-fold IgA-memory ASC responses (Fig. 3C) and IgG-ASC responses (Fig. 3D), respectively. Notably, baseline memory B cell response was extremely high in the nonresponder group (Fig. 3C, 3D, white boxes), reflecting their residual immunity to influenza.

Ag-specific effector CD4 T cell immunity after A(H1N1)pdm09 vaccination (Fig. 4) was observed in more than half of the donors, who had increased A(H1N1)pdm09-specific cytokine-secreting CD4 T cell responses at either D21 or M4 (53.5%; Fig. 4A). Although the overall frequency of A(H1N1)pdm09-specific CD8 T cells did not change for the cohort as a whole (Fig. 1G), we found that 36.4% of individuals had increased frequencies of A(H1N1)pdm09-specific cytokine-producing CD8 T cells, more than 2-fold from baseline (17.2 at D21 and 19.2% at M4) (Fig. 4B). Similarly, 41.4% of our population subjects had ≥2-fold increase in their A(H1N1)pdm09-specific CD107a+ CD8 cells (Fig. 4C).

At D21, IFN-γ– and/or TNF-α–secreting CD4 T cells had increased significantly (Fig. 4D, middle radar chart). However, the A(H1N1)pdm09-specific CD4 effector/memory T cells at M4 were IFN-γ–producing cells (Fig. 4D, right radar chart). SP (IFN-γ– and TNF-α–secreting) CD8 T cells were the major populations increasing significantly at D21 (p < 0.001; Fig. 4E). The major population that did increase significantly at M4 was that composed of CD107a+ cells (Fig. 4C).

We observed that the baseline multifunctionality of both CD4 and CD8 T cells differed significantly in each group (Fig. 4F). As the histogram analysis shows, the nonresponder group had significantly higher levels of double-positive (IFN-γ–IL-2+ or IFN-γ–TNF-α–) and SP (IFN-γ– or IL-2– or TNF-α–) cytokine CD4 cells (Fig. 4F, left histogram) and SP cytokine-secreting CD8 T cells (Fig. 4F, right histogram) than did the responders at D0.

Overall, the level of baseline immunity (T cell intensity and quality) was found to have a significant impact on the magnitude of the response after vaccination.

**Fine-tuning of immune response to influenza vaccine defined clusters of individuals with differential magnitude of influenza-specific immunity**

To extract relevant information related to all immune parameters amplified after vaccination and subjects, we used PCA as a visualization tool to better understand the underlying structure of the data in an unsupervised way. The PCA is a mathematical operation used to extract the main factors that contribute to the variance.
resulting in a reduction of the dimensionality of the data. It is a simple nonparametric method of extracting relevant information from a confusing/complex data set (29, 30). Thus, we conducted an integrative analysis of the trends of multiple immune parameters (HI titers, IgA+ and IgG+ ASCs, cytokine-producing CD4 and CD8 T cells, CD107a+ CD8 T cells), all of them available at all time points in 79 donors. The analysis by PCA (Fig. 5A) and hierarchical clustering (Fig. 5B) is based on the fold increase of immune responses between D0 and D21, described earlier. For each segregated cluster, radar-chart analyses of immune parameters at D0 and D21 postvaccination were shown side by side to the clustergrams representation of the cluster (Fig. 5). The analyses defined five clusters of donors, each characterized by mobilization of one or more particular immune compartments at D21 post-vaccination compared with D0. One group of individuals had predominantly humoral responses (HI titers, IgA+ and IgG+ ASCs; cluster 1, \( n = 24 \)) amplified after vaccination without amplification of T cell responses, whereas other subjects developed humoral responses and CD8 and/or CD4 T cell responses (cluster 4, \( n = 16 \); cluster 5, \( n = 13 \)). Clusters 2 (\( n = 17 \)), 3 (\( n = 9 \)), and 5 (\( n = 13 \)) were also characterized by an amplification of the CD8 T cell response (Fig. 5B). Furthermore, this cluster analysis confirmed that when donors had low baseline levels of one immune variable, that parameter was highly amplified after vaccination, and thus confirmed the results presented in Figs. 3 and 4.

Our study raises the question of the role of comobilization of multiple immune compartments in the efficacy of vaccine-induced immunity.

**Discussion**

Our work strengthened the requirement of multiparametric analysis of the global immune response against influenza vaccine and the comobilization of multiple immune compartments in the efficacy of influenza vaccine–induced immunity. Our initial global analysis of the entire cohort pointed out the predominance of humoral response and influenza-specific effector CD4 T cell responses together, with the lack of change in A(H1N1)pdm09-specific effector/memory CD8 responses in this healthy population vaccinated against A(H1N1)pdm09. Four decades ago, Hobson et al. (1) suggested that the deficiency of one type of anti-influenza immune response (HI) could be counterbalanced by other actors of this specific immune system (1). At that time, immunological tools were limited to Ab response.

We used PCA to visualize and to better understand the underlying structure of the data in an unsupervised way, by reducing multidimensional data sets to lower dimensions (29, 30). It allows taking into account the similarities between subjects in order to have a robust informative viewpoint while preserving a percent of the variation of the initial data set. In our data, the first three principal components allow us to see 79 subjects who were in the...
FIGURE 4. Fine-tuning of the quality of influenza-specific CD4 and CD8 T cellular responses at baseline determined the intensity and quality of postvaccination immune responses. (A–C) One hundred subjects were evaluated for A(H1N1)pdm09-specific cytokine+ CD4 (A) or CD8 (B) T cells or CD107a+ CD8 T cells (C). Different groups were defined based on maximum of fold changes between D0 and either D21 or M4 after vaccination: nonresponders (white) and responders (fold change between D0 and either D21 or M4 ≥2, red and blue, respectively). Box and whiskers plots with 10th–90th percentiles are presented for each parameter and log 10 scale of intensity of immune responses (except for CD107a expression). The percentage of subjects in each group is indicated below the graph. (D and E) Radar charts presenting minimum and maximum values of each cytokine+ CD4 (D) and CD8 (E) T cell response with the mean of these responses (D0: black line, D21: red line, M4: blue line). (F) Frequencies of A(H1N1)pdm09-specific CD4 (left) and CD8 (right) T cells producing single, double, or triple cytokines are presented for each group at baseline (D0): nonresponders (no change from baseline; white) and responders (red and blue, as indicated). Statistical analyses were performed at D0 with the Mann–Whitney U test. Statistical analyses were performed between D0 and D21 or D0 and M4 with the Wilcoxon matched pairs test and Bonferroni correction. Statistical significance is indicated: **p < 0.001, ***p < 0.0001, ****p < 0.00001.
FIGURE 5. Differential mobilization of immune response after A (H1N1)pdm09 influenza vaccination.
(A) The analysis by PCA and hierarchical clustering is based on the fold increase of immune responses between D0 and D21. PCA of immune response revealed that 79 subjects were segregated on the basis of log 10 fold increases (D21/D0) into 5 clusters: cluster 1 (n = 21; green), cluster 2 (n = 17; blue), cluster 3 (n = 9; red), cluster 4 (n = 16; yellow), and cluster 5 (n = 13; pink). (B) For each segregated cluster, radar-chart (right) analyses of immune parameters were shown side by side to the clustergrams representation of the cluster (left) representing a hierarchical clustering of subjects of immune responses. The radar chart (right) presents the minimum and maximum values of each assay as indicated in log10 scale. The mean of influenza-specific immune responses is presented at D0 (black) and D21 (red). The dominant immune compartments mobilized in each cluster are indicated in bold.

original space in 6 dimensions (6-fold increased variables). This reduction takes into account 80.5% of the variation of the initial information, thus allowing for conserving most information. This mathematical procedure can be seen as a simple visual way to reveal the internal “hidden” structure of the data.

Using PCA, we demonstrated that anti-influenza immunity is the result of a balance between the different immune compartments for each cluster of individuals. Our longitudinal integrative study of multiple immune parameters before and after A(H1N1)pdm09 vaccination (HI titers, IgA+ and IgG+ ASCs, cytokine+ CD4 and CD8 T cells, CD107a+ CD8 T cells) has allowed us to define different profiles of immune responses represented by five clusters of subjects. Each cluster was characterized by an important fold change for one or more particular immune components.

Predominance of humoral responses early after influenza A (H1N1)pdm09 vaccination is consistent with previous data, including validation of vaccine efficacy in healthy individuals (25, 26, 28, 31). This predominance of HI titers is observed in all five clusters; this is not surprising because the influenza A(H1N1) pdm09 vaccine is adjuvanted and has been designed to induce high HI titers. The presence of adjuvant might shape the immunity toward the humoral responses (31). In addition, the route of administration will also impact the immunological outcomes (32, 33). In accordance, we also observed increased serum avidity directed against A(H1N1)pdm09 vaccine and increased influenza-specific neutralizing Abs. A(H1N1)pdm09-specific memory B cells producing IgG or IgA were positively correlated with the fold increase (D21/D0) in HI titers (p < 0.01).

We demonstrated a significant amplification of the effector CD4 T cell response, predominantly IFN-γ+–producing CD4 T cells, in the first weeks after A(H1N1)pdm09 vaccination, and it positively correlated with the increased HI titers at D21 (p = 0.0048). Previous studies suggest that CD4 T cells might exert antiviral activities via effector functions mediated by the production of IFN-γ.
and perforin, and the activation of innate responses in infected tissue (34–36). Two independent studies have shown that one dose of adjuvanted subunit vaccine containing proteins from either H5N1 or A(H1N1)pdm09 virus is sufficient to induce amplification of specific circulating CD4 T cells in the first weeks post-vaccination (31, 37). The study of donors vaccinated with H5N1 vaccine showed that the expansion of specific activated CD4 T cells predicted the subsequent increase of neutralizing Abs after booster immunization and their persistence at 6 months (37). Clustering analyses showed that different combinations of HI titers, IgA– and IgG3–ASCs, and/or CD107a+ CD8 T cells increased highly in these individuals by D21 even if CD4 cell frequencies did not change (clusters 1, 2, 3 and 5). One cluster of individuals (cluster 4) showed an increased frequency of vaccine-specific CD4 T cells together with vaccine-specific CD8 T cells.

Although we observed no significant change in either the magnitude or the quality of the Ag-specific CD8 T cell response in the cohort as a whole, we did distinguish in this study ~37% of subjects who had >2-fold increase in A(H1N1)pdm09-specific cytokine+ CD8 T cells at D21 and at M4. At the three study points, A(H1N1)pdm09-specific CD8 T cells were predominantly TNF-α-, IFN-γ-, or both. In addition, we found a sharp increase in CD107a+ T cells, which reached a very high frequency at M4, suggesting a continuous differentiation into a memory CD8+ T cell pool. These results are particularly important and call into question the effect of late induction of cytotoxic CD8 cells upon vaccination of elderly individuals, and its potential impact in the severity of influenza illness for individuals vaccinated later during the vaccination season. One could also hypothesize that vaccinated individuals were re-exposed to circulating influenza A H1N1pdm 09 virus that could boost their immune system. However, we found that individuals with high CD107a+ CD8 T cells did not present higher HI titers at M4 that would witness a potential Ag re-exposure.

In accordance with the literature, we also found that fold change in HI titers was inversely correlated to the age of the subject \( (p = 0.0293; r = -0.1877) \); however, we did not find any correlation between the age of the subject and fold change (D21/D0) of other immune parameters.

Vaccination campaign with A(H1N1)pdm09-adjuvanted vaccine in our clinical trial began in week 43 of 2009 (October 21, 2009) and lasted until week 51 of 2009 (December 16, 2009). In France, influenza-illness incidence, predominantly infections with the A (H1N1)pdm09 strain, has been shown to achieve the epidemic threshold between weeks 42 and 53 (38). However, the number of cases reported revealed to be much lower than the initial estimation. For our study, weekly telephonic surveys have shown that only 10 donors of the cohort have influenza-illness symptoms over the period of our study. However, all were negative for A(H1N1) pdm09 virus–specific PCR excluding an impact of potential recent infection with influenza virus on the immune status of the donors before and after vaccination. In addition, the level of HI titers did not increase at M4. However, we cannot exclude potential re-encountering of influenza viruses during the season.

Our data also suggest that the anti-influenza immune status before vaccination influences humoral and cellular outcomes, and that might explain, in part, the intraindividual heterogeneity of immune responses. Most adults have memory immunity against influenza Ags, typically established after Ag encounter at seasonal vaccination or during infection. The emergence of the new virus A (H1N1)pdm09 strain, genetically and antigenically distinguishable from previously circulating seasonal viruses, has provided an opportunity to assess the cross-reactivity that might help to limit disease severity and to mount effective immune responses (39, 40).

Broadly cross-reactive Abs, directed against the stem region of HA and derived from memory B cells, are protective against A (H1N1)pdm09 and other heterosubtypic influenza viruses (3). A recent study showed the presence of pre-existing serum anti-influenza Abs that cross-reacted with, but did not protect against, A(H1N1)pdm09 virus in middle-aged adults with severe influenza disease (5). The avidity of these nonprotective Abs for A(H1N1) pdm09 influenza Ags was low; indeed, the Abs were associated with the formation of low-avidity deleterious pulmonary immune complexes.

The heterogeneity of specific immune responses and individual capacity to mobilize/recall one or more particular immune components in response to vaccine also results from host factors such as age, global immune status, and genetic characteristics. The basal variations in the healthy human immune system and the complexity of its evaluation also probably contribute to the difficulty in predicting specific immune responses to vaccines (41). Some recent studies using high-throughput technologies and systems biology have led to progress in identifying genes, molecules, and networks of molecules involved in the immune response to vaccines and early predictive molecular signatures (42–47). Our longitudinal integrative analysis of the intensity and quality of multiple immune parameters induced shortly or several months after vaccination is a complementary and relevant tool, not only for assessing a given vaccine’s immunogenicity, but also for understanding the underlying mechanisms of the immunity induced by vaccine.

Our work proposes an overall evaluation of immunity to influenza vaccine that could be further extended to other immune compartments (mucosal immunity) for future vaccine design and evaluation of protective capacity. Challenging vaccination-induced efficacy to identify correlates of long-term protection is a key issue in vaccinology and often also a complicated step in the development of vaccination strategies against specific diseases.

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References


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