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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: 623–631.**

The principal reference criterion for evaluating the efficacy of influenza vaccination in clinical trials is the magnitude of the Ag-specific Ab titer. Ever since Hobson et al. (1) determined the threshold of hemagglutination inhibition (HI) Ab titers for protection after influenza infection in 1972, these titers have been regarded as the cornerstone of an anti-influenza immune response. However, both the quality and the quantity of humoral responses such as avidity, mucosal responses, or frequencies of Ab-secreting B cells (ASCs) (2, 3) are important immunological parameters of protection (4–6). Khurana et al. (7) observed qualitatively superior humoral responses in elderly individuals with pre-existing immunity after pandemic 2009 A(H1N1) (A[H1N1] pdm09) vaccination. Although humoral responses have been defined as the sole indicators of the correlates of protection in seasonal influenza vaccination (1, 8), renewed attention is being paid to T cell responses because of their role in decreasing disease severity and their capacity for long-term maintenance after immunization (9–11). In the context of pandemics, where no or few pre-existing Abs can rapidly control the infection, T cells might mediate protection or limit the severity of the influenza-associated illness in humans (12–14) and murine models (15, 16). T cells remain highly cross-reactive among influenza strains and also recognize constant epitopes of influenza proteins (17, 18).

Assessing the potency and immunogenicity of vaccines would be essential in designing novel vaccination strategies. Global description of immunogenicity of vaccines is a laborious and cost-effective task that is partly due to the multiplicity of phenotypes and functions that can be studied for each immune compartment. We took advantage of the influenza A(H1N1)pdm09 vaccination to perform a multiparametric analysis of effector and memory

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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 preexisting immunity and immune response to influenza upon 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 $\pm$ 3.00 seasonal influenza vaccinations during their life (A[H1N1]pdm09 vaccination (Supplemental Table II). Blood samples at day 0 (D0), day 21 (D21), and month 4 (M4) after A(H1N1)pdm09 vaccination were collected for immunological analyses. PBLs were isolated on Ficoll gradients (Eurobio, Courtaboeuf, France). Sera and PBL were frozen, and samples (D0, D21, and M4) were treated simultaneously for each subject.

### *Ethics committee approval and health authorities*

The trial was conducted in accordance with the latest version of the Declaration of Helsinki, Good Clinical Practice, and International Conference on Harmonisation regulatory guidelines. The study protocol and patient information forms were approved by the Ethics Committee of Pitié-Salpêtrière Hospital. Written informed consent was obtained from each volunteer before study entry. This trial is registered with ClinicalTrials.gov: NCT01063608.

### *Hemagglutination inhibition assay*

Serum Abs against nonadjuvanted A(H1N1)pdm09 influenza vaccine (Panenza; Sanofi Pasteur) were measured by a microtiter HI assay modified from Kendal et al. (19). In brief, after treatment by receptor-destroying enzyme, serial 2-fold dilutions of serum (from 1:10) were tested against 4 hemagglutinin (HA) units of Ag, on human O Rh-RBCs. The HI titers were defined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

### *Microneutralization assay*

Neutralizing Abs titers were measured with standard techniques by microneutralization (MN) assays. Sera were first heat-inactivated at 56°C for 30 min. Serial 2-fold dilutions of serum (from 1:10) were added with $10^3$ $TCID_{50}$ A/California/07/2009 (H1N1) influenza virus and incubated at 37°C for 2 h before being transferred onto 96-well microtiter plates containing confluent MDCK cells. The neutralization titer is expressed as the reciprocal of the highest serum dilution at which virus infection is blocked after 3 d of culture.

### *Serum avidity assay*

Serum avidity of anti-HA Abs was evaluated by ELISA (20). Sera from donors were incubated with recombinant HA from A/California/07/2009 (H1N1) influenza virus (Protein Sciences) coated on ELISA plates at a dilution equivalent to 2 μg/ml HA, in the presence of serial dilutions of sodium thiocyanate (Sigma). HRP-conjugated mouse anti-human IgG (Southern Biotech) was incubated before revelation with *o*-phenylenediamine peroxidase substrate buffer. Reaction was stopped with 2N HCl, and OD was red with a GENios lector using the Xfluor4 software at 492 nm. Serum avidity was defined as the concentration of sodium thiocyanate required to induce a 50% inhibition of Ab binding.

### *ASC detection*

Differentiation of memory B cells into ASCs was induced after 6 d of PBL culture in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS [PAA], L-glutamine, and antibiotics [Life Technologies BRL, Life Technology]) supplemented with 55 μM 2-ME (Sigma-Aldrich) and a mix of PWM, protein A from *Staphylococcus aureus* (Sigma-Aldrich), and CpG oligodesoxyribonucleotides (InvivoGen). This method was previously described by Crotty et al. (21). ELISPOT plates were coated with Pandemrix vaccine (without adjuvant) at a dilution equivalent to 2 μg/ml HA or PBS (background). $IgA^+$ or $IgG^+$ ASCs were detected with alkaline phosphatase–conjugated goat anti-human IgA or IgG Abs (Sigma-Aldrich), were revealed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma-Aldrich), and were counted with an automated microscope (Zeiss, Le Pecq, France). ELISPOT readouts were expressed as the number of A(H1N1)pdm09-specific IgA or IgG ASCs/$10^6$ PBLs.

### *Intracellular cytokine assay*

Frozen PBLs were available for further analysis of A(H1N1)pdm09-specific T cells. Cells were stimulated for 16 h at 37°C with or without (background) Pandemrix vaccine (without adjuvant) at a dilution equivalent to 60 ng/ml HA. Brefeldin A and monensin (Sigma-Aldrich) in the presence of CD107a-PE-Cy5 mAbs (BD Biosciences) were added during the last 14 h. Cells were washed and stained in PBS-2% FCS at 4°C with CD3-AmCyan, CD4-Pacific Blue, CD8-allophycocyanin-H7, CD27-PE (BD), and CD45RA-ECD (Beckman Coulter) mAbs. Cytofix/Cytoperm kit (BD) was used to permeabilize cells, according to the manufacturer's instructions, before staining with IL-2–FITC, IFN-γ–Alexa Fluor 700 and TNF-α–PE–Cy7 (BD) mAbs. Flow cytometry was performed with an LSRII flow cytometer (BD). At least 1,000,000 live events were accumulated 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, 1E, 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).

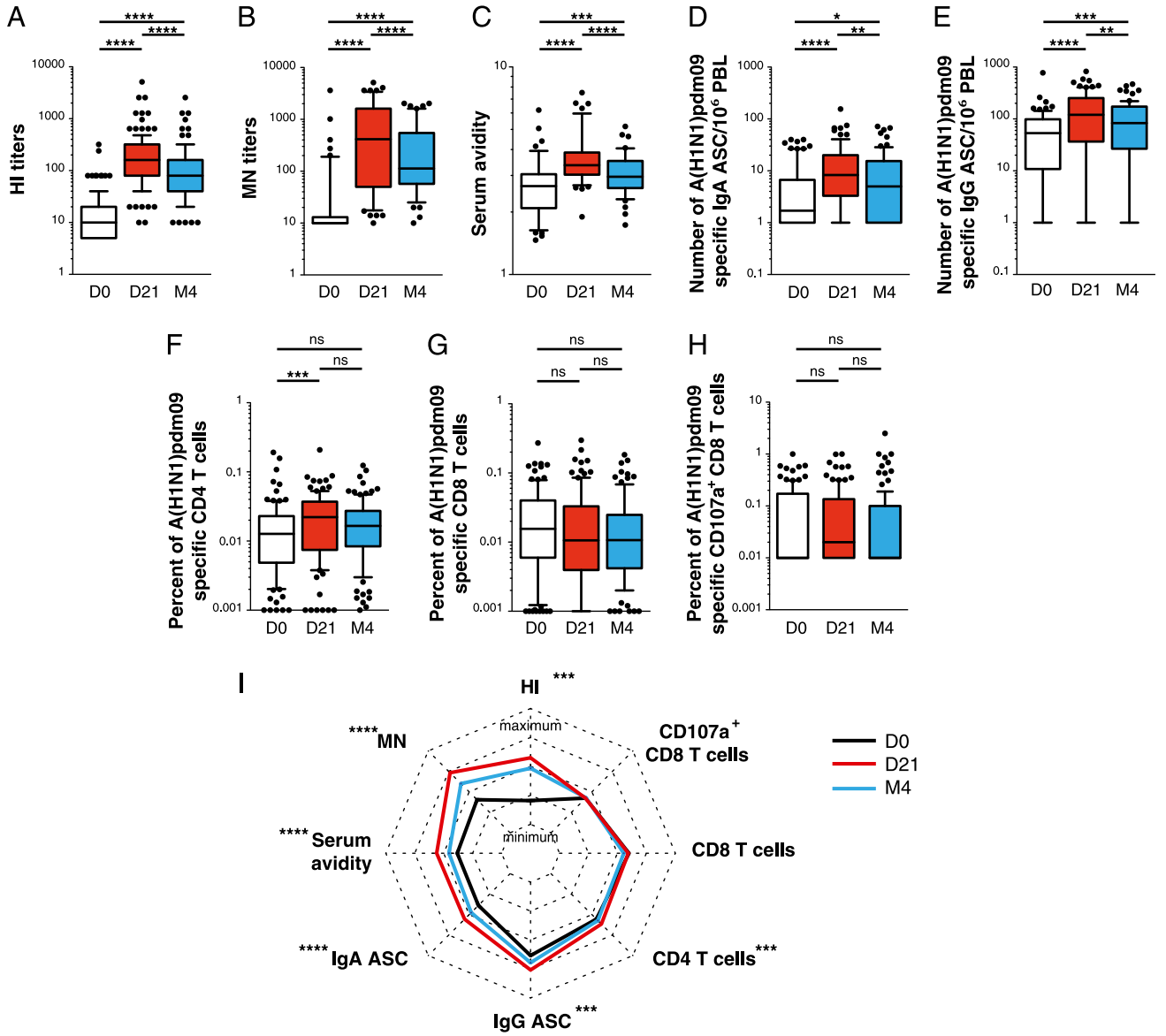


**FIGURE 1.** A(H1N1)pdm09 influenza vaccine elicited heterogeneous humoral and cellular immune responses. Immune responses were evaluated in blood before (D0) and at D21 and M4 after Pandemrix vaccination. Box and whisker plots with 10th and 90th percentiles are depicted for each parameter with log10 scale of intensity of immune responses. (**A**–**C**) Humoral response was assessed by HI assay ($n$ = 137) (A), MN assay ($n$ = 51) (B), and serum avidity assay ($n$ = 46) (C). (**D** and **E**) A(H1N1)pdm09-specific memory B cell responses were measured by ELISPOT assays: A(H1N1)pdm09-specific IgA ASCs (D) and IgG ASCs (E) ($n$ = 79). (**F**–**H**) A(H1N1)pdm09-specific T cell responses were measured by intracellular cytokine staining (Boolean gating of IFN-γ, IL-2, and/or TNF-α) in CD4 T cells (F), CD8 T cells (G), or by degranulation marker expression (CD107a) (H) ($n$ = 100). (**I**) Radar chart presents the minimum and maximum values for each assay as indicated in log10 scale. The mean influenza-specific immune responses are presented at D0 (black), D21 (red), and M4 (blue). Statistical analyses were performed with the Wilcoxon matched pairs test. Statistical significance is indicated as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant.

As expected, HI titers increased significantly between D0 (geometric mean titer [GMT] = 11.45, 17.52% HI titers ≥ 40) and D21 (GMT = 133.5, 94.81% HI titers ≥ 40) and then decreased at M4 (GMT = 72.44, 81.75% HI titers ≥ 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 ≥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-ASC (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-γ. Accordingly, we analyzed the longitudinal frequency of single-positive (SP; IFN-$\gamma^+$IL-2$^-$ TNF-$\alpha^-$, or IFN$\gamma^-$IL-2$^+$TNF-$\alpha^-$ or 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-$\alpha^+$), and triple-positive (IFN-$\gamma^+$IL-2$^+$TNF-$\alpha^+$)

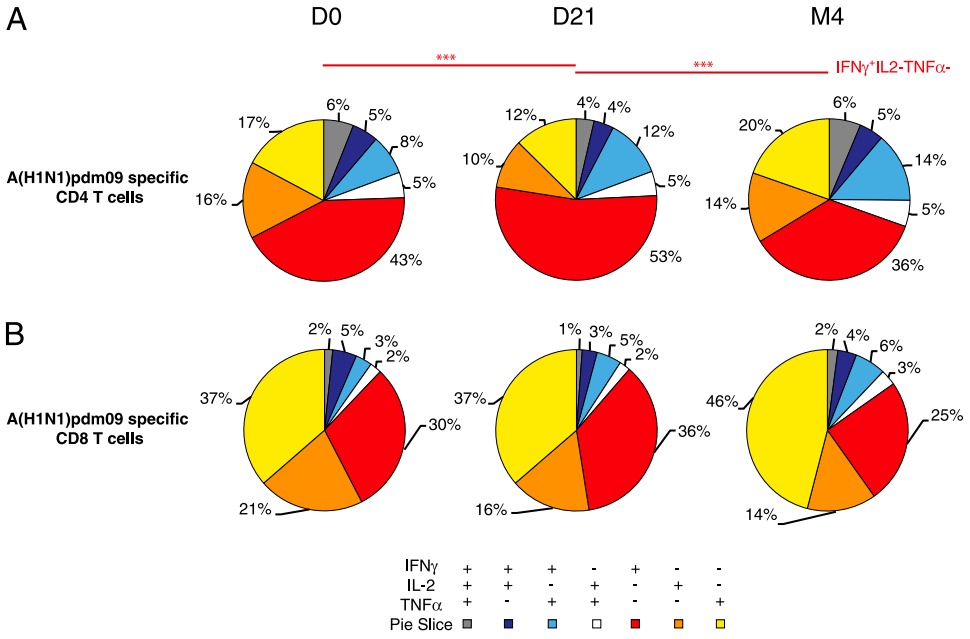


**FIGURE 2.** Heterogeneity in quality of A(H1N1)pdm09 vaccine–specific T cell responses. Multifunctionality of A(H1N1)pdm09-specific CD4 T cells (**A**) and CD8 T cells (**B**) was assessed by analyzing IFN-γ, IL-2, and TNF-α response patterns at D0, D21, and M4. Pie charts present the fraction of cytokine-secreting cells among total A(H1N1)pdm09-specific T cell response. Cytokine$^+$ CD4 and CD8 T cell response patterns are color-coded: IFN-γ$^+$IL-2$^+$ TNF-α$^+$ (gray), IFN-γ$^+$IL-2$^+$TNF-α$^-$ (dark blue), IFN-γ$^+$IL-2$^-$TNF-α$^+$ (light blue), IFN-γ$^-$IL-2$^+$TNF-α$^+$ (white), IFN-γ$^+$IL-2$^-$TNF-α$^-$ (red), IFN-γ$^-$IL-2$^+$TNF-α$^-$ (orange), and IFN-γ$^-$IL-2$^-$TNF-α$^+$ (yellow). Statistical analyses were performed with the Wilcoxon matched pairs test with a Bonferroni correction to compare groups. Statistical significance is indicated: ***$p < 0.001$.

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

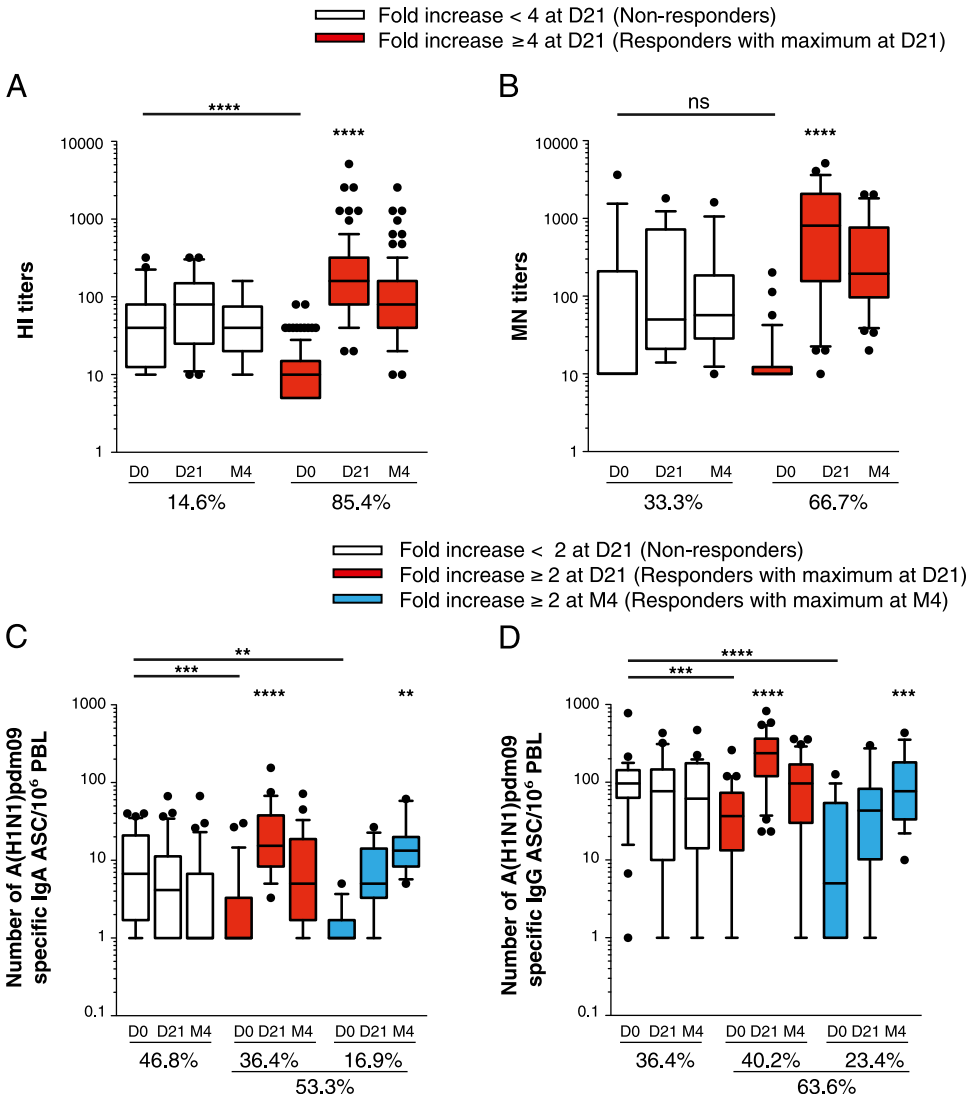


**FIGURE 3.** A(H1N1)pdm09-specific immunity at baseline influenced the intensity of both postvaccination Ab and memory B cell responses. We defined groups of individuals based on the maximum of fold changes between D0 and D21 and between D0 and M4 after vaccination: nonresponders (white) and responders at D21 (red) and M4 (blue) (≥4-fold change for HI and MN titers, and ≥2-fold change for cellular responses). Box and whisker plots with 10th and 90th percentiles are presented for each parameter and log 10 scale of intensity of immune responses. The percentage of subjects for each group is indicated for HI titers (**A**) ($n$ = 137), MN titers (**B**) ($n$ = 51), IgA-ASCs (**C**), and IgG-ASCs (**D**) ($n$ = 77). Statistical analyses were performed at D0 with the Mann–Whitney $U$ test. Statistical significance is indicated: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. ns, not significant. Statistical analyses compared values at D0 and at either D21 or M4 with the Wilcoxon matched pairs test. Statistical significances are indicated: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

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 postvaccination 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 cells (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

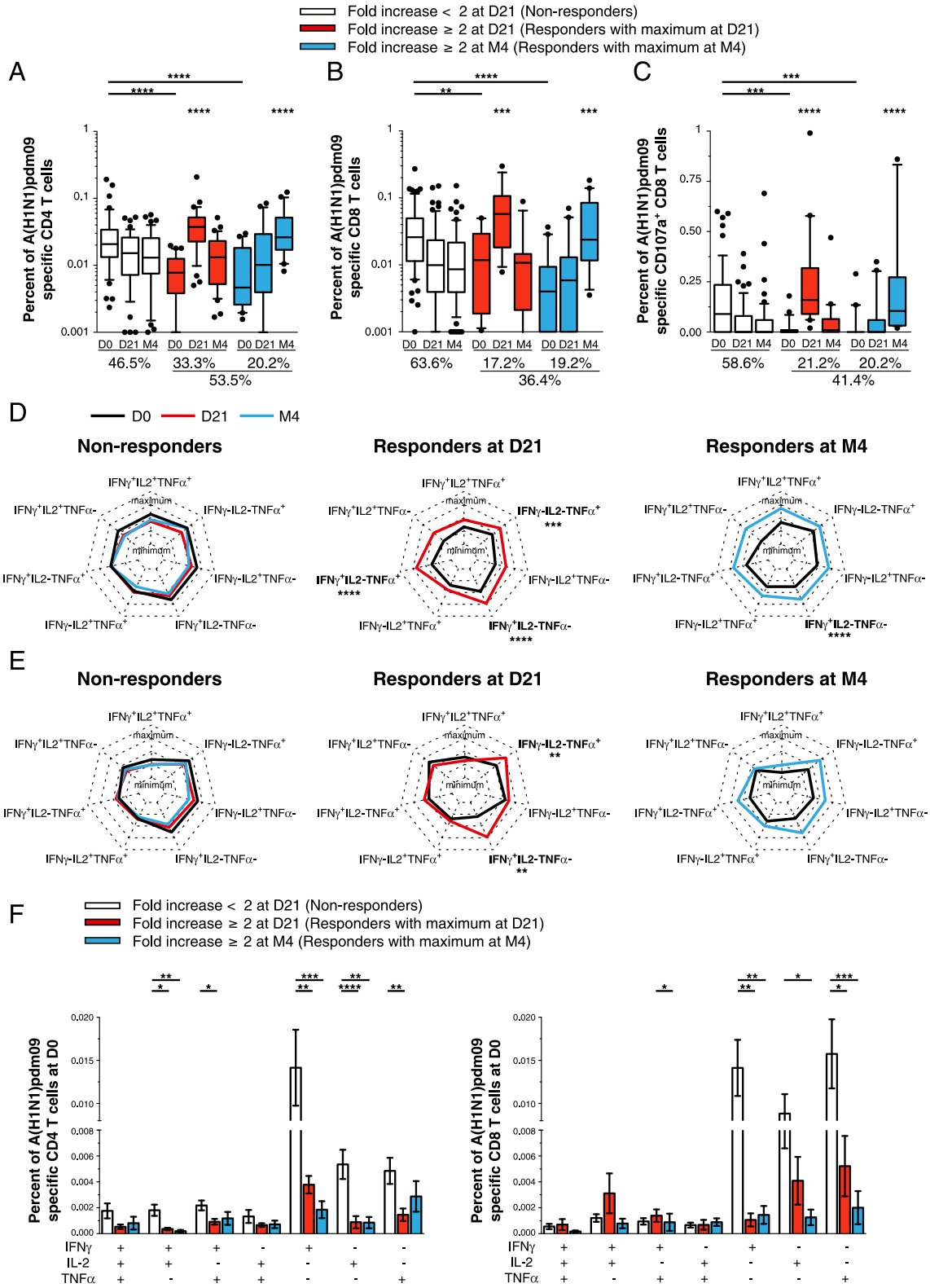


**FIGURE 4.** Fine-tuning of the quality of influenza-specific CD4 and CD8 T cellular responses at baseline determined the intensity and quality of postvaccination immune responses. (**A**–**C**) One hundred subjects were evaluated for A(H1N1)pdm09-specific cytokine$^+$ CD4 (A) or CD8 (B) T cells or 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: $^{**}p < 0.001$, $^{***}p < 0.0001$, $^{****}p < 0.00001$.

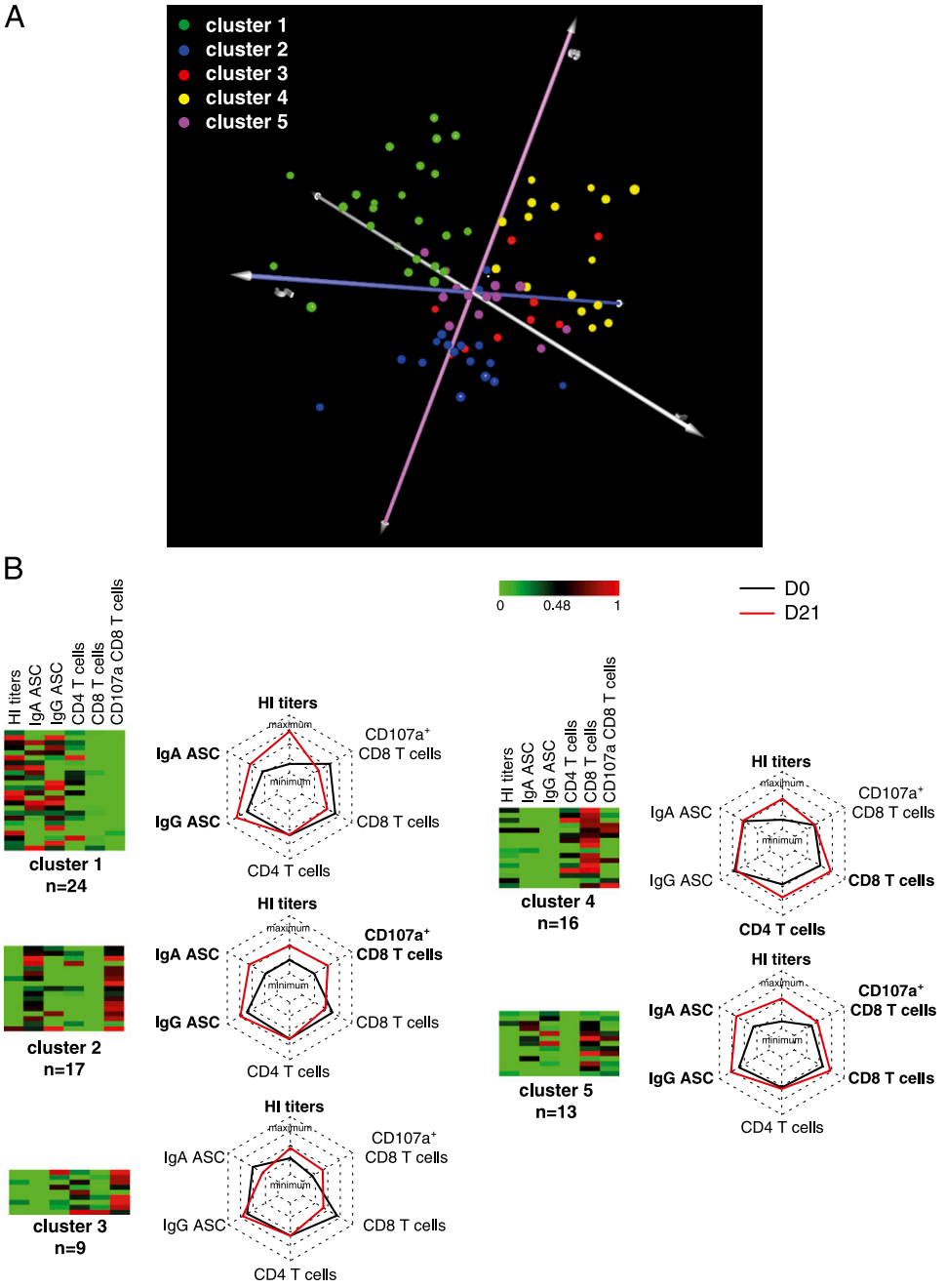


**FIGURE 5.** Differential mobilization of immune response after A (H1N1)pdm09 influenza vaccination. (**A**) The analysis by PCA and hierarchical clustering is based on the fold increase of immune responses between D0 and D21. PCA of immune response revealed that 79 subjects were segregated on the basis 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-$\gamma^+$–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-$\gamma$

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 $IgG^+$-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-$\alpha^+$, IFN-$\gamma^+$, 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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F.C. has received consulting fees from Roche, Aventis, and Chiron-Novartis, and has attended sponsor-funded meetings. B.C. has received consulting fees from Sanofi-Pasteur MSD, Pfizer, and Merck, and has attended sponsor-funded meetings. S.v.d.W. has received support from GlaxoSmithKline for meeting attendance, attended sponsor-funded meetings, and through her research unit received consulting fees from Danone. O.L. has participated as an investigator in vaccine studies sponsored by Sanofi-Pasteur MSD, and GlaxoSmithKline, and has attended sponsor-funded meetings. P.L. has participated as an investigator in vaccines studies sponsored by Sanofi-Pasteur MSD and GlaxoSmithKline, and has attended sponsor-funded meetings. The other authors have no financial conflicts of interest.

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