Age-Associated Cross-reactive Antibody-Dependent Cellular Cytotoxicity Toward 2009 Pandemic Influenza A Virus Subtype H1N1

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Background. During the 2009 pandemic of influenza A virus subtype H1N1 (A[H1N1]pdm09) infection, older individuals were partially protected from severe disease. It is not known whether preexisting antibodies with effector functions such as antibody-dependent cellular cytotoxicity (ADCC) contributed to the immunity observed.

Methods. We tested serum specimens obtained from 182 individuals aged 1–72 years that were collected either immediately before or after the A(H1N1)pdm09 pandemic for ADCC antibodies to the A(H1N1)pdm09 hemagglutinin (HA) protein.

Results. A(H1N1)pdm09 HA-specific ADCC antibodies were detected in almost all individuals aged >45 years (28/31 subjects) before the 2009 A(H1N1) pandemic. Conversely, only approximately half of the individuals aged 1–14 years (11/31) and 15–45 years (17/31) had cross-reactive ADCC antibodies before the 2009 A(H1N1) pandemic. The A(H1N1)pdm09-specific ADCC antibodies were able to efficiently mediate the killing of influenza virus–infected respiratory epithelial cells. Further, subjects >45 years of age had higher ADCC titers to a range of seasonal H1N1 HA proteins, including from the 1918 virus, compared with younger individuals.

Conclusions. ADCC antibodies may have contributed to the protection exhibited in older individuals during the 2009 A(H1N1) pandemic. This work has significant implications for improved vaccination strategies for future influenza pandemics.

Keywords. ADCC; influenza; NK cells; swine-origin influenza virus.

The pandemic emergence of a swine-origin influenza A virus subtype H1N1 during 2009 (A[H1N1]pdm09) resulted in significant human morbidity worldwide [1–5]. Interestingly, individuals born before 1957 had lower rates of infection and less severe disease than individuals born after this time [2, 6–10], presumably from prior exposure to seasonal influenza A virus subtype H1N1 (sH1N1) [11–15]. The mechanism of natural protection against A(H1N1)pdm09 infection has been largely attributed to cross-neutralizing antibodies and/or T-cell responses induced following prior exposure to sH1N1 strains [11, 12, 16–22], but other antibody effector mechanisms may be involved and are unexplored.

Influenza virus hemagglutinin (HA)–specific antibodies have been mainly characterized for their neutralizing capacity. However, these antibodies have other functions, including complement fixation [23, 24], phagocytosis [25], and antibody-dependent cytotoxicity (ADCC) [26–30]. While neutralizing antibodies may target more variable regions found on the globular head of the viral HA, ADCC antibodies may target more conserved regions of the influenza virus HA and other surface proteins [31, 32]. Recently, we developed new assays to measure ADCC antibodies against multiple influenza virus strains [30]. We previously intensively studied ADCC responses in 10 healthy adults to a broad range of influenza virus strains and detected

ADCC antibodies to various influenza virus strains in the absence of detectable neutralizing antibodies. However, our previous small human cohort study was limited by unclear exposure to specific influenza viruses. The emergence and rapid spread of A(H1N1)pdm09 provided an opportunity to study the cross-reactivity of ADCC antibodies against influenza virus that were present before the pandemic. We hypothesized that ADCC antibodies to A(H1N1)pdm09 were present in older individuals and may have potentially played a role in reducing the severity of disease due to A(H1N1)pdm09. We studied serum specimens obtained from 93 subjects before the 2009 H1N1 pandemic and from 89 separate unpaired subjects after the 2009 H1N1 pandemic for ADCC to A(H1N1)pdm09.

METHODS

Serum Samples and Donors

Serum samples collected from 93 individuals immediately before the 2009 A(H1N1) pandemic winter season in Australia (21 November 2008–13 May 2009) and from 89 individuals immediately following the pandemic (3 August 2009–16 November 2009), were provide by the World Health Organization Collaborating Centre for Reference and Research on Influenza and approved by institutional review boards at each contributing institution. The Alfred Hospital ethics committee approved the use of human donor blood (number 248/06). All subjects provided written informed consent.

Influenza Virus Antigens and Hemagglutination Inhibition (HI) Assay

Mammalian-cell-expressed recombinant HA proteins were purchased from Sinobiologicals (Shanghai, China). The influenza virus used was a reassortant virus propagated in embryonated hen's eggs expressing the HA and neuraminidase (NA) from A/Auckland/01/2009 (A/California/07/2009-like 2009 pandemic H1N1) with the internal genes from A/Puerto Rico/8/1934 (H1N1). HI assays were performed as previously described [9].

Plate-Bound ADCC Natural Killer (NK) Cell Activation Assay

We recently described novel ADCC assays that measure antibody-mediated NK cell activation [30], developed as modifications of human immunodeficiency virus–specific ADCC assays [33, 34]. Briefly, 96-well ELISA plate (Nunc, Rochester, NY) were coated overnight at 4°C with purified influenza protein (400 ng/well) in phosphate-buffered saline (PBS). Wells were washed with PBS and incubated with heat-inactivated plasma/sera for 2 hours at 37°C. Plates were washed with PBS, and 10^6 freshly isolated human healthy donor peripheral blood mononuclear cells (PBMCs) were added to each well. Brefeldin A (5 μg/mL, Sigma, St. Louis, MO), monensin (5 μg/mL, BD Bioscience, San Jose, CA), and CD107a-APC-H7 (H4A3 clone, BD Bioscience, San Jose, CA) were added, and plates were incubated for 5 hours at 37°C with 5% CO_2. Cells

were then incubated with surface antibodies CD3-PerCP (clone SP34–2), CD14-PE-Cy7 (clone M5E2), and CD56-APC (clone B159, all from BD Bioscience) for 30 minutes at room temperature in the dark. Cells were fixed with 1% formaldehyde (Sigma, St. Louis, MO) for 10 minutes and permeabilized using Permeabilizing Solution 2 for 10 minutes (BD Bioscience). Finally, cells were incubated at room temperature for 1 hour with interferon γ (IFN-γ)–AlexaFluor700 (clone B27, BD Bioscience).

Antibody-Dependent Cellular Viral Elimination Assay, Using a Human Respiratory Epithelial Cell Line

As previously described [30], confluent A549 cells (ATCC no. CCL-185) were washed once with PBS and infected with 10 multiplicities of infection of A(H1N1)pdm09 for 1 hour at 37°C in 10% CO_2, washed with RF10, and incubated for 3 hours at 37°C in 5% CO_2. Cells were removed from the monolayer by use of trypsin versene, washed, resuspended in 200 μL, and incubated with 50 μL of phycoerythrin-conjugated anti–major histocompatibility complex class I antibody (clone W6/32, eBioscience, San Diego, CA) for 20 minutes to facilitate identification of target cells by a fluorescence-activated cell sorter. Following washing, 6×10^5 infected A549 cells were combined with 2×10^5 freshly isolated human PBMCs (E:T, 1:3) in a 96-well U-bottomed plates, with or without 10 μL of heat-inactivated plasma and CD107a-APC-H7 (H4A3 clone, BD Bioscience, San Jose, CA), and incubated for 6 hours at 37°C in 5% CO_2. Following incubation, cells were stained with surface antibodies anti-human CD3, CD14, and CD56 as described above, permeabilized, intracellularly stained with fluorescein isothiocyanate–conjugated anti-influenzavirus nucleoprotein (431 clone, Abcam, Cambridge, MA) antibody, fixed, and acquired by flow cytometry. The reduction in infected cells was calculated as [(percentage of nucleoprotein-positive cells or total number of nucleoprotein-positive cells in the presence of plasma) – (percentage of nucleoprotein-positive cells or total number of nucleoprotein-positive cells in the absence of plasma)]/[percentage of nucleoprotein-positive cells or total number of nucleoprotein-positive cells in the absence of plasma] × 100.

Statistical Analyses

Statistical analyses used SPSS, version 18, software (IBM, Armonk, NY) and Prism GraphPad, version 6 (GraphPad Software, San Diego, CA). Data were analyzed by the t test (Figure 1B and 1C, Figure 2A, and Figure 3), 1-way analysis of variance (ANOVA) together with Tukey post hoc tests (Figure 1D and 1E), 2-way ANOVA together with Bonferroni comparisons (Figure 5A), and Pearson product-moment correlation coefficient (Figure 4 and Figure 5B and 5C). Statistically significant outliers were determined by GraphPad Outlier calculator (α = 0.05). Before 1-way or 2-way ANOVA, data were log_{10}-transformed to equalize variances, according to the Levene test.
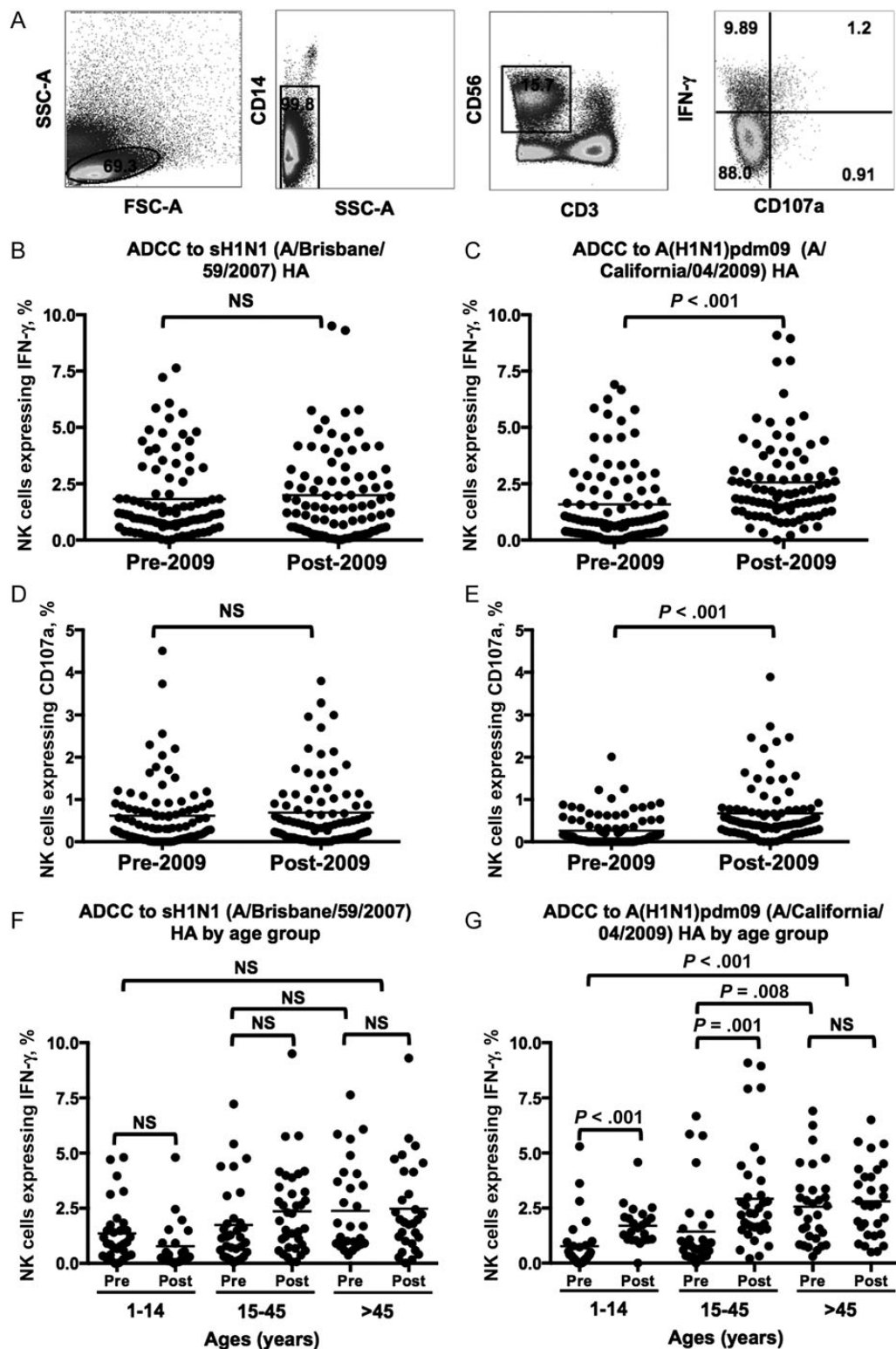
Figure 1. Antibody-dependent cellular cytotoxicity (ADCC)–mediated natural killer (NK) cell activation to seasonal influenza A virus subtype H1N1 (sH1N1) and 2009 pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09). *A*, Gating strategy of NK cell activation assay. Intracellular expression of interferon γ (IFN-γ) was determined in CD14−CD3−CD56+ lymphocytes. *B–E*, The NK cell expression of IFN-γ and CD107a in response to plate-bound sH1N1 HA (*B* and *D*; A/Brisbane/59/2007) or A(H1N1)pdm09 HA (*C* and *E*; A/California/04/2009) proteins in the presence of either sera collected before the 2009 A(H1N1) pandemic (Pre-2009) or after the 2009 H1N1 pandemic (Post-2009). A total of 93 Pre-2009 specimens and 89 Post-2009 specimens were evaluated. Comparisons were made using *t* tests. *F* and *G*, NK cell expression of IFN-γ in response to sH1N1 (*F*) or A(H1N1)pdm09 (*G*) hemagglutinin (HA) protein

RESULTS

Subjects and Influenza Virus HI Antibodies

It is not clear whether antibody effector mechanisms, including ADCC antibodies, contributed to reduced A(H1N1)pdm09-associated disease observed during the 2009 A(H1N1) pandemic. To study this, serum and plasma samples were collected from 93 individuals immediately before the occurrence of the 2009 A(H1N1) pandemic winter season in Australia and from 89 individuals immediately following the 2009 A(H1N1) pandemic season and analyzed for ADCC-based immunity to pandemic influenza virus. All samples were first tested by HI assays for reactivity toward seasonal (A/Brisbane/59/2007) or pandemic (A/California/7/2009) H1N1 viruses (Table 1). Samples collected from individuals before the 2009 A(H1N1) pandemic had undetectable HI titers against A/California/07/2009 (<10), and samples collected after the 2009 A(H1N1) pandemic were recruited on the basis of positive HI titers against this strain (>40). Because previous studies indicated that individuals <45 years of age showed a higher incidence of A(H1N1)pdm09 infection and a greater frequency of hospitalization with severe influenza [2, 6, 7], we prospectively divided the samples into groups on the basis of age (ie, 1–14, 15–45, and >45 years; Table 1).

A(H1N1)pdm09-Specific ADCC Antibodies in Serum Obtained From Individuals Before and After the 2009 A(H1N1) Pandemic

We first assessed ADCC by using a plate-bound NK cell activation assay [30] (Figure 1A). Studying the entire cohort, we found that ADCC-mediated NK cell activation (as measured by both IFN-γ and CD107a expression) against A(H1N1)pdm09 (A/California/04/2009) HA protein was significantly higher in sera collected after the 2009 A(H1N1) pandemic, compared with sera collected before the 2009 A(H1N1) pandemic (mean IFN-γ expression [±SEM] by NK cells, 1.59% ± 0.18% before the 2009 pandemic vs 2.57% ± 0.20% after the 2009 pandemic; $P < .001$, by the t test; Figure 1C). We observed no significant increase in antibody-mediated NK cell IFN-γ or CD107a expression against sH1N1 (A/Brisbane/59/07) HA protein between samples taken before and those taken after the 2009 A(H1N1) pandemic (mean IFN-γ expression [±SEM] by NK cells, 1.83% ± 0.18% before the 2009 pandemic vs 1.99% ± 0.21% after the 2009 pandemic; $P = .54$, by the t test; Figure 1B). These data confirm that A(H1N1)pdm09 infection results in enhanced A(H1N1)pdm09-specific ADCC antibodies.

During the 2009 A(H1N1) pandemic, older individuals had a reduced incidence of infection with A(H1N1)pdm09, suggestive of a degree of protection against A(H1N1)pdm09 infection [2,

6–10]. To determine whether older subjects had higher levels of ADCC antibodies before the 2009 A(H1N1) pandemic, we prospectively sorted our data into 3 distinct age groups (1–14, 15–45, and >45 years). For each age group, ADCC responses to sH1N1 (A/Brisbane/59/07) HA protein were similar in samples taken before and after the 2009 A(H1N1) pandemic (Figure 1F). In contrast, ADCC-mediated NK cell activation against A(H1N1)pdm09 HA protein was significantly increased for individuals aged 1–14 years ($P < .001$) and those aged 15–45 years ($P < .002$) following the 2009 A(H1N1) pandemic (Figure 1G). However, similar levels of antibody-mediated NK cell activation against A(H1N1)pdm09 HA protein was observed in samples taken before and after the 2009 A(H1N1) pandemic from individuals >45 years of age (mean IFN-γ expression [±SEM] on NK cells, 2.56% ± 0.31% before the 2009 pandemic vs 2.80% ± 0.30% after the 2009 pandemic; $P = .993$; Figure 1G). In addition, samples collected before the 2009 A(H1N1) pandemic from individuals >45 years of age showed higher levels of NK cell activation, compared with samples taken before the 2009 A(H1N1) pandemic from individuals aged 1–14 years ($P < .001$) and those aged 15–45 years ($P = .008$). Thus, before the 2009 A(H1N1) pandemic, individuals >45 years of age commonly possessed preexisting cross-reactive ADCC antibodies against A(H1N1)pdm09.

Increase in A(H1N1)pdm09–Specific ADCC Titer Following the 2009 A(H1N1) Pandemic

The percentage of NK cells expressing IFN-γ at a single serum dilution is an effective means of comparing ADCC activity between different serum samples but may not accurately quantify the level of ADCC antibodies. Therefore, serial dilutions of each sample were performed, and we determined the dilution at which NK cell activation falls below a threshold of 3 times the background level of activation. We used a randomly selected subset of 30 sera samples collected before the 2009 A(H1N1) pandemic and 24 sera samples collected after the 2009 A(H1N1) pandemic (from individuals aged 15–45 years and those aged >45 years only). ADCC titers against A(H1N1)pdm09 HA were significantly higher in samples taken after the 2009 A(H1N1) pandemic, compared with samples taken before the 2009 A(H1N1) pandemic (mean titer, 95.7 before the 2009 pandemic vs 364.2 after the 2009 pandemic; Figure 2A). The increased ADCC titers following the 2009 A(H1N1) pandemic suggests the hypothesis that preexisting ADCC antibodies may assist in clearing pandemic influenza virus infection and reducing illness in these individuals.

Figure 1 continued.   in samples collected before the 2009 A(H1N1) pandemic and after the 2009 A(H1N1) pandemic from individuals aged 1–14, 15–45, and >45 years. All samples were corrected for background on the basis of their response to wells containing sera but with no plate-bound antigen. The number of specimens range from 23 (Post-2009; ages 1–14 years) to 46 (Post-2009; ages 15–45 years). Lines depict the mean of each group. Comparisons were made using 1-way analysis of variance followed by the Tukey post hoc test on $\log_{10}$-transformed data.
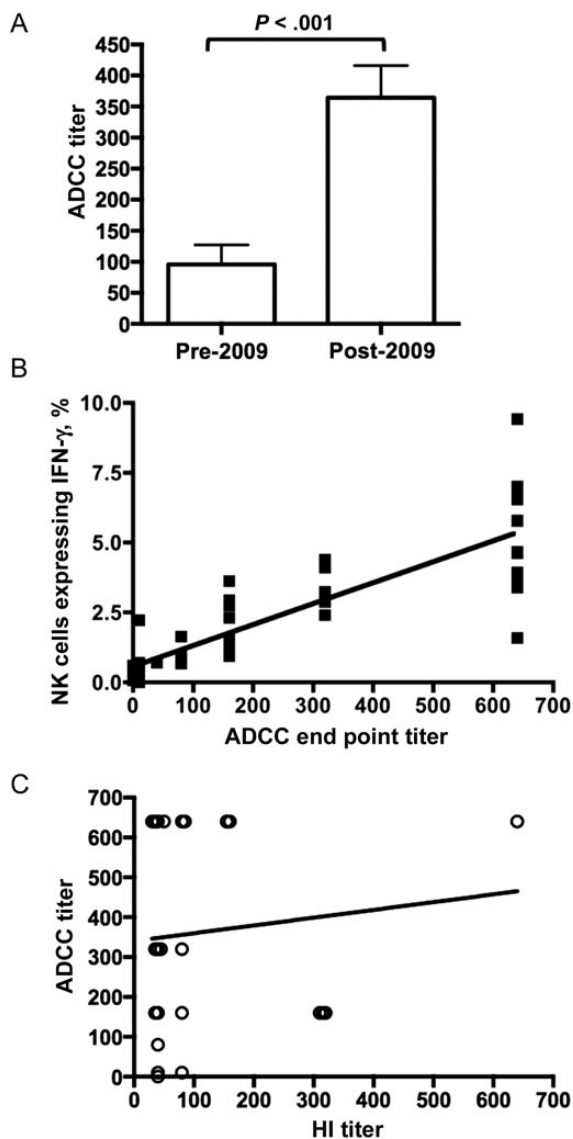
Figure 2. Higher antibody-dependent cellular cytotoxicity (ADCC) titers following 2009 pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09) infection and correlation between ADCC and hemagglutination inhibition (HI) titer. *A*, ADCC antibody end point titers to A(H1N1)pdm09 (A/California/04/2009) hemagglutinin (HA) were determined by measuring the percentage of natural killer (NK) cells expressing interferon γ (IFN-γ) at 2-fold dilutions of subject sera (1:10–1:640). End point titers were presented as mean + standard error of the mean (n = 30 before the 2009 pandemic [Pre-2009] and n = 24 for after the 2009 pandemic [Post-2009]). Groups were compared by the *t* test. A response was deemed positive when the frequency of NK cells expressing IFN-γ was at least 3 times background (without HA protein but with subject sera). *B*, Comparison of the frequency of NK cells expressing IFN-γ in response to plate-bound A(H1N1)pdm09 HA protein in the presence of 1:10 dilution of sera, compared with the end point titers to A(H1N1)pdm09 HA, using the same sera (n = 54). *C*, Comparison of end point ADCC titer and HI titer to A(H1N1)pdm09 (A/California/04/2009 and A/California/07/2009 respectively; n = 24). Dots representing subjects with the same HI and ADCC titers have been adjusted (for HI titer) to visualize individual data points.

Previous influenza virus ADCC studies assumed that the proportion of NK cells activated by ADCC antibodies corresponds with the end point titer of ADCC antibodies measured in this assay, but direct evidence is lacking [30]. To determine whether the level of NK cell activation correlates with the ADCC end point titer, we compared the percentage of ADCC-activated NK cells at a 1:10 dilution to the ADCC end point titer for a number of different serum samples. A clear positive association was observed between NK cell activation at a 1:10 dilution and the end point ADCC titer (Figure 2*B*).

In vaccination and infection studies, a HI antibody titer of >40 is generally regarded as protective [35]. Recently, we demonstrated that the presence of cross-reactive ADCC antibodies to influenza virus can be present in the absence of neutralizing antibodies [30]. Interestingly, across this cohort there was no relationship between the ADCC titer to A(H1N1)pdm09 HA and the HI titer against A(H1N1)pdm09 (Figure 2*C*). Eleven of 24 individuals recorded a HI titer of 40 but an ADCC titer of ≥160. Thus, the HI titer does not reflect the level of HA-specific ADCC antibodies, highlighting a potential limitation of solely using HI titer to identify predictive correlates of protection. It is also notable that HI titers to the sH1N1 A/Brisbane/59/07 were higher in the samples obtained before the 2009 pandemic, but ADCC levels to A/Brisbane/59/07 were similar before and after the 2009 pandemic (Figure 1*B*), further emphasizing the poor correlation between HI titers and ADCC antibodies.

ADCC Antibodies Eliminate A(H1N1)pdm09–Infected Respiratory Epithelial Cells

We next evaluated the functional capacity of ADCC antibodies in sera obtained after the 2009 A(H1N1) pandemic to clear A(H1N1)pdm09-infected airway epithelial cells. Figure 3*A* shows an example of the frequency of influenza virus nucleoprotein-containing cells in the absence (58.9%) or presence of a sera samples from either before the 2009 A(H1N1) pandemic (60.9%) or after the 2009 A(H1N1) pandemic (29.1%). We calculated a reduction in the number of influenza-virus-infected respiratory cells, from 14 308 (no serum) to 4967 (after the 2009 A(H1N1) pandemic), for an absolute reduction of 65.3%. In the same well, we observed a marked increase in the expression of the degranulation marker CD107a on NK cells, from 2.8% in the absence of sera to 25.6% in the presence of sera, in a sample obtained after the 2009 A(H1N1) pandemic (Figure 3*A*). Comparison of the reduction of the number of A(H1N1)pdm09-infected respiratory cells in the presence of either sera obtained before or after the 2009 A(H1N1) pandemic showed that there was a significant increase in the elimination of virus-infected cells in sera collected after the 2009 A(H1N1) pandemic (mean absolution reduction [±SEM] of virus-infected cells, 5.9% ± 3.0% before the 2009 pandemic and 36.4% ± 5.8% after the 2009
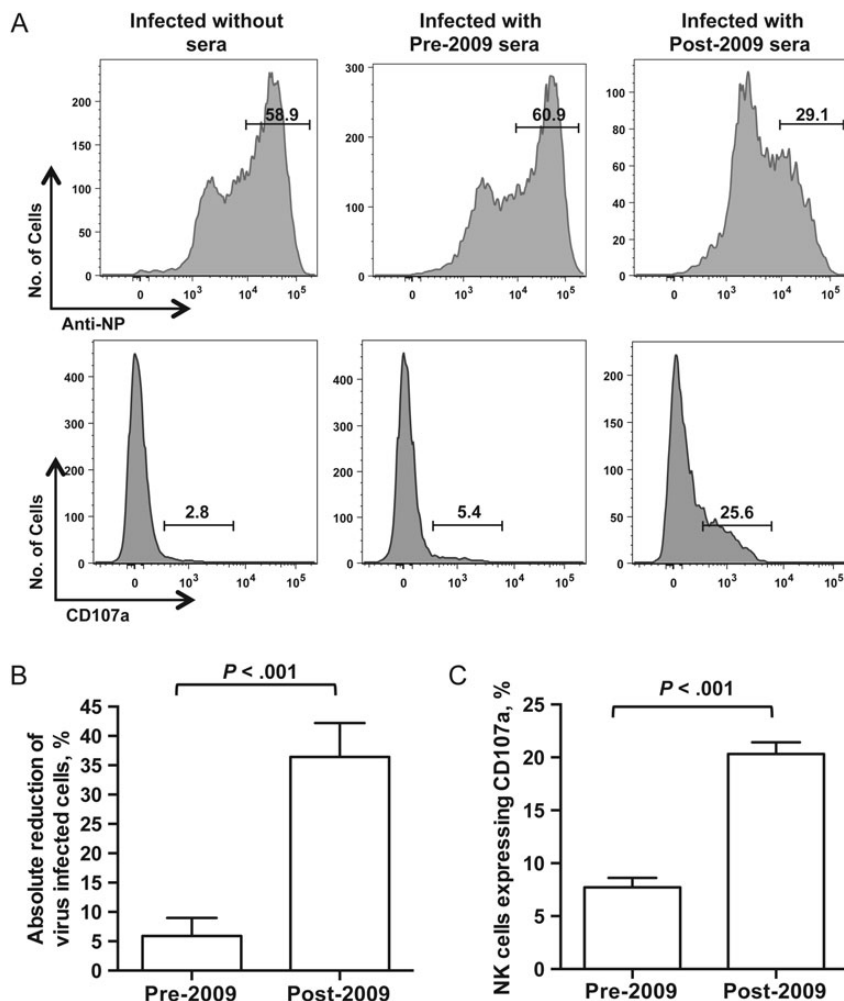
Figure 3. Antibody-dependent cellular cytotoxicity (ADCC)–mediated elimination of influenza virus–infected respiratory cells. Human airway epithelial cell line (A549) was infected with influenza virus (A/Auckland/01/2009-PR8) at a multiplicity of infection of 10. After 4 hours incubation, infected or uninfected A549 cells were combined with healthy donor peripheral blood mononuclear cells at 1:3 (E:T ratio) in the presence or absence of a 1:10 dilution of sera. Representative plots of A549 cells expressing influenza virus nucleoprotein (NP; top) and CD56+CD3− natural killer (NK) cells expressing CD107a (bottom), with or without sera collected from individuals before the 2009 A(H1N1) pandemic (Pre-2009) or after the 2009 A(H1N1) pandemic (Post-2009). *B* and *C*, The absolute reduction in A549 cells (*B*; n = 30 for Pre-2009 and n = 24 for Post-2009) and natural killer (NK) cell CD107a expression (*C*; n = 30 for Pre-2009 and n = 23 for Post-2009) was measured in the presence of sera from individuals obtained before the 2009 A(H1N1) pandemic and after the 2009 A(H1N1) pandemic. Data are presented as mean + standard error of the mean. Groups were compared by the *t* test.

pandemic; $P < .001$, by the *t* test; Figure 3*B*). Concomitantly, there was increased CD107a expression on NK cells exposed to sera obtained after the 2009 A(H1N1) pandemic, compared with sera obtained before the 2009 A(H1N1) pandemic (Figure 3*C*). Together, this shows that ADCC antibodies after the 2009 A (H1N1) pandemic can mediate the elimination of A(H1N1) pdm09-infected respiratory cells.

ADCC to sH1N1 Correlate With ADCC to A(H1N1)pdm09 in Older Individuals

Protection of older individuals from A(H1N1)pdm09 infection has been attributed to previous infections with 1918-like

sH1N1 [11, 12, 17]. We compared antibody-mediated NK cell activation against HA proteins from 2 sH1N1 vaccine strains (A/New Caledonia/20/99 and A/Brisbane/59/07) to the antibody-mediated NK cell activation against A(H1N1)pdm09 HA (A/California/04/09) protein, using all 89 sera samples collected immediately before the 2009 A(H1N1) pandemic (all individuals aged 1–14, 15–45, and >45 years). We found weak or no association between ADCC responses (NK cell activation) to sH1N1 HA proteins and ADCC responses to A(H1N1) pdm09 HA protein for individuals aged 1–14 years ($P = .01$ and $r^2 = 0.20$ for A/New Caledonia/20/99; $P = .34$ and $r^2 = 0.34$ for Brisbane/59/07; Figure 4*A* and 4*B*) and those aged 15–45 years
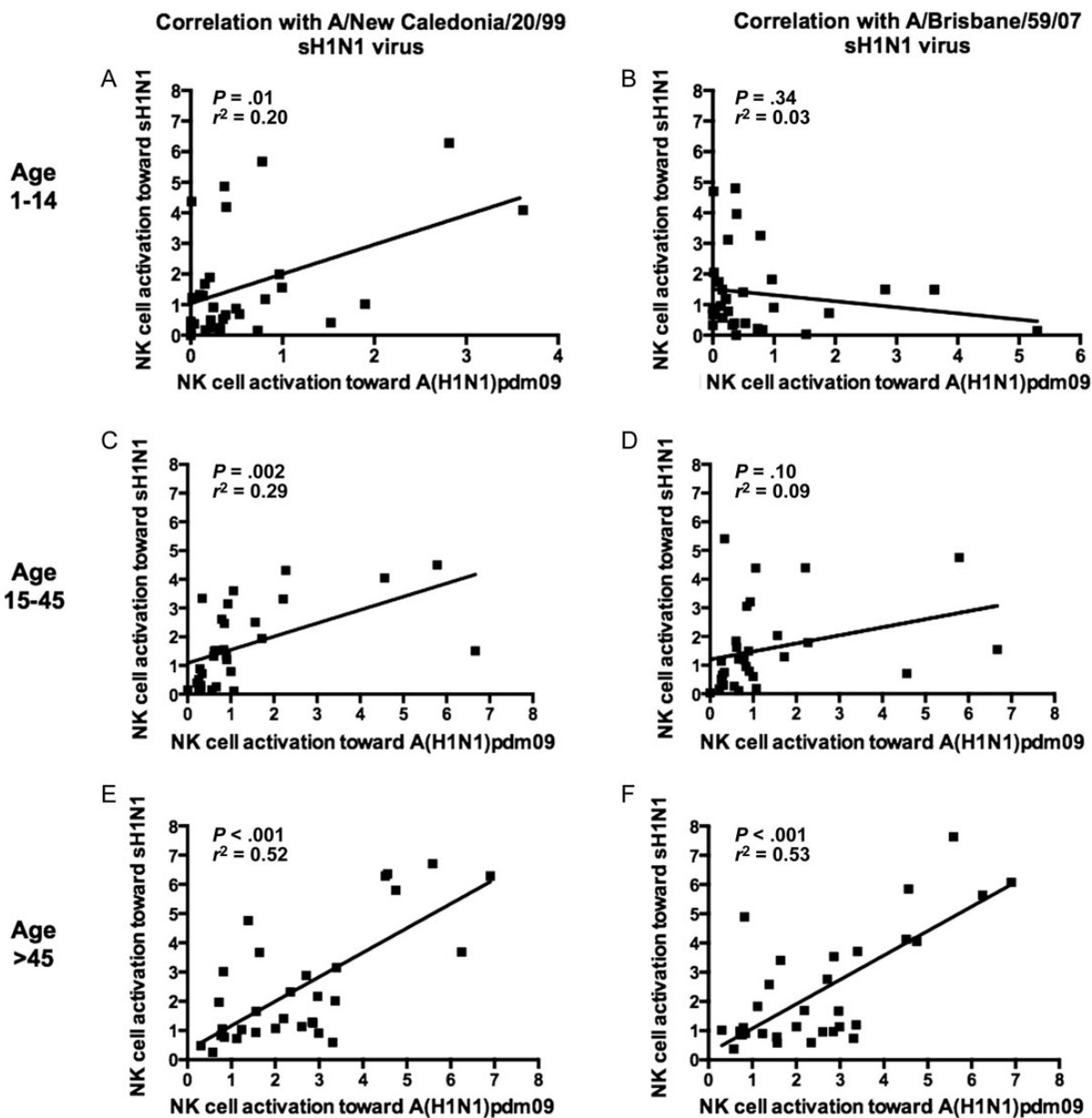
Figure 4. Correlation of antibody-dependent cellular cytotoxicity (ADCC) antibody responses to 2009 pandemic influenza A virus subtype H1N1 (A[H1N1] pdm09) and seasonal H1N1 viruses (sH1N1). Natural killer (NK) cell activation (measured as expression of interferon γ [IFN-γ] by $CD56^+CD3^-$ cells) against A(H1N1)pdm09 (A/California/04/2009) hemagglutinin (HA) was compared to NK cell activation against sH1N1 HA from A/New Caledonia/20/99 in the presence of serum samples collected before the 2009 A(H1N1) pandemic (Pre-2009) from individuals aged 1–14 years (A), 15–45 years (C), or >45 years (E). NK cell activation against A(H1N1)pdm09 (A/California/04/2009) HA was compared to NK cell activation against sH1N1 HA from A/Brisbane/59/07 in the presence of serum samples collected before the 2009 pandemic from individuals aged 1–14 years (B), 15–45 years (D), and >45 years (F). The Pearson product-moment correlation coefficient was used to determine when significant correlations occurred. The number of specimens ranged from 31 (Pre-2009; ages 15–45 years) to 46 (after the 2009 pandemic; ages 15–45 years).

($P = .002$ and $r^2 = 0.29$ for A/New Caledonia/20/99; $P = .10$ and $r^2 = 0.09$ for A/Brisbane/59/07; Figure 4C and 4D). However, there was a strong correlation between ADCC responses against sH1N1 HA proteins and ADCC responses against A(H1N1) pdm09 HA proteins for individuals >45 years of age ($P < .001$

and $r^2 = 0.52$ for A/New Caledonia/20/99; $P < .001$ and $r^2 = 0.53$ for A/Brisbane/59/07; Figure 4E and 4F). This suggests that previous sH1N1 infections in older subjects contributed to the development of cross-reactive antibodies against H1N1 A (H1N1)pdm09.
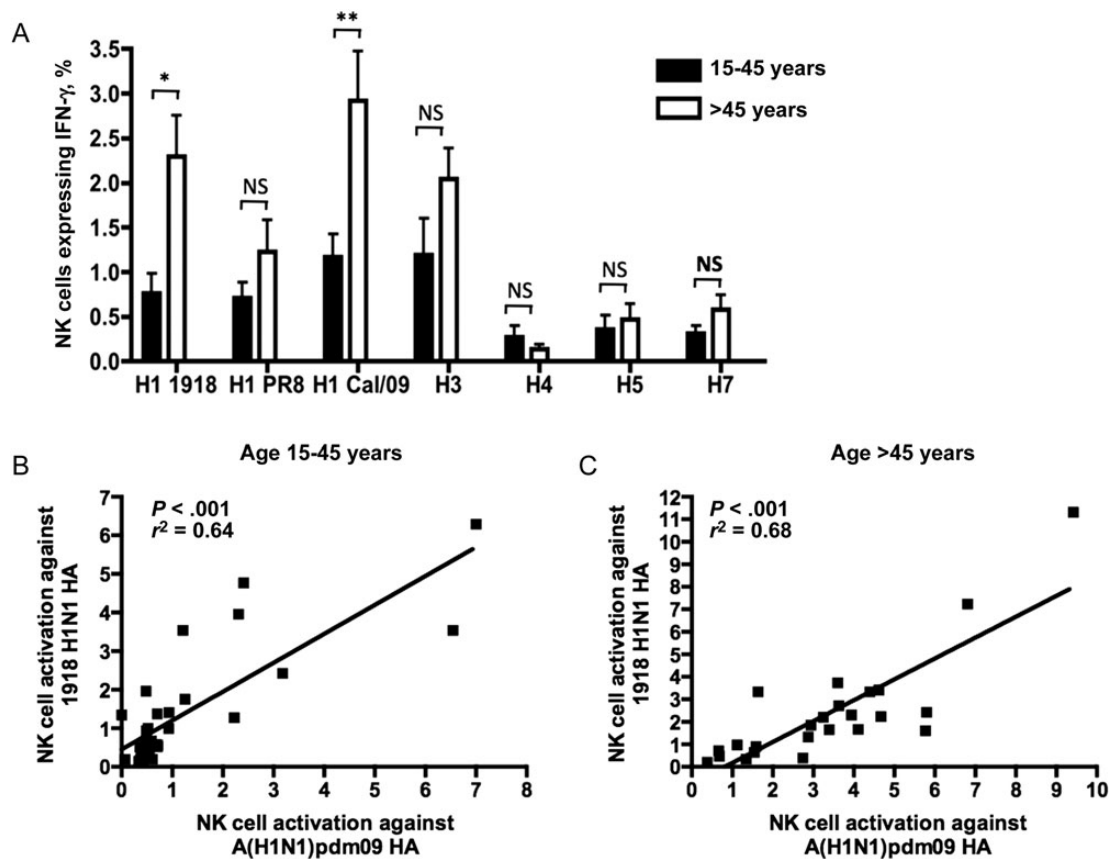
Figure 5. Antibody-dependent cellular cytotoxicity (ADCC) responses to 2009 pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09) hemagglutinin (HA) correlates with ADCC antibodies to 1918-like H1N1 virus HA. *A*, Comparison of the frequency of natural killer (NK) cells expressing interferon γ (IFN-γ) in response to plate-bound 1918 H1 HA (A/Brevig Mission/1/1918), PR8 H1 HA (/Puerto Rico/8/1934), 2009-H1N1 HA (A/California/04/2009), H3 HA (A/Brisbane/10/2007), H4 HA (A/Swine/Ontario/01911–1/99), H5 HA (A/Anhui/1/2005), or H7 HA (A/chicken/Netherlands/1/03) in the presence of a 1:40 dilution of sera from individuals aged 15–45 years or >45 years. Log$_{10}$-transformed data were analyzed by 2-way analysis of variance, followed by Bonferroni comparisons. *$P < .05$; **$P < .01$; n = 7 for each group. NS, nonsignificant. *B* and *C*, Comparison of NK cell activation to 1918 H1 HA (A/Brevig Mission/1/1918) and NK cell activation to A(H1N1)pdm09 HA(A/California/04/2009), both in the presence of 1:40 dilution of sera collected from individuals aged 15–45 years (*B*) or >45 years (*C*) after the 2009 A(H1N1) pandemic, analyzed by Pearson product-moment correlation coefficient (n = 54).

Cross-reactive ADCC Toward 1918-Like Influenza Viruses

We further hypothesized that infection with A(H1N1)pdm09 might also increase the immunity to related H1N1 influenza viruses by inducing cross-reactive ADCC antibodies in older subjects. We compared antibody-mediated NK cell activation against HA proteins from both the 1918 H1N1 HA protein and the 1934 H1N1 strain (A/Puerto Rico/8/1934) HA protein, using a subset of 9 sera taken from individuals aged 15–45 years and 15 sera taken from individuals aged >45 years immediately following the 2009 A(H1N1) pandemic. There was higher antibody-mediated NK cell activation in response to HA proteins from H1N1 viruses from 1918 (A/Brevig Mission/1/1918) and 2009 (A/California/04/2009) in individuals aged >45 years, compared with those aged 15–45 years (2-way ANOVA with Bonferroni comparisons, $P < .05$ for A/Brevig Mission/1/1918 and $P < .01$ for A/California/04/2009; Figure 5*A*). However,

there was no significant difference in antibody-mediated NK cell activation between the 2 age groups when comparing HA proteins from H1N1 (A/Puerto Rico/8/1934) virus and HA proteins derived from H3N2 (A/Brisbane/10/2007), H4N6 (A/Swine/Ontario/01911–1/99), H5N1 (A/Anhui/1/2005), and H7N7 (A/chicken/Netherlands/1/03) viruses (Figure 5*A*).

If indeed A(H1N1)pdm09 infection boosted ADCC antibodies to 1918-like H1N1 strains, a correlation might be predicted between antibody-mediated NK cell activation to HA proteins from A(H1N1)pdm09 and 1918 H1N1. We compared antibody-mediated NK cell activation in response to A(H1N1) pdm09 HA protein to that against 1918 (A/Brevig Mission/1/ 1918) HA protein in individuals aged 15–45 years and those aged >45 years after the 2009 A(H1N1) pandemic. Interestingly, ADCC responses to 1918 HA protein correlated strongly with ADCC responses to A(H1N1)pdm09 HA protein in

Table 1. Age-Stratified Summary of Serum Hemagglutination Inhibition (HI) Titers in the Study Cohort Before and After the 2009 Pandemic of Influenza A Virus Subtype H1N1 Infection

Time, Age	Subjects, No.	Age, Years, Median (Range)	HI Titer, Geometric Mean (95% CI)	
			Seasonal (A/Brisbane/59/2007)	Pandemic (A/California/07/09)
Before 2009[a]				
Overall	93	23 (1–59)	31.5 (−10.6 to 73.7)	5.1 (4.9–5.3)
1–14 y	31	4 (1–14)	163.6 (56.7–270.6)	5.2 (4.8–5.7)
15–45 y	31	23 (15–46)	26.2 (−5.2 to 57.5)	5.1 (4.8–5.4)
>45 y	31	52 (46–59)	7.3 (.7–14.0)	5
After 2009[b]				
Overall	89	22 (1–72)	8.0 (−1.3 to 17.2)	97.2 (46.2–148.2)
1–14 y	23	6 (1–14)	5	146.2 (30.3–262.0)
15–45 y	36	21 (16–45)	8.3 (−13.0 to 29.6)	95.1 (−1.6 to 191.9)
>45 y	30	63 (46–72)	11.5 (−.8 to 23.8)	72.9 (41.3–104.6)

Abbreviation: CI, confidence interval.

[a] Samples were collected between 21 November 2008 and 13 May 2009, before the pandemic.

[b] Samples were collected between 3 August 2009 and 16 November 2009, after the pandemic. Samples were selected on the basis of the presence of HI titers of >40 to A/California/07/09.

individuals aged 15–45 years and those aged >45 years ($P < .001$; $r^2 = 0.64$ and $r^2 = 0.68$, respectively; Figure 5B and 5C). This suggests that infection with A(H1N1)pdm09 significantly boosts cross-reactive ADCC antibodies to 1918-like viruses.

DISCUSSION

Novel pandemic influenza viruses emerge regularly, leading to widespread mortality and morbidity. The recent 2009 A(H1N1) pandemic affected individuals <40 years of age more severely than older individuals [2, 6–10]. This suggests that there is some level of cross-protection against A(H1N1)pdm09 in older individuals that is not found in younger individuals. Although studies have shown that neutralizing antibodies and T-cell responses are critical in cross-protection [12, 13, 17, 21, 36], here we show that other antibody effector mechanisms, such as ADCC, may play an important role. Sera from individuals >45 years of age commonly contained preexisting ADCC antibodies to A(H1N1)pdm09. These ADCC antibodies not only activated NK cells but also eliminated A(H1N1)pdm09-infected respiratory cells. Indeed, levels of A(H1N1)pdm09-specific ADCC antibodies were significantly increased following the 2009 A(H1N1) pandemic. The ability of ADCC antibodies to protect against novel influenza viruses is an important area for future research.

Recent animal model studies found that seasonal influenza virus infection provides some protection from challenge with A(H1N1)pdm09 and that nonneutralizing antibodies may be involved [11, 17, 37–39]. A higher titer of A(H1N1)pdm09-specific binding antibodies is induced by infection with sH1N1 that circulated before 1950 [11]. sH1N1 infection induced partial protection against subsequent challenge with A(H1N1)pdm09 among macaques in the absence of detectable HI antibodies [17]. Although T-cell immunity likely plays a role in the protection observed, subsequent analysis of serum samples from that macaque study by our group found that ADCC antibodies induced by the initial sH1N1 infection were cross-reactive with A(H1N1)pdm09 (unpublished data). We found that ADCC responses to 2 distinct sH1N1 viruses correlated with A(H1N1)pdm09-specific ADCC in older individuals. This suggests that prior exposure to seasonal influenza virus infections in older individuals induces cross-reactive A(H1N1)pdm09-specific ADCC. Future studies in animal models can now focus on passive transfer of monoclonal nonneutralizing antibodies that mediate ADCC, to define a role for ADCC in protective immunity.

HA-specific neutralizing antibodies generally recognize highly variable antigenic regions on the globular head of HA and tend to be highly specific for a particular strain of influenza virus. We speculate that ADCC antibodies against conserved regions of influenza virus HA may provide partial cross-protection from a range of influenza virus strains. We found no correlation between HI titers and HA-specific ADCC antibodies; this was expected since HI assays assess the binding of the antibody Fab region to bind antigen, whereas the NK cell assay measures binding of the antibody Fc region to CD16 on NK cells. Antibodies frequently target regions outside the receptor-binding domain of HA, and elderly individuals have a greater antibody diversity and higher affinity for the HA1 region of A(H1N1)pdm09 HA, compared with younger individuals [37, 38]. ADCC antibodies directed to non-HA influenza virus proteins

such as the NA protein and M2 proteins may also participate in cross-reactive immunity [31, 32].

We recognize that there are several limitations to our study that warrant future study. First, we had only a small number of individuals >70 years old; future studies should expand the number of such subjects. Second, individuals aged 1–14 years in our study had high preexisting sH1N1 HI titers. We felt that for the fairest comparison of ADCC responses it was important to ensure that younger individuals who were tested had at least some exposure to influenza virus and were not influenza naive before the 2009 A(H1N1) pandemic. Third, the unpaired nature of our samples meant we were unable to directly assess the effects of A(H1N1)pdm09 infection in each individual. Fourth, the viral elimination assays were only performed using A(H1N1)pdm09-infected respiratory epithelial cells. Future studies could analyze ADCC-mediated clearance of cells infected with a range of influenza virus strains.

In summary, sera from individuals >45 years of age commonly had preexisting ADCC activity to A(H1N1)pdm09 HA before the 2009 pandemic. Both the titer of ADCC antibodies to A(H1N1)pdm09 and the elimination of virus-infected respiratory cells significantly increased following the 2009 A(H1N1) pandemic. The correlation between ADCC responses to sH1N1 and A(H1N1)pdm09 in older individuals implicates sH1N1 infection in generating cross-reactive ADCC antibodies toward the A(H1N1)pdm09. We speculate that the induction of long-lived cross-reactive ADCC antibodies through vaccination may provide a level of protection from emerging influenza viruses.

Notes

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