Longevity and Determinants of Protective Humoral Immunity after Pandemic Influenza Infection

Saranya Sridhar¹, Shaima Begom¹, Katja Hoschler², Alison Bermingham², Walt Adamson³, William Carman³, Steven Riley⁴, and Ajit Lalvani¹

¹Section of Respiratory Infections, National Heart and Lung Institute, and ⁴Department of Infectious Disease Epidemiology, Imperial College London, London, United Kingdom; ²Respiratory Virus Unit, Centre for Infections, Public Health England, London, United Kingdom; and ³West of Scotland Specialist Virology Centre, Glasgow, Scotland, United Kingdom

Abstract

Rationale: Antibodies to influenza hemagglutinin are the primary correlate of protection against infection. The strength and persistence of this immune response influences viral evolution and consequently the nature of influenza epidemics. However, the durability and immune determinants of induction of humoral immunity after primary influenza infection remain unclear.

Objectives: The spread of a novel H1N1 (A[H1N1]pdm09) virus in 2009 through an unexposed population offered a natural experiment to assess the nature and longevity of humoral immunity after a single primary influenza infection.

Methods: We followed A(H1N1)pdm09-seronegative adults through two influenza seasons (2009–2011) as they developed A(H1N1)pdm09 influenza infection or were vaccinated. Antibodies to A(H1N1)pdm09 virus were measured by hemagglutination-inhibition assay in individuals with paired serum samples collected preinfection and postinfection or vaccination to assess durability of humoral immunity. Preexisting A(H1N1)pdm09-specific

multicytokine-secreting CD4 and CD8 T cells were quantified by multiparameter flow cytometry to test the hypothesis that higher frequencies of CD4⁺ T-cell responses predict stronger antibody induction after infection or vaccination.

Measurements and Main Results: Antibodies induced by natural infection persisted at constant high titer for a minimum of approximately 15 months. Contrary to our initial hypothesis, the fold increase in A(H1N1)pdm09-specific antibody titer after infection was inversely correlated to the frequency of preexisting circulating A(H1N1)pdm09-specific CD4⁺IL-2⁺IFN- γ ⁺TNF- α ⁺ T cells ($r = -0.4122$; $P = 0.03$).

Conclusions: The longevity of protective humoral immunity after influenza infection has important implications for influenza transmission dynamics and vaccination policy, and identification of its predictive cellular immune correlate could guide vaccine development and evaluation.

Keywords: pandemic influenza; immunology; antibodies; T cells; epidemiology

Neutralizing antibodies against the surface glycoproteins, hemagglutinin and neuraminidase, of influenza virus are the primary mediators of protective immunity against influenza infection (1). Antigenic viral evolution and thereby global influenza circulation patterns are critically influenced by the nature of host humoral immunity.

Recent work has shown that multiple influenza exposures, through infection or vaccination, can maintain antibody responses over a prolonged duration of time (2, 3). However, the durability of antibody responses after a single primary infection, as opposed to multiple infections, remains uncertain.

A previous study during the reemergence of 1977 H1N1 virus reported detectable levels of antibodies up to 3 years after infection in most children, although kinetics of antibody titer was not reported (4). Reports of the kinetics of antibody responses in serum collected from ill patients seeking medical care during the

(Received in original form October 8, 2014; accepted in final form December 4, 2014)

A.L. is a Wellcome Trust Senior Research Fellow in Clinical Science and NIHR Senior Investigator.

Author Contributions: S.S. and A.L. conceived and designed the study. S.S., S.B., K.H., A.B., W.A., and W.C. performed the experiments. S.S. and S.R. analyzed the data. S.S., S.R., A.L., and S.B. interpreted the data. S.S., A.L., S.B., K.H., and S.R. contributed to writing of the manuscript.

Correspondence and requests for reprints should be addressed to Saranya Sridhar, M.B. B.S., M.S., D.Phil., Department of Respiratory Medicine, National Heart and Lung Institute, Imperial College London, Norfolk Place, London W2 1PG, UK. E-mail: s.sridhar@imperial.ac.uk; or Ajit Lalvani, M.A., D.M., Department of Respiratory Medicine, National Heart and Lung Institute, Imperial College London, Norfolk Place, London W2 1PG, UK. E-mail: a.lalvani@imperial.ac.uk

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 191, Iss 3, pp 325–332, Feb 1, 2015

Copyright © 2015 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201410-1798OC on December 15, 2014

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Protection against influenza is primarily mediated by neutralizing antibodies and their persistence in populations determines the rate of viral evolution and global influenza transmission patterns. Recent work has shown that multiple influenza exposures maintain antibodies for a prolonged duration of time. However, how long antibodies can last after a single infection, as opposed to multiple infections, remains uncertain.

What This Study Adds to the

Field: We exploited the natural experiment of an influenza pandemic to investigate persistence of antibodies after natural community-acquired influenza infection. We determined that in individuals previously unexposed to an influenza strain, antibodies to influenza are maintained at protective levels for up to 15 months after a single influenza infection. We also identify, for the first time, a cellular immune correlate for the induction of such long-lasting naturally acquired protective antibodies in humans.

recent 2009 H1N1 pandemic (A[H1N1] pdm09) have varied between rapid decline within 6 months to maintenance up to 1 year after symptom onset (5–7). Thus, the question of durability of antibody responses induced by a single influenza infection remains unresolved and, to our knowledge, there is no prospective study reporting the long-term persistence of humoral immunity after natural A(H1N1)pdm09 infection.

Although the importance of sustaining protective humoral immunity is well recognized, the immune determinants of the longevity of these antibody responses remain unclear. Preexisting memory B cells, natural killer cells, and CD4 helper and CD8 cytotoxic T cells have been reported to be associated with vaccine-induced humoral immunity (8–11). However, the relationship between preexisting immune responses and antibody responses induced by infection is unknown.

Characterizing antibody durability and its determinants after natural infection

would advance the understanding of influenza epidemiology and could inform the design and evaluation of influenza vaccines. However, defining the evolution of immune responses in humans to a single primary influenza infection is challenging because of prior exposures and preexisting humoral immunity. Ideally, individuals should be naive to the infecting strain but this requires studying infants or awaiting the emergence of novel virus strains to which individuals lack any previous exposure. Biologic samples before and after incident infection in conjunction with reliable information on subsequent vaccination or reinfection are required for each individual. We therefore exploited the unique opportunity offered by the 2009 pandemic to initiate a prospective cohort study of A(H1N1)pdm09-seronegative subjects to investigate the durability of humoral immunity induced by natural influenza infection. We also assessed whether preexisting T-cell responses impact the nature of infection-induced antibodies, hypothesizing that a higher frequency of influenza-specific CD4⁺ helper T cells

would correlate with a stronger durable antibody response.

Methods

Study Design and Cohort

As the 2009 H1N1 pandemic evolved in the United Kingdom, we initiated this pandemic response-mode research study after approval by the North West London Research Ethics Committee. Healthy adult (>18 yr) staff and students of Imperial College London were invited to participate as previously described (12). Participants were rapidly recruited between September 13 and November 6, 2009 and followed through the 2009–2010 and 2010–2011 influenza seasons with blood collected at the start and end of each season (Figure 1). Participants were provided with nasal swab packs, self-swabbing instructions, and requested to record temperature, self-sample, and return nasal swabs when experiencing any influenza-like symptoms. The date of infection or vaccination was estimated as previously described (12).

Figure 1. Schematic of study outline. Healthy adults were recruited after the first wave of the pandemic had passed in the United Kingdom and followed over two influenza seasons with peripheral blood mononuclear cells and serum samples collected prior to and at the end of each influenza winter season. Nasal swabs were collected by participants if symptomatic and returned to the laboratory. Infection was defined by detection of A(H1N1)pdm09 virus in returned nasal swab or a fourfold rise in A(H1N1)pdm09 hemagglutination inhibition (HAI) titer in paired serum samples. Arrows between boxes denote longitudinal progression of individuals during the study; white boxes indicate A(H1N1)pdm09-uninfected individuals; light green boxes indicate A(H1N1)pdm09-infected individuals; blue boxes indicate individuals who were vaccinated. In a subset of infected individuals reliable determination of the date of infection was possible if a temporal relationship between illness episode and return of a positive nasal swab or if a single illness episode was experienced during the season. Median duration and interquartile range (IQR) between date of infection and collection of serum samples at different follow-up time points is shown.

Briefly, using symptom questionnaires in a subset of infected individuals with a single influenza-like illness episode or where a positive nasal swab was temporally associated with symptom onset, we determined the date of infection. Date of vaccination was estimated using date of symptom questionnaire in which individuals reported receiving influenza vaccination. Time between infection or vaccination and study time points was estimated by subtracting date of study follow-up from date of infection or vaccination. Written informed consent was obtained from all participants.

Sample Collection and Processing

Blood was collected for isolation of peripheral blood mononuclear cells (PBMCs) and serum. PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) density centrifugation, washed twice in RPMI 1640 (Sigma-Aldrich, Gillingham, UK), and suspended in RPMI 1640 supplemented with 10% fetal calf serum (Invitrogen, Paisley, UK). PBMCs were cryopreserved in heat-inactivated fetal calf serum supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich) at -180°C in liquid nitrogen. All assays were undertaken using cryopreserved PBMCs.

Laboratory Assays

Antibody titers to A(H1N1)pdm09 were measured by the hemagglutination inhibition (HI) assay used for UK national surveillance (13) with seroconversion defined as a fourfold rise in HI titer on paired serum samples (i.e., titer rise from below detection limit to titer $\geq 1:32$ or significant/fourfold rise in titer) taken before and after each influenza season. Presence of virus in nasal swabs was confirmed by a multiplex real-time reverse-transcriptase polymerase chain reaction assay using standard methods by Public Health England (formerly the Health Protection Agency, England) (13) and Scotland (14). Influenza A(H1N1)pdm09-infected individuals were defined as A(H1N1)pdm09-unvaccinated persons showing antibody seroconversion and/or detection of viral genome in nasal swabs.

Flow Cytometry Assay

PBMCs were stimulated with media (negative control), phorbol myristate acetate/ionomycin (positive control), live A(H1N1)pdm09 virus, and cytomegalovirus lysate for 18 hours

and cells were stained for surface markers and intracellular cytokines as previously described with at least 1 million live cells collected for all samples (12). Data were acquired using the BD LSR Fortessa (BD Biosciences, Oxford, UK) machine and analyzed using FlowJo (FlowJo, Ashland, OR) software.

Statistical Analysis

Antibody titers below the detection level (titers $<1:8$) were given an arbitrary value of 4 for the purposes of statistical analysis with fold change defined as a ratio of titers. Antibody titers, fold change, and frequencies of T-cell responses were normalized by log transformation and normality tested using Shapiro-Wilk test. Student *t* test or Kruskal-Wallis test was used to identify statistically significant differences between groups, accounting for multiple comparisons. For the analysis of flow cytometry results, because of the sample size, results from the different cytokine subsets were compared using two-sided matched pair nonparametric one-way analysis of variance with Dunn post-test comparison accounting for multiple comparisons. Correlations between preexisting T-cell responses and fold change in antibodies used Spearman rank correlation test. Multivariate stepwise regression with forward selection was undertaken with fold change as the dependent variable and age, sex, and frequencies of CD4 T-cell cytokine-secreting cells as independent variables. Graphpad Prism version 4 (GraphPad Software Inc., La Jolla, CA) and Stata v11 (StataCorp, College Station, TX) was used for statistical analysis.

Results

Study Population

To determine the longevity of humoral immunity after a primary influenza infection, only individuals seronegative to A(H1N1)pdm09 (HI titers $\leq 1:8$) at baseline (prior to infection or vaccination episode) and with at least one paired serum sample after infection or vaccination were included in this analysis. Fifty-three eligible individuals developed incident A(H1N1)pdm09 infection. Although our primary aim was to characterize humoral immunity after influenza infection, we also analyzed antibody responses in 32 eligible individuals who were vaccinated. Eleven infected individuals had serum samples

collected at baseline and at each subsequent study time point with no evidence of reinfection or vaccination in the subsequent season, enabling longitudinal assessment of humoral immunity after a single primary infection. Among vaccinated individuals with baseline samples who were not subsequently revaccinated, 11 had samples at two subsequent follow-up time-points and two at each of the three follow-up time-points (Figure 1). The median age of the participants included in this analysis was 32 years (interquartile range, 18–64). In a subset of individuals, the date of infection or vaccination episode was estimated as previously described (12), which allowed us to calculate the time between infection or vaccination and study time points (Figure 1). There was no statistically significant difference in duration of follow-up between vaccinated and infected individuals.

Induction and Durability of Antibody Responses

Approximately 3 months after infection with A(H1N1)pdm09 the geometric mean HI titer in previously seronegative individuals was 186 (95% confidence interval [CI], 153.03–228.23). In only 2 of 53 individuals who were infected (identified by polymerase chain reaction–confirmed nasal swabs), no antibody seroconversion was observed. The lack of induction of antibodies in these two individuals was not associated with the timing of serum sampling because sera were collected at 90 and 113 days postinfection. However, a delayed twofold rise in antibody titer was observed in one of these individuals (Table 1, F075). At the first follow-up time point, no difference was observed in mean antibody titer or fold change in titer induced by A(H1N1)pdm09 infection or vaccination (Figure 2; see Figure E1 in the online supplement). In our cohort of predominantly young adults, there was no difference in the mean titer induced by A(H1N1)pdm09 infection between adults aged younger and older than 40 years (Figure 2A).

We assessed durability of antibody responses by primary influenza infection in 11 individuals who developed incident A(H1N1)pdm09 infection and had serum available from all three postinfection study time points. There was no significant decline in the mean titer over time because infection with the mean HI titer maintained higher than the protective threshold of 1:32 for up

Table 1. Hemagglutination-Inhibition Titers in Infected and Vaccinated Individuals at Each Time Point with Fold Change in Titer between the Different Time Points

Infected Individuals								Vaccinated Individuals							
Study ID	HI Titer				Fold Change			Study ID	HI Titer				Fold Change		
	Preinfection	T1	T2	T3	T1-T0	T2-T1	T3-T2		Preinfection	T1	T2	T3	T1-T0	T2-T1	T3-T2
F071	4	32	128	128	6.4	4.0	1.0	F040	4	4	4		1.0	1.0	
F075	4	4	16	16	1.0	3.2	1.0	F066	4	16	16	16	3.2	1.0	1.0
F009	4	32	64	64	6.4	2.0	1.0	F082	4	32	32		6.4	1.0	
F155	4	32	32	32	6.4	1.0	1.0	F033	4	32	32		6.4	1.0	
F007	4	32	32	32	6.4	1.0	1.0	F288	4	32	32		6.4	1.0	
F291	4	64	64	64	12.8	1.0	1.0	F164	4	512	512		64.0	1.0	
F162	4	128	128	128	25.6	1.0	1.0	F045	4	64	32		12.8	0.5	
F093	4	128	128	128	25.6	1.0	1.0	F012	4	64	32		12.8	0.5	
F229	4	128	64	64	25.6	0.5	1.0	F403	4	128	64		25.6	0.5	
F196	4	128	64	64	25.6	0.5	1.0	F077	4	16	4		3.2	0.3	
F179	4	128	4	4	25.6	0.04	1.0	F280	8	1,024	256	256	128.0	0.3	1.0
Average					15.2	1.4*	1.0						24.5	0.7	1.0

Definition of abbreviation: HI = hemagglutination inhibition.

* $P = 0.05$ comparing average fold change between infected and vaccinated individuals. Statistical analysis of comparison of fold change was undertaken using nonparametric Mann-Whitney U test.

Fold change was calculated as ratio of titers.

to 480 days postinfection (geometric mean titer, 47.66; 95% CI, 24.45–92.89) (Figure 2B). In only one individual did antibody titers decline to baseline levels within 300 days postinfection (F179; Table 1). We also analyzed antibody titers in 11 vaccinated individuals with serum available from each of the two post-vaccination time points. The magnitude of antibody titers was significantly lower ($P = 0.02$) in serum collected at approximately

300 days (T2) compared with approximately 90 days (T1) post-vaccination (Figure 2B), although mean titers remained just above the protective threshold of 1:32 (geometric mean titer, 35.49; 95% CI, 13.81–91.23). The fold increase in antibody titer between the first (~90 d) and second (~300 d) follow-up time points was significantly higher ($P < 0.05$) in the infected group compared with the vaccinated group (Table 1).

Correlation of Preexisting CD4⁺ T Cells with Induction of Antibody Responses

We investigated the cellular immune determinants of this naturally acquired durable humoral immunity hypothesizing that higher frequencies of preexisting CD4⁺ T cells would be associated with stronger induction and more durable antibody responses. We characterized the frequency and functionality of preinfection CD4⁺

Figure 2. Induction and durability of antibodies after A(H1N1)pdm09 infection or vaccination. Hemagglutination-inhibition assay titers were evaluated against the A(H1N1)pdm09 (A/England/195/09) virus in paired serum samples collected over the duration of the study. (A) Geometric mean titer (in natural log scale) at baseline (T0) and the first time point (T1) after A(H1N1)pdm09 infection or vaccination in 53 infected individuals and 32 vaccinated individuals who had A(H1N1)pdm09 hemagglutination-inhibition titers less than 8 at baseline. The blue lines represent titers in individuals younger than age 40 and the green lines represent titers in those older than age 40. Error bars represent 95% confidence intervals. (B) Geometric mean titer (in natural log scale) in 11 infected individuals who had serum samples at each of the study time points (left) and 11 vaccinated individuals who had serum samples at three study time points (right). Individuals were infected or vaccinated between T0 and T1. Statistical analysis comparing the mean titers was undertaken using the nonparametric Wilcoxon signed rank-sum test. Error bars represent 95% confidence intervals.

T cells by measuring IFN- γ , IL-2, and tumor necrosis factor (TNF)- α cytokine secretion to A(H1N1)pdm09 virus in 33 eligible individuals with PBMCs available prior to development of infection. A total of 27 of 33 (82%) individuals had detectable ($>0.001\%$) antigen-specific cytokine-secreting CD4 $^{+}$ T cells to at least one of the three IFN- γ , IL-2, or TNF- α cytokines. Boolean analysis of the different cytokine-

secreting subsets revealed that single-cytokine-secreting cells (IFN- γ^{+} TNF- α^{-} IL-2 $^{-}$, IFN- γ^{-} TNF- α^{+} IL-2 $^{-}$, IFN- γ^{-} TNF- α^{-} IL-2 $^{+}$) were in significantly higher frequency than triple-cytokine-secreting CD4 $^{+}$ T cells and double-cytokine-secreting CD4 $^{+}$ T cells (Figure 3B). There was no statistically significant difference between frequencies of total IFN- γ or IL-2 or TNF- α secreting

A(H1N1)pdm09-specific CD4 $^{+}$ T cells (Figure 3A) or between frequencies of the single-cytokine-secreting T-cell populations (Figure 3B).

We correlated the preinfection frequency of antigen-specific CD4 $^{+}$ cytokine-secreting T cells with the fold-change in antibody titer postinfection in the 27 individuals who had a A(H1N1)pdm09-specific cytokine response. An inverse

Figure 3. Preexisting A(H1N1)pdm09-specific CD4 $^{+}$ cytokine-secreting T cells predict rise in antibodies after infection. The magnitude of preexisting A(H1N1)pdm09 virus-specific CD4 $^{+}$ cells was determined using multiparameter flow cytometry after 18-hour stimulation of peripheral blood mononuclear cells with live A(H1N1)pdm09 virus. Antigen-specific frequencies of CD4 $^{+}$ IFN- γ^{+} , CD4 $^{+}$ IL-2 $^{+}$, and CD4 $^{+}$ TNF- α^{+} T cells (A) and CD4 $^{+}$ cytokine-secreting subsets of cells (B) were assessed in baseline (T0) samples of individuals developing incident A(H1N1)pdm09 infection ($n = 33$). Symbols represent responses for each individual with the line depicting the median response. Correlation between the fold change in hemagglutination inhibition (HI) titer preinfection (T0) and postinfection (T1) and frequency of preexisting virus-specific CD4 $^{+}$ IL-2 $^{+}$ (C) and CD4 $^{+}$ IL-2 $^{+}$ IFN- γ -TNF- α - (D) T cells. Only individuals with a detectable antigen-specific response ($>0.001\%$) were included ($n = 27$). Multivariate analysis of fold change in antibody titers with cytokine-secreting CD4 $^{+}$ T-cell frequencies adjusting for age and sex showed an association of only CD4 $^{+}$ IL-2 $^{+}$ T cells ($P = 0.04$) and CD4 $^{+}$ IL-2 $^{+}$ IFN- γ -TNF- α - T cells ($P = 0.008$). The fold change and frequency of response was log transformed with r indicating the Spearman rank correlation coefficient. TNF = tumor necrosis factor.

correlation was found between the frequencies of total IL-2 (Spearman rank coefficient, -0.3764 ; $P = 0.05$) and single-cytokine IL-2⁺IFN- γ ⁻TNF- α ⁻ (Spearman rank coefficient, -0.4122 ; $P = 0.03$) secreting T cells and the fold-change in antibody titer (Figure 3C). Preexisting frequencies of CD4⁺TNF- α (Spearman rank coefficient, -0.4116 ; $P = 0.03$) and single-cytokine-secreting TNF- α ⁺IL-2-IFN- γ ⁻ (Spearman rank coefficient, -0.4071 ; $P = 0.04$) were also inversely correlated with rise in antibody titer after infection. Multivariate analysis adjusting for sex and age undertaken for total and single-cytokine-secreting CD4⁺ T cells found only total IL-2 ($P = 0.04$) and single-cytokine-secreting IL-2⁺IFN- γ ⁻ TNF- α ⁻ T cells ($P = 0.008$) to have a significant inverse correlation with the magnitude of antibody rise after infection.

As a sensitivity analysis, we used data from all 33 individuals including the six individuals lacking any detectable A(H1N1)pdm09-specific cytokine response. Multivariate analysis confirmed the association of antibody rise with total IL-2 ($P = 0.005$) and IL-2-only ($P < 0.001$) and also showed significant associations with total IFN- γ ($P = 0.04$) and IFN- γ -only ($P = 0.01$) CD4⁺-secreting T-cell responses.

As a control, antigen-specific CD4⁺ cytokine-secreting responses to cytomegalovirus lysate were not associated with rise in A(H1N1)pdm09-specific antibodies postinfection (see Figure E2). We undertook a similar analysis for responses to vaccination and found no association between frequencies of antigen-specific cytokine-secreting CD4⁺ T cells and rise in antibody titer after vaccination (see Figure E3), although we cannot exclude the possibility of having underpowered this analysis. Although not our primary hypothesis, we also tested whether CD8⁺ antigen-specific responses were associated with rise in antibody titer after infection or vaccination and found antigen-specific cytokine-secreting CD8⁺ T cells not to be associated (see Figure E4).

Discussion

We investigated the development and persistence of humoral immunity to a single influenza exposure by following individuals through a pandemic as they developed incident influenza infection. We found that

natural influenza infection maintains antibodies at a constant titer, above the protective threshold, for at least 1.5 years and that this induction of durable antibodies was inversely associated with preexisting frequencies of influenza-specific CD4⁺IL-2⁺ T cells.

Durable maintenance of protective titers of HI antibodies for 1.5 years has implications for understanding patterns of influenza transmission. Several populations have experienced severe follow-up waves of A(H1N1)pdm09 infection in the post-pandemic season in the absence of any reported antigenic drift (15, 16), with waning humoral immunity raised as a potential explanation (17). Our results, using longitudinal data from individuals, are the first to provide robust evidence that repeat pandemic waves prior to antigenic drift (18) are not driven by waning humoral immunity. Rather, our results suggest that third waves of A(H1N1)pdm09 may have been caused by not-yet-characterized large susceptible populations at the end of initial pandemic waves, possibly in addition to other explanations, such as increased intrinsic transmissibility (19). Furthermore, if similar patterns of antibody persistence occur during the interpandemic period, they may explain the apparent cycling of H1N1 and H3N2 subtypes (20) and consequently the persistence of H1N1 as a minor subtype. A caveat to these observations is that our study was restricted to healthy adults and therefore the findings may not extend to children, the elderly, or other groups at higher risk of generating clinical cases.

Despite years of study, the longevity of influenza antibodies after infection remains controversial. A major confounding factor is the effect of preexisting humoral immunity. The emergence of a novel influenza strain in 2009 (A(H1N1)pdm09) provided a unique opportunity to overcome this challenge by studying the dynamics of humoral immunity in serologically naive individuals. The only studies to investigate A(H1N1)pdm09 antibody durability after infection used samples collected after illness onset not accounting for the presence of preexisting antibodies, perhaps explaining the contradictory results among these studies (5–7). Moreover, these studies undertaken in patients attending medical care is in contrast to our study reflecting the spectrum of community-acquired influenza illness in healthy adults (i.e., with

predominantly mild-to-moderate illness or asymptomatic infection). To robustly characterize the persistence of antibody responses after a single exposure, we prospectively followed individuals as they developed incident infection in the absence of subsequent reinfection and vaccination although subclinical exposure without an associated rise in HI titers cannot be ruled out. This is critically important because recent work has shown that antibody responses are continually boosted by multiple influenza exposures (2, 3), implying that antibody persistence after a single infection cannot be accurately determined by repeated cross-sectional surveys or through longitudinal studies lacking reliable individual subject clinical data and vaccination status.

Our study encompassed two influenza seasons and therefore whether antibodies induced by natural infection are maintained for longer periods of time remains an open question. Indeed, a study in seronegative children infected with the 1978 H1N1 strain suggested that antibodies were maintained for up to 3 years after infection, although the magnitude of antibody titers or whether the levels declined below the protective threshold was not reported (4). Our study complements this historical work by reporting the maintenance of antibody levels above the putative protective threshold of greater than or equal to 1:32 for at least one subsequent influenza season.

Despite extensive study, the determinants of long-lasting humoral immunity to influenza remain unclear. Our study identifies preexisting CD4⁺ as natural determinants of durable influenza-specific humoral immunity. Contrary to our original hypothesis, lower frequencies of preexisting CD4⁺IL-2⁺ and CD4⁺IL-2⁺TNF- α ⁻IFN- γ ⁻ influenza-specific T cells were associated with a stronger induction of antibodies after natural influenza infection. Although vaccine studies that explored the association of vaccine-induced antibodies with preexisting cellular immune responses found variable results reporting a positive (21), inverse (9), or no correlation (22), the cellular immune determinants of humoral immunity induced by natural influenza infection have not, to the best of our knowledge, hitherto been assessed. We found an unexpected inverse correlation between preexisting influenza-specific memory CD4⁺IL-2⁺ T cells and magnitude of antibody induction.

One explanation for this negative effect on antibody responses may lie in the proposed protective role of CD4⁺ T cells in limiting viral replication, either through direct killing (23, 24) or by induction of greater frequencies of natural killer cells that limit antigen-presentation to antibody-producing B cells (25, 26). This would be consistent with reduced antibody levels in individuals after antiviral therapy (27). However, if this were the case, it did not lead to observable changes in the severity of outcome in this cohort: we found no association between CD4⁺ T-cell frequencies and severity of illness (12) or between the severity of illness and rise in antibody titer (see Figure E5). Therefore, we suggest a different explanation. CD4⁺ICOS⁺IL-21⁺ T follicular helper cells are specialized for providing cognate B cell help and after influenza vaccination, it is the induction of these cells, rather than preexisting frequencies, that is associated with antibody induction (28, 29). Interestingly, work in animal models revealed that IL-2 suppresses the differentiation of T follicular helper cells and negatively impacts influenza-specific long-lived antibody responses, a finding consistent with our observation (30, 31). The strength and consistency of our finding of a particular subset of cytokine-secreting CD4⁺ T cells, namely IL-2⁺TNF- α ⁺IFN- γ ⁺, leads us to favor this explanation,

although the mechanism needs further study.

Determining the immunologic basis for long-term antibody persistence is particularly important for vaccination strategies. In our cohort, the kinetics of antibody responses induced by inactivated influenza vaccines was different to that induced by infection. We observed a decline in the magnitude of titer within 9 months post-vaccination, earlier than natural infection, although these antibodies remained at a protective titer. Our findings suggest potential differences between maintenance of humoral immunity post viral infection of the respiratory tract and parenteral administration of inactivated proteins. Our findings of the long-term durability of antibodies after natural infection in conjunction with the finding of CD4⁺IL-2⁺ T cells relating to the strength of antibody rise provides both a potential biomarker and a probable biologic mechanism to exploit in future vaccine designs for generating sustainable humoral immunity.

Our study has limitations inherent in undertaking longitudinal cohort studies in real-time during a pandemic. We were only able to measure HI antibodies and whether antibodies to neuraminidase or mucosal antibodies have similar dynamics remains to be studied. Our stringent criteria for eligibility in this analysis restricted our

sample size particularly for vaccinated individuals, most of whom did not have longer follow-up because they were excluded for being revaccinated. Although seronegative individuals in this analysis were defined by absence of HI antibodies, we cannot rule out the possibility that other serologic assays, such as ELISA, may have detected influenza-specific antibodies in individuals seronegative by HI assay. We defined infection as antibody seroconversion and therefore we cannot know whether infected individuals who do not seroconvert have different antibody dynamics, although such instances are reported in approximately 10% of individuals (32).

In conclusion, our prospective cohort study defining the longevity of protective antibodies after natural infection will advance the understanding of global influenza epidemiology and evaluation of vaccination strategies. The unexpected role of preexisting cellular immunity in determining antibody responses to influenza may help to guide design and evaluation of new improved influenza vaccines. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors acknowledge the support of the NIHR Health Protection Research Unit in Respiratory Infections.

References

- Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)* 1972;70: 767–777.
- Miller MS, Gardner TJ, Krammer F, Aguado LC, Tortorella D, Basler CF, et al. Neutralizing antibodies against previously encountered influenza virus strains increase over time: a longitudinal analysis. *Sci Transl Med* 2013;5:198ra07.
- Lessler J, Riley S, Read JM, Wang S, Zhu H, Smith GJ, Guan Y, Jiang CQ, Cummings DA. Evidence for antigenic seniority in influenza A (H3N2) antibody responses in southern China. *PLoS Pathog* 2012;8: e1002802.
- Grilli EA, Davies JR, Smith AJ. Infection with influenza A H1N1. 1. Production and persistence of antibody. *J Hyg (Lond)* 1986;96:335–343.
- Chan KH, To KK, Hung IF, Zhang AJ, Chan JF, Cheng VC, Tse H, Che XY, Chen H, Yuen KY. Differences in antibody responses of individuals with natural infection and those vaccinated against pandemic H1N1 2009 influenza. *Clin Vaccine Immunol* 2011;18:867–873.
- Wang M, Yuan J, Li T, Liu Y, Wu J, Di B, Chen X, Xu X, Lu E, Li K, et al. Antibody dynamics of 2009 influenza A (H1N1) virus in infected patients and vaccinated people in China. *PLoS ONE* 2011;6:e16809.
- Liu W, Ma MJ, Tang F, He C, Zhang XA, Jiang LF, Xin DS, Hu CY, Looman C, Cao WC. Host immune response to A(H1N1)pdm09 vaccination and infection: a one-year prospective study on six cohorts of subjects. *Vaccine* 2012;30:4785–4789.
- Furman D, Jojic V, Kidd B, Shen-Orr S, Price J, Jarrell J, Tse T, Huang H, Lund P, Maecker HT, et al. Apoptosis and other immune biomarkers predict influenza vaccine responsiveness. *Mol Syst Biol* 2013;9:659.
- He XS, Holmes TH, Zhang C, Mahmood K, Kemble GW, Lewis DB, Dekker CL, Greenberg HB, Arvin AM. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *J Virol* 2006;80:11756–11766.
- Nakaya HI, Wrammert J, Lee EK, Racioppi L, Marie-Kunze S, Haining WN, Means AR, Kasturi SP, Khan N, Li GM, et al. Systems biology of vaccination for seasonal influenza in humans. *Nat Immunol* 2011;12: 786–795.
- Tan Y, Tamayo P, Nakaya H, Pulendran B, Mesirov JP, Haining WN. Gene signatures related to B-cell proliferation predict influenza vaccine-induced antibody response. *Eur J Immunol* 2014;44:285–295.
- Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, Bean T, Barclay W, Deeks JJ, Lalvani A. Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat Med* 2013;19:1305–1312.
- Miller E, Hoschler K, Hardelid P, Stanford E, Andrews N, Zambon M. Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study. *Lancet* 2010;375: 1100–1108.
- Adamson WE, McGregor EC, Kavanagh K, McMenamin J, McDonagh S, Molyneux PJ, Templeton KE, Carman WF. Population exposure to a novel influenza A virus over three waves of infection. *J Clin Virol* 2011;52:300–303.

15. Dávila J, Chowell G, Borja-Aburto VH, Viboud C, Grajales Muñiz C, Miller M. Substantial morbidity and mortality associated with pandemic A/H1N1 influenza in Mexico, winter 2013–2014: gradual age shift and severity. *PLoS Curr* 2014;6:6.
16. Hoschler K, Thompson C, Andrews N, Galiano M, Pebody R, Ellis J, Stanford E, Baguelin M, Miller E, Zambon M. Seroprevalence of influenza A(H1N1)pdm09 virus antibody, England, 2010 and 2011. *Emerg Infect Dis* 2012;18:1894–1897.
17. Camacho A, Cazelles B. Does homologous reinfection drive multiple-wave influenza outbreaks? Accounting for immunodynamics in epidemiological models. *Epidemics* 2013;5:187–196.
18. Miller MA, Viboud C, Balinska M, Simonsen L. The signature features of influenza pandemics—implications for policy. *N Engl J Med* 2009; 360:2595–2598.
19. Dorigatti I, Cauchemez S, Ferguson NM. Increased transmissibility explains the third wave of infection by the 2009 H1N1 pandemic virus in England. *Proc Natl Acad Sci USA* 2013;110:13422–13427.
20. Goldstein E, Cobey S, Takahashi S, Miller JC, Lipsitch M. Predicting the epidemic sizes of influenza A/H1N1, A/H3N2, and B: a statistical method. *PLoS Med* 2011;8:e1001051.
21. Wagar LE, Rosella L, Crowcroft N, Lowcock B, Drohomysky PC, Foisy J, Gubbay J, Rebbapragada A, Winter AL, Achonu C, et al. Humoral and cell-mediated immunity to pandemic H1N1 influenza in a Canadian cohort one year post-pandemic: implications for vaccination. *PLoS ONE* 2011;6:e28063.
22. Nayak JL, Fitzgerald TF, Richards KA, Yang H, Treanor JJ, Sant AJ. CD4+ T-cell expansion predicts neutralizing antibody responses to monovalent, inactivated 2009 pandemic influenza A(H1N1) virus subtype H1N1 vaccine. *J Infect Dis* 2013;207:297–305.
23. McKinstry KK, Strutt TM, Kuang Y, Brown DM, Sell S, Dutton RW, Swain SL. Memory CD4+ T cells protect against influenza through multiple synergizing mechanisms. *J Clin Invest* 2012;122:2847–2856.
24. Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, Lambkin-Williams R, Gilbert A, Oxford J, Nicholas B, et al. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nat Med* 2012;18:274–280.
25. He XS, Holmes TH, Sasaki S, Jaimes MC, Kemble GW, Dekker CL, Arvin AM, Greenberg HB. Baseline levels of influenza-specific CD4 memory T-cells affect T-cell responses to influenza vaccines. *PLoS ONE* 2008;3:e2574.
26. Horowitz A, Hafalla JC, King E, Lusingu J, Dekker D, Leach A, Moris P, Cohen J, Vekemans J, Villafana T, et al. Antigen-specific IL-2 secretion correlates with NK cell responses after immunization of Tanzanian children with the RTS,S/AS01 malaria vaccine. *J Immunol* 2012;188:5054–5062.
27. Aoki FY, Hayden FG, Dolin R. Antiviral drugs (other than antiretrovirals). In: Mandell GL, Bennett JE, Dolin R, editors. *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*, 6th ed. Philadelphia: Churchill Livingstone/Elsevier; 2009. p. 541.
28. Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med* 2013; 5:176ra32.
29. Spensieri F, Borgogni E, Zedda L, Bardelli M, Buricchi F, Volpini G, Fragapane E, Tavarini S, Finco O, Rappuoli R, et al. Human circulating influenza-CD4+ ICOS1+IL-21+ T cells expand after vaccination, exert helper function, and predict antibody responses. *Proc Natl Acad Sci USA* 2013;110: 14330–14335.
30. León B, Bradley JE, Lund FE, Randall TD, Ballesteros-Tato A. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun* 2014;5:3495.
31. Ballesteros-Tato A, León B, Graf BA, Moquin A, Adams PS, Lund FE, Randall TD. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* 2012;36: 847–856.
32. Horby P, Mai Q, Fox A, Thai PQ, Thi Thu Yen N, Thanh T, Le Khanh Hang N, Duong TN, Thoang DD, Farrar J, et al. The epidemiology of inter-pandemic and pandemic influenza in Vietnam, 2007–2010: the Ha Nam household cohort study I. *Am J Epidemiol* 2012;175: 1062–1074.