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# Infection with 2009 H1N1 Influenza Virus Primes for Immunological Memory in Human Nose-Associated Lymphoid Tissue, Offering Cross-Reactive Immunity to H1N1 and Avian H5N1 Viruses

Waleed H. Mahallawi,<sup>a</sup> Anand V. Kasbekar,<sup>b</sup> Maxwell S. McCormick,<sup>c</sup> Katja Hoschler,<sup>d</sup> Nigel Temperton,<sup>e</sup> Samuel C. Leong,<sup>c</sup> Helen Beer,<sup>c</sup> Francesca Ferrara,<sup>e</sup> Paul S. McNamara,<sup>f</sup> Qibo Zhang<sup>a</sup>

Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom<sup>a</sup>; ENT Department, Alder Hey Children's Hospital, Liverpool, United Kingdom<sup>b</sup>; ENT Department, Royal Liverpool University Hospital, Liverpool, United Kingdom<sup>c</sup>; Respiratory Virus Unit, Health Protection Agency, London, United Kingdom<sup>d</sup>; Viral Pseudotype Unit, School of Pharmacy, University of Kent, Kent, United Kingdom<sup>e</sup>; Institute of Child Health, University of Liverpool, Liverpool, United Kingdom<sup>f</sup>

**Influenza is a highly contagious mucosal infection in the respiratory tract. The 2009 pandemic H1N1 (pH1N1) influenza virus infection resulted in substantial morbidity and mortality in humans. Little is known on whether immunological memory develops following pH1N1 infection and whether it provides protection against other virus subtypes. An enzyme-linked immunosorbent spot assay was used to analyze hemagglutinin (HA)-specific memory B cell responses after virus antigen stimulation in nose-associated lymphoid tissues (NALT) from children and adults. Individuals with serological evidence of previous exposure to pH1N1 showed significant cross-reactive HA-specific memory B cell responses to pH1N1, seasonal H1N1 (sH1N1), and avian H5N1 (aH5N1) viruses upon pH1N1 virus stimulation. pH1N1 virus antigen elicited stronger cross-reactive memory B cell responses than sH1N1 virus. Intriguingly, aH5N1 virus also activated cross-reactive memory responses to sH1N1 and pH1N1 HAs in those who had previous pH1N1 exposure, and that correlated well with the memory response stimulated by pH1N1 virus antigen. These memory B cell responses resulted in cross-reactive neutralizing antibodies against sH1N1, 1918 H1N1, and aH5N1 viruses. The 2009 pH1N1 infection appeared to have primed human host with B cell memory in NALT that offers cross-protective mucosal immunity to not only H1N1 but also aH5N1 viruses. These findings may have important implications for future vaccination strategies against influenza. It will be important to induce and/or enhance such cross-protective mucosal memory B cells.**

Influenza is a highly contagious and acute respiratory infection caused by influenza virus in the mucosa of the respiratory tract (1). Both seasonal and pandemic influenza virus infections continue to cause substantial morbidity and mortality in humans. The 2009 pandemic H1N1 (pH1N1) influenza virus and the potential of a highly pathogenic pandemic avian H5N1 (aH5N1) influenza virus highlighted the need for effective preventative strategies. Understanding the development of natural immunity following the pH1N1 pandemic may provide important information on host protective immunity in humans, which could inform future vaccination strategies against influenza.

The pH1N1 virus was antigenically different from seasonal H1N1 (sH1N1) viruses and affected large population groups who were immunologically naïve to the virus (2–4). Little is known on the development of immunological memory following the pH1N1 virus infection, how it interacts with other influenza viruses, and whether this memory provides any protective immunity to aH5N1 virus, a pathogen with considerable potential to cause a future pandemic.

Surface hemagglutinin (HA) is a major virulence factor crucial for virus binding to host cell membrane and essential in the induction of host protective immunity. HA-specific antibodies play a key role in protection against influenza (5, 6). During the 2009 pH1N1 pandemic, older people (>65 years) were protected because they had existing anti-HA antibodies induced by previous exposure to antigenically related H1N1 strains, e.g., pandemic A/H1N1 1918 virus or strains circulating before 1957 (4, 7, 8). Structurally, HA consists of two domains: a globular head, com-

posed of part of HA1, and a stalk structure, composed of portions of HA1 and all of HA2 (9). The globular head contains the variable region of HA and is the major target for neutralizing antibodies that inhibit virus binding to target cells. These neutralizing antibodies are traditionally detected by hemagglutination inhibition assay (HAI). The stalk domain is more conserved. Recent studies have suggested that antibodies targeting the stalk region may also have neutralizing activity and may contribute to the cross-reactive immunity to different influenza viruses induced by either infection or vaccination (10–13). There are 16 different influenza virus subtypes of HA, and they are clustered into two groups based on the molecular relatedness of the HA sequences, group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16) and group 2 (H3, H4, H7, H10, H14, and H15) (14).

Influenza virus is transmitted through airborne droplets and infects human nasopharyngeal mucosa. Human adenoids and tonsils are major components of nose-associated lymphoid tissues (NALT) which are considered to be an important part of the mucosal immune system (15–17). However, studies have shown there

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Address correspondence to Qibo Zhang, qibo.zhang@liv.ac.uk.

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are some major differences between human NALT in the nasopharynx and other mucosal compartments such as Peyer's patches in the intestine. B cells in the former predominantly produce IgG, whereas the majority of B cells in the latter produce IgA (18, 19). We demonstrated previously that pneumococcal protein antigens elicited a predominantly IgG memory B cell response in human NALT presumably primed by previous colonization (20, 21). The NALTs are considered to be important induction sites for both mucosal and systemic immunity to upper respiratory pathogens, including influenza virus (16, 22–24). The induction of immunological memory against influenza virus most likely involves these immunocompetent NALTs, where antigen-specific memory B cells are primed. However, limited data exist on the development and function of such memory B cells in humans. Recent studies using monoclonal antibodies from B cells isolated from patients infected with either the 1918 or 2009 pandemic H1N1 viruses suggest the presence of memory B cells (25–27). It was also reported that some HA-specific monoclonal antibodies isolated from these patients were cross-reactive with the stalk regions of HAs of a number of different influenza virus strains (13, 28).

In this study, we investigated the HA-specific memory B cell responses in human NALT to pH1N1, sH1N1, sH3N2, and aH5N1 viruses. We demonstrated that patients who had serological evidence of previous exposure to pH1N1 virus showed memory B cell response in NALTs that produce cross-reactive neutralizing antibodies against a number of influenza virus subtypes upon pH1N1 virus antigen stimulation. The result suggests that the 2009 pH1N1 infection primed human host with cross-reactive mucosal memory responses to other H1N1 and the highly pathogenic aH5N1 virus strains. These findings may have important implications in future vaccination strategies against influenza.

(This study was presented in part at the European Conference of Immunology, 5 to 8 September 2012, Glasgow, United Kingdom [abstract P-0445] [29]).

## MATERIALS AND METHODS

**Patients and samples.** Adenoids and tonsils were obtained from children and adults (3 to 30 years of age) undergoing adenoidectomy and/or tonsillectomy between March 2011 and March 2012. A venous blood sample was obtained. Patients who were previously vaccinated against influenza or who were immunocompromised in any way were excluded. The study was approved by the local ethics committee (Liverpool Pediatric Research Ethics Committee) and written, informed consent obtained from each patient/parent as appropriate.

**Influenza virus antigens.** Influenza virus antigens for cell stimulation experiments were  $\beta$ -propiolactone-inactivated, partially purified whole-virus antigens from the National Institute for Biological Standards and Control (NIBSC, United Kingdom) and were used following a standard procedure as described previously (30). The pH1N1, sH1N1, sH3N2, and aH5N1 virus antigens were derived from A/California/04/2009, A/Brisbane/59/2007, A/Brisbane/10/2007, and A/Vietnam/1203/2004 virus strains, respectively.

**Recombinant HA.** Purified recombinant HA proteins of pH1N1 (A/California/04/2009), sH1N1 (A/Brisbane/59/2007), sH3N2 (A/Brisbane/10/2007), aH5N1 (A/Vietnam/1203/2004), H2N2 (A/Singapore/1/57), and H7N3 (A/Canada/RV444/04) virus were from the Biodefense and Emerging Infections Research Resources Repository, ATCC (Manassas, VA). The recombinant HAs of pH1N1 and sH1N1 contain a C-terminal histidine tag and were produced in High Five insect cells using a baculovirus expression vector system (31). The HAs were purified from cell culture supernatant by immobilized-metal affinity chromatography (IMAC) and contain a trimerizing (foldon) domain (31). The recombi-

nant HAs of sH3N2, aH5N1, H2N2, and H7N3 viruses were full-length glycosylated HAs that were produced in Sf9 insect cells using a baculovirus expression vector system, membrane extracted from infected cells, and purified by affinity chromatography under native conditions that preserved their biological activity and tertiary structure. The purified HA forms trimers (32).

**Cell separation.** Adenoidal and tonsillar tissues were transported to the laboratory in Hanks buffered salt solution supplemented with glutamine and antibiotics (penicillin, 100 U/ml; streptomycin, 100  $\mu$ g/ml). Mononuclear cells (MNC) from adenoids and tonsils were isolated using Ficoll density centrifugation following methods described previously (20, 33). In some experiments, memory T cells (CD45RO<sup>+</sup>) or memory B cells (CD27<sup>+</sup>) were depleted from adenotonsillar MNC using magnetic-activated cell sorting (MACS) with magnetic microbeads (Miltinyi) before cell stimulation (20, 33).

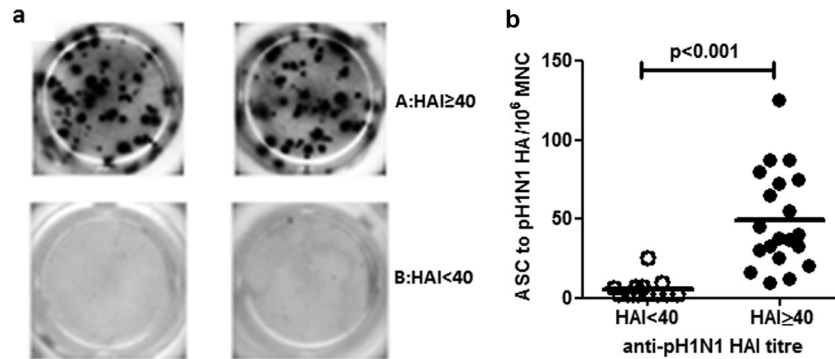
**Cell culture and stimulation by influenza virus antigens.** Adenotonsillar MNC were cultured at  $4 \times 10^6$ /ml in RPMI medium containing glutamine, penicillin, streptomycin, and 10% fetal bovine serum (FBS), with and without a predetermined optimal concentration of influenza virus antigens. For enumerating antibody-secreting cells (ASC) by enzyme-linked immunospot (ELISpot) assay, adenotonsillar MNC were cultured for 5 days before being transferred to ELISpot plates. Cell culture supernatants were collected at day 7 and stored at  $-70^\circ\text{C}$  until assay for measuring antibodies by enzyme-linked immunosorbent assay (ELISA).

Paired experiments in adenoidal and tonsillar MNC revealed no difference in memory B cell responses activated by influenza virus antigens (data not shown). Therefore, data derived from tonsillar MNC only are presented in this paper.

**Measurement of memory B cell response by ELISpot assay.** HA-specific memory B cell responses following individual virus antigen stimulations were analyzed using an ELISpot assay to enumerate HA-specific ASC as described previously (34). Briefly, ELISpot plates (Millipore, United Kingdom) were coated overnight with optimized concentrations of recombinant HAs in phosphate-buffered saline (PBS). Plates were washed and blocked by incubation with RPMI medium containing 10% FBS at  $37^\circ\text{C}$  for 2 h. Antigen-stimulated MNC were added to the plates and incubated overnight at  $37^\circ\text{C}$ . Plates were washed and incubated with biotinylated anti-human IgG/IgA antibody (Invitrogen, United Kingdom) for 30 min at room temperature. After washing, avidin D-horseradish peroxidase (HRP) conjugate (Vector Laboratories) was added and the mixture was incubated. Colored spots were developed with the addition of substrate (3-amino-9-ethylcarbazole; Sigma) and counted using an automated ELISpot reader (AID; Autoimmune Diagnostika GmbH, Germany). The ELISpot assay shows the predominance of HA-specific IgG memory B cell responses following stimulation by influenza virus antigens, so only IgG ASC results are shown.

**Measurement of HA-specific antibodies by ELISA.** HA-specific IgG antibodies were analyzed following a standard ELISA procedure as previously described (35). In brief, ELISA plates were coated with recombinant HAs and incubated overnight at  $4^\circ\text{C}$ . After washing, plates were blocked with 10% FBS followed by incubation of cell culture supernatants at predetermined optimized dilutions for 1.5 h. Alkaline phosphatase-conjugated anti-human IgG (Sigma) was then incubated for 1.5 h. After washing, p-nitrophenyl phosphate substrate was applied. Optical density was measured at 405 nm, and data were analyzed using DeltaSoft microplate analysis software (BioMetallics Inc.). Sandoglobulin (Sandoz, United Kingdom), which contains high titers of antibodies to sH1N1 and sH3N2 HA, was used as a reference standard for measurement of antibodies to sH1N1 and sH3N2. A human convalescent-phase serum sample from a subject with confirmed pH1N1 infection (BEI Resources, ATCC) was used as a standard for measurement of anti-pH1N1 HA antibodies. Both reference standards were arbitrarily assigned an antibody titer of 5,000 U/ml.

**HAI assay.** Hemagglutination inhibition (HAI) assays were performed following standard methods (8) at the Microbiology Services—



**FIG 1** pH1N1 virus antigen induces a strong HA-specific memory B cell response. (a) Panels A and B show representative samples from patients with HAI  $\geq 40$  and HAI  $< 40$ , respectively. (b) Numbers of HA-specific IgG ASC in tonsillar MNC enumerated by ELISpot assay after stimulation by pH1N1 virus antigen in subjects with serum anti-pH1N1 HAI titer  $\geq 40$  ( $n = 20$ ) and low HAI titers ( $< 40$ ,  $n = 14$ ). Horizontal lines represent the means of the numbers of HA-specific ASC ( $P < 0.001$  compared with those with HAI  $< 40$ ).

Colindale, Health Protection Agency (London, United Kingdom). The virus strains used included the following. For the pandemic H1N1 virus, NIBRG122 virus is a reassortant prepared from A/England/195/2009 (H1N1v), the prototype United Kingdom isolate antigenically and genetically closely related to A/California/4/2009; for the seasonal H1N1 virus, the A/H1N1/Brisbane/59/2007 strain was used; for the seasonal H3N2 virus, the A/H3N2/Brisbane/10/2007 strain was used; and for the avian H5N1 virus, NIBRG-14 virus, a reassortant prepared from A/H5N1/Vietnam/1194/2004 virus, was used.

#### Influenza pseudotype virus production and neutralization assay.

The construction of lentiviral pseudotypes with an HA envelope glycoprotein derived from the highly pathogenic H5N1 avian influenza virus (A/Viet Nam/1194/04) has been described previously (36). H1N1 HA-expressing plasmids were constructed for A/Brisbane/59/2007 (H1N1) and A/South Carolina/1/18 (H1N1) virus using analogous methodologies. Pseudotype viruses were produced by cotransfection of HEK293T/17 cells with the respective HA plasmids, the HIV gag-pol plasmid p8.91, and the reporter plasmid pCSFLW (expressing firefly luciferase) using Fugene-6 transfection reagent (Roche, United Kingdom). For the production of the H1N1 pseudotypes, protease-expressing plasmid was also added to the transfection mixture as described previously (11). The HA content was normalized via a surrogate readout of the firefly relative light units (RLU)/ml for each virus. For the virus neutralization assays, cell culture supernatant samples were 2-fold serially diluted in culture medium and mixed with each pseudotype virus ( $1 \times 10^6$  RLU firefly luciferase input) at a 1:1 (vol/vol) ratio. After incubation at 37°C for 1 h,  $1 \times 10^4$  HEK293T cells were added to each well of a white 96-well flat-bottomed tissue culture plate. Firefly RLU values were determined 72 h later by luminometry using a Bright-Glo assay system (Promega, United Kingdom).

**Statistical analysis.** Differences in memory response or antibody titers between different groups were analyzed by analysis of variance and Student's *t* test. Association between two factors was analyzed by Pearson's correlation. A *P* value of  $< 0.05$  was considered statistically significant. Statistical analysis was performed using SPSS software (version 16).

## RESULTS

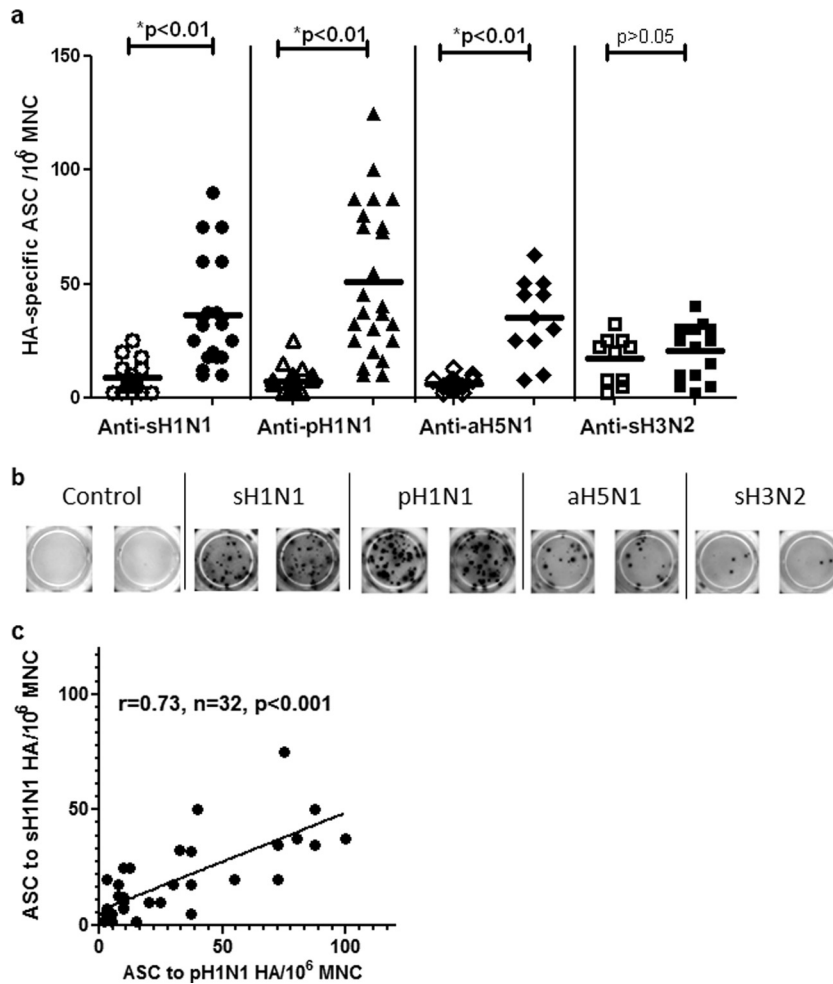
**The 2009 pH1N1 virus induces memory B cell responses that cross-react with sH1N1 and aH5N1 viruses.** To analyze pH1N1 HA-specific memory B cell responses in tonsillar MNC, an ELISpot assay was performed to enumerate numbers of HA antigen-specific ASC after stimulation with pH1N1 virus antigen. Large numbers (mean ASC/ $10^6$  MNC, 50.0) of HA-specific IgG ASC responding to pH1N1 virus were found after pH1N1 virus antigen stimulation in subjects with a serum anti-pH1N1 HAI

titer  $\geq 40$ , whereas minimal numbers (5.5) of ASC were seen in those with low ( $< 40$ ) HAI titers (Fig. 1;  $P < 0.01$ ).

In contrast, when the subjects were divided into two groups with serum HAI titers  $< 40$  and  $\geq 40$  against either sH1N1 or sH3N2 viruses, there was no difference between the two groups in the numbers of pH1N1 HA-specific IgG ASC after pH1N1 antigen stimulation ( $P > 0.05$ ; data not shown).

To determine whether this pH1N1 HA-specific memory B cell response cross-reacted with other influenza A virus subtypes, memory B cell responses to sH1N1, sH3N2, and aH5N1 HAs were also analyzed following tonsillar MNC stimulation with pH1N1 virus antigen. Numbers of HA-specific IgG ASC responding to sH1N1 and aH5N1 virus after the antigen stimulation in subjects with serum anti-pH1N1 HAI titer  $\geq 40$  were significantly higher than in those who had anti-pH1N1 HAI titer  $< 40$  (36.2 versus 8.7 and 35.0 versus 6.1 for anti-sH1N1 and -aH5N1 ASC, respectively) (Fig. 2a and b;  $P < 0.01$ ). Further analysis revealed a good correlation ( $r = 0.73$ ,  $P < 0.001$ ) between the number of HA-specific ASC responding to pH1N1 and that responding to sH1N1 virus (Fig. 2c). However, no difference between subjects with anti-pH1N1 HAI titer  $\geq 40$  and those with HAI titer  $< 40$  was found in the numbers of specific IgG ASC responding to sH3N2 HA after pH1N1 virus antigen stimulation (Fig. 2b).

**pH1N1 virus elicits stronger cross-reactive memory B cell responses than sH1N1 and sH3N2 virus antigens.** To compare pandemic and seasonal influenza A virus-induced memory B cell responses and their cross-reactivities, HA-specific memory B cell responses in tonsillar MNC following stimulation with sH1N1 and sH3N2 virus antigens were analyzed. Stimulation with the sH1N1 virus antigen (A/Brisbane/59/2007) elicited a modest increase in the number of HA-specific ASC responding to sH1N1 (mean ASC/ $10^6$  MNC, 24.8) and pH1N1 (26.5), but no increase was seen in the number of specific ASC responding to sH3N2 and aH5N1 HAs (Fig. 3a) either in patients with anti-sH1N1 HAI  $\geq 40$  or in those with anti-sH1N1 HAI  $< 40$  (data not shown). Stimulations with the A/New Caledonia/20/99 H1N1 virus antigen resulted in similar numbers of HA-specific ASC responding to sH1N1 (mean, 22.8) and pH1N1 (21.0). This was in contrast to the stronger cross-reactive memory B cell responses elicited by pH1N1 antigen stimulation not only in response to pH1N1 (50.8) but also in response to sH1N1 (38.0) and aH5N1 (32.7) viruses in patients



**FIG 2** pH1N1 virus antigen elicits memory B cell responses that cross-react with sH1N1 and avian H5N1 viruses. (a) The magnitudes of HA-specific IgG memory B cell responses to sH1N1 (○), pH1N1 (△), aH5N1 (◇), and sH3N2 (□) in tonsillar MNC were analyzed after pH1N1 virus antigen stimulation and compared between subjects with serum anti-pH1N1 HAI titer ≥ 40 (filled symbols) and those with HAI < 40 (open symbols) (\*, *P* < 0.01). (b) ELISpot images of HA-specific ASC responding to sH1N1, pH1N1, aH5N1, and sH3N2 in tonsillar MNC from one representative patient after pH1N1 antigen stimulation. Control, negative control with no specific HA antigen coating in ELISpot assay. (c) There was a good correlation between the numbers of HA-specific ASC responding to pH1N1 and that responding to sH1N1 after pH1N1 antigen stimulation (*r* = 0.73, *P* < 0.001).

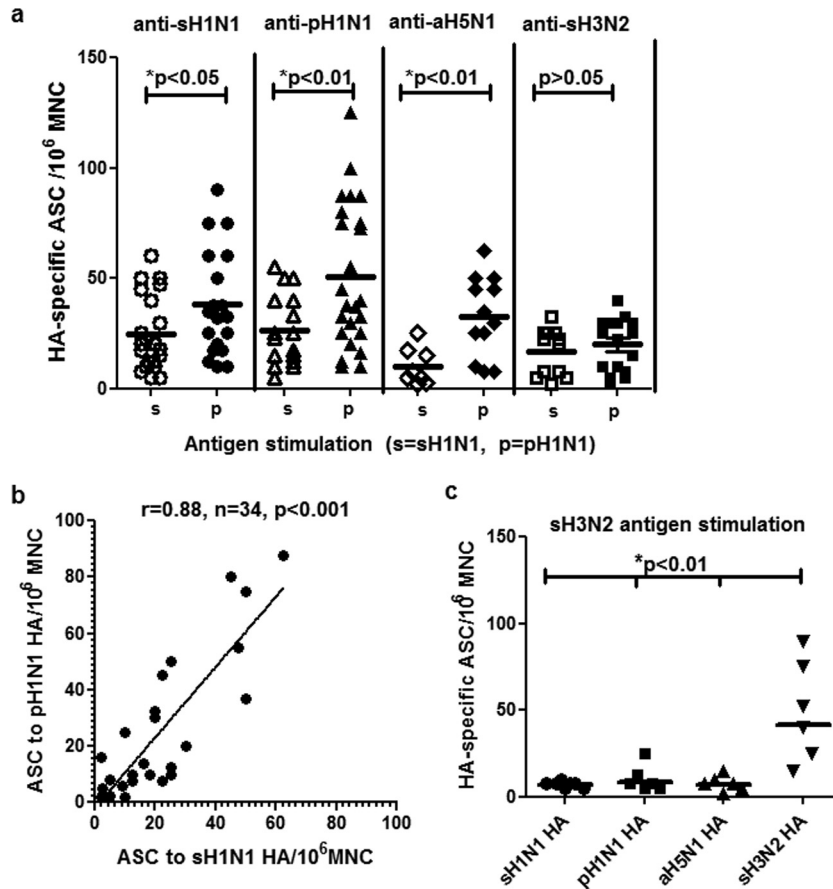
with anti-pH1N1 HAI titer ≥ 40 (Fig. 3a). Nevertheless, there was a positive correlation between the pH1N1 HA-specific memory B cell response activated by the pH1N1 antigen and the sH1N1 HA-specific memory response elicited by the sH1N1 antigen stimulation (Fig. 3b; *r* = 0.88; *P* < 0.001). In comparison, stimulation with the sH3N2 antigen did not induce an increase in the number of HA-specific ASC responding to the sH1N1, pH1N1, and aH5N1 virus, although it did induce a strong increase in the number of ASC responding to sH3N2 HA (49.6) (Fig. 3c).

**Avian H5N1 virus antigen elicits a cross-reactive memory B cell response similar to that seen with pH1N1 antigen.** We reasoned that if pH1N1 infection in patients induced memory B cells cross-reactive to aH5N1, these cells should mount a memory response upon an antigenic challenge by aH5N1 virus. Tonsillar MNC were stimulated with aH5N1 virus antigen followed by analysis of HA-specific ASC. Indeed, this stimulation elicited memory B cell responses, with mean numbers of IgG ASC responding to sH1N1 (20.8) and pH1N1 virus (50.8) HAs similar to the numbers induced by pH1N1 antigen in patients who had an anti-pH1N1

HAI titer ≥ 40 (Fig. 4a). A moderate response to aH5N1 (14.6) but not sH3N2 virus HA was also observed in these patients (Fig. 4a). Figure 4b shows that the numbers of pH1N1 HA-specific ASC elicited by aH5N1 antigen stimulation correlated well with that elicited by pH1N1 antigen stimulation (*r* = 0.85; *P* < 0.01). No significant ASC response to HA of any of the four viruses was found after aH5N1 antigen stimulation in subjects with an anti-pH1N1 HAI titer < 40.

Further analysis using memory T cell (CD45RO<sup>+</sup>)- or memory B cell (CD27<sup>+</sup>)-depleted tonsillar MNC failed to show any significant numbers of HA-specific ASC by ELISpot assay after each virus antigen stimulation (data not shown). This suggests that the HA-specific ASCs detected were derived from memory rather than naive B cells in tonsillar MNC.

**pH1N1 virus activates memory B cell responses that produce cross-reactive neutralizing antibodies.** As expected, there was a good correlation between the numbers of pH1N1 HA-specific IgG ASC in tonsillar MNC and anti-HA IgG antibody titers in cell culture supernatants after pH1N1 antigen stimulation (Fig. 5a;



**FIG 3** pH1N1 virus elicits a stronger cross-reactive memory B cell response than seasonal H1N1 virus antigen. (a) HA-specific IgG memory B cell responses to sH1N1 (○), pH1N1 (△), aH5N1 (◇), and sH3N2 (□) in tonsillar MNC were analyzed, and sH1N1 antigen (open symbols)- and pH1N1 antigen (filled symbols)-induced responses in subjects with serum anti-pH1N1 HAI titer  $\geq 40$  were compared. (b) A good correlation was shown between HA-specific memory B cell responses to pH1N1 and sH1N1 virus induced by pH1N1 and sH1N1 antigen stimulation, respectively. (c) sH3N2 virus antigen stimulation induced HA-specific memory B cell response to H3N2 but not H1N1 and H5N1 viruses ( $n = 6$ ). Horizontal bars represent the mean numbers of HA-specific ASC.

$r = 0.78$ ;  $P < 0.001$ ). To determine whether pH1N1 virus antigen-activated memory B cells produce cross-reactive neutralizing antibodies, cell culture supernatants were analyzed for virus-neutralizing activity. In subjects from whom a memory B cell response to pH1N1 HA was detected, high levels of neutralizing antibodies against sH1N1 (A/Brisbane/59/2007) and 1918 H1N1 (A/South Carolina/1/18) pseudotype viruses were detected after stimulation with both pH1N1 and aH5N1 virus antigens, but only a low level of the neutralizing activity was induced by sH1N1 virus antigen (Fig. 5b;  $P < 0.001$ ). Similarly, neutralizing activity against the aH5N1 pseudotype virus was also detected in cell culture supernatants after stimulation by pH1N1 or aH5N1 virus antigen (Fig. 5c;  $P < 0.001$ ), whereas no neutralizing activity against aH5N1 virus was detected in cell culture supernatants after stimulation by sH1N1 virus antigen (Fig. 5c). No neutralizing activity against sH1N1, 1918 H1N1, and aH5N1 viruses was detectable in subjects in whom no memory B cell response to pH1N1 HA was detected (data not shown).

## DISCUSSION

The 2009 pH1N1 virus caused a global pandemic in 2009 which infected an estimated 11% to 21% of the world population and resulted in considerable morbidity and mortality (37). It remains unclear

whether the pH1N1 virus infection induced mucosal B cell memory in the infected population and whether this memory provides cross-protective immunity to different types of influenza viruses.

In this study, we showed a significant HA-specific memory B cell response to pH1N1 virus in tonsillar cells from individuals with serological evidence of prior exposure to pH1N1 virus (serum HAI  $\geq 40$ ), whereas no such memory response was found in those with serum HAI  $< 40$ . We also showed that stimulation with pH1N1 virus antigen activated an IgG memory B cell response, with production of HA-specific antibodies against not only pH1N1 but also sH1N1 and aH5N1 viruses. In addition, abundant anti-H2N2 HA IgG antibody production was also elicited in tonsillar cell culture supernatants after pH1N1 antigen stimulation in these subjects (with a mean titer [U/ml] of 4.5 compared to 0.8 in those with HAI  $< 40$ ). This suggests that 2009 pH1N1 infection primed or activated cross-reactive memory B cells in human NALT in response to HAs of different influenza viruses. There was a good correlation between the numbers of HA-specific ASC responding to pH1N1 and that responding to sH1N1, as shown after stimulation with pH1N1 and sH1N1 antigens, respectively (Fig. 3b). This suggests that these NALT memory B cells were likely to be primed by the same antigenic epitopes derived from both pH1N1 and sH1N1 viruses.

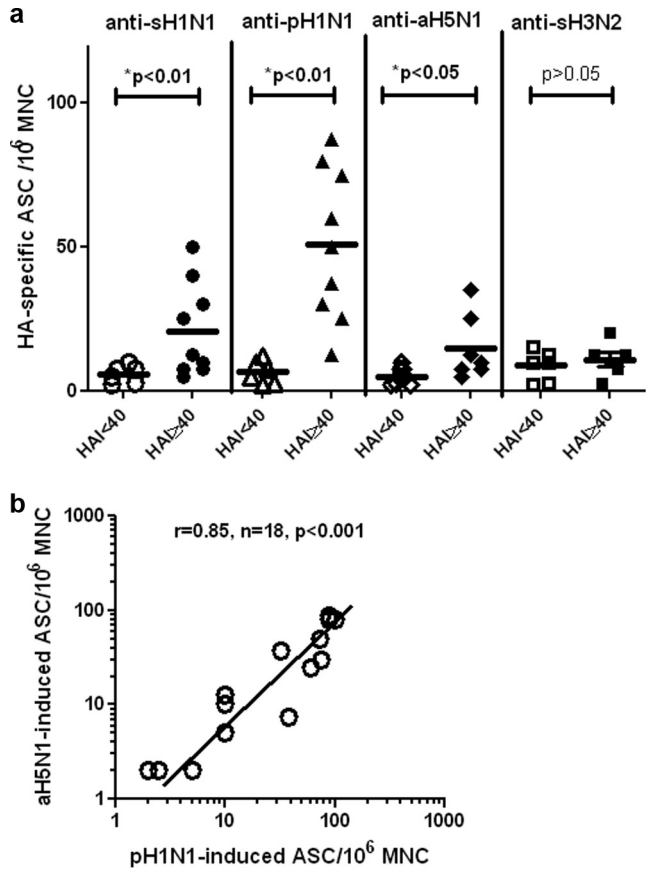


FIG 4 Avian H5N1 virus antigen elicits cross-reactive memory B cell responses. (a) HA-specific IgG memory B cell responses in tonsillar MNC to HAs of sH1N1 (○), pH1N1 (△), aH5N1 (◇), and sH3N2 (□) viruses after stimulation with avian H5N1 virus antigen were analyzed and compared between subjects with serum anti-pH1N1 HAI titer  $\geq 40$  (filled symbols,  $n = 9$ ) and those with HAI < 40 (open symbols) ( $n = 9$ ). (b) There was a good correlation between numbers of anti-pH1N1 HA-specific ASC induced by pH1N1 and that induced by avian H5N1 antigens ( $r = 0.85$ ,  $P < 0.001$ ).

The finding that the pH1N1 virus antigen-activated memory B cell response was cross-reactive to sH1N1 and aH5N1 HAs, but not sH3N2 HA (Fig. 2a), is consistent with previous studies evaluating the cross-reactivity of serum antibodies in patients infected with pH1N1 virus (12). This is likely due to the structural similarities between the group 1 HAs, including H1, H2, and H5 subtypes, which are phylogenetically different from group 2 HAs, including the H3 and H7 subtypes. Indeed, no detectable levels of anti-H7N3 HA IgG antibodies were observed in the tonsillar cell culture supernatants after stimulation with pH1N1, and we found no significant production of the antibody following stimulation by sH3N2 virus antigen in this study.

This is the first report to demonstrate a significant memory B cell response to pH1N1 virus in human NALT 1 to 2 years after the 2009 pH1N1 pandemic. Upon pH1N1 antigen stimulation, the memory B cell response produces cross-reactive antibodies against HAs of a number of different influenza virus strains. These results are consistent with the presence of plasmablasts secreting cross-reactive neutralizing antibodies in patients infected with pH1N1 (11, 25, 26, 28, 38–40) and are in agreement with the hypothesis that pH1N1 infection may acti-

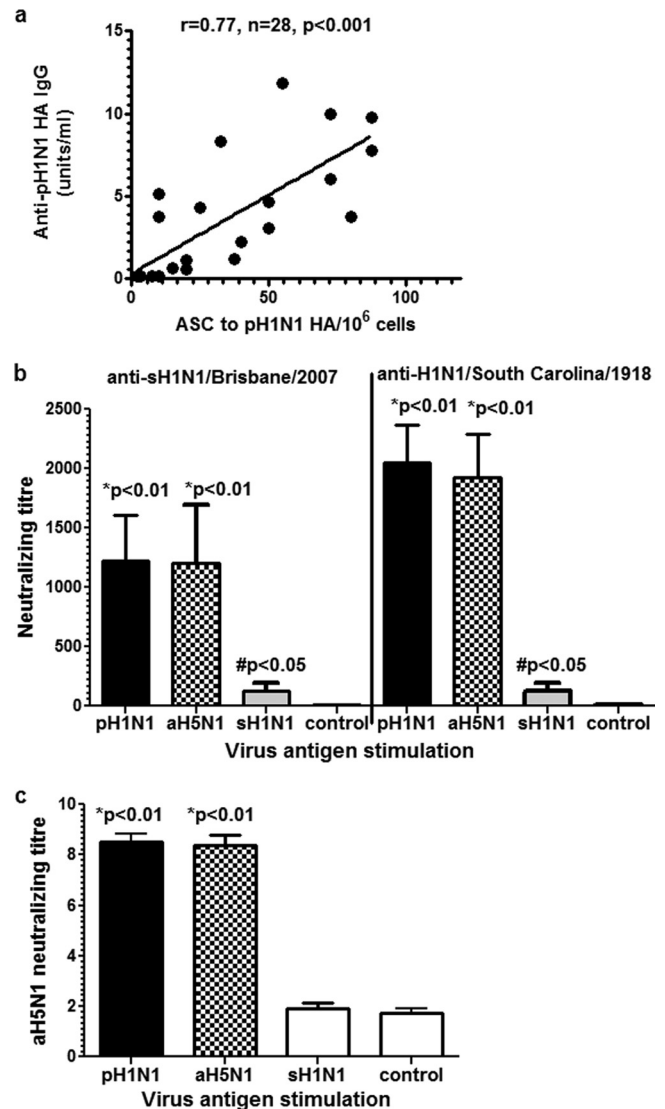


FIG 5 pH1N1 virus antigen activates a memory B cell response that produces cross-reactive neutralizing antibodies. (a) Correlation between numbers of HA-specific IgG ASC after stimulation by pH1N1 virus antigen and anti-pH1N1 HA IgG antibody titers in tonsillar cell culture supernatants ( $r = 0.77$ ,  $P < 0.001$ ). (b and c) Virus neutralization activities against sH1N1 and 1918 H1N1 (b) and aH5N1 (c) pseudotype viruses in tonsillar cell culture supernatants after cell stimulation by pH1N1, aH5N1, and sH1N1 virus antigens, respectively (\*,  $P < 0.01$  compared with sH1N1 stimulation; #,  $P < 0.05$  compared with unstimulated control;  $n = 6$ ).

vate pre-existing memory B cells targeting conserved regions of HA molecule (12, 13).

It could be argued that if previous infection with seasonal viruses (e.g., sH1N1) had induced cross-reactive B cell memory through repeated exposure, then most individuals would have had developed immunity to pH1N1 virus before the pandemic. We show here that there is a significant difference between pH1N1 and sH1N1 virus antigens in the capacity to activate cross-reactive memory B cell responses and to produce neutralizing antibodies. The former (pH1N1) virus activated a cross-reactive memory response and neutralizing antibodies, whereas the latter (sH1N1) virus elicited only a moderate

memory response and a low level of cross-reactive neutralizing antibodies (Fig. 3a and 5b). This relative inability of sH1N1 virus antigen to activate memory B cells to produce cross-reactive neutralizing antibodies may help explain the failure of previous sH1N1 infections to induce immunological protection against the pH1N1 infection (41). The reasons for the difference between pH1N1 and sH1N1 viruses in the ability to activate cross-reactive memory B cells are not clear. It is likely due to the difference in the host immunogenicity of the two viruses, including innate immunity. Recent studies showed that cross-reactive anti-HA stalk antibodies were boosted following both 2009 pH1N1 infection and the pH1N1 influenza virus vaccination in humans (13, 42). It has been postulated that cross-reactive memory B cells specific for conserved regions of the HA stalk of sH1N1 virus were selectively boosted by pH1N1, whereas repeated seasonal H1N1 infection tended to stimulate memory B cells that target the head of HA which were less cross-reactive (12).

The cross-reactive memory response to aH5N1 HA in individuals with previous exposure to pH1N1 virus is of particular interest and may have important implications given that aH5N1 is a highly pathogenic virus and a potential cause of future influenza pandemics. It remains to be seen whether this cross-reactive memory induced by natural infection alone offers any protection against aH5N1 virus infection, as the neutralization activity of the memory B cell response to aH5N1 virus appears to be modest compared to its high neutralizing activity against H1N1 strains. However, it is plausible to enhance such cross-reactive B cell memory by vaccination through, e.g., intranasal mucosal immunization to boost this natural immunity. The ability of pH1N1 virus antigen to elicit a strong HA-specific memory B cell response and cross-reactive neutralizing antibodies suggests that it may be possible to utilize pH1N1 HA or conserved HA regions in an influenza vaccine to induce cross-reactive immunity to influenza viruses, including aH5N1.

Considering that none of the subjects in this study had been exposed to aH5N1 virus, it is intriguing that aH5N1 virus antigen could induce a memory B cell response to pH1N1 and sH1N1 virus HAs (Fig. 4). The finding that this memory response was detected only in those who had previous pH1N1 exposure suggests that pH1N1 infection primed the host for cross-reactive memory for different virus strains, including aH5N1. It was reported previously that serum antibodies in an aH5N1 virus-infected patient bind to a variety of conserved peptides in the stem region of HA (43), so it is possible that there are cross-reactive epitopes in the HAs of pH1N1 and aH5N1 viruses.

The pH1N1 virus caused an influenza pandemic which spread rapidly worldwide in 2009. The predominant virus circulating in the subsequent influenza season in 2010 to 2011 was pH1N1, which essentially replaced the previously circulating sH1N1 viruses (12, 44). This phenomenon is similar to that described following the previous influenza pandemics in 1957 and 1968, when circulating virus strains disappeared after the emergence of the pandemic strains (45). It has been hypothesized that the induction of cross-reactive antibodies may contribute to the disappearance of the circulating strains (12). The cross-reactive memory B cell response activated by pH1N1 virus as described in this study may contribute to the reduction of sH1N1 and help explain the low

influenza activity in the 2011 to 2012 influenza season in the United Kingdom (46).

It is generally considered that IgA antibodies are predominant at the mucosal level. However, the question of whether mucosal IgA memory can be induced in humans either through natural infection or vaccination is being debated. A number of studies have shown that antigen-specific mucosal IgA responses are short-lived and that reimmunization does not reliably induce memory-type IgA responses (47, 48). Although IgA ASC numbers were reported to increase in tonsillar cells after influenza vaccination, they were likely to represent mainly a primary rather than a memory IgA response (49). The predominance of antigen-specific IgG memory B cell responses to influenza virus HA in tonsillar tissues shown in this study is concordant with previous studies demonstrating the predominance of IgG memory B cell responses to protein antigens in human NALT (18, 19, 21).

Taking the results together, we present evidence that pH1N1 infection in humans primed the host with cross-reactive memory B cells in NALTs that can respond strongly to stimulation by both pH1N1 and aH5N1 virus antigens to produce cross-reactive neutralizing antibodies. These findings may have important implications for future vaccination strategies against influenza. It will be important to induce and/or enhance such cross-protective mucosal memory B cells. The ability of pH1N1 and aH5N1 virus antigens to stimulate cross-reactive memory B cell responses in human NALT warrants efforts to explore the conserved regions of these HA as components of future vaccines, for example, in intranasal mucosal vaccination, to induce broad immunity to influenza.

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