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F. Eun-Hyung Lee, Jessica L. Halliley, Edward E. Walsh, Andrew P. Moscatiello, Brittany L. Kmush, Ann R. Falsey, Troy D. Randall, Denise A. Kaminiski, Richard K. Miller and Iñaki Sanz

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Circulating Human Antibody-Secreting Cells during Vaccinations and Respiratory Viral Infections Are Characterized by High Specificity and Lack of Bystander Effect

F. Eun-Hyung Lee,* Jessica L. Halliley,* Edward E. Walsh,[†] Andrew P. Moscatiello,*
Brittany L. Kmush,* Ann R. Falsey,[†] Troy D. Randall,[‡] Denise A. Kaminiski,[‡]
Richard K. Miller,[§] and Iñaki Sanz[‡]

Surges of serum Abs after immunization and infection are highly specific for the offending Ag, and recent studies demonstrate that vaccines induce transient increases in circulating Ab-secreting cells (ASCs). These ASCs are highly enriched but not universally specific for the immunizing Ag, suggesting that a fraction of these ASCs could arise from polyclonal bystander stimulation of pre-existing memory cells to unrelated Ags. This model is proposed to explain maintenance of long-lived serological memory in the absence of Ag exposure. To test this model, we measure the ability of respiratory syncytial virus and influenza virus infection or immunizations to influenza virus, tetanus toxoid, hepatitis B Ag, and human papillomavirus to stimulate bystander memory cells specific for other major environmental Ags that represent a large fraction of the preexisting memory B compartment. Bystander or nonspecific ASC responses to respiratory syncytial virus and tetanus could not be detected above the background levels in healthy adults, despite the presence of circulating memory B cells specific for the corresponding Ags. Nonspecific ASC responses in the healthy subjects and cord blood samples were similar. In contrast, both vaccination and infection induce massive expansion of circulating Ag-specific ASCs without significant increases in the frequencies of ASCs against unrelated Ags. Hence, nonspecific stimulation of memory B cells is unlikely to contribute to the mechanisms of long-term serological memory against major human pathogens. Additionally, high specificity of circulating ASCs after antigenic challenge highlights the diagnostic value of interrogating ASCs as an ideal single-time-point diagnostic immune surrogate for serology during acute infection. *The Journal of Immunology*, 2011, 186: 000–000.

A long-standing immunological observation of major clinical consequence is that both vaccination and infection induce substantial increases in the levels of serum Abs specific for the inciting Ags. Recent studies, including our own (1, 2), have demonstrated that these serological surges are mediated by a dramatic expansion of Ag-specific Ab-secreting cells (ASCs) that are readily detected in the peripheral blood a few days after antigenic stimulation. Such Ag-specific ASC expansions

have been demonstrated after vaccination with each Ag tested to date including influenza, tetanus, diphtheria, and meningococcus (3–10). Of great significance for our understanding of serological memory, the antigenic specificity of early ASC expansions is consistent with the lack of increases of Abs against unrelated Ags (11–13). Our recent demonstration that circulating ASC responses precede and correlate with serological responses also supports the notion that the characteristics of early ASC responses should reflect and predict the subsequent serological responses (1).

Notably, despite the tremendous enrichment (several hundredfold) in Ag-specific ASC typically observed in early responses, a significant fraction (ranging from 10 to 80% depending on the specific Ag, timing, and individual donor) of all circulating ASCs do not appear to produce Abs against the immunizing Ag. This observation, suggestive of nonspecific bystander stimulation, has been documented both by ELISPOT assay measurements and by generation of mAbs (5, 7).

Nonetheless, the contribution of bystander, nonspecific polyclonal stimulation of memory B cells to the homeostatic maintenance of both cellular and serological memory remains controversial. Several human studies argue against a bystander model (9, 11–13), and a mouse study shows that memory cells may not be needed to maintain the plasma cell compartment (14). By contrast, the potential contribution of nonspecific polyclonal memory B cell activation to the maintenance of serological memory has been suggested by other relevant studies (5). The latter mechanism is also supported by elegant experiments showing that memory B cells in vitro efficiently differentiate into

*Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Rochester Medical Center, Rochester, NY 14642; [†]Division of Infectious Diseases, Department of Medicine, University of Rochester Medical Center, Rochester, NY 14642; [‡]Division of Allergy, Immunology and Rheumatology, Department of Medicine, University of Rochester Medical Center, Rochester, NY 14642; and [§]Department of Obstetrics and Gynecology, University of Rochester Medical Center, Rochester, NY 14642

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Address correspondence and reprint requests to Dr. F. Eun-Hyung Lee, Division of Pulmonary and Critical Care Medicine, University of Rochester Medical Center, 601 Elmwood Avenue, Box 692, Rochester, NY 14642. E-mail address: eunhyung_lee@urmc.rochester.edu

Abbreviations used in this article: ASC, Ab-secreting cell; HPV, human papillomavirus; RSV, respiratory syncytial virus; RSV F, RSV fusion protein; TIV, trivalent influenza vaccine.

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plasma cells through noncognate, BCR-independent, TLR- or IFN-mediated stimulation (5, 15–18). It should be noted, however, that in vivo studies supporting nonspecific bystander polyclonal activation have been limited in scope (5).

To address formally the question of bystander Ag specificity, we studied the ASC surge after immunization with four different vaccines to determine the relative contribution of cells specific for the corresponding Ag compared with cells specific to Ag-unrelated responses against prevalent agents representing a substantial fraction of the preexisting human memory B cell repertoire. Our results confirm the high specificity of the ASC response for the immunizing Ag and argue against universal stimulation of other memory cells in response to immunization. The vaccination results were also confirmed in more than 20 adults with respiratory viral infections likely to induce more vigorous and broader responses than vaccines. Our results bear important clinical and immunological implications that will be discussed in detail in this article.

Materials and Methods

Subjects

A total of 97 adult subjects aged 20–96 y (mean \pm SD, 43 \pm 19 y) were enrolled in this study during the period 2006–2009. Thirty-seven subjects were men, and 60 were women. In addition, 12 cord blood samples were obtained. The 97 adult subjects included 19 vaccinated healthy individuals; 28 asymptomatic healthy control subjects without current illness or significant medications; 28 additional asymptomatic healthy controls enrolled for the analysis of memory B cell frequencies; and 22 adults with confirmed respiratory syncytial virus (RSV) or influenza virus infections. All studies were approved by the University of Rochester and Rochester General Hospital Institutional Review Board.

Vaccinated subjects. We enrolled 19 subjects total. These 19 subjects, between the ages of 21 and 60 y (mean \pm SD, 38 \pm 17 y), received the following vaccines: 10 subjects received the trivalent influenza vaccine (TIV) 2008 (A/Brisbane/57/2007, A/Brisbane/10/2007, B/Florida/4/2006; Sanofi Pasteur, Swiftwater, PA) or 2009 (A/Brisbane/57/2007, A/Brisbane/10/2007, B/Brisbane) vaccination, 1 subject received hepatitis B vaccination (Merck, West Point, PA), 1 subject received human papillomavirus (HPV) vaccination (Merck), and 7 subjects received tetanus toxoid vaccination (Sanofi Pasteur). Blood was drawn at 6–7 d postvaccination. In one subject who received tetanus vaccination, blood was also obtained prior to and on days 5–9, 15, and 28 after immunization.

Subjects with respiratory infections. All subjects with respiratory viral infections were recruited from the Rochester General Hospital outpatient and inpatient facilities in Rochester, N.Y. Eleven adult subjects (ages 50–94 y) with respiratory symptoms such as fever, cough, sore throat, rhinorrhea, and dyspnea who were RSV RT-PCR positive (19) were recruited during winter 2007–2008. In addition, 11 subjects (ages 21–96 y) with influenza-like illness were recruited in the period 2007–2009. Influenza B infection and pandemic (H1N1) influenza A infection were diagnosed by RT-PCR. From all patients with respiratory infections, nasal swabs and blood were drawn during the acute illness between days 2 and 11.

Healthy control subjects. Fifty-six healthy adults aged 20–63 y (mean \pm SD, 36 \pm 11 y) without concurrent infection or recent vaccination were enrolled as control asymptomatic healthy subjects during the spring and summer months of 2007–2009. Subjects were considered immunologically “healthy” as defined by a modified Senieur clinical survey (20). Half of the controls were randomly assigned to measure specificity of ASC, whereas 28 were used to measure memory B cells to common Ags.

Cord blood samples. Cord blood was isolated from 12 fresh placentas of healthy newborn deliveries at University of Rochester Medical Center during the period 2006–2009.

RT-PCR for RSV and influenza viruses

The initial nasal swab samples were screened for the presence of RSV RNA by RT-PCR using a nonquantitative multiplex group A and B RSV-specific RT-PCR assay as previously described (21, 22). Influenza B RT-PCR was also performed on samples from selected individuals according to published methods (23), and pandemic 2009 H1N1 RT-PCR results were obtained using procedures provided by the Centers for Disease Control and Prevention (S. Lindstrom, personal communication).

ASC ELISPOT assay

The frequency of Ag-specific ASCs was measured by ELISPOT assay as previously described (24). Briefly, 96-well ELISPOT plates (MAIPS-4510 96-well) were coated overnight at 4°C in a humidified chamber with the following Ags: tetanus toxoid (1 Lf/ml; Cylex, Columbia, MD), TIV vaccine 2007 (A/Solomon Island/3/2006, A/Wisconsin/67/2005, B/Malaysia/2506), 2008 (A/Brisbane/57/2007, A/Brisbane/10/2007, B/Florida/4/2006), or 2009 (A/Brisbane, A/Brisbane, B/Brisbane) (6 μ g/ml; Sanofi Pasteur), purified HA H1:New Caledonia/06/09, H1:Solomon Islands/03/06, H1:Brabant/59/07, H1:Brabant/10/07, or H7:A/Netherlands/219/03 (3 μ g/ml; Protein Sciences, Meriden, CT), purified H1:2009 California (3 μ g/ml; Immune Technology, New York, NY), purified NP_A proteins (20 μ g/ml) (25), RSV fusion protein (RSV F) (26), recombinant surface hepatitis B Ag 10 μ g/ml (Albervon, Fargo, ND), recombinant HPV vaccine (20 μ g/ml; Gardasil; Merck), or anti-human IgG (5 μ g/ml; Jackson ImmunoResearch Laboratories, West Grove, PA). BSA 2% (MP Biomedicals, Solon, OH) in sterile PBS was used as an irrelevant Ag. Plates were incubated at 37°C for 18–20 h with serially diluted numbers of PBMCs. After incubation, wells were washed, and bound Abs were detected with alkaline phosphatase-conjugated anti-human IgG Ab (1 μ g/ml; Jackson ImmunoResearch Laboratories) for 2 h and developed with VECTOR Blue, Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA). Spots in each well were counted using the CTL immunospot reader (Cellular Technologies, Shaker Heights, OH). For analysis, background spots from wells without any capture Ag were subtracted from each well.

Enumerating memory B cells (memory B cell ELISPOT assay)

Total PBMCs were cultured in vitro as previously described using protocols shown to induce differentiation of CD27⁺ memory B cells into ASCs (17). Briefly, PBMCs were stimulated with PWM (gift from Shane Crotty, University of California, San Diego), IL-2 (10 pg/ml; PeproTech, Rocky Hill, NJ), and CpG (ODN 7909, 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3') (2.5 μ g/ml; Oligos Etc., Wilsonville, OR) for 6 d. ASC ELISPOT assays were performed as described earlier from the proliferated in vitro cultures. For cord blood, total IgM was also measured using anti-human IgM 5 μ g/ml (Invitrogen, Carlsbad, CA) and detected with alkaline phosphatase-conjugated anti-human IgM 1 μ g/ml (Jackson ImmunoResearch Laboratories).

Statistical analysis

Comparison of the ASC frequencies between in vitro proliferation of memory B cells or direct ex vivo frequencies of cord blood and healthy adult subjects was performed using Mann–Whitney two-tailed unpaired *t* tests, as variances were not assumed to be equal. Similar analyses were used to compare direct ex vivo ASC frequencies of nonspecific Ags in subjects during vaccination or infection with those of healthy controls.

Results

Adult memory B cell compartment contains high frequencies of cells specific for universally exposed Ags

Our studies were designed to test the specificity of the ASC response after immunization or infection and the degree of expansion of cells specific for prevalent but unrelated Ags both as a proof of specificity and a measure of potentially universal bystander effect. We reasoned that if the latter mechanism makes a consistent and substantial contribution to maintaining serological memory, then immunization with one Ag should trigger an expansion of cells specific for other unrelated Ags that represent a significant frequency of the preexisting memory compartment. To test this hypothesis, we chose a small number of important Ags (included tetanus, hepatitis B, RSV, and influenza virus), because nearly all adults have had a history of exposure to these Ags either through childhood vaccination or infection (27–30). All adults have had exposure to RSV and influenza virus (27), and 81 and 100% of our subjects reported having received hepatitis B and tetanus vaccines, respectively.

The frequency of circulating memory B cells specific to two of these Ags, RSV and tetanus, was measured in a sample of healthy adults using the memory B cell ELISPOT assay. Memory B cells to

tetanus were readily detected in all 16 adults with frequencies ranging from 0.52 to 40 per 1000 total IgG (mean $8.5 \pm 12.5/1000$ total IgG) (Fig. 1A). Memory B cells to RSV were observed in 27 of 28 subjects with frequencies ranging from 0 to 52 per 1000 total IgG (mean 4.9 ± 10 cells/1000 total IgG) (Fig. 1B). The subject without memory RSV B cells had evidence of memory B cells to tetanus suggesting that all healthy adults tested had memory B cells to either RSV and/or tetanus. As expected, memory IgG B cell frequencies to RSV and tetanus were not detected in seven cord blood samples (Fig. 1). Therefore, functional memory B cells against both RSV and tetanus are readily detectable from healthy adults. These data indicate that bystander stimulation would be likely to impact these responses and that the contribution of bystander stimulation to the maintenance of serological memory should be measurable in most healthy subjects.

Direct ex vivo ASC specificity after vaccination

After immunization, recently proliferated ASCs typically appear in the circulation on day 4 and peak between days 5 and 8 with secondary exposure (1). Accordingly, ASC ELISPOT assays were performed with unstimulated PBMCs to assess the frequency of both Ag-specific and nonspecific responses 6–7 d after vaccination. As expected, a brisk expansion of total IgG ASC (5- to 10-fold from baseline) was observed after immunization with four different Ags (influenza, tetanus, hepatitis B, and HPV). Strikingly, in all cases, we readily detected significant frequencies of ASCs reactive with the corresponding vaccine but no responses above background levels to any of the other four Ags (Fig. 2). For example, increased influenza-specific ASCs after TIV are shown with undetectable ASCs to the unrelated Ags. Similar specificities were detected with ASCs against tetanus, hepatitis B, and HPV Ags although the magnitude of the responses was variable (Fig. 2). Tetanus responses were particularly striking as nearly all ASCs are Ag-specific accounting for 97% of the total IgG frequencies. Hepatitis B and HPV

vaccination showed similar results but lower frequencies compared with those for influenza or tetanus. Importantly, the nonspecific bystander ASC responses in adults to universally exposed RSV protein are not detectable with influenza, tetanus, hepatitis B, and HPV vaccination. These data are representative of lack of bystander ASC responses to at least one of the nonspecific Ags in an additional nine and five patients receiving influenza or tetanus vaccine, respectively. The nonspecific RSV ASC frequencies during immunization were similar to asymptomatic responses ($p = 0.08$). In addition, ASCs to these five Ags could not be detected in an asymptomatic healthy adult control (Fig. 2).

The lower percentage of Ag-specific ASC responses to hepatitis B and HPV vaccines may be due to several reasons. First, fewer Ag epitopes may be exposed when the proteins are coated onto the polyvinylidene difluoride membrane in the ASC ELISPOT assay. Second, all healthy asymptomatic adult subjects have low levels of total IgG ASC responses circulating at steady state. A small rise of ASC numbers in response to the vaccine above this steady-state of total IgG responses may demonstrate only a small proportion of the total IgG frequencies but may represent a large fraction of the total IgG frequencies that proliferated due to the vaccine.

Because one of the adults tested did not have evidence of memory B cells to RSV (Fig. 1A), the lack of nonspecific ASC responses could have been related to a deficiency of memory B cells to these Ags in the vaccine recipients. Therefore, memory B cells were measured prior to immunization from the blood of this subject prior to influenza vaccination, and despite memory B cell frequencies of 6, 4, 0.2, and 0.2 per 1000 IgG-producing cells for RSV, tetanus, hepatitis B, and HPV, respectively, direct ex vivo ASC specificities were not detectable. Hence, the lack of bystander ASCs in the blood after vaccination was not due to a deficiency of circulating memory B cells to these individual Ags.

Direct ex vivo ASC specificity in asymptomatic healthy controls

Direct ex vivo ASC specificities could not be detected in 28 asymptomatic adult subjects without a recent history of infection or vaccination. The mean frequencies for ASC to TIV, RSV F, tetanus, hepatitis B, and HPV were 2.4 ± 3.7 , 0.8 ± 1.2 , 0.2 ± 1.2 , 0.0 ± 1.0 , and 0.4 ± 1.2 spots/ 10^6 PBMCs, respectively (Fig. 3A). The total IgG ASC frequencies are also much lower at steady state (mean \pm SD, 134 ± 118 spots/ 10^6 PBMCs) than those after the surges during vaccination or acute infection consistent with other studies (31).

Direct ex vivo ASC specificity in cord blood samples

In cord bloods, the mean frequencies of direct ex vivo ASC frequencies to TIV, RSV F, tetanus, hepatitis B, and HPV were 0.0 ± 0.7 , 0.1 ± 1.2 , 0.0 ± 0.3 , 0.1 ± 0.3 , and 0.1 ± 0.6 spot/ 10^6 PBMCs, respectively (Fig. 3B). Spontaneous total IgG frequencies were much lower in the cord blood (mean \pm SD, 5.7 ± 6.7 spots/ 10^6 PBMCs) compared with those of the blood of adults. Viability of the cord blood cells was assured in all samples with trypan blue exclusion (>99%) and/or total memory IgM responses (>1000/ 10^6 cord blood mononuclear cells).

Notably, no statistical differences were found between the spontaneous Ag-specific ASC frequencies of cord blood and adult samples for TIV, RSV F, tetanus, hepatitis B, and HPV ($p = 0.06$, 0.44, 0.58, 0.90, 0.52, respectively; Mann–Whitney). The ASC frequencies to influenza in asymptomatic adults and cord blood samples were 2.4 ± 3.7 and 0.0 ± 0.7 spots/ 10^6 PBMCs, respectively, and thus it is possible that this difference could have reached statistical significance with a larger sample size. Importantly, this difference was not noted with the other four Ags suggesting that it was not a universal phenomenon. One ex-

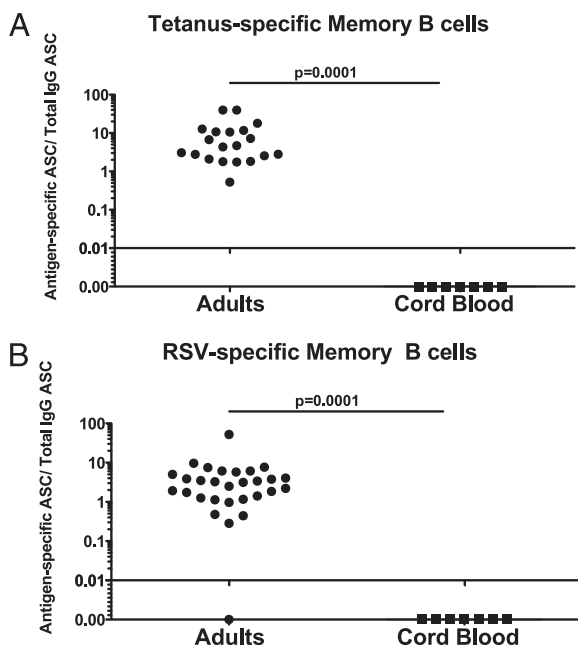


FIGURE 1. Memory IgG B cell frequencies in healthy adults and cord blood. For these experiments, PBMCs were stimulated in vitro for 6 d, then replated for ASC ELISPOT assays as described in *Materials and Methods*. A, Tetanus-specific memory B cell frequencies in 16 adults compared with that in 7 cord blood samples. B, RSV-specific memory B cell frequencies in 29 adults compared with that in 7 cord blood samples. p values, Mann–Whitney U test.

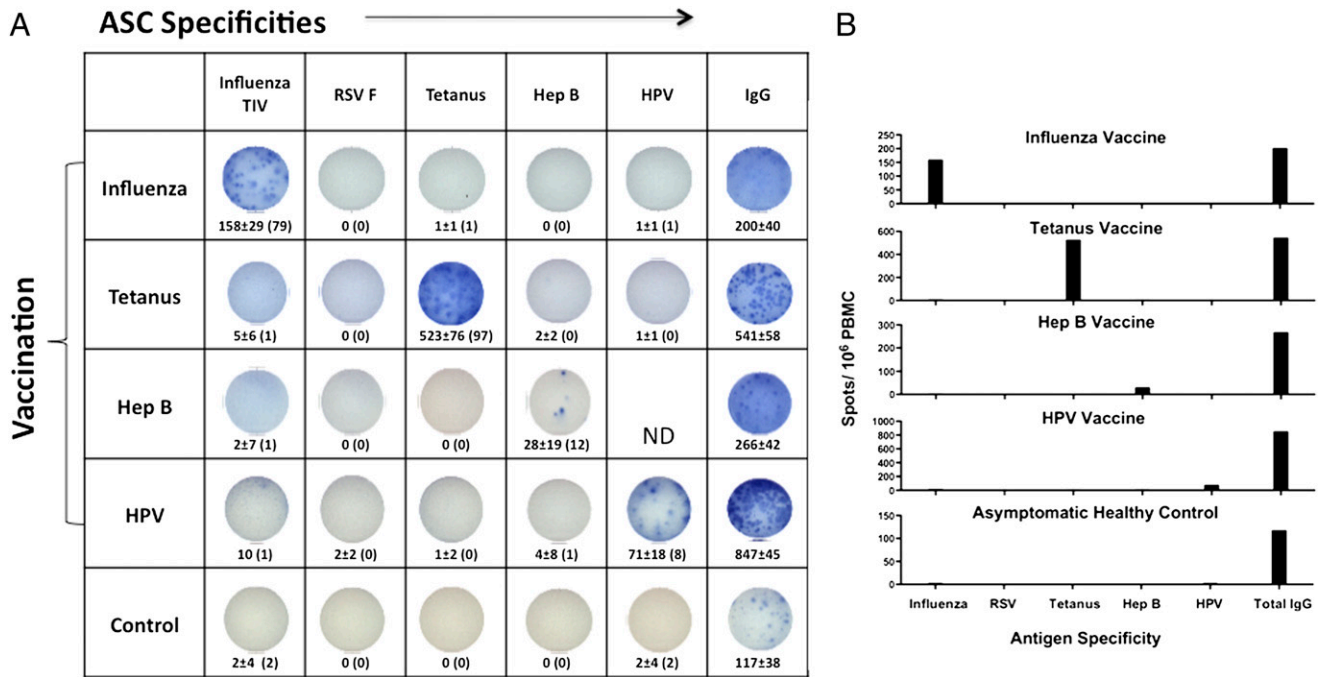


FIGURE 2. High specificity of the ASC ELISPOT assay is consistently detected in subjects after immunization for the corresponding Ags with the absence of cross-reactivity to nonspecific pathogens. For these experiments, ASC ELISPOT assays were performed directly ex vivo without in vitro proliferation. *A*, Representative wells of the ELISPOT assay. The sample in each row is representative blood from four subjects with indicated vaccination. *Bottom row* is representative of an asymptomatic healthy subject at steady state. Columns represent ASC assays specific for the following Ags: TIV, RSV F, tetanus toxoid (Tet), hepatitis B vaccine, and HPV vaccine. PBLs (PBMCs) were incubated at 300,000 cells/well directly ex vivo (without stimulation). Spots were detected with anti-human IgG. Total IgG ASCs are shown in the *far right column*. Numbers below each well represent mean \pm SD of triplicate wells. Numbers in parentheses indicate percentage of Ag-specific to total IgG spots. *B*, Quantitation of data based on the assay in *A*. ND, not done.

planation could be the complex nature and large numbers of different influenza Ags in the trivalent influenza vaccine, as purified individual hemagglutinin proteins did not give the same responses (data not shown). Collectively, these data suggest that memory B cells do not affect the occasional low-level spontaneous production of Ag-specific ASCs to the above-mentioned Ags.

Kinetics of bystander ASC responses

It is possible that the kinetics of bystander responses may be different from Ag-specific responses. Therefore, we measured both ASC to tetanus (Ag-specific) and nonrelevant Ags (influenza, RSV, hepatitis B, and HPV) on days 0, 5–9, 15, and 28 in one subject (Fig. 4). Again, we found no ASC responses to influenza, RSV, hepatitis B, and HPV above background levels of the asymptomatic healthy subjects (mean \pm SD, 1.7 ± 1.6 , 0 ± 0 , 0 ± 0 , 0 ± 0 ; $p = 0.60$, 0.12 , 0.85 , 0.41 , respectively; Mann–Whitney). This lack of bystander ASCs at these additional time points suggests that timing does not affect ASC specificities.

Bystander and specific ASC responses during acute viral infections

Compared with inactivated or purified protein vaccines, live virus infections are likely to generate a more vigorous, sustained, and complex response and may stimulate the generation of nonspecific ASCs from bystander memory B cells due to TLR and cytokine-mediated mechanisms (5, 32). Yet, circulating ASCs detected during acute respiratory viral infections also possessed very high specificity and recognized only the pathogens of exposure. For instance, in the blood of a patient with influenza infection, only ASCs reacting to influenza proteins, but not to control Ags, were detectable (Fig. 5A). Similar findings were noted with RSV infection with detection of only RSV-specific ASCs on day 2 of symptom onset. Specificities to the bystander Ags (influenza,

tetanus, hepatitis B, and HPV) were not detectable above background responses.

These findings were consistent in all 11 patients with acute influenza virus infection on days 4–11 after symptom onset (mean \pm SD, 7 ± 2 d). Five had influenza B infection in 2007, and six had pandemic H1N1 infection in spring 2009. All 11 patients had influenza-specific ASCs in the blood at a single time point during the acute illness and undetectable frequencies of RSV-specific ASC responses (Fig. 5B). The same was true regarding ASC specificity during acute RSV infections on days 2–10 (mean \pm SD, 7 ± 2 d). All 11 patients (6 outpatients and 5 inpatients) with acute RSV infection had only RSV-specific ASCs detected. Ten of the 11 had undetectable influenza-specific ASCs (Fig. 5C).

ASC responses in patients with acute 2009 pandemic influenza and influenza B infections were detected using the 2009 and 2007 trivalent influenza vaccine, respectively. Mouse studies demonstrated that Abs produced in response to primary infection with 2009 H1N1 virus were cross-reactive with older H1N1 influenza viruses (33). The nucleoprotein of the 2009 virus had nearly 95% conserved amino acid sequences to seasonal H1N1 viruses. To be sure that cross-reactive H1:Brisbane and NP_A ASC responses could be measured using TIV as the Ag, we measured ASC responses directly ex vivo (no proliferation) to individual influenza proteins in several subjects with 2009 pandemic H1N1 infection. We detected ASC responses to influenza NP_A Ags, H1:2009 California, H1:New Caledonia, H1:Solomon Islands, H1:Brisbane, but not to H3 or H7, which are closely related HA proteins (Fig. 6). Hence, the H1:Brisbane and NP Ags contained in the TIV preparation were actual Ab epitopes that were contained in the 2009 H1N1 virus.

One patient with an RSV-specific ASC response also had a substantial expansion of influenza-specific ASCs from the same

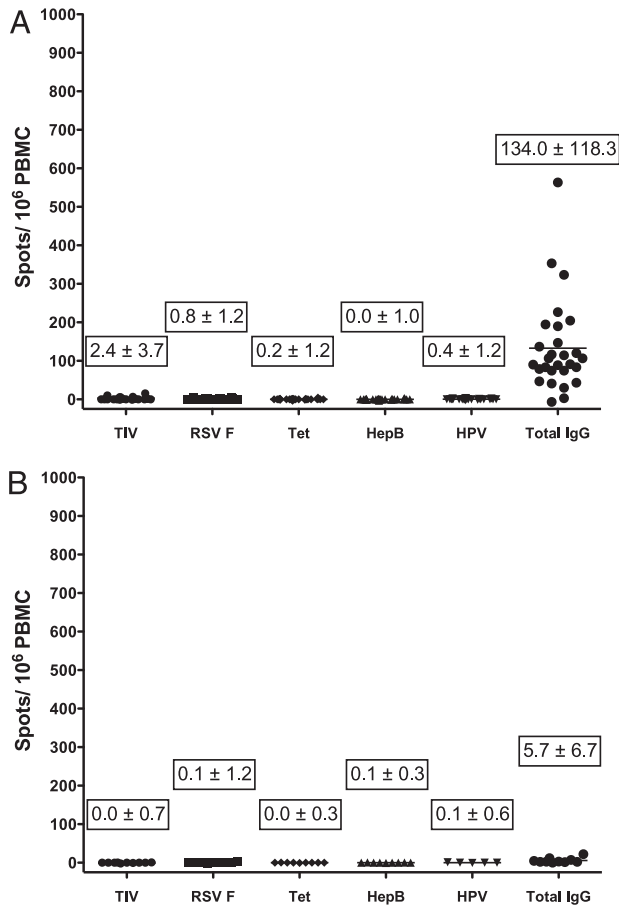


FIGURE 3. Baseline Ag-specific ASCs from PBMCs of 28 healthy human subjects at steady state (A) and 9 cord blood samples (B). ASC assays specific for TIV, RSV F, tetanus, hepatitis B, HPV, and total IgG. Boxes show mean ± SD. For these experiments, ASC ELISPOT assays were performed directly ex vivo without in vitro proliferation.

blood sample (Fig. 7). These findings suggested two possibilities: a unique massive bystander response or a simultaneous RSV and influenza virus infection. Additional nasopharyngeal PCR tests revealed a coinfection with influenza B (and not influenza A) virus confirming the ASC specificities. In conclusion, similar to the vaccine responses, ASCs during an acute respiratory viral infection are highly specific to only the pathogen of recent exposure, and when dual specificities are noted, the results are due to true coinfections.

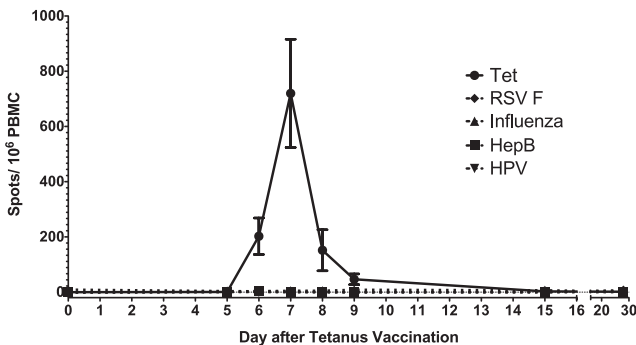


FIGURE 4. Kinetics of tetanus and bystander (RSV F, influenza, hepatitis B, HPV)-specific ASC frequencies from one subject prior to and after tetanus vaccination on days 5–9, 15, and 30. For these experiments, ASC ELISPOT assays were performed directly ex vivo without in vitro proliferation.

Discussion

Collectively, our data show that the massive expansion of ASCs detected in the blood after vaccination is highly enriched for Ag-specific clones and that bystander ASC specificities to unrelated Ags are not detected above background responses. Notably, a bystander ASC response was not observed with immunization despite the near universal presence of memory B cells to the Ags tested. Similarly, during acute viral infections, only pathogen-specific responses to the exposed microorganism are detected, with the lack of ASCs with unrelated specificities. Because measuring specificities to all previously exposed Ags is not possible, we chose a few universally exposed Ags such as tetanus, RSV, and influenza virus to serve as surrogates.

These results begin to address the cellular underpinning of the maintenance of long-lived Ab memory. Several in vitro studies including our work also demonstrate the proliferation of memory B cells with noncognate polyclonal activation presumably through TLR or cytokine-related activation in in vitro culture systems (17). In this study, from a collection of healthy adults, RSV-specific memory B cell frequencies ranged from 0 to 52 per 1000 IgG producers (mean 5/1000 total IgG). If equal bystander proliferation occurred during acute influenza viral infections, expected RSV-specific ASC frequencies would reach 52–572 RSV-specific ASCs per 10⁶ PBMCs when total IgG ASCs reached 1000–11,000 per 10⁶ PBMCs as demonstrated in virus-infected patients in this study. Our results do not reveal bystander RSV ASC levels close to those frequencies.

The low percentages of influenza- and RSV-specific ASC responses (37.4 ± 26.4% and 15.6 ± 6.4% of total IgG ASCs, respectively) during these acute infections are concerning because a large fraction of the ASCs are unaccountable. However, several possible explanations may account for this result. First, loss of epitopes may occur when proteins bind to the PVDF membranes in the ASC ELISPOT assay. Second, for influenza infections, there may be poor matching of the Ags in the TIV vaccine to the strains causing the infection. Third and most important, not all viral proteins were coated on the ASC ELISPOT well. For example, RSV has eight structural proteins that are highly immunogenic for Ab responses, but we coated with only RSV F. Despite low percentage of Ag-specific responses to total IgG responses, the lack of bystander responses to unrelated Ags is consistent during vaccination and respiratory viral infection.

Nonspecific ASC responses using mAb generation after influenza vaccine have been shown (7). Both specific (71% or 61 of 86 Abs) and noninfluenza-specific (29%) mAbs were generated from circulating ASCs after influenza vaccination (7). However, those authors also remark that the specificities to noninfluenza protein are likely to be explained by multiple causes such as technical errors introduced in cloning, specificities to denatured nonvaccine components (7), or even long-lived plasma cells released from the bone marrow (34) proposing strong possibilities for the lack of bystander responses.

The implications of the bystander concept are controversial, and one may argue that despite high memory B cell frequencies, the activation of unrelated memory B cells occurs at extremely low levels relative to the Ag-specific frequencies (5). This phenomenon does not appear to be the case because the data demonstrate similar frequencies of spontaneous Ag-specific ASCs in the adult blood (which contains high frequencies of memory B cells) and cord blood samples (that contain no memory IgG B cells). Although no statistical differences were found in ex vivo ASC responses to all five Ags between the adult and cord bloods, a *p* value equal to 0.06 for influenza-specific ASC responses raises the

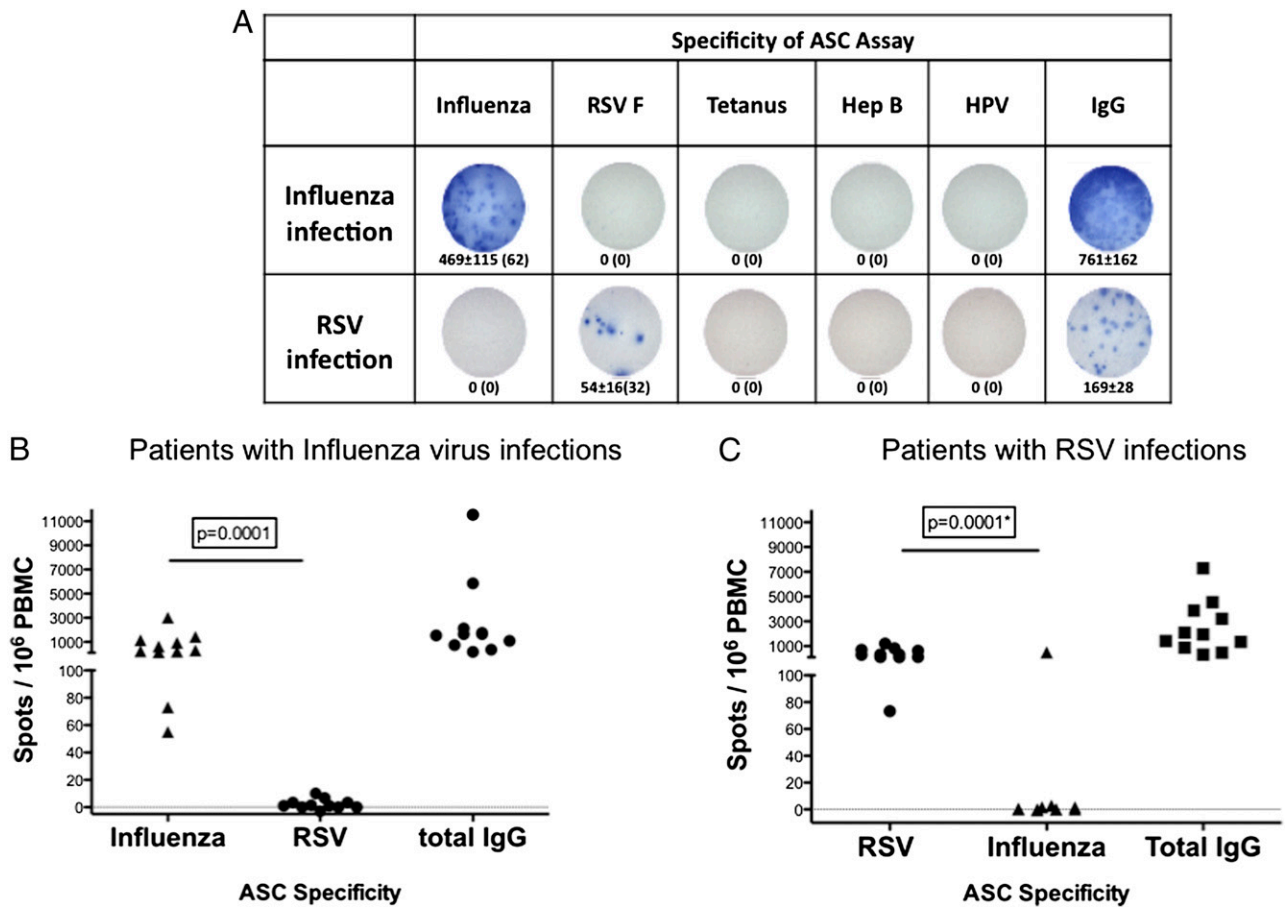


FIGURE 5. ASC specificity in the blood against influenza, RSV F, tetanus, hepatitis B, HPV, and total IgG during acute respiratory viral infections. *A*, Wells of 300,000 PBMCs plated from blood of patients with acute infection with influenza A virus (*top panel*) and RSV (*bottom panel*). *B* and *C*, ASC specificity for RSV, influenza, and total IgG in (*B*) 11 patients with acute influenza A 2009 pandemic H1N1 and B infections and (*C*) 11 patients with acute RSV infection (*comparison of ASC responses with RSV and influenza excluding the patient with dual infection in Fig. 7). For these experiments, ASC ELISPOT assays were performed directly ex vivo without in vitro proliferation (*p* values, Mann-Whitney *U* test).

possibility that influenza Ags may be maintained by this model. However, this trend was not seen with RSV, tetanus, hepatitis B, or HPV Ags suggesting that a universal model for bystander proliferation for plasma cell maintenance could not be applied to all Ags. More than likely, influenza Ags from formalin-inactivated vaccine preparation may yield higher background responses because individual influenza protein preparations did not yield this background response. Thus, more than likely, extremely low levels of Ag-specific ASCs directly ex vivo of 0.1 to 1 spot/ 10^6 PBMCs are the technical limits of the ELISPOT assay sensitivity, and these low frequencies are difficult to reproduce.

The specificity of the recently blasted ASCs found in the blood after Ag exposure could be used to identify the Ag or pathogen causing illness. We have recently demonstrated this concept with acute RSV infections (2). Currently available immune assays

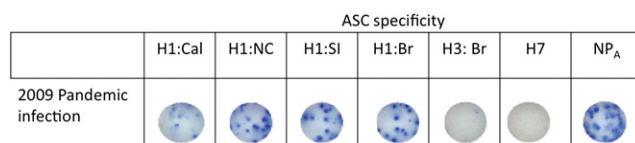


FIGURE 6. Cross-reactivity of direct ex vivo circulating ASC responses in one patient with confirmed 2009 pandemic H1N1 infection. HA epitopes for H1:California 2009, H1:New Caledonia, H1:Solomon Islands, H1: Brisbane, H3:Brisbane, H7:Netherlands, and NP_A are shown. ASC assays were performed directly ex vivo without in vitro proliferation.

preclude their routine use for diagnosing acute illness. For example, IgM serology offers low diagnostic yields with frequent false positives (35), and a single IgG level is not helpful in diagnosing secondary respiratory infections in adults. A single elevated serum Ab titer to multiple Ags could reflect several possible scenarios: 1) a new encounter with the pathogen; 2) persistence of long-lived bone marrow ASCs producing Abs demonstrating infection, which may have occurred long ago; 3) sustained production of Abs due to persistence of chronic infections; or 4) the presence of acute coinfection by more than one organism. Therefore, serum Abs require longitudinal changes to

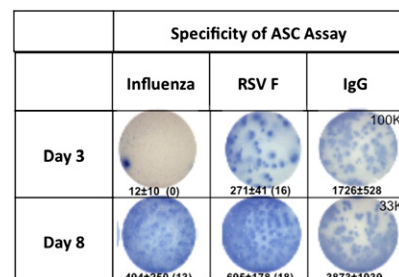


FIGURE 7. Patient with circulating ASCs specific for both RSV and influenza as enumerated in Fig. 5*B*. Patient with dual RSV and influenza virus B infection confirmed by nasopharyngeal PCR. ASC ELISPOT assays were performed directly ex vivo without in vitro proliferation.

distinguish among new acute infections, exacerbations of chronic infections, and past pathogen exposures. These ambiguities could be resolved by examination of circulating ASC specificity.

This pathogen-specific ASC found in the blood as we demonstrate in this study could function as a serologic surrogate with similarly high specificities of Abs, but with one major advantage: the pathogen-specific ASCs require only a single time point during the acute illness. Additionally, the ASC assay may also have greater benefits over the single-IgM serology because the assay detects high-affinity IgG or IgA Abs. Evidence of bystander proliferation and differentiation of memory B cells to ASC would undoubtedly complicate the application of the test by “falsely” elevating the level of ASC with antigenic specificities unrelated to the current infection. However, this study demonstrates that minimal non-specific ASC response during respiratory viral infections occurs. Clearly, further evaluation is needed to determine the kinetics of the circulating ASC during acute viral infections before it can be used as a diagnostic test, but the lack of bystander nonspecific ASCs detected in this assay demonstrates its potential.

In conclusion, we demonstrate high specificity of recently blasted ASCs after four different vaccines and two respiratory viral infections and that bystander ASC responses are not observed. Whether this finding can be translated to all infections including bacterial and fungal pathogens needs further investigation. Clearly, active respiratory viral infections do not appear to significantly increase the frequency of circulating ASCs against other Ags. Although serum Abs narrate a tale of a patient’s life history of pathogen exposure, the circulating ASCs can be instructive of only the most recent microbial exposure and may be an ideal measure for a novel diagnostic assay for acute infections.

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