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Influenza Virus-Specific Neutralizing IgM Antibodies Persist for a Lifetime

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Detection of immunoglobulin M (IgM) antibodies has long been used as an important diagnostic tool for identifying active viral infections, but their relevance in later stages has not been clearly defined in vivo. In this study, we followed the kinetics, longevity, and function of influenza virus-specific IgM antibodies for 2 years following sublethal infection of mice with live mouse-adapted A/PR/8/34 virus or immunization with formalin-inactivated virus. These groups mounted robust protective immune responses and survived lethal challenges with 50 X 50% lethal dose (LD50) mouse-adapted A/PR/8/34 virus 600 days after the primary exposure. Surprisingly, the virus-specific IgM antibodies persisted along with IgG antibodies, and we found a significantly higher number of IgM-positive (IgM+) virus-specific plasma cells than IgG+ plasma cells that persisted for at least 9 months postexposure. The IgM antibodies were functional as they neutralized influenza virus in the presence of complement just as well as IgG antibodies did.

Influenza is a common infectious disease of the respiratory system caused by a negative-sense, single-stranded RNA virus, which belongs in the Orthomyxoviridae family. The most virulent strains belong to type A influenza, and their classification is based on the antigenic differences of their major surface antigens, hemagglutinin (HA) and neuraminidase (NA), which arose from the gene segment exchange between the avian and human pools.

The humoral immune responses to influenza comprise neutralizing antibodies against HA, NA, matrix protein M1, and nucleoprotein (NP), whereas the cellular immune responses are against HA, NP, matrix protein M2, and RNA polymerase PB2 (1). Vaccination or infection of human populations with inactivated influenza vaccine induces long-lived IgG-secreting plasma cells and influenza-specific B memory cells in the bone marrow. During the initial acute phase of microbial and viral infections, a component of the rapidly induced humoral immune response derives from IgM production as a first line of defense as IgM is expressed without isotype switching (2). These antibodies are of usually lower affinity since B cells have not undergone somatic hypermutation. Nonetheless, the pentameric nature of IgM molecules compensates for the low affinity by binding simultaneously to multivalent antigens and conferring high overall avidity. In addition, IgM antibodies, following binding to antigen, activate the complement cascade. Complement is a complex network of plasma and membrane-associated serum proteins which can elicit highly efficient and tightly regulated inflammatory and cytolytic immune responses to infectious organisms (bacteria, viruses, and parasites), injured tissue, and other surfaces identified as “non-self” (3). Although the presence of IgM has long been used as an important diagnostic tool for identifying individuals with active infections, the relevance of IgM in later stages of many viral infections even after the resolution of infection has not been clearly defined in vivo.

In this study, we explored the kinetics of virus-specific IgM responses and determined whether they were functional. The high avidity of IgM due to its pentameric nature along with its ability to activate the complement cascade might potentially be effective at controlling virus spread. Here we show that (i) complete protection against a high lethal dose of virus occurs 600 days after the primary antigenic exposure, (ii) virus-specific IgM responses persist at least up to 540 days, and (iii) IgM antibodies in the presence of complement can neutralize influenza virus just as efficiently as IgG antibodies.

MATERIALS AND METHODS

Cell lines and viruses. Madin-Darby canine kidney (MDCK) cells (ATCC CCL 34; American Type Culture Medium, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech, Manassas, VA) containing 10% fetal bovine serum (HyClone, Thermo Fisher Scientific, Rockford, IL). Influenza virus stocks (A/PR/8/34, A/California/10/78, A/Chile/1/83, A/New Caledonia/20/09, A/Brisbane/59/07 and A/California/04/09, A/Aichi/2/68, A/Orlando/07/1/90, A/Hong Kong/04/81, A/Hong Kong/1/68, A/Victoria/3/75, A/Wyoming/03/03, and A/Perth/16/2009) were prepared, purified, and characterized as described previously (4, 5). Inactivation of the purified virus was carried out with β-propiolactone and confirmed by plaque assay in MDCK cells (5).

Measurement of lung titers by plaque assay. Mouse-adapted A/PR/8/34 and A/Brisbane/59/07 stocks were propagated in BALB/c mouse lungs by intranasal infection. Viral titers of lung lysates were assessed by plaque assay and the 50% lethal dose (LD50) was calculated with the Reed-Muench formula (6).

Immunizations/infections. Six- to 8-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were housed under patho-
gen-free conditions at the Emory Vaccine Center and the Whitehead Building facilities of the Emory University School of Medicine. All animal studies were approved by the Emory University’s institutional animal care and use committee (IACUC). Three cohorts of mice were used for the first part of this study. The first group was vaccinated intramuscularly with 1,400 hemagglutinin units (HAU) of inactivated virus. The second group was infected intranasally under isoflurane anesthesia with 0.01 × LD₅₀ mouse-adapted live virus and held separately under pathogen-free conditions in biocontainment level 2 (BSL-2). The third group was the negative control of the study and received phosphate-buffered saline (PBS) intranasally. In the second part of the study, three cohorts were either vaccinated intramuscularly with 3 μg whole inactivated influenza virus (A/Brisbane/59/07), sublethally infected with 0.5 × LD₅₀ mouse-adapted homologous strain, or received PBS intranasally. For the A/PR/8/34 study, at least 15 animals were used per cohort for humoral immune responses and challenge studies at 20 months postinfection or postvaccination. A separate cohort of at least 30 mice per group (infected, vaccinated, or mock treated with PBS) was used for T cell immune responses, and another cohort of 30 mice per group was used for B cell responses. Finally, a large cohort of infected mice (at least 10 per group) was used for terminal bleedling: 12 months postinfection for serum collection and purification of IgG and IgM antibodies. For the A/Brisbane/59/07 study, a total of 15 mice (infected, vaccinated, or naive) were used for mucosal and cellular immune responses.

Lethal challenge of mice with influenza virus. All groups were challenged for survival by intranasal instillation of 50 × LD₅₀ mouse-adapted A/PR/8/34 influenza virus under isoflurane anesthesia 18 months postinfection or postvaccination and monitored daily for signs of morbidity (body weight changes, fever, and hunched posture) and mortality for 14 days. A weight loss exceeding 20% in all challenged mice was used as the experimental endpoint, and mice reaching this endpoint were euthanized according to IACUC guidelines.

Sample collection. Mice vaccinated or infected with the A/PR/8/34 influenza strain were bled at designated time points (1, 3, 6, 9, 12, and 18 months) postvaccination or postinfection. Blood was collected by submandibular bleeding. Bone marrow and spleens were collected at 1, 3, 6, 9, 12, and 18 months immediately after bleeding for enzyme-linked immunosorbent assays (ELISA), neutralization assays, and hemagglutination inhibition assays. Single-cell suspensions from bone marrow were analyzed for virus-specific B and T cells using enzyme-linked immunosorbent spot (ELISPOT) assays (7). Tissue samples for B cell assays were collected at 1, 3, 6, 9, 12, and 18 months after infection for serum collection and purification of IgG and IgM antibodies. For the A/Brisbane/59/07 study, a total of 15 mice (infected, vaccinated, or naive) were used for mucosal and cellular immune responses.

Complement treatment. Mouse sera or purified IgG and IgM fractions were heated inactivated at 56°C for 30 min. The samples were incubated with equal volumes of mouse complement serum (Rockland Immunochemicals Inc., Gilbertsville, PA) for 60 min at 37°C (8).

Evaluation of humoral immune responses. Influenza-specific IgG and IgM titers were determined quantitatively in sera, BAL fluid, or lung suspensions by ELISA as described previously (9). Purified mouse Ig(H+L), IgG, and IgM standards and goat anti-mouse IgG-horseradish peroxidase (HRP) and IgM-HRP were purchased from Southern Biotechnology Associates (Birmingham, AL). Nunc MaxiSorp plates (Thermo Scientific, Waltham, MA) were coated with either 2 μg/ml Ig(H+L) or with 5 μg/ml whole inactivated A/PR/8/34 or A/Brisbane/59/07 virus for quantitation of influenza-specific binding antibodies. Hemagglutination assays were carried out using sera treated with receptor-destructing neuraminidase (RDE) (Roche Diagnostics, Indianapolis, IN) according to the WHO protocol (4, 7), and microneutralization assays were performed with heat-inactivated sera at 56°C or purified IgG and IgM serum fractions (4, 8). The hemagglutination inhibition (HAI) titers were read as the reciprocal of the highest dilution of serum that conferred inhibition of hemagglutination. The highest serum dilution that generated ≥50% specific signal was considered to be the neutralization titer. The values were expressed as the geometric mean ± standard error of the mean.

Purification of IgG and IgM antibodies from immune mouse sera. Serum samples from immune mice infected with A/PR/8/34 1 year earlier were diluted in Tris-HCl (pH 7.5 [running buffer]) and run with Sepharclay column chromatography (GE Healthcare Life Sciences). IgG and IgM standards were run before the test sera to identify the position of IgG and IgM peaks. Out of the two peaks obtained, the first peak was IgG and the second one was IgM as confirmed by ELISA. Their neutralizing activities against A/PR/8/34 were assessed with HAI. The IgM samples were further purified using a MBA IgM purification kit (Thermo Scientific, Waltham, MA). The purified IgG and IgM fractions were pooled separately and concentrated through Amicon centrifuge columns (Millipore, Bedford, MA). The contents of IgG and IgM were quantified by protein assays (Bio-Rad, Hercules, CA) and ELISAs against total IgG and IgM and for influenza specificity in unseparated and in purified samples after separation. Nunc MaxiSorp plates were coated with either 2 μg/ml Ig(H+L) or with 4 μg/ml whole inactivated A/PR/8/34 virus for the quantitation of total and influenza-specific binding antibodies with ELISAs. The samples were analyzed for HAI and neutralizing antibody titers against A/PR/8/34 in the absence or presence of complement.

Avidity determination of IgM- and IgG-purified fractions from mouse sera. The antibody avidity was determined in purified IgM and IgG serum fractions collected from mice infected 1 year ago with 0.01 × LD₅₀ mouse-adapted A/PR/8/34 influenza virus. Equivalent concentrations of IgG and IgM antibodies (0.02 mg/ml) were diluted in 0.1 M sodium phosphate–4 mM EDTA (PB-EDTA), and 1.0 M 2-mercaptoethanol (2-ME) was added to a final concentration of 0.01 M. Antibodies (Abs) were assessed for influenza virus reactivity by ELISAs after treatment with 2-ME. A mild reduction with 2-ME treatment causes 19S pentameric IgM to dissociate into 7S H₃L₂ subunits that remain capable of binding antigen (10). Reduced and mock-treated samples (receiving an equal volume of PB-EDTA) were incubated at 37°C for 2 h and serially in A/Brisbane/59/07-coated ELISA plates (4 μg/ml), followed by anti-Ig(H+L) HRP to measure Ab binding.

Evaluation of cellular immune responses. Spleen cell suspensions (3 × 10⁶/well) in complete RPMI (cRPMI) medium were stimulated in vitro in the presence of the 2 μg/ml hemagglutinin (HA) or nucleoprotein (NP) peptide stimulants of A/PR/8/34 virus in cRPMI medium as previously described (11). Class I ovalbumin and class II ovalbumin peptides were used as negative controls. A mixture of phorbol 12-myristate 13-acetate (PMA) (Sigma) (1 ng/ml) and ionomycin (ION) (Sigma) (5 ng/ml) was used as a positive control. The A/PR/8/34 B cell epitope derived from nucleoprotein NP₁₄₇–₁₅₈ (R156 del) was included as an irrelevant stimulant. The peptides were synthesized using a peptide synthesizer following standard protocols according to published sequences for influenza A/PR/8/34. They were purified by reverse high-performance liquid chromatography (HPLC) and checked by mass spectrometry. The mouse gamma interferon (IFN-γ) monoclonal antibody (MAb) and biotinylated anti-IFN-γ for the ELISPOT assay were purchased from Mabtech (Sweden).

Quantification of anti-A/PR/8/34 antibody-secreting cells. Virus-specific antibody-secreting plasma cells (ASC) in the bone marrow were determined by a B cell ELISPOT assay as described previously (12). MultiScreen HA plates (Millipore) were coated overnight at 4°C with purified inactivated A/PR/8/34 virus at a concentration of 300 ng/well. Bone marrow cell suspensions in cRPMI were applied to the coated plates and incubated for 16 h at 37°C in a 5% CO₂ atmosphere. Anti-A/PR/8/34 ASC were detected after incubation with biotinylated anti-mouse IgG or IgM antibodies (eBioscience, San Diego, CA) followed by streptavidin–alkaline phosphatase (AP) (eBioscience). The blue spots were developed with alkaline phosphatase substrate (Vector Laboratories) and enumerated in an
ELISPOT reader (Cellular Technology, Shaker Heights, OH). The results are shown as the number of ASC per 10^6 cells.

Quantification of anti-A/Brisbane/59/07 antibody-secreting cells. Virus-specific ASC in the bone marrow from mice infected or vaccinated with A/Brisbane/59/07 influenza strain were determined as previously described (13). Millipore MultiScreen HA plates were coated overnight at 4°C with purified inactivated A/Brisbane/59/07 virus at a concentration of 1,000 ng/well. Bone marrow cell suspensions in cRPMI were applied to the plates and incubated for 16 h at 37°C in a 5% CO2 incubator. Anti-A/Brisbane/59/07 ASC were detected after incubation with anti-IgG or anti-IgM HRP (eBioscience). Red spots were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma) and enumerated in an ELISPOT reader (Cellular Technology). The results are shown as the number of ASC per 10^6 cells.

Statistics. The statistical significance of the difference was calculated by a two-tailed unpaired Student t test and one-way or two-way analysis of variance [ANOVA], including the Bonferroni multiple-comparison test. Differences were considered statistically significant at P values ≤0.05.

RESULTS
Influenza virus-specific humoral and cellular immune responses induced by both infection and vaccination are long-lived. First, we looked at the magnitude and longevity of influenza-specific antibody responses to live infection or vaccination. The antibody responses were analyzed by hemagglutination inhibition (HAI) (Fig. 1a) and neutralizing antibody (NT) (Fig. 1b) assays. We observed that the HAI titers in both cohorts increased over time, reaching a peak at 6 months (Fig. 1a). In both groups, the antibody titers were maintained as late as 18 months postinfection or postimmunization although the HAI titers produced in the infected group were significantly higher than those in the vaccinated cohort at most time points, the highest difference being observed at 9 months postexposure to the virus. We also compared the virus-neutralizing antibody titers against A/PR/8/34 (Fig. 1b). The data demonstrate that similarly to the HAI findings, both vaccinated and infected animals produced neutralizing antibodies that persisted at elevated levels up to 18 months postimmunization. The titers were comparable in both groups. Taken together, our results show that both vaccination and sublethal infection induce persistent and high levels of A/PR/8/34-specific functional antibodies.

We next compared the longevity and magnitude of virus-specific T cell responses in the infected group versus those in the immunized group. Both groups expressed virus-specific CD4^+ and CD8^+ T cells, and, not surprisingly, the CD4^+ and CD8^+ T cell responses in the infected group were significantly higher than those in the immunized cohort of mice at all time points (Fig. 1c and d). Nevertheless, our results showed that both vaccination...
and infection can induce long-lasting influenza-specific T cell responses, which are more pronounced in the CD8\(^+\) T cell population, and that these responses are stronger in live virus infections. Sublethally infected or vaccinated mice are protected from lethal challenge 600 days after exposure to influenza antigen. Since virus-specific humoral and cellular responses were sustained up to 18 months postexposure to influenza, we next determined whether these mice were protected from lethal challenge against the homologous strain. The results demonstrated that pre-exposure to antigen, whether it was infection or vaccination, conferred complete protection against infection with 50\(\times\) LD\(_{50}\) doses of homologous virus. In addition, the morbidity was minimal in both groups as they showed \(<10\%\) reduction of initial body weights by day 6 although the infected group showed better recovery to the original weight. Animals in the naive unexposed control group had significant weight reduction and had to be euthanized by day 4 postchallenge (Fig. 2a and b).

Long-lived influenza virus-specific IgG\(^+\) and IgM\(^+\) plasma cells persist in the bone marrow following infection or vaccination. Since A/PR/8/34 virus exposure induced robust and long-lasting NT and HAI titers, we investigated the kinetics of B cell responses in both infected and vaccinated cohorts of mice. It is known that persisting antibodies are produced by long-lived plasma cells in the bone marrow (7, 12); hence, we analyzed the bone marrow from sublethally infected or vaccinated mice at 1, 3, 6, 9, 12, and 18 months postexposure and quantitated the influenza-specific IgG\(^+\) and IgM\(^+\) plasma cell numbers by ELISPOT analysis using naïve mice as negative controls.

Both cohorts maintained high numbers of influenza-specific IgG\(^+\) plasma cells in the bone marrow for \(>18\) months (Fig. 3a). The magnitudes of influenza-specific IgG plasma cells were comparable at all time points between groups and peaked at 6 months, correlating with the levels of functional antibodies (Fig. 1a and b). The infected group exhibited significantly higher numbers of plasma cells than the vaccinated cohort only at 3 months after virus exposure (\(P = 0.025\)). Surprisingly, virus-specific, IgM\(^+\)–secreting plasma cells also persisted in the bone marrow of the sublethally infected versus immunized cohorts, and their numbers were at similar levels and comparable at all time points analyzed (Fig. 3b).

In the presence of complement, IgM antibodies efficiently neutralize influenza virus. To determine the extent to which IgM antibodies produced were functional in neutralizing the virus, we collected sera from mice sublethally infected 1 year earlier and separated the IgG and IgM fractions. We achieved 89.2\% recovery of the IgG fraction after the one-step purification procedure and

![FIG 2](image-url) The persistence of functional antibodies in both infected and vaccinated cohorts confers complete protection against 50\(\times\) LD\(_{50}\) A/PR/8/34 20 months after influenza virus exposure. Cohorts of infected or vaccinated mice were challenged 20 months after influenza exposure with 50\(\times\) LD\(_{50}\) mouse-adapted homologous virus and were monitored for survival (a) and morbidity (b), reflected in the body weight (BW) changes for 14 days postchallenge. Nine-month-old naïve mice were used as a negative-control group challenged with the same virus lethal dose. A weight loss exceeding 20\% in all infected mice was used as the experimental endpoint. The graph in panel b represents the means ± SEM of body weight changes of the survivors.

![FIG 3](image-url) Influenza virus-specific IgM\(^+\) and IgG\(^+\) plasma cells are maintained in bone marrow (BM) up to 18 months following infection and vaccination. Bone marrow from infected, vaccinated, or naïve BALB/c mice was collected at 1, 3, 6, 9, 12, and 18 months after virus exposure for B cell ELISPOT analysis. Bone marrow cells were added to plates coated with 0.5 μg/well of inactivated A/PR/8/34 influenza virus. The number of anti-influenza IgG-secreting plasma cells (a) and anti-influenza IgM-secreting plasma cells (b) were plotted per 10\(^6\) bone marrow cells. Graph bars denote the means ± SEM. The data are the averages from three separate experiments.
73% of the IgM fraction after the two-step purification procedure as measured by ELISAs (Fig. 4a and b). First, we compared the relative affinities of IgM and IgG fractions following reduction with 2-ME. Our data show that for both IgM and IgG antibodies, treatment with 2-ME showed reductions in binding, but these reductions were comparable for IgM and IgG. On average, following 2-ME treatment, the percent reductions in binding for IgM and IgG were 35% and 28%, respectively. The percent reduction in antigen-binding for IgM versus IgG following 2-ME treatment was not statistically significant (Fig. 4c and d).

Next, we tested the antibody fractions for functional antibody titers against the A/PR/8/34 strain by HAI. When the HAI titers were normalized per the protein concentrations of the fractions, the purified IgG fraction exhibited 3-fold higher HAI activity than the IgM fraction (Fig. 4e). The IgG fraction also demonstrated 4-fold higher influenza-specific neutralizing antibody titers detected in the IgG and IgM fractions were plotted in the absence or presence of complement. Each data point represents an individual animal. The data are the averages from triplicate experiments. The graphs represent G_{mean} ± 95% CI.
titers than the IgM fraction (Fig. 4f). Since the preparation of serum for the hemagglutination inhibition assay and neutralization assay involves a step of heating the sera at 56°C for 30 min to inactivate the neuraminidase activity, complement in the serum samples will be destroyed as well. Hence, we investigated whether the addition of complement back to our fractions might improve their ability to neutralize viruses. As shown in Fig. 4D, the addition of complement enhanced the ability of the IgG fractions to neutralize live A/PR/8/34 virus in vitro by 2.5-fold, whereas the neutralizing activity of IgM showed an impressive 4.5-fold increase.

Next, we compared the breadth of the neutralizing activities of the IgG and the IgM fractions against that for four seasonal H1N1 influenza strains (A/California/10/78, A/Chile/1/83, A/New Caledonia/20/99, and A/Brisbane/59/07) and the swine-origin 2009 pandemic H1N1 strain (A/California/04/09). Since the immunizing strain was the H1N1 influenza virus that circulated in 1934, to test the cross-reactivity we selected newer H1N1 strains that circulated in humans between 1970 and 2009. We found that the IgG fractions in the presence of complement had the highest neutralizing antibody titers against the chronologically closer seasonal influenza viruses and the lowest against the more distant ones, A/California/10/78 (geometric mean \[G_{\text{mean}}\] titer of 211), and A/Chile/1/83 (\[G_{\text{mean}}\] titer of 40), whereas there was no neutralizing activity against A/New Caledonia/20/99 (\[G_{\text{mean}}\] titer of 10) or A/Brisbane/59/07 (\[G_{\text{mean}}\] titer of 13). In agreement with our previous findings, the IgG fraction from A/PR/8/34-infected mice cross-neutralized the pandemic A/California/04/09 virus, producing a mean titer of 52 (Fig. 5a). The IgM fractions neutralized all viruses but A/New Caledonia/20/99, with mean antibody titers ranging from 180 for A/California/10/78 to 56 for A/Chile/1/83 226 to 64 for A/Brisbane/59/07 and to 127 for the reassortant A/California/04/09, suggestive of the protective role of IgM antibodies against a broader range of H1N1 influenza viruses (Fig. 5b). We also tested the breadth of immunities of IgG and IgM fractions (raised against A/PR/8/34 H1N1) in the presence or absence of complement against five H3N2 viruses (A/Aichi/2/68, A/Udorn/307/72, A/Victoria/3/75, A/Wyoming/03/03, and A/New Caledonia/20/99, and A/Brisbane/59/07) in the presence of complement. A/PR/8/34 virus used for infection and vaccination was the positive control. Each data point represents an individual animal. The data are the average of triplicate experiments. The graphs represent Gmean ± 95% CI.

Influenza-specific IgM antibodies are detected in lungs of mice 18 months after exposure to the vaccine or the live virus. Secretory immunoglobulins present at mucosal surfaces protect the host against infections. These include IgA, IgM, and even IgG. Secreted IgA and IgM antibodies are capable of transcytosis across the epithelial barriers via the poly(Ig) receptors. In addition, IgG antibodies are also secreted into the nasal secretions and intestinal lumen of humans (14). Here we tested whether virus-specific IgM antibodies persisted in the lungs of BALB/c mice either immunized with whole inactivated A/Brisbane/59/07 influenza strain or sublethally infected with the mouse-adapted virus. We found that the IgM titers in the mucosal secretions were similar in the infected and vaccinated mice and considerably higher than those in the negative-control group (\(P < 0.05\)) (Fig. 6a). In contrast, the IgG titers of the infected group were 2-fold higher than those in the vaccinated cohort in the lung suspensions (\(P = 0.06\)) and the BAL fluid (\(P = 0.02\)) (Fig. 6b). Contrary to serum influenza-specific IgG titers which persisted for at least 18 months in vaccinated (\(P = 0.008\)) or infected mice (\(P = 0.02\)) (Fig. 6d), influenza-specific IgM antibodies waned to undetectable levels (Fig. 6c) but notably were still present in the lungs of both infected and vaccinated animals. The differences in IgM titers between the systemic and respiratory compartments suggest the independent production and role of the secretory IgM in the mucosa (15, 16). Interestingly, the IgM and IgG antibodies in the respiratory compartment correlate with the numbers of influenza IgM- and IgG-secreting cells in the bone marrow of the cohorts (Fig. 6e and f).

DISCUSSION

IgM antibodies are considered mainly as indicators of acute inflammation (17–19), appearing early in the host immune response to pathogens and waning shortly after the appearance of IgG antibodies. In the present study, we show that influenza-virus-specific IgM antibodies persist along with IgG antibodies for the lifetimes of mice (>600 days). Both vaccination and infection induced comparable levels of humoral immune responses as shown by the influenza-specific functional antibodies in terms of magnitude and longevity, with the hemagglutination inhibition titers and the neutralizing antibody titers persisting up to 18 months and conferring complete protection against a 50× LD\(_{50}\) lethal challenge with live virus 600 days after primary antigen exposure. The sustained antibody titers can be attributed to the long-lived populations of influenza-specific IgG- and IgM-secret-
ing plasma cells detected at comparable numbers in the bone marrow of both cohorts throughout the 18 months of our study. Vaccination produced IFN-γ-secreting CD4+ and CD8+ T cell memory responses sustained at high levels only up to 6 months after antigen exposure followed by a sharp decline of the numbers by 12 months. Both CD8+ and CD4+ memory T cell responses were stronger and detectable for a longer period of time in the infected group. Interestingly, the IFN-γ CD4+ memory T cell response, although weaker than the CD8+ T response, was detectable up to 12 months after virus exposure, whereas the CD8+ T cell response was measurable up to 18 months. Thus, our findings are in agreement with recent reports on the impact of early antigenic exposure by vaccination or infection to the generation of effector and memory CD8 responses (20).

In our experience, a HAI or NT titer of ≥40 in mice is indicative of protection. Our results showed that the purified IgM fractions lacked virus-neutralizing activity as the NT titers were significantly <40, but the addition of the complement restored the functional properties, inducing a 6-fold increase in IgM neutralizing titers. On the other hand, the neutralizing titers of the IgG fractions went up 2-fold in the presence of complement. Although the numbers of viruses tested are limited, we found that the addition of complement to the purified IgG fractions assisted in the cross-neutralization of three out of five H1N1 viruses tested, namely, A/California/10/78, A/Chile/1/83, and the swine-origin A/California/04/09, whereas complement with the purified IgM fraction neutralized four out of five of the tested viruses (A/California/10/78, A/Chile/1/83, A/Brisbane/59/07, and A/California/04/09), showing similar titers in all of them.

Our studies are also in agreement with other studies that have shown that IgM antibodies can be protective: Harada and colleagues used influenza virus infection of activation-induced cytidine deaminase (AID)-knockout mice, which are deficient in IgM to IgG class switching and somatic hypermutation. They demonstrated that unmutated IgM antibodies were sufficient to protect from death against both primary and secondary infections with A/PR/8/34 albeit with higher morbidity. Although high-affinity IgG clearly plays an important role in influenza immunity, the level of protection provided by IgM was remarkable in AID-deficient mice (21). IgM antibodies have also been shown to play an important role in host defense against other viral infections. Diamond et al. examined the role of IgM in protection against West

**FIG 6** Long-lived mucosal humoral immune responses are attributed to IgM persistence. Lungs and BAL fluid were collected from BALB/c mice 18 months after sublethal infection with mouse-adapted A/Brisbane/59/07 virus or intramuscular vaccination with 3 μg of whole inactivated A/Brisbane/59/07 virus or from naive mice and tested for influenza-specific IgM (a) and IgG (b) titers. Serum samples were collected at the same time point and tested for IgM (c) and IgG (d) titers. The data are the averages from triplicate experiments. Bone marrow cells were isolated from euthanized vaccinated, infected, and naive mice and were assayed for influenza-specific IgM-secreting cells (e) and IgG-secreting cells (f) by B cell ELISPOT assays. Graph bars denote means ± SEM.
Nile virus (WNV) infection and reported that the induction of a specific, neutralizing IgM response early in the course of WNV infection limits viremia and viral dissemination into the central nervous system and protects against lethal infection (22). Immune IgM antibodies have also been shown to play a role in protection against yellow fever virus (23), polyomavirus (24), vesicular stomatitis virus (25, 26), herpes simplex hominis (27), and enterovirus (28) infections.

Goudsmit and colleagues generated a set of human monoclonal antibodies from human IgM+ memory B cells of volunteers who were vaccinated with seasonal influenza vaccine. One of these IgM monoclonal antibodies, CR621, conferred complete protection against lethal H1N1 and H5N1 virus infection in mice (29). In a recent study, it was demonstrated that the breadth of cross-reactivity of influenza-specific IgM-secreting cells is broader than that of their IgG counterparts, which is in agreement with our findings (30). The authors proposed that upon vaccination, some IgM memory B cells are directly differentiated into highly cross-reactive IgM-secreting plasmablasts to confer the host early line of cross-protection, while the rest of the IgM memory B cells are recruited to the secondary response. Together, these studies and ours demonstrate that IgM plays a larger role in immunity to viral infections than originally suggested.

The lifelong persistence of virus-specific IgM antibodies is intriguing. The precise role of long-lived IgM antibodies is unclear, but it is possible that IgM antibodies might provide a broader extent of cross-reactivity against related viruses. IgM antibodies might also be key in regulating IgG responses. Chen and colleagues showed that mice lacking secreted IgM antibodies exhibited impaired IgG responses (31). IgM Abs might also directly regulate B cells as these cells express FcγR, the receptor for the Fc portion of IgM antibodies. Thus, IgM-antigen (Ag) complexes might have an direct role in B cells. In support of this, mice that are deficient in FcγR upon immunization exhibited enhanced germinal center B cell and plasma cell responses (32).

The limitation of the study is that we used bulk IgM antibody fractions that are directed not only against influenza virus but also to other organisms that the animal may have encountered in its lifetime. With this, it is also difficult to map the fine specificities of the IgM antibodies: globular head versus stem binding. In addition, to carefully test cross-protection against influenza viruses, it is important to passively transfer the antibodies and perform challenge studies. The quantities necessary to perform such passive transfer experiments. Another limitation is that we do not know whether the IgM antibodies have undergone somatic hypermutation and affinity-driven selection of the higher-affinity antibody clones. We are in the process of cloning Ig heavy and light chains from individual sorted plasma cells to recreate monoclonal antibodies so that the aforementioned issues can be addressed to the fullest extent.

In conclusion, we demonstrate for the first time that following vaccination or infection, long-lived, influenza virus-specific IgM-secreting plasma cells persist in the bone marrow for a lifetime. The virus-specific IgM antibodies effectively neutralize virus in the presence of complement, and most interestingly, IgM antibodies were present in the mucosal compartment up to 18 months after the initial antigen exposure. This study raises the possibility that virus-specific IgM titers might be an important correlate of protection against influenza viruses.

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