Memory B cells in the lung participate in protective humoral immune responses to pulmonary influenza virus reinfection

Taishi Onoderaa, Yoshimasa Takahashia−1, Yusuke Yokotab,c, Manabu Atod, Yuichi Kodamae, Satoshi Hachimurab, Tomohiro Kurosakic,d, and Kazuo Kobayashia

aDepartment of Immunology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan; bResearch Center for Food Safety, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan; cLaboratory of Lymphocyte Differentiation, World Premier International Immunology Frontier Research Center, and Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka 565-0871, Japan; and dLaboratory for Lymphocyte Differentiation, RIKEN Research Center for Allergy and Immunology, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

Edited by Michel C. Nussenzweig, The Rockefeller University, New York, NY, and approved December 29, 2011 (received for review September 18, 2011)

After pulmonary virus infection, virus-binding B cells ectopically accumulate in the lung. However, their contribution to protective immunity against reinfecting viruses remains unknown. Here, we show the phenotypes and protective functions of virus-binding memory B cells that persist in the lung following pulmonary infection with influenza virus. A fraction of virus-binding B-cell population in the lung expressed surface markers for splenic mature memory B cells (CD73, CD80, and CD273) along with CD69 and CXCR3 that are up-regulated on lung effector/memory T cells. The lung B-cell population with memory phenotype persisted for more than 5 mo after infection, and on reinfection promptly differentiated into plasma cells that produced virus-neutralizing antibodies locally. This production of local IgG and IgA neutralizing antibody was correlated with reduced virus spread in adapted hosts. Our data demonstrate that infected lungs harbor a memory B-cell subset with distinctive phenotype and ability to provide protection against pulmonary virus reinfection.

B-cell memory is bipartite, consisting of both long-lived plasma cells and memory B cells. Immediate protection against re-infection is mediated by long-lived plasma cells that are present in the bone marrow and secrete antibodies in an antigen-independent fashion. Recall responses are mediated by memory B cells that rapidly proliferate and differentiate in response to antigenic stimulation (1, 2). The accessibility of memory B cells to reinfecting pathogens is, therefore, likely a significant factor in determining the effectiveness of humoral protection against reinfection. Thus, it is fundamentally important to determine the protective functions of pathogen-specific memory B cells that reside at the sites of infection after the resolution of a primary infection.

In the case of influenza virus, the initial infection and replication occur in the respiratory tract. These elicit immune responses in associated secondary lymphoid organs, e.g., mediastinal lymph nodes (MLNs), which support the initial rounds of B-cell priming that follow a pulmonary infection (3). In addition, nonlymphoid organs, including the lungs, participate in these primary responses. Indeed, after primary infection with influenza virus, infected lungs often support the development of ectopic tertiary lymphoid structures known as induced bronchus-associated lymphoid tissue (iBALT) that contain germinal centers (GCs) and plasma cells (4). Moreover, infected lungs harbor the precursors of virus-binding plasma cells as revealed after in vitro stimulation of lung cells (5, 6), suggesting the existence of virus-binding memory B cells in the lungs. However, virus-binding memory B cells in the lungs have not been indentified at cellular level, thereby their phenotypic and functional characterization is still lacking.

In this study, we characterized the phenotypes and functions of class-switched, influenza-specific B cells in the lungs. We show that a fraction of class-switched, influenza-specific B cells in the lungs possess a memory phenotype, persist for a long period, and respond to virus reinfection by promoting rapid viral clearance. Our data demonstrate that local tissues are important sites for the maintenance and reactivation of protective humoral memory responses.

Results

Infection with Influenza Virus Induces Antigen-Specific Memory-Like B-Cell Population in Lung. Pulmonary infection with influenza viruses induces precursors of antigen-specific, class-switched plasma cells in the lungs (5, 6); however, the phenotypes of these cells have not been determined. To characterize the phenotype and persistence of influenza-specific B cells in lungs, we labeled B cells recovered from lung tissue with recombinant hemagglutinin (rHA) conjugated to PE. Non-B cells, transitional B cells, B1 cells, and plasma cells were excluded from our analyses by colabeling with 12 mAbs specific for their surface markers (SI Materials and Methods and Fig. S1). IgM/D+ cells were also excluded from the present analysis to reduce the risk of including naïve HA-binding B cells present in the preimmune repertoire (7). This staining procedure resulted in the clear visualization of HA-binding, class-switched B cells in mice infected with the X31 influenza virus but not with other influenza virus subtypes (Fig. S1), confirming our methods’ specificity and sensitivity. Among the HA-binding IgM+/D+ lung B cells was a CD38 subset that could represent a memory B-cell population (8, 9). We first traced the numbers of both CD38+ and CD38− B cells in lung, MLN, and spleen for 160 d after a primary infection (Fig. 1 A and B). The numbers of HA-binding IgM+CD38− B cells rapidly but transiently increased in lung, MLN, and spleen. However, CD38− B cells in MLNs persisted for a longer period than those in lungs and spleens, similar to splenic or MLN GCs following vesicular stomatitis or influenza virus infection, respectively (10, 11).

HA-binding IgM+/CD38− B cells were found in the lung, MLN, and spleen, but lung CD38− B cells required more time to reach equilibrium than that required for CD38+ B cells in other organs (Fig. 1B). This process resembles the slower accumulation of plasma cells in the lungs after influenza virus infection (11) and may reflect a requirement for structural alteration and/or niche formation in the infected lungs for B-cell localization.

Author contributions: Y.T., M.A., S.H., T.K., and K.K. designed research; T.O., Y.T., Y.Y., and Y.K. performed research; T.O. and Y.T. analyzed data; and Y.T., T.K., and K.K. wrote the paper.

The authors declare no conflict of interest.

*a Direct Submission article had a prearranged editor.

1To whom correspondence should be addressed. E-mail: y-takahashi@nih.go.jp.

This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1115369109/DCSupplemental.
Once generated, HA-binding IgM/D^+ CD38^+ B cells stably persisted for 160 d after infection in all organs. Although definitive markers of murine memory B cells remain to be identified (12, 13), CD73, CD80, and CD273 (PD-L2) are expressed at higher levels on splenic memory than on naïve B cells (14, 15). It is also postulated that the proportions of CD80^+ and/or CD273^+ cells reflect the maturation of memory B cells, because CD80^+ and/or CD273^+ cells express isotype-switched, somatically mutated B cell receptors more frequently than CD80^− CD273^− cells (15). HA-binding IgM/D^+ CD38^+ B cells in the lung, MLN, and spleen expressed elevated levels of CD73, CD80, and CD273 than naïve B cells (IgD^+ CD38^− B220^+ ) from the same tissue (Fig. S2). The phenotypic similarity of these HA-binding B cells to hapten-binding memory B cells (14, 15) supports our hypothesis that HA-binding IgM/D^+ CD38^+ B cells represent a memory population. Significantly, HA-binding IgM/D^+ CD38^+ lung B cells expressed CD73, CD80, and CD273 at higher frequencies than comparable populations in the MLN and spleen (Fig. S2); however, CD80 expression in the lung and MLN did not differ significantly. Considering the slow and steady accumulation of HA-binding IgM/D^+ CD38^+ lung B cells, they may suggest that these lung B cells develop after progressive acquisition of mature memory phenotypes.

Further characterization revealed distinctive features of HA-binding IgM/D^+ CD38^+ lung B cells. These lung B cells showed elevated expression of CD69 and CXCR3, a chemokine receptor governing effector T-cell migration to inflamed lung tissue (16), compared with the comparable populations in MLN and spleen (Fig. 1C and Fig. S3). Notably, Lee et al. (17) recently suggested that CD69 regulates lung localization of CD8^+ T cells following influenza virus infection. Thus, HA-binding IgM/D^+ CD38^+ lung B cells expressed elevated levels of localization factors that direct the infiltration and residence of T cells in response to lung inflammation; however, the contribution of these to lung B-cell localization is not yet known. Together, phenotypic characterization of HA-binding IgM/D^+ CD38^+ lung B cells revealed their unique phenotypes sharing surface markers for murine memory B cells with lung localization factors. Hereafter, we putatively define HA-binding IgM/D^+ CD38^+ B-cell population as memory-like B-cell population.

After pulmonary influenza virus infection, IgA-secreting plasma cells develop in the lung concomitantly with the presence of IgA Ab in bronchoalveolar lavage fluids (BALFs) (6, 18). To know the relative distribution of virus-specific IgA^+ B cells in lung and other organs, we compared the frequencies of IgA^+ cells among HA-binding IgM/D^+ CD38^+ B cells in lung, MLN, and spleen. As expected, the memory-like B-cell population in lung expressed IgA isotype more frequently than the comparable populations in MLN and spleen; however, the average frequency of IgA^+ cells represented only 7% of the lung B-cell population (Fig. 1C and Fig. S3). The minor composition of IgA^+ cells among IgM/D^− memory-like B cells in lungs is also supported by the previous estimation of IgA:IgG ratio (~1:10) in the precursors of plasma cells in lungs (6). This result suggests that IgA switching is enhanced but is not a major event during the development of the lung memory-like B-cell population following primary infection.

**Memory-Like B-Cell Population in Lung Rapidly Differentiates into IgG- or IgA-Secreting Plasma Cells on Pulmonary Challenge.** Accelerated responses to antigen challenge are a defining feature of memory B cells. To examine whether the memory-like B-cell population in lung are indeed responding to secondary infection, we detected lung B cells proliferating shortly after virus challenge by BrdU-incorporation assay. The memory-like B-cell population in the lungs did not incorporate detectable levels of BrdU at day 80 after primary infection (labeling period: 2 d) (Fig. 2A), consistent with the maintenance as a stable B-cell population in the absence of frequently dividing precursors. In contrast, by 2–3 d after secondary infection, BrdU^+ memory-like B-cell populations appeared in the lungs and MLNs, but not in the spleens (Fig. 2B). By day 4 postinfection, HA-binding IgM/D^+ CD138^+ cells expressing plasmablasts/plasma cell markers accumulated in the lungs, and about half of these expressed IgA (Fig. 2B). ELISPOT analysis confirmed the prompt appearance of plasma cells in the lungs consisting of comparable frequencies of IgG- or IgA-secreting plasma cells (Fig. S4). These results suggest that, in response to virus reinfection, the memory-like B-cell population in lung divided and developed into plasma cells. In addition, regardless of infrequent expression of surface IgA on the memory-like B-cell populations, HA-binding class-switched plasma cells expressed IgG or IgA isotypes at similar frequencies following secondary challenge.

---

**Fig. 1.** Kinetics and surface phenotypes of HA-binding IgM/D^+ B-cell populations. (A) Cells recovered at the indicated time points after infection were subjected to flow-cytometric analysis (n=4–5). Representative flow data for HA-binding/CD38 expression by IgM/D^− B cells (Fig. S1) are shown. (B) Absolute cell number of CD38^+ and CD38^− cells within the lymphocyte gate was plotted. (C) The frequencies of cells expressing CD69, CXCR3, or IgA are plotted. In B and C, each circle represents the result for an individual mouse (lung and spleen) and two to four pooled mice (MLN). **P < 0.01.
Protective Function of the Memory-Like B-Cell Population in Lung. To examine the protective capacity of the memory-like B-cell population against reinfection, HA-binding IgM/D^CD38^+ B cells were highly purified from the lungs and spleens of the mice >2 mo after primary infection, and then transferred into scid mice together with CD4^+^ T cells isolated from the same donors. MLNs provided too few cells for adoptive transfer experiments and were not used. Accumulating evidence indicates that iBALT serves not only a site for initiating respiratory immune responses but also as a homing site for plasma cells (18, 19). Therefore, we considered that preforming iBALT structure might be required for reconstitution of local, secondary Ab responses to virus infection in adoptive hosts. To generate iBALTs in recipient mice before memory B-cell transfer, recipient scid mice were subjected to intranasal CpG treatment and i.v. transfer of spleen cells. As expected, this treatment generated peribronchial B-cell clusters 10 d later (Fig. 3 A and B). The treated mice were inoculated with challenging viruses 1 d after transfer of purified HA-binding IgM/D^CD38^+ B cells (Fig. 3C), and then we determined virus titers in BALFs 6 d after infection. Remarkably, the mice reconstituted with the memory-like B-cell population in lung significantly reduced virus titers in BALFs, whereas those given splenic counterparts were similar to controls (Fig. 3D). Moreover, the protective ability of lung memory-like B-cell population was not observed in recipient mice without pre-CpG treatment (Fig. 3E).

To address whether adoptive transfer of the memory-like B-cell population in lung accelerated the Ab production in the respiratory tract, the numbers of HA-binding lung plasma cells and the levels of anti-HA Abs in BALFs were evaluated at the same time point. Adoptive transfer of lung memory-like B-cell population generated sixfold more IgG- and IgA-secreting plasma cells in the lungs compared with splenic counterparts in a manner depending on pre-CpG treatment of recipient mice (Fig. 3 F and G). Consistent with the results in secondary challenged BALB/c mice, the frequencies of IgA-secreting plasma cells were comparable to those of IgG-secreting plasma cells. These results support the contention that the enhanced IgA response following secondary infection reflects the increased supply of IgA-secreting plasma cells from memory and not naïve B cells. In accordance with ELISPOT data, anti-HA IgG and IgA Ab titers in BALFs were elevated in the mice reconstituted with the lung B-cell population after CpG treatment but not in those given the splenic B-cell population (Fig. 3F). Together, these data indicate that HA-binding IgM/D^CD38^+ lung B cells are able to generate large amounts of Abs at the site of virus replication to confer protection more effectively than splenic counterparts. Moreover, given the successful reconstitution of local, secondary Ab responses in adoptive hosts, we conclude that HA-binding IgM/D^CD38^+ B cells represent memory B cells in both phenotypes and functions.

Protective Functions of IgG Versus IgA Abs in Respiratory Tracts. Although it is established that secretory IgA can provide protection more effectively than IgG in upper respiratory tracts (20, 21), it is not known whether IgA in lower respiratory tracts contributes to protection in the presence of IgG. To estimate the contribution of IgG and IgA Abs in BALFs to virus neutralization, we first determined the titers of HA-binding IgG and IgA Abs. Consistent with comparable accumulation of IgG- and IgA-secreting plasma cells in the lungs (Figs. 2 and 3), BALFs in secondary challenged mice contained both HA-binding IgG and IgA Abs (Fig. 4A). The relative contributions of IgG and IgA to virus neutralization were estimated using BALFs depleted of either IgG or IgA Abs by affinity chromatography. We observed that removal of either IgG or IgA reduced virus-neutralizing activity to partial, but not complete, reduction in activity. Moreover, removal of both IgG and IgA reduced virus-neutralizing activity to levels close to the detection limit. These data suggest that concomitant production of IgG and IgA Abs is required to achieve maximum neutralization activity.

**Virus Particles Enhance Secondary IgA Response by Recruiting IgA-Secreting Plasma Cells from IgA^− Memory B Cells.** To explore the mechanisms for enhanced IgA secretion following secondary infection, we first examined whether the enhanced IgA response depends on intranasal infection by live viruses. Similar to Fig. 3, scid mice were reconstituted with either lung or splenic memory B cells and then subjected to i.p. boosting with formalin-inactivated...
viruses, which are defective in replication but retain structure of virus particles. Reactivated lung memory B cells generated IgA-secreting plasma cells at increased frequencies of 40% among class-switched plasma cells (Fig. 5A and B). Although the numbers were lower than those of lung memory B cells, reactivated splenic memory B cells also increased the frequencies of IgA-plasma cells in the lungs of CpG-treated mice (Fig. 5A) or untreated (Fig. 5B) CB17-scid mice were reconstituted with either lung or splenic memory-like B-cell population (3,000 cells per mouse) together with splenic B cells from naïve mice and CD4+ T cells from infected mice. Splenic naïve B cells were added to prevent the loss of small numbers of memory-like B-cell population after sorting. On day 6 postinfection, virus titers in BALFs were determined. (F and G) HA-binding IgG and IgA-secreting plasma cells in the lungs of CpG-treated (F) or untreated (G) mice were enumerated by ELISPOT. (H) HA-binding IgG and IgA Ab titers in BALFs of CpG-treated mice were estimated by ELISA. In D-H, each circle represents the result for an individual mouse. *P < 0.05; **P < 0.01.

Discussion

Here, we have demonstrated that infected lungs harbor antigen-specific, class-switched B cells expressing CD38, CD73, CD80, and CD273, which are the most reliable surrogate markers for murine memory B cells. Moreover, two lines of evidences support that they promptly differentiate into mature plasma cells upon reinfection. First, lung memory B cells started to proliferate on day 2 after reinfection, when memory B cells in MLNs and spleens were still inert (Fig. 2A). Second, transferred lung memory B cells were able to generate IgG and IgA-secreting plasma cells in the lungs of adopted hosts after reinfection. Together, we conclude that antigen-specific memory B cells localize and respond to antigenic challenge in the lungs following pulmonary influenza virus infection.

The most striking feature of lung memory B cells is their ability to reduce virus spread in lower respiratory tracts of recipient mice. Given that lung memory B cells provided local IgG and IgA Ab in recipient mice following virus challenge, it is conceivable that both isotypes contribute to virus neutralization in situ. This idea is supported by the fact that virus neutralization depends on both IgG and IgA Abs in vitro (Fig. 4). Dispensability of IgA for providing protection in the lower respiratory tracts was previously suggested as vaccinated, IgA-deficient mice were found to be fully resistant to pulmonary influenza virus infection (22). However, IgA-deficient mice might use compensatory mechanisms that lead to altered expression of other Ab isotypes (23, 24). Given that systemic injection of anti-HA IgA
mAbs was effective to prevent the initial infection in lung airways (25), we prefer the idea that both IgG and IgA Abs in lung airways contribute to virus neutralization.

The mechanism underlying the ability of lung memory B cells to supply local IgG and IgA Abs remains an important question to be addressed. Lung memory B cells express CD69 and CXCXR3, possible mediators of lung localization. Thus, one possibility is that transferred lung memory B cells home back to the lung, wherein they can generate IgG- and IgA-secreting plasma cells at virus replication sites. This possibility is supported by requirements for intranasal CpG treatment before memory B-cell transfer. CpG-induced inflammation would trigger the local expression of several ligands (e.g., CXCL9 and CXCL10) for chemokine and/or homing receptors expressed on lung memory B cells. However, without pre-CpG treatment, the reduction of virus spread was not observed in the mice reconstituted with lung memory B cells (Fig. 3 D–G). The alternative possibility is that transferred lung memory B cells home to the MLNs, where they supply plasma cells in the lungs upon reinfection. Although we made tremendous efforts for in vivo tracing of transferred memory B cells, our attempts were unsuccessful due to a paucity of memory B cells.

B-cell intrinsic recognition of intact viruses is often hampered by the tissue tropisms of virus replication in nonlymphoid organs. Following pulmonary influenza virus infection, lung localization of memory B cells is one way to facilitate B cell intrinsic recognition for shaping the magnitude and quality of protective effector functions. Better understanding of the generation, maintenance, and reactivation of memory B cells in lung provides important insights for the development of vaccines for protection against influenza virus and other respiratory pathogens.

Materials and Methods

Mice and Viruses. Mice and viruses used in this study are described in SI Materials and Methods.

Cell Preparation and Flow Cytometry. Lung cells were isolated by Percoll gradient centrifugation after digestion with collagenase D and DNase I. Single-cell suspensions from lungs, MLNs, and spleens were stained with mixtures of biotinylated mAbs, followed by fluorescence-conjugated mAbs. BrdU-labeled cells were detected by using BrdU Flow kit (BD Biosciences). Stained cells were analyzed or purified using FACS Canto II or FACS Aria (BD Biosciences). Detailed methods are included in SI Materials and Methods.

Quantification of Anti-HA Abs and Plasma Cells. HA-binding Abs and plasma cells were quantified by ELISA and ELISPOT using rHA as coating antigens and anti-mouse IgG- or IgA Abs as secondary Abs. Virus-neutralization Ab titers were quantified by microneutralization assay using MDCK cells and X31 virus (105 TCID50) (26). Detailed methods are included in SI Materials and Methods.

Statistical Analyses of Data. Statistical significance was determined by an unpaired two-tailed Student t test. P < 0.05 were considered significant.

ACKNOWLEDGMENTS. We thank Dr. G. Kelsoe (Duke University) for critical reading of the manuscript; Dr. T. Tsutaya (Tokyo Medical and Dental University) for providing the X31 virus; and Ms. E. Watanabe, E. Izumiyama, and K. Fukuhara for technical assistance. This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology in Japan (to Y.T. and T.K.), the Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (to Y.T. and T.K.); and the Emerging and Re-Emerging Infectious Diseases and Regulatory Science of Pharmaceuticals and Medical Devices of the Ministry of Health, Labour and Welfare in Japan (to Y.T. and K.K.).


Supporting Information

Onodera et al. 10.1073/pnas.1115369109

SI Materials and Methods

Mice and Viruses. Female BALB/c and CB17-scid mice were maintained under specific pathogen-free conditions and used in experiments at the age of 7–12 wk. The influenza A viruses X31 (H3N2) and A/New Caledonia/20/99 (H1N1) were grown in a 10-d-old embryonated hen eggs and were purified through a 10–50% sucrose gradient as described (1). BALB/c mice, anesthetized by i.p. injection with sodium pentobarbital, were inoculated intranasally with 0.1 lethal dose50 (LD50) for the primary challenge or 10 LD50 for the secondary challenge in a volume of 20 μL. In some experiments, CB17-scid mice were injected with 15 μg of ODN-DNA bearing CpG-motifs intranasally (2) and 1 × 106 BALB/c splenocytes intravenously. All mice were used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases, Japan.

Cell Preparation. Single-cell suspensions were prepared from spleens and MLNs in DMEM containing 2% (vol/vol) FCS, 2 mM t-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5 × 10−5 M β-mercaptoethanol as described (3). For isolation of lung cells, mice were perfused with PBS in the right ventricle to clear the lungs of blood. Lungs were minced and incubated at 37 °C for 30 min in 1 mg/mL collagenase D (Roche) and 10 μg/mL DNase I (Roche) and then disrupted between the frosted ends of glass slides. After 70%/44% (vol/vol) Percoll gradient centrifugation, the cells between the 70%/44% interface were recovered.

Preparation of Recombinant HA Protein. cDNA encoding 1–529 amino acid residues of X31 HA was amplified by PCR using high fidelity DNA polymerase (KOD-Plus-Ver.2, Toyobo). During the PCR amplification, 6× His tag sequence was attached to C-terminal sequence of the truncated HA. BamHI I and NotI sites were also incorporated into its N- and C-terminal sequence, respectively. Amplified cDNA was inserted into cloning site of pBacPAK8 expression vector (Clontech) and transfected into Sf21 (Spodoptera frugiperda) insect cells. Recombinant HA proteins tagged with 6× His were produced in Sf21 cells adapted to FCS-free conditions and purified from culture supernatant using TALON columns (Clontech), in accordance with the manufacturer’s protocol.

Abs. Anti-FcyRII/III (2.4G2) mAbs were purified in our laboratory. Recombinant HA was conjugated with PE in our laboratory. Anti-IgM (II/41)-biotin, anti-IgD (11-26c)-biotin, anti-B220/CD45R (RA3-6B2)-biotin, anti-CD93 (AA4.1)-biotin, anti-Thy1.2/CD90.2 (53-2.1)-biotin, anti-CD3 (145-2C11)-biotin, anti-Gr-1 (RB6-8C5)-biotin, anti-F4/80 (BM8)-biotin, anti-CD11b (M1/70)-biotin, anti-CD11c (N418)-biotin, anti-CD117 (2B8)-biotin, anti-CD8α (53-6.7)-biotin, anti-TER-119 (TER-119)-biotin, anti-CD5 (53-7.3)-biotin, anti-CD4 (GK1.5)-biotin, anti-CD27 (122)-FITC, and anti-IgD (11-26c)-PE were purchased from eBioscience. Anti-CD43 (S7)-biotin, anti-CD138 (281-2)-biotin, anti-CD19 (1D3)-biotin, and streptavidin (SA)-PE-TexasRed were purchased from BioLegend. SA-Tricolor/AlexaFluor555 and Image-iT FX Signal Enhancer were purchased from Invitrogen. Goat anti-mouse IgG2a-alkaline phosphatase (AP), goat anti-mouse IgG2b-AP, goat anti-mouse IgM-AP, goat anti-mouse IgA-horseradish peroxidase (HRP), goat anti-mouse IgG1-HRP, goat anti-mouse IgG3-HRP, and goat anti-mouse IgG-HRP were purchased from Southern Biotech.

Flow Cytometry and Cell Sorting. Cells were pretreated with anti-FcγRII/III mAb and then incubated with biotinylated mAbs against IgM, IgG, CD43, CD138, CD5, CD93, CD90, CD3, F4/80, CD11b, CD117, CD11c, Gr-1, and TER-119 for memory B cells and IgM, IgD, CD90, CD3, F4/80, and Gr-1 for plasma cells. This was followed by staining with anti-B220-Pacific Blue, rHA-PE, SA-Tricolor, propidium iodide (PI), and anti-CD38-APC or anti-B220-Pacific Blue, anti-CD38-FITC, rHA-PE, SA-PE-TexasRed, and PI (memory B cells). Plasma cells were stained by anti-B220-Pacific Blue, anti-IgA-FITC, rHA-PE, SA-PE-TexasRed, PI, and anti-CD138-APC. BrdU-labeled cells were detected by using BrdU Flow Kit (BD Biosciences). For sorting memory B cells, cells that were stained with biotinylated Abs were depleted using a MACS column (Miltenyi Biotech), followed by staining with fluorescence-conjugated reagents. Stained cells were analyzed or purified using FACS Canto II or FACS Aria (BD Bioscience). A total of >200,000 events were collected, and data were analyzed with FlowJo software (TreeStar).

Detection of Anti-HA Abs and Plasma Cells. For detection of anti-HA plasma cells by ELISPOT, nitrocellulose membranes were coated with 20 μg/mL rHA, and cells were incubated on the membranes for 2 h at 37 °C. After the cells were washed off, the membranes were incubated with anti-mouse IgG2a-AP/anti-mouse IgA-HRP, anti-mouse IgG2b-AP/anti-mouse IgG1-HRP, or anti-mouse IgM-AP/anti-mouse IgG3-HRP. AP and HRP activities were visualized as described (3). For detection of anti-HA Ab titers, ELISA was performed using anti-mouse IgG-HRP or anti-mouse IgA-HRP as described (1). IgG and IgA was removed from BALFs by passage through Sepharose columns conjugated to goat anti-mouse IgG and IgA, respectively. Virus-neutralization Ab titers in BALFs were determined by microneutralization assay using MDCK cells (1). In brief, serially diluted BALFs were incubated with X31 virus (100 TCID50) for 30 min at 37 °C and then added to MDCK cells together with acetyltyrpsin (Sigma). After 3-d incubation, the dilution at which 50% of the cultures were protected from infection were determined and plotted as virus-neutralization Ab titers.

Histology. Lung tissues were recovered after intratracheal injection of 50% OCT compound in PBS. After freezing, sections (5 μm) were deposited on slides and fixed in ice-cold acetone. For immunohistochemistry, sections were pretreated with Image-iT FX Signal Enhancer (Invitrogen). Endogenous biotin was blocked using a streptavidin/biotin blocking kit (Vector Laboratories). Sections were then stained with anti-CD90-biotin, followed by anti-B220/AlexaFluor647, SA-AlexaFluor555, and DAPI. Stained sections were scanned under a confocal laser-scanning microscope LSM 510 (Carl Zeiss).

Adaptive Cell Transfer. B cells and CD4+ T cells were purified from pooled spleens using a MACS system with biotinylated mAbs against CD3, CD90, CD4, F4/80, Gr-1, CD11b, CD43, and CD138 (B cells) and CD19, B220, IgM, IgD, F4/80, Gr-1, CD11b, and CD8 (CD4+ T cells), followed by SA-microbeads (Miltenyi Biotech). B cells (2 × 106 per head) from naïve mice and CD4+ T cells (1 × 106 per head) from infected mice were i.v. injected into CB17-scid mice with or without sorted memory B cells. On the next day, the recipient mice were intraperitoneally
boosted with inactivated viruses (20 μg) or intranasally infected with X31 (5 LD_{50}).

**Single Cell RT-PCR.** Single HA-binding IgM/D⁺IgA⁺CD38⁺B220⁺ cells or HA-binding IgM/D⁺IgA⁺CD38⁺B220⁺ cells was directly sorted into 10 μL H₂O containing 50 ng carrier RNA (Qiagen). RT-PCR mixture (OneStep RT-PCR kit, Qiagen) was prepared to include primer pairs for β-actin and J_{H}-C_{α} and dispersed at 15 μL per well. Primers used for PCR were β-actin sense 5’-AC-TATTGGCAACGAGCGGTTC, and β-actin antisense 5’-CCAGGCTACATCATCAGCGG, and C_{α} antisense 5’-CCCAGGTCACATTCCATCGTGCG. One-step RT-PCR was performed by 1 cycle of 30 min at 50 °C, 1 cycle of 15 min at 95 °C, 40 cycles of 10 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C. The PCR products were separated by a 1.5% agarose gel electrophoresis and the samples including DNA bands of β-actin (284 bp) and J_{H}-C_{α} (155-160 bp) were judged as positive for J_{H}-C_{α}, and those including only β-actin as negative.

Fig. S2. Surface expression of memory markers in HA-binding IgM/D−CD38+ B cells in lung, MLN, and spleen. (A) Cells were recovered from pooled organs (n > 3) at day 70–80 after infection and subjected to flow-cytometric analysis. Surface expression of CD73, CD80, and CD273 in HA-binding IgM/D−CD38+ B220+ cells (solid) and IgD−CD38+B220+ cells (dashed) is shown. (B) The frequencies of positive cells from three independent experiments are plotted. Error bars indicate SD. *P < 0.05; **P < 0.01.
Fig. S3. Surface expression of CD69, CXCR3, and IgA in HA-binding IgM/D~CD38~ B cells in lung, MLN, and spleen. Cells were recovered from pooled organs (n > 3) at day 70–80 after infection and subjected to flow-cytometric analysis. Representative flow data for CD69, CXCR3, and IgA expression in HA-binding IgM/D~CD38~ B cells are presented.

Fig. S4. Quantification of HA-binding IgG- or IgA-secreting plasma cells after primary and secondary infection by ELISPOT. (A) The numbers of HA-binding IgG⁺ and IgA⁺ plasma cells in the lungs were counted by ELISPOT at the indicated time points after primary and secondary infection. Histograms represent mean ± SD (n = 4). (B) The frequencies of IgA⁺ cells among class-switched plasma cells are shown.
Fig. S5. Single-cell RT-PCR analysis for the detection of rearranged JH-Cα mRNA. (A) Lung cells were recovered from the mice (n = 4) at day 65–82 after infection and IgA+ and IgA− cells among HA-binding IgM+CD38−B220− populations were sorted into 96 well plates at 1 cell per well. (A, B) One-step RT-PCR was performed to amplify β-actin (284 bp) and rearranged JH-Cα mRNA (155-160 bp) in the same tubes from IgA+ (A) and IgA− (B) cells and PCR product was separated on 1.5% agarose gel. (C) The frequencies of JH-Cα+ among β-actin+ wells were calculated from 72 (IgA+) and 374 (IgA−) single-sorted cells.