An Early CD4+ T Cell–dependent Immunoglobulin A Response to Influenza Infection in the Absence of Key Cognate T–B Interactions

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Abstract

Contact-mediated interactions between CD4+ T cells and B cells are considered crucial for T cell–dependent B cell responses. To investigate the ability of activated CD4+ T cells to drive in vivo B cell responses in the absence of key cognate T–B interactions, we constructed radiation bone marrow chimeras in which CD4+ T cells would be activated by wild-type (WT) dendritic cells, but would interact with B cells that lacked expression of either major histocompatibility complex class II (MHC II) or CD40. B cell responses were assessed after influenza virus infection of the respiratory tract, which elicits a vigorous, CD4+ T cell–dependent antibody response in WT mice. The influenza-specific antibody response was strongly reduced in MHC II knockout and CD40 knockout mice. MHC II–deficient and CD40-deficient B cells in the chimera environment also produced little virus-specific immunoglobulin (Ig)M and IgG, but generated a strong virus-specific IgA response with virus-neutralizing activity. The IgA response was entirely influenza specific, in contrast to the IgG2a response, which had a substantial nonvirus-specific component. Our study demonstrates a CD4+ T cell–dependent, antiviral IgA response that is generated in the absence of B cell signaling via MHC II or CD40, and is restricted exclusively to virus-specific B cells.

Key words: B lymphocytes • T lymphocytes • antibody formation • immunoglobulin A • mucosal immunity

Introduction

The standard model for the induction of T-dependent Ab responses to protein Ags commences with Ag uptake by professional APCs, such as DCs, which migrate to secondary lymphoid tissues and present peptide fragments of processed Ag on their surface in the context of MHC class II (MHC II) molecules (1). Specific CD4+ T cells are activated and undergo clonal expansion after TCR recognition of peptide/MHC II on the APC, and the subsequent binding of costimulatory molecules, such as CD28, on the T cell with ligands on the APC (2). Meanwhile, Ag-specific naive B cells internalize and process Ag bound by surface Ig receptors, leading to surface presentation of antigenic peptides in the context of MHC II molecules. The T help necessary to drive the B cell response is delivered by the expanded population of specific CD4+ T cells, and is initiated by TCR engagement of peptide/MHC II on the B cell. Subsequently, up-regulated CD40 ligand (CD40L) on the T cell delivers an essential proliferative signal to the B cell via CD40, and T cell–derived cytokines drive B cell proliferation and direct Ig isotype switching (3–6).

In this model, cognate, Ag-specific recognition of B cells by T helper cells is a requirement for B cell proliferation and differentiation, but this imposes a significant delay on humoral responses. The frequencies of Ag-specific T and B cells are low at the time of initial Ag encounter, and the expectation is that specific CD4+ T cell numbers must be

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Abbreviations used in this paper: AFC, Ab-forming cell; ALP, alkaline phosphatase; CD40L, CD40 ligand; GC, germinal center; i.n., intranasal(ly); MDCK, Madin-Darby canine kidney; MHC II, MHC class II; MLN, mediastinal LN.

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expanded to generate sufficient cells to permit the likelihood of cognate T–B interactions. This scenario appears inconsistent with the strong humoral responses that are rapidly generated against many pathogens (7–9). It has been proposed (10) that cognate T–B interactions might be facilitated by an early, T cell–independent phase of B cell proliferation and IgM secretion driven by repetitive antigenic structures.

The rapidity of humoral responses to infection may also be due in part to the ability of activated T cells to provide bystander or nonspecific help for B cell responses. Such help is independent of cognate, Ag-specific T–B interactions, and might be mediated by secreted cytokines or nonspecific molecular interactions between adjacent cells. There is considerable in vitro evidence that in the presence of appropriate B cell activation signals, the cytokine products of activated T cells will promote B cell proliferation and the production of switched Ab isotypes (11). However, the significance of bystander help in vivo is not known. It is noteworthy that DCs have been shown to retain Ag in a form that is recognized by B cells (12) and also provide signals that direct isotype switching in T-dependent humoral responses (12, 13). The precise contribution of T cells in this situation is uncertain (14), and a role for bystander help by T cells should be considered.

This study was initiated to assess the physiological relevance of bystander help for the B cell response to influenza virus infection in mice. Radiation BM chimeras were constructed in which virus-specific $CD4^+$ T cell activation occurred, but cognate, Ag-specific recognition of B cells by $CD4^+$ T cells was prevented by the absence of MHC II expression on B cells. Thus, $CD4^+$ T cell help for B cell responses could only be delivered through alternative, non-Ag–specific cognate interactions, or via soluble factors. The results of this analysis lead us to apply the chimera approach to determine whether activated $CD4^+$ T cells could also drive virus-specific Ab responses in the absence of signaling to B cells via CD40. In parallel studies, the radiation BM chimeras were used to investigate the requirements for nonvirus-specific Ab induction by influenza virus. Collectively, our findings point to a mechanistic distinction between the early, influenza-specific IgA response, and the IgM and IgG responses.

Materials and Methods

Mice. The C57BL/6J (B6) and C57BL/6J-*Igh*^{a} Thy1^a Gpi1^a (B6-*Igh*^{a}) mice, and mice deficient for CD40 (B6.129P2-*Tnfrsf5*^{tm1Kik}; reference 15) or both the αβ and γδ T cell receptor (B6.129P2-*Tcrb*^{tm1Mom} Tcrd^{tm1Mom}; reference 16) were purchased from The Jackson Laboratory. MHC II–deficient (I-A$^{b-/-}$) mice (17) and B cell–deficient C57BL/6-Igh-6 (μMT) mice (18) were bred at Charles River Laboratories.

Radiation BM chimeras in which all B cells were deficient in the expression of MHC II were generated by i.v. injection of equal numbers of T cell–depleted I-A$^{b-/-}$ and μMT BM cells ($1–2 \times 10^7$ total injected cells) into 8–10-wk-old B6-*Igh*^{a} mice that had been lethally irradiated (950 rad) 1 d previously. Chimeric mice in which all B cells were $CD40^{-/-}$ were generated in the same way, except that $CD40^{-/-}$ BM was used instead of I-A$^{b-/-}$ BM. Control chimeras with WT B cells were made using a combination of B6 and μMT donor BM. Two sets of additional control chimeras were produced by reconstituting B6-*Igh*^{a} recipients with μMT BM alone or with a mixture of μMT and B6.*Igh*^{a} BM. Recipient mice were held for at least 10 wk before virus challenge. During this period, the characteristics of peripheral blood lymphocytes were verified by flow cytometry (19). Chimeric mice were also analyzed by flow cytometry at the time of sampling. The staining reagents were FITC-conjugated mAbs to B220 (RA3-6B2), CD4 (RM4-5), CD40 (HM40-3), IgM^a (DS-1), and IgM^b (AF6-78); PE-conjugated mAbs to B220, CD8α (53-6.7), CD90.1 (OX-7), CD90.2 (53-2.1), and I-A^b (AF6-120.1); and APC-conjugated mAbs to B220 and CD3ε (145-2C11; BD Biosciences).

Mice were housed under specific pathogen-free conditions until virus infection and thereafter in BL2-level containment. Females were used in all studies and, with the exception of the BM chimeras, were infected at 8–12 wk of age. The Animal Care and Use Committee of St. Jude Children's Research Hospital approved all animal procedures.

Infection and Sampling. Mice were anesthetized with Avertin (2,2,2-tribromoethanol) given i.p., and then infected intranasally (i.n.) with $10^{6.8}$ 50% egg infectious doses of the H3N2 influenza A virus HKx31 (30 μl in PBS). Anesthetized mice were exsanguinated via the retro-orbital plexus before sampling. Nasal cavities were washed with PBS/0.1% BSA as described by Nedrud et al. (20). Centricon YM-30 centrifugal filter devices (Millipore) were used as instructed by the manufacturer to concentrate pooled nasal washings to ~5% of the starting volume. Sera and nasal washings were stored at −20°C for later determination of Ab titers. The right posterior mediastinal LN (MLN) and spleen were collected and disrupted to generate single cell suspensions (21).

In Vivo $CD4^+$ T Cell Depletion. Mice were depleted of $CD4^+$ T cells using the CD4-specific mAb GK1.5 as previously described (19).

ELISPOT Assay for Ab-forming Cells (AFCs). Influenza-specific AFCs were enumerated by ELISPOT assay. Plates were coated with purified influenza HKx31 (Charles River Laboratories) and single cell suspensions were plated and incubated as previously described (21). Replicate plates were developed with an allotype-specific set and a nonallotype-specific set of detection Abs. Biotinylated allotype-specific mAbs to IgM^a (DS-1), IgM^b (AF6-78), IgG1^a (10.9), IgG1^b (B68-2), IgG2a^a (8.3), and IgG2a^b (5.7) purchased from BD Biosciences, and biotinylated anti-IgA^a (Hy-16) and anti-IgA^b (HIS-M2) were diluted optimally in PBS plus 2% FCS and added to the plates. After overnight incubation at 4°C, plates were washed and incubated with peroxidase-labeled goat anti–biotin (Vector Laboratories) at 5 μg/ml in PBS-Tween (0.05%)-FCS (1%) for 1–2 h at room temperature. Plates were washed thoroughly and incubated at room temperature with the developing substrate 3-amino-9-ethylcarbazole (Sigma-Aldrich) to allow spot formation. The nonallotype-specific reagents, alkaline phosphatase (ALP)-conjugated goat anti–mouse Abs with specificity for IgM, IgA, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Inc.), were used in combination with the substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) to generate spots (21). Plates were washed and dried after optimal spot development, and spots representing individual AFCs were counted using an Olympus SZH Stereozoom microscope. Comparable results were obtained when AFCs producing a particular isotype were detected using either the allotype-specific mAb, or the nonallotype-specific goat anti–mouse Ab. Al-

though Abs detected using the anti-IgG2a^b mAb or the nonallotype-specific anti-IgG2a reagent are referred to as IgG2a, it should be noted that IgG2a expressed by B6 mice is now recognized as a distinct isotype designated IgG2c (22).

Total AFC numbers were determined using assays specific for the Igh^b allotype. Plates were coated with mAbs to IgA^b (HIS-M2) or IgG2a^b (5.7; BD Biosciences), and bound Abs were detected using ALP-conjugated goat anti–mouse IgA or IgG2a (Southern Biotechnology Associates, Inc.), respectively. Spots were visualized with the 5-bromo-4-chloro-3-indolyl phosphate substrate.

ELISA for Virus-specific Ab. Influenza-specific Ab levels in sera and nasal washes were determined by ELISA (21) using plates coated with 0.5 μg/well of purified, detergent-disrupted influenza HKx31 (Charles River Laboratories). Bound Ab was detected with ALP-conjugated goat anti–mouse Abs specific for IgM, IgA, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Inc.), followed by *p*-nitrophenylphosphate substrate (Sigma-Aldrich). Influenza-specific Ab concentrations in arbitrary units per milliliter were calculated from standard curves constructed using HKx31-immune serum (for IgA) or HKx31-specific mAbs (provided by R. Webster, St. Jude Children's Research Hospital, Memphis, TN) representing the IgM, IgG1, IgG2a, IgG2b, and IgG3 isotypes.

Virus Neutralization Assay. Madin-Darby canine kidney (MDCK) cells in MEM (Invitrogen) containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 5% FCS were grown in flat-bottom 96-well tissue culture plates (Sarstedt). Influenza A virus HKx31 was diluted in MEM supplemented with L-glutamine, antibiotics, 1 μg/ml TPCK-trypsin (Sigma-Aldrich), and 0.3% BSA to give 4×10^2 50% tissue culture infectious dose/ml, and then mixed with equal volumes of nasal wash concentrates. After incubation for 2 h at 37°C in a 5% CO_2 atmosphere, the virus/sample mixtures were added to washed MDCK monolayers in the 96-well plates. Plates were incubated for 3 d at 37°C in a 5% CO_2 atmosphere, and wells that were positive for virus growth were identified by hemagglutination of chicken RBCs.

Assaying Virus-specific CD4$^+$ T Cell Activation. Influenza-specific CD4$^+$ T cell frequencies were determined as previously described (19). In brief, splenic CD4$^+$ T cells were enriched by negative selection and incubated in vitro with either HKx31-infected or -uninfected irradiated splenocytes. The frequency of virus-specific CD4$^+$ T cells was calculated from the number of IFN-γ–producing cells measured by ELISPOT assay after 2–3 d incubation.

Statistics. Statistical comparisons of mean values were performed using the nonparametric Mann-Whitney U test for unpaired samples.

Results

The Influenza-specific B Cell Response Is CD4$^+$ T Cell Dependent. Influenza A virus infection of the respiratory tract in mice generates a rapid and vigorous humoral response in regional LNs, particularly the MLN that drains the lower respiratory tract (8). Initial experiments characterized the Ab response elicited in the MLN and established its T cell dependence.

i.n. infection with influenza virus generated a response in the MLN characterized by the early appearance of virus-specific IgM AFCs, followed by increased numbers of cells producing virus-specific IgA and the IgG subclasses (Fig. 1 A). A strong, virus-specific IgA response developed early and peaked on day 7, whereas the near maximal virus-specific IgG response on day 7 was sustained for at least 2 more days. The specificity of the AFC responses in the MLN of influenza-infected mice was demonstrated in parallel assays using plates coated with Sendai virus Ags. No spots representing IgA or IgG AFCs developed on the Sendai-coated plates, and only ~3% of the IgM AFCs induced by influenza infection were cross-reactive (not depicted). Subsequent experiments focused on the influenza-specific response in the MLN on days 7 and 9 after infection.

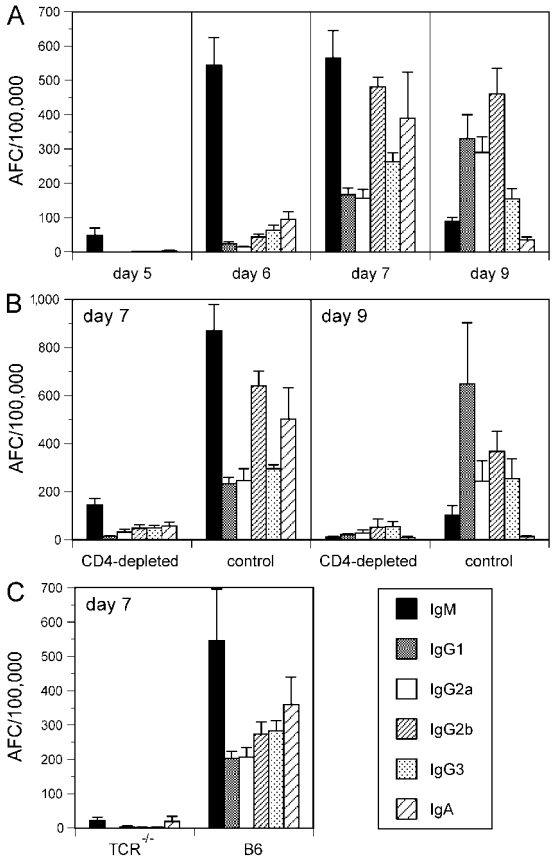

Figure 1. The influenza-specific AFC response in the MLN is CD4$^+$ T cell dependent. A kinetic analysis of the early, influenza-specific AFC response in the MLN of B6 mice was performed (A). Mice were infected i.n. with influenza virus and the ELISPOT assay was used to measure virus-specific AFC frequencies at intervals after infection. The influenza-specific AFC response in the MLN was then analyzed at selected times after infection in CD4$^+$ T cell–depleted B6 mice and mock-depleted controls (B), and in αβ and γδ T cell receptor–deficient mice (TCR$^{-/-}$) and WT B6 controls (C). Results are expressed as the number of AFCs/10^5 nucleated cells. The mean ± SE is shown for three to nine individual mice per group.

The depletion of CD4$^+$ T cells resulted in a dramatic reduction in the number of cells producing virus-specific Ab of each isotype (Fig. 1 B), indicating that the humoral response in this model is largely dependent on CD4$^+$ T cell help. Essentially identical results were obtained when the B cell response to influenza infection was compared in I-A$^{b-/-}$

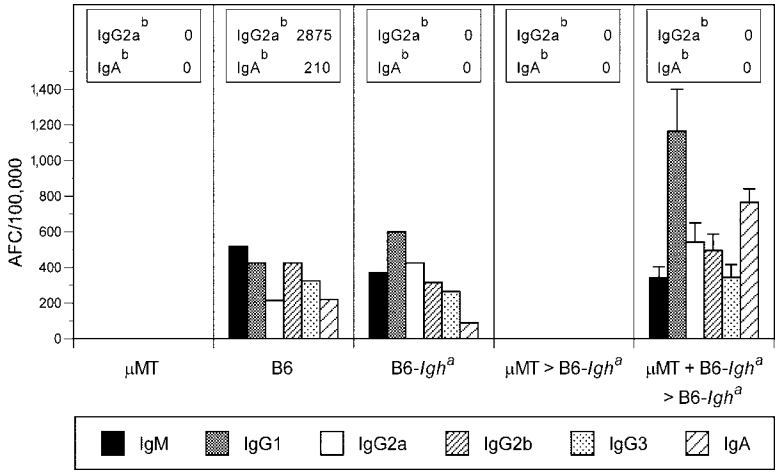

Figure 2. Cells derived from μMT BM do not produce Abs in response to influenza infection. Mice were infected i.n. with influenza virus and sampled after 7 d. Virus-specific AFC frequencies in the MLN are shown for B cell–deficient (μMT), B6, and B6-*Igh*^{a} mice, and the radiation BM chimeras μMT → B6-*Igh*^{a} and μMT plus B6-*Igh*^{a} → B6-*Igh*^{a}. The numbers of cells in MLN cell suspensions producing virus-specific IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA were determined by nonallotype-specific ELISPOT assays. In parallel, total IgG2a^b and IgA^b AFC numbers were determined by ELISPOT assays specific for the Igh^b allotype (the allotype of μMT and B6 mice). Results are expressed as the number of AFCs/10^5 nucleated cells. The mean ± SE is shown for five individual mice in the mMT group and the chimera groups. B6 and B6-*Igh*^{a} results are for pools of two mice.

mice (CD4$^+$ T cell–deficient) and WT controls (unpublished data). The virus-specific Ab response was almost completely absent in mice lacking all T lymphocytes (Fig. 1 C).

Bystander CD4$^+$ T Cell Help Contributes Only to the Virus-specific IgA Response. Having established the CD4$^+$ T cell dependence of the early B cell response to influenza infection, the question was whether a component of this essential T cell help could be delivered in bystander fashion without classical cognate, Ag-specific recognition of B cells. To address this question, radiation BM chimeras were constructed by reconstituting lethally irradiated recipient mice with a combination of BM from I-A$^{b-/-}$ mice and MHC II$^{+/+}$ B cell–deficient (μMT) mice. The chimeric mice would thus carry MHC II$^{-/-}$ B cells and MHC II$^{+/+}$ DCs and macrophages necessary for normal CD4$^+$ T cell activation. Effective priming of specific CD4$^+$ T cells does not require MHC II–expressing B cells (23), and can even be achieved in the complete absence of B cells when influenza virus infection is the stimulus (24). Control WT chimeras were constructed using donor BM from B6 mice and μMT mice. Congenic, Ig heavy chain allotype-disparate WT mice with normal lymphoid tissue microarchitecture were used as BM recipients so that allotype-specific single cell assays could be used to distinguish Ig production by donor-derived (Igh^b) and host-derived (Igh^a) cells.

Influenza infection did not result in a virus-specific Ab response in the MLNs of μMT mice or mice reconstituted with only μMT BM (Fig. 2). In addition, Igh^b B cells (potentially of μMT origin) did not contribute to the strong influenza-specific response in the recipients of μMT and B6-*Igh*^{a} BM (Fig. 2). These experiments established that cells derived from donor μMT BM in the chimera system did not produce Ab in response to infection.

Flow cytometric analysis of chimeric mice before virus challenge established that circulating B cells were MHC II$^{-/-}$ in chimeras constructed with I-A$^{b-/-}$ plus μMT BM, and MHC II$^{+/+}$ in the control chimeras (Fig. 3 A). There was no evidence of residual recipient Igh^a B cells in any chimeric mice (Fig. 3 B), a result supported by phenotyping of MLN cells at intervals after virus infection (unpublished data). Analysis of MLN cells after infection also established that the frequencies of B cells (B220$^+$), CD4$^+$ T cells, and CD8$^+$ T cells were not significantly different in the chimeras with MHC II$^{-/-}$ B cells compared with the control chimeras (unpublished data).

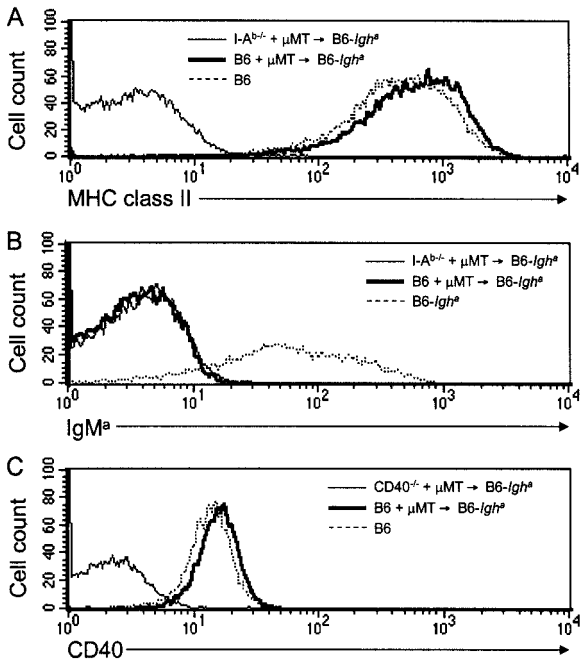

Figure 3. Flow cytometric analysis of peripheral blood B cells in chimeric mice. Peripheral blood lymphocytes were obtained from individual radiation BM chimeras I-A$^{b-/-}$ plus μMT → B6-*Igh*^{a}, CD40$^{-/-}$ plus μMT → B6-*Igh*^{a}, and B6 plus μMT → B6-*Igh*^{a}, and from B6 and B6-*Igh*^{a} mice as staining controls. The I-A$^{b-/-}$ plus μMT → B6-*Igh*^{a} chimeras have CD4$^+$ T cells, MHC II–deficient B cells of the Igh^b allotype (I-A$^{b-/-}$), and MHC II$^{+/+}$ macrophages and DCs (μMT). The CD40$^{-/-}$ plus μMT → B6-*Igh*^{a} chimeras have CD4$^+$ T cells, CD40-deficient B cells of the Igh^b allotype (CD40$^{-/-}$), and CD40$^{+/+}$ macrophages and DCs (μMT). The B6 plus μMT → B6-*Igh*^{a} WT control chimeras have WT Igh^b B cells. Residual recipient WT B cells in the chimeras would be Igh^a. Representative staining is shown for gated B cells (B220$^+$) analyzed for the expression of MHC II (A), IgM^a (B), or CD40 (C).

The activation of influenza-specific $CD4^+$ T cells in the spleen on days 7 and 9 after infection was evaluated by IFN-γ ELISPOT assay. There were insufficient cells for an analysis of both B cell and $CD4^+$ T cell responses in the MLN of individual mice. Frequencies of IFN-γ spot-forming cells per 10^6 splenic $CD4^+$ T cells (mean ± SE) were 90 ± 24 (day 7) and 166 ± 51 (day 9) for chimeras with MHC $II^{-/-}$ B cells, and 349 ± 73 (day 7) and 142 ± 65 (day 9) for WT control chimeras. IFN-γ spot-forming cells did not develop in parallel control cultures incubated with mock-infected stimulator cells (unpublished data). This analysis demonstrates that virus-specific $CD4^+$ T cells were activated in the chimeras with MHC $II^{-/-}$ B cells.

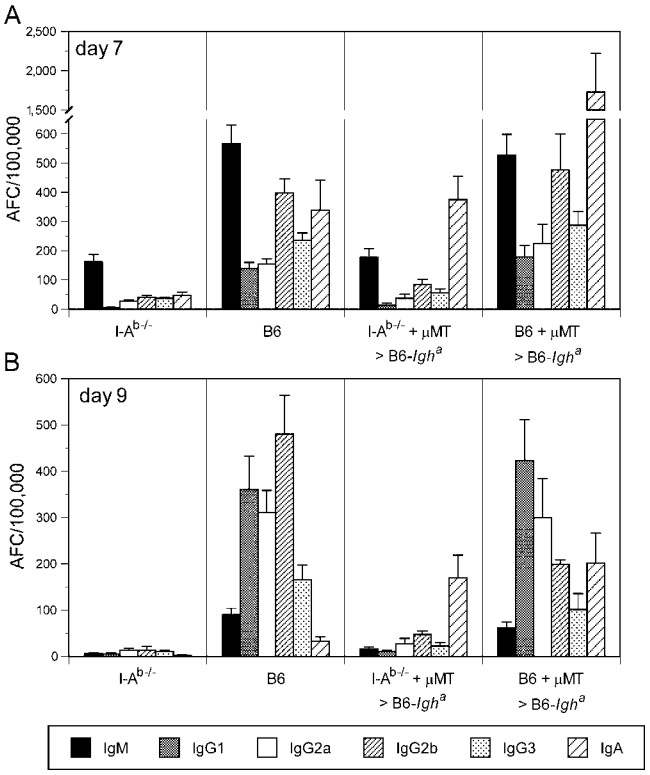

Figure 4. MHC II–deficient B cells generate a vigorous, influenza-specific IgA response in the MLN of chimeric mice. Mice were infected i.n. with influenza virus and sampled after 7 (A) or 9 d (B). Virus-specific AFC frequencies are shown for MHC II–deficient mice ($I\text{-}A^{b-/-}$), B6 mice, and the radiation BM chimeras $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a} and B6 plus μMT → B6-*Igh*^{a} described in the legend to Fig. 3. Responses were analyzed using a combination of nonallotype-specific ELISPOT assays and ELISPOT assays that were specific for the donor (Igh^b) allotype in the BM chimeras. Importantly, ELISPOT assays specific for IgM^a, $IgG1^a$, $IgG2a^a$, or IgA^a (the allotype of the recipient B6-*Igh*^{a} mice) were always applied in analyzing the responses of the BM chimeras and were invariably negative. Results are expressed as the number of AFCs/10^5 nucleated cells. The mean + SE is shown for 9–23 and 7–8 individual mice per group on days 7 and 9, respectively. B6 compared with $I\text{-}A^{b-/-}$: $P < 0.0001$ (day 7) and $P < 0.005$ (day 9) for IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA. $I\text{-}A^{b-/-}$ compared with $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a}: $P < 0.0001$ (day 7) and $P < 0.005$ (day 9) for IgA; $P < 0.05$ (days 7 and 9) for IgG2b; $P < 0.05$ (day 9) for IgM. B6 compared with $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a}: $P < 0.0001$ (day 7) for IgM, IgG1, or IgG3; $P < 0.0005$ (day 7) for IgG2a or IgG2b. B6 compared with B6 plus μMT → B6-*Igh*^{a}: $P < 0.005$ (day 7) for IgA.

Virus-specific B cell responses in the MLN were compared after influenza infection of the chimeric mice, $I\text{-}A^{b-/-}$ mice, and B6 mice (Fig. 4). Strong, virus-specific IgM (day 7) and IgG (days 7 and 9) responses were of similar magnitude in the control chimeras and B6 mice. In contrast, IgM and IgG responses were substantially diminished in the chimeras with MHC $II^{-/-}$ B cells and in $I\text{-}A^{b-/-}$ mice, indicating a requirement for cognate T–B collaboration mediated by B cell expression of MHC II. Surprisingly, on both days 7 and 9, a vigorous, virus-specific IgA response developed in the chimeras with MHC $II^{-/-}$ B cells. This response was significantly elevated compared with the response in $I\text{-}A^{b-/-}$ mice, and not significantly different from that in B6 mice. Apparently, MHC $II^{-/-}$ B cells can be driven to produce substantial virus-specific IgA in the presence of activated $CD4^+$ T cells. This bystander help contributed only to the IgA response and had little, if any, impact on IgM or IgG production by MHC $II^{-/-}$ B cells. Although there was a small but consistent increase in the IgG2b response in the chimeras with MHC $II^{-/-}$ B cells compared with $I\text{-}A^{b-/-}$ mice, the levels remained substantially lower than in B6 mice. The virus-specific IgA response in the MLN on day 7 was also enhanced in the WT control chimeras compared with B6 mice. The reason for this is unclear, but might be related to increased T and/or B cell activation because we noted perivascular lymphoid

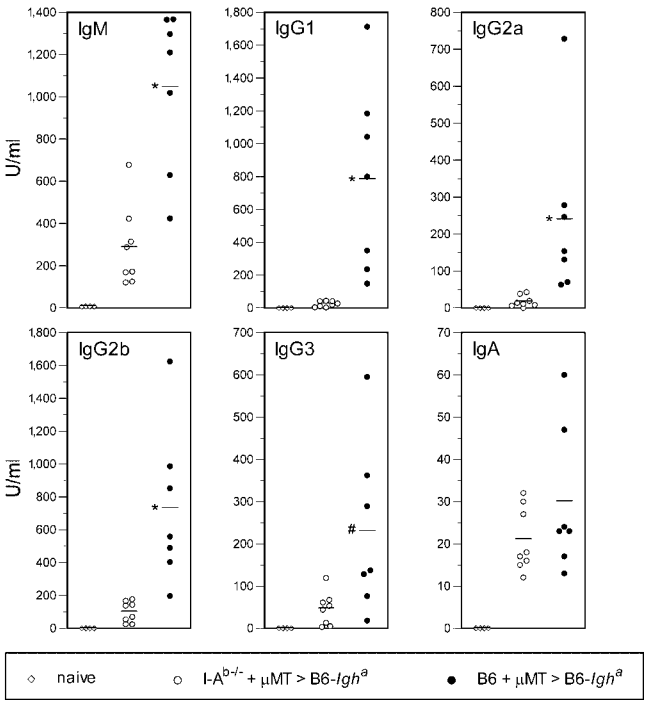

Figure 5. Serum levels of influenza-specific IgA are similar in chimeric mice with MHC $II^{-/-}$ B cells and in WT control chimeras. Sera were collected 9 d after i.n. influenza infection of the radiation BM chimeras $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a} and B6 plus μMT → B6-*Igh*^{a} described in the legend to Fig. 3. Virus-specific serum Ab titers in arbitrary units per milliliter were determined by ELISA. Titers are shown for individual mice, and a horizontal bar identifies the mean. *, $P < 0.005$ and #, $P < 0.05$ compared with $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a} chimeras.

accumulations in the lungs of uninfected control chimeras that were not present in B6 mice or in chimeras with MHC $II^{-/-}$ B cells (unpublished data).

Serum levels of virus-specific Abs in the chimeric mice on day 9 after infection (Fig. 5) were consistent with the response profiles measured at the single cell level in the MLN (Fig. 4).

Bystander CD4+ T Cell Help Drives Physiologically Relevant Virus-specific IgA Production. Influenza infection of the respiratory tract rapidly leads to the appearance of virus-specific IgA in the nasal passages and airways, where it contributes to virus clearance (25–27). Therefore, we assessed the physiological significance of virus-specific IgA production driven by bystander $CD4^+$ T cell help by examining virus-specific IgA recovered in nasal washings.

A kinetic study was undertaken comparing virus-specific IgA levels in nasal washings from I-$A^{b-/-}$ mice and B6 mice. In addition, nasal washings were collected on day 9 after infection from chimeras with MHC $II^{-/-}$ B cells and control chimeras. Virus-specific IgA was detected in the nasal wash of B6 mice as early as day 7 after infection (Fig. 6 A), correlating well with the early induction of IgA AFCs in the MLN (Fig. 1 A). Nasal wash levels in B6 mice continued to increase to a plateau by day 14. In contrast, virus-specific IgA was almost absent in the nasal washes from I-$A^{b-/-}$ mice, indicating a dependence on $CD4^+$ T cells. The levels of virus-specific IgA in nasal washings from chimeras with MHC $II^{-/-}$ B cells were significantly higher than in those from I-$A^{b-/-}$ mice, and not significantly different from the levels in the control chimeras or B6 mice. Thus, bystander $CD4^+$ T cell help was responsible for the early appearance of virus-specific IgA at a significant site of influenza virus replication (27, 28).

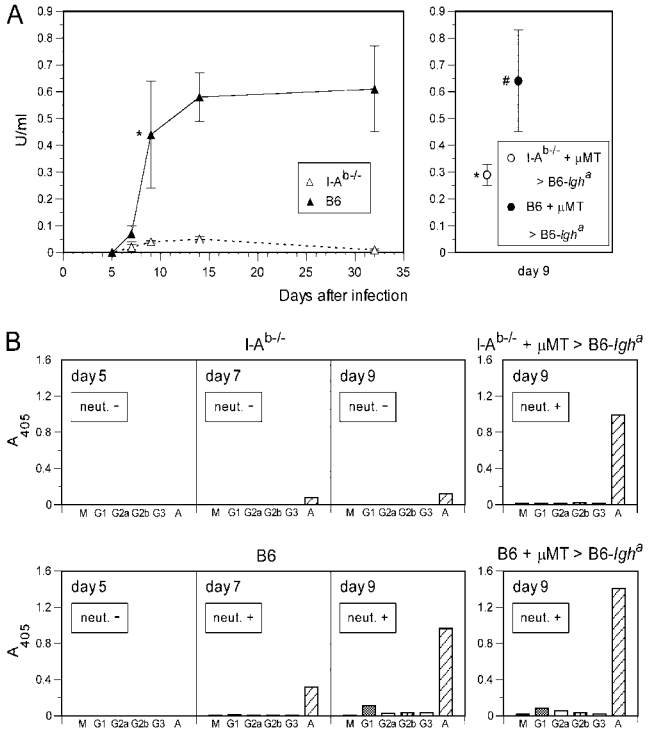

Figure 6. Analysis of IgA in nasal washings. Mice were infected i.n. with influenza virus. Nasal washings were collected at intervals from MHC II–deficient (I-$A^{b-/-}$) mice and B6 mice, and on day 9 after infection from the radiation BM chimeras I-$A^{b-/-}$ plus µMT → B6-*Igh*^{a} and B6 plus µMT → B6-*Igh*^{a} described in the legend to Fig. 3. (A) Influenza-specific IgA levels in nasal washings. Levels of virus-specific IgA in arbitrary units per milliliter were measured by ELISA. Each point represents the mean for three to six individual mice. Statistics are only shown for day 9 comparisons. *, $P < 0.01$ and #, $P < 0.05$ compared with I-$A^{b-/-}$ mice. (B) Influenza-neutralizing activity and Ab isotype composition of nasal washings. Pooled washings from individual mice were concentrated to ~5% of the starting volume. Nasal wash concentrates that completely blocked the replication of influenza virus in MDCK cells were scored as positive (+) for virus neutralizing activity (neut). Levels of influenza-binding Abs in the nasal wash concentrates were measured by ELISA and are expressed as the absorbance of a 1 in 20 dilution.

Importantly, IgA produced by MHC $II^{-/-}$ B cells in the chimera environment displayed virus-neutralizing activity. Influenza virus growth in vitro was completely blocked by day 9 nasal wash concentrates from B6 mice, WT control chimeras, and chimeras with MHC $II^{-/-}$ B cells (Fig. 6 B). In contrast, the day 9 concentrate from I-$A^{b-/-}$ mice was not neutralizing. Analysis of nasal wash concentrates for influenza-specific Abs demonstrated that IgA was the only isotype present at significant levels, and prominent IgA titers correlated well with the development of virus-neutralizing activity (Fig. 6 B). Therefore, we conclude that the neutralizing activity was a function of IgA.

T Cell Activation Drives Virus-specific IgA Production in the Absence of CD40 Signaling to B Cells. The delivery of a proliferative signal to the B cell after CD40–CD40L interaction is considered an essential requirement for T-dependent humoral responses (5). Having demonstrated the ability of MHC $II^{-/-}$ B cells to mount a vigorous, virus-specific IgA response in the context of $CD4^+$ T cell activation, we next asked whether $CD4^+$ T cell activation could also facilitate virus-specific Ab production by $CD40^{-/-}$ B cells.

Analysis of the influenza-specific AFC response in the MLN on day 7 after infection demonstrated that the production of all Ab isotypes was substantially diminished in $CD40^{-/-}$ mice compared with WT controls (unpublished data), a result consistent with previous studies of other T-dependent Ab responses in $CD40^{-/-}$ mice (15, 29). However, $CD4^+$ T cell activation by professional APCs is dependent on CD40–CD40L interactions (30), and the level of influenza-specific $CD4^+$ T cell activation was, as expected, much reduced in mice deficient in CD40 or CD40L (unpublished data).

The radiation BM chimera approach previously described was therefore modified to allow analysis of the antiviral responses of $CD40^{-/-}$ B cells in the context of $CD4^+$ T cell activation. Lethally irradiated recipient mice were reconstituted with BM from both $CD40^{-/-}$ mice and µMT mice to generate chimeras carrying $CD40^{-/-}$ B cells, and the $CD40^{+/+}$ professional APCs necessary for normal $CD4^+$ T cell activation. Flow cytometric analysis of peripheral blood lymphocytes before virus challenge (Fig. 3 C)

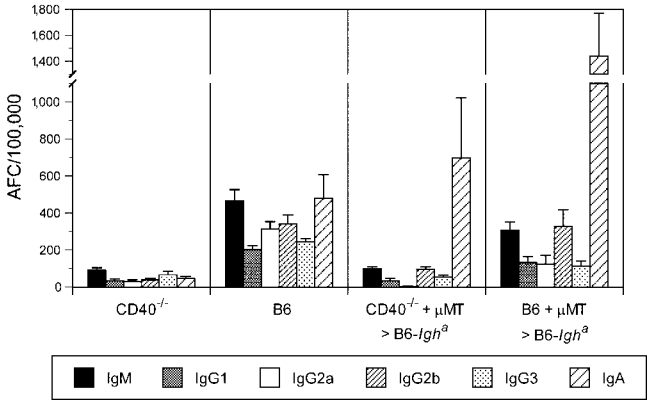

Figure 7. CD40-deficient B cells generate a vigorous, influenza-specific IgA response in the MLN of chimeric mice. Mice were infected i.n. with influenza virus and sampled after 7 d. Virus-specific AFC frequencies are shown for $CD40^{-/-}$ mice, B6 mice, and the radiation BM chimeras $CD40^{-/-}$ plus μMT → B6-*Igh*^{a} and B6 plus μMT → B6-*Igh*^{a} described in the legend to Fig. 3. The samples (from 6–15 individual mice per group) were analyzed as described in the legend to Fig. 4. B6 compared with $CD40^{-/-}$: $P < 0.0001$ for IgM, IgG1, IgG2a, or IgG2b; $P < 0.0005$ for IgA or IgG3. $CD40^{-/-}$ compared with $CD40^{-/-}$ plus μMT → B6-*Igh*^{a}: $P < 0.001$ for IgA; $P < 0.005$ for IgG2a; $P < 0.01$ for IgG2b. B6 compared with $CD40^{-/-}$ plus μMT → B6-*Igh*^{a}: $P < 0.005$ for IgM, IgG1, IgG2a, IgG2b, or IgG3.

and MLN cells 7 d after infection (unpublished data), identified only B cells of donor origin in chimeric mice reconstituted with $CD40^{-/-}$ and μMT BM. In addition, allotypic differences between donor (Igh^b) and recipient (Igh^a) mice, and the application of allotype-specific assays to analyze AFC responses in the MLN, ensured that the measured Ab responses were by donor ($CD40^{-/-}$) B cells. Analysis of splenic $CD4^+$ T cells by IFN-γ ELISPOT assay established that influenza infection activated virus-specific $CD4^+$ T cells in the chimeras with $CD40^{-/-}$ B cells (unpublished data).

Influenza-specific AFC responses in the MLN on day 7 after infection were compared for the chimeric mice, $CD40^{-/-}$ mice, and B6 mice (Fig. 7). Virus-specific IgM and IgG responses in the chimeras with $CD40^{-/-}$ B cells were substantially decreased compared with those in B6 mice and the control chimeras, and were generally not significantly stronger than in $CD40^{-/-}$ mice. In striking contrast, the chimeras with $CD40^{-/-}$ B cells produced a vigorous, virus-specific IgA response that far exceeded the response in $CD40^{-/-}$ mice, and was not significantly different from the IgA responses in B6 mice and the control chimeras. Thus, the early, virus-specific IgA response is distinct from the IgM and IgG responses and can be driven by $CD4^+$ T cell activation in the absence of CD40 signaling to B cells.

Comparative Analysis of the Virus-specific and Total Ab Response to Influenza Infection: Signaling Requirements for Nonspecific B Cell Activation. The B cell response to many virus infections includes the production of large quantities of Abs that are not specific for virus-encoded molecules (31). Studies of a range of DNA and RNA virus models indicate that nonspecific Ab induction is $CD4^+$ T cell dependent and reflects polyclonal B cell activation (21, 32, 33), but the molecular mechanisms remain unclear. The construction of chimeric mice in which $CD4^+$ T cells can be activated, but B cells are MHC $II^{-/-}$ or $CD40^{-/-}$, provided an opportunity to evaluate the ability of activated $CD4^+$ T cells to drive nonvirus-specific Ab production in the absence of key cognate T–B interactions.

A massive increase in total IgG2a AFC numbers dwarfed the virus-specific IgG2a response in WT control chimeras and B6 mice (Fig. 8 A), indicating that influenza virus, like many other viruses (31), elicits a vigorous nonvirus-specific IgG2a response. The increase in total IgG2a AFCs in chimeric mice with either MHC $II^{-/-}$ or $CD40^{-/-}$ B cells was substantially smaller than in WT mice, and no greater than the response in MHC $II^{-/-}$ or $CD40^{-/-}$ mice. Thus,

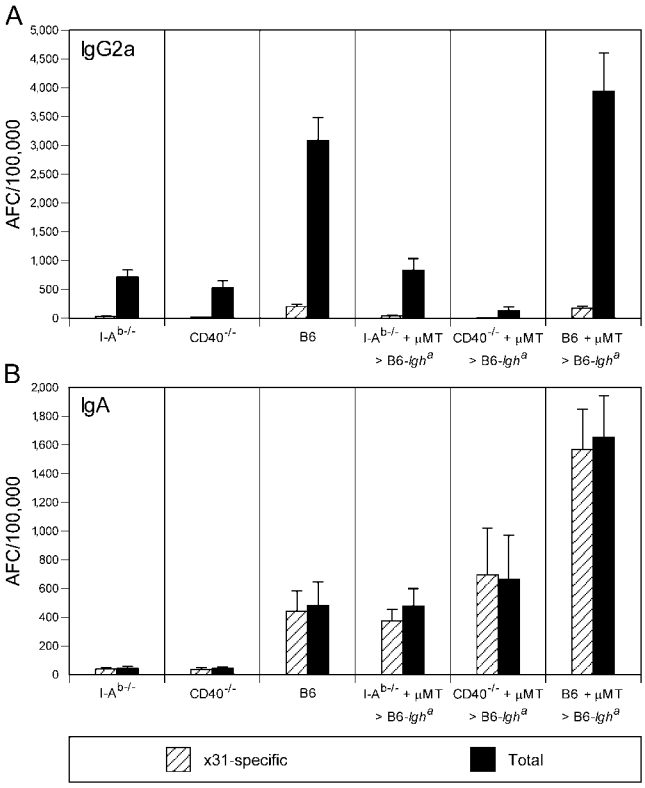

Figure 8. The influenza-specific IgG2a response, but not the IgA response, includes a nonspecific component. Mice were infected i.n. with influenza virus and sampled after 7 d. Virus-specific and total IgG2a AFCs (A) and IgA AFCs (B) in the MLN are shown for MHC II–deficient mice ($I\text{-}A^{b-/-}$), CD40-deficient mice ($CD40^{-/-}$), B6 mice, and the radiation BM chimeras $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a}, $CD40^{-/-}$ plus μMT → B6-*Igh*^{a}, and B6 plus μMT → B6-*Igh*^{a} described in the legend to Fig. 3. Virus-specific AFC numbers were determined as described in the legend to Fig. 4. Total AFC numbers were determined in parallel with the analyses presented in Figs. 4 and 7 using ELISPOT assays specific for IgG2a^b or IgA^b. The mean ± SE is shown for 6–20 individual mice per group. Baseline levels of AFCs in the MLN of uninfected mice were negligible for $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a} chimeras and 18 ± 7.4 (IgG2a) and 18 ± 7.1 (IgA) for B6 mice and B6 plus μMT → B6-*Igh*^{a} control chimeras. B6 compared with $I\text{-}A^{b-/-}$ or $CD40^{-/-}$, and B6 plus μMT → B6-*Igh*^{a} compared with $I\text{-}A^{b-/-}$ plus μMT → B6-*Igh*^{a} or $CD40^{-/-}$ plus μMT → B6-*Igh*^{a}: $P < 0.0005$ for total IgG2a AFCs.

activated $CD4^+$ T cells are insufficient to drive nonvirus-specific IgG2a production in the absence of signaling to B cells via MHC II or CD40.

In contrast, the total IgA AFC number closely approximated the virus-specific IgA AFC number in each mouse sampled (Fig. 8 B), demonstrating that the virus-specific IgA response does not have a nonspecific component. This analysis reinforces the finding that activated $CD4^+$ T cells drive extensive virus-specific IgA production by MHC $II^{-/-}$ or $CD40^{-/-}$ B cells and emphasizes the distinction between the IgA and IgG responses. In addition, a form of compartmentalization between the IgA and IgG2a responses is suggested by the observation that substantial numbers of nonvirus-specific B cells were activated to produce IgG2a, but IgA production was confined to virus-specific B cells.

Discussion

This study demonstrates that a $CD4^+$ T cell–dependent, virus-neutralizing IgA response to influenza infection can be generated in the absence of cognate interactions involving MHC II or CD40 on the B cell. Therefore, the mechanism for the production of this IgA does not fit with the generally accepted model for the delivery of $CD4^+$ T cell help in T-dependent B cell responses. In this model, key elements of cognate T–B collaboration are TCR recognition of an antigenic peptide–MHC II complex on the B cell, and the interaction of CD40L on the T cell with B cell–expressed CD40 (3, 15, 29, 34). In contrast to our findings for IgA, our observations regarding the T-dependent induction of influenza-specific IgM and IgG are consistent with this model.

In chimeric mice with activated, virus-specific $CD4^+$ T cells, substantial virus-specific IgA was produced by MHC $II^{-/-}$ or $CD40^{-/-}$ B cells (Figs. 4–7). Thus, the IgA response was driven by nonspecific or bystander help because Ag-specific recognition of B cells by T cells was not a prerequisite. A strong IgA response by $CD40^{-/-}$ B cells in the chimeras indicates that bystander help was not the result of CD40L up-regulation on activated $CD4^+$ T cells. However, we have not determined whether B cells that are doubly deficient in MHC II and CD40 expression are able to produce virus-specific IgA in the chimera model, and there might be some redundancy in the signaling requirements for IgA responses. Our studies raise the possibility that other contact-mediated interactions between T and B cells are of particular importance in the influenza-specific IgA response. Indeed, multiple molecules expressed by B cells transmit B cell activation and differentiation signals following the engagement of ligands on T cells (3).

We demonstrated influenza-specific IgA with virus-neutralizing activity in concentrated washings from the nasal passages of WT mice and also chimeric mice in which the responding B cells were MHC $II^{-/-}$ (Fig. 6 B). Thus, bystander help is sufficient to drive the rapid appearance of virus-neutralizing IgA in the nasal passages, a location where it can have a direct antiviral effect (27, 28). Our data demonstrate a correlation between a strong, virus-specific IgA response in the MLN and the presence of virus-specific IgA in the nasal passages, suggesting that IgA effector cells generated in the MLN migrate to the lamina propria of the respiratory tract. Generation of an early, virus-neutralizing IgA response in the MLN, as indicated by our studies, is supported by the finding that hybridomas derived from the MLN of mice as early as 6 d after i.n. influenza infection secreted IgA that neutralized the homologous virus strain (Coleclough, C., personal communication).

The role of the germinal center (GC) reaction in the generation of the early IgA response to influenza infection is unclear. GCs are considered critical for the affinity maturation of Ab responses, a process likely to be required if antiviral Abs are to have neutralizing capability. Our findings indicate that influenza-neutralizing IgA was generated early in the response by MHC $II^{-/-}$ B cells, and this may also have been the case for $CD40^{-/-}$ B cells. However, additional studies are required to determine whether this IgA is indeed the product of affinity maturation in responding B cells. It is generally believed that T–B interactions involving MHC II and CD40 are required for effective GC development (35). However, there is evidence that at least in some systems, optimal $CD4^+$ T cell activation can promote GC formation and function, even in the absence of signaling via CD40 (36). It also remains a possibility that the early, influenza-specific IgA response is independent of conventional GC reactions. Sealy et al. (37) recently found that i.n. influenza infection of WT mice induced IgA AFCs in the MLN before GCs could be identified.

Our analysis of total AFC numbers in parallel with measurements of the virus-specific AFC response (Fig. 8) clearly showed that nonvirus-specific IgG2a production, like the virus-specific IgG2a response, was dependent on cognate T–B interactions involving B cell–expressed MHC II or CD40. Unexpectedly, these studies demonstrated yet another distinctive feature of the IgA response, namely, that it did not include a nonvirus-specific component. This was the case even though the magnitude of the virus-specific IgA response often greatly exceeded the magnitude of the virus-specific IgG2a response in the MLN. It is surprising that large numbers of nonvirus-specific B cells can be activated to produce IgG2a, and yet none of these cells are induced to switch to IgA expression in a LN microenvironment that supports a strong, virus-specific IgA response. These findings further emphasize distinctions between the IgA and IgG responses and strongly suggest a form of compartmentalization that limits IgA switch signals only to virus-specific B cells. It remains to be determined whether a similar situation exists in the gastrointestinal tract that, unlike the respiratory tract, is a site of chronic immune stimulation and IgA induction that could potentially promote nonspecific IgA production in association with Ag-specific responses.

The cellular interactions and specific signals that are required for rapid IgA responses in the respiratory tract remain unclear. There is evidence that TGF-β1 is a key fac-

tor inducing B cells to switch to IgA expression (38, 39). However, it is likely that optimal IgA responses depend on a complex pattern of B cell stimulation involving TGF-β1 and other cytokines in combination with cross-linking of surface Ig receptors and other mitogenic signals (40). Our observation that IgA switch signals were only delivered to virus-specific B cells even though there was extensive, nonspecific B cell activation in the MLN of WT mice, indicates a requirement for close cellular interactions. Full development of the early, virus-specific IgA response was dependent on $CD4^+$ T cells, but surprisingly, classical Ag-specific T–B collaboration was not required. This raises the possibility that $CD4^+$ T cells may not provide the key IgA switch signal, but simply drive the proliferation and differentiation of IgA-expressing B cells. There is evidence that DCs may present native Ag directly to B cells and promote isotype switching, including switching to IgA expression (12, 13, 41). Recent in vitro studies by Litinskiy et al. (41) demonstrate that BLyS and APRIL, up-regulated on DCs by stimuli such as IFN-α, bind to receptors on B cells and induce class switch recombination to Cγ and Cα in the presence of IL-10 or TGF-β. However, Ab secretion requires additional B cell stimulation via B cell receptor cross-linking, and is enhanced by IL-15 or IL-2. A role for DC–B cell interactions in the generation of IgA responses in the respiratory tract is not yet established, but it might be relevant that influenza virus–infected DCs remain viable and express virion surface glycoproteins on the cell membrane for a prolonged period (42). Such an array of viral glycoproteins may provide the basis for cognate DC interactions with virus-specific B cells as well as deliver a potent stimulatory signal to the B cells via B cell receptor cross-linking. This idea is consistent with evidence that the early IgA response in the MLN after influenza infection is dependent on virus replication (8) and directed exclusively against virion envelope glycoproteins (37).

It is clear that at least the IgM and IgG2a component of the specific response to influenza infection is produced by follicular B-2 cells, and not by cells of the B-1 lineage (43). However, a role for B-1 cells in the influenza-specific IgA response has not been ruled out. Indeed, B-1 cells contribute significantly to secreted IgA in the intestine and generate specific, T cell–independent IgA responses to intestinal bacteria (44). Furthermore, we show that at least a component of the early, virus-specific IgA response to influenza infection is mechanistically distinct from the IgM and IgG responses, and this could be taken to indicate IgA production by a distinct B cell lineage with different activation requirements, such as B-1 cells (45). Nevertheless, we believe that the following points argue against a large role for B-1 cells in the influenza-specific IgA response. (a) The early, influenza-specific IgA response was $CD4^+$ T cell dependent, whereas IgA production by B-1 cells, including Ag-specific IgA responses, does not require T cell help (44). (b) A vigorous, influenza-specific IgA response developed in CBA/CaHN-*Btk*^{xid}/J mice (unpublished data), even though this strain is deficient in B-1 cells (46). (c) B-1 cells are largely undetectable in the LNs of normal mice (47). (d) The chimeric mice used in our studies were constructed by transferring adult BM cells to irradiated recipients, a process that preferentially reconstitutes B-2 rather than B-1 cells (48 and unpublished data).

In our studies, irradiated recipient mice were reconstituted with BM from μMT mice in combination with BM from another donor strain. Recently, Macpherson et al. (49) demonstrated IgA production in μMT mice with the B6 genetic background, raising the possibility that the transplanted μMT BM contributed B cells capable of producing IgA in response to influenza infection. However, comprehensive control experiments (Fig. 2) clearly established that Ab responses in chimeric mice were not generated by cells derived from μMT BM. Moreover, the anti-influenza IgA response characterized in our studies is substantially dependent on $CD4^+$ T cells, whereas the production of IgA by μMT mice is T cell independent and apparently represents a response to intestinal bacteria (49).

Our demonstration that a $CD4^+$ T cell–dependent IgA response to influenza infection can be generated in the absence of signaling to B cells via MHC II establishes that the process is driven by non-Ag–specific bystander help. It follows that the necessary T cell help might be provided not only by virus-specific $CD4^+$ T cells that are responding to infection, but also by activated $CD4^+$ T cells that are specific for heterologous Ags. Indeed, an activated $CD4^+$ T cell population might be a feature of the lung in nonspecific pathogen-free hosts because the airways are continuously exposed to environmental Ags, and activated, virus-specific $CD4^+$ T cells persist in lung tissues for long periods after infection (50). Influenza-specific memory $CD4^+$ T cells (51) may also provide bystander help to drive rapid, virus-specific IgA production by naive B cells, a potentially important mechanism because polarized Th1 memory $CD4^+$ T cells (52) may tend to promote switching to IgG isotypes if they engaged B cells in Ag-specific cognate interactions. In this way, memory $CD4^+$ T cells may contribute to the so-called heterosubtypic immunity that is broadly protective against multiple influenza A subtypes (28, 53).

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