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The Journal of experimental medicine, 198(7)

0022-1007

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2003-10-01

10.1084/jem.20021745

Peer reviewed
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
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-Ighb+ Gpi1a (B6-Ighb) mice, and mice deficient for CD40 (B6.129P2-Tnfrsf5−/−; reference 15) or both the αβ and γδ T cell receptor (B6.129P2-Tnfrsf5−/− I-Aa−/−; reference 16) were purchased from The Jackson Laboratory. MHC II–deficient (I-Aβ−/−) 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-Aβ−/− and μMT BM cells (1–2 × 107 total injected cells) into 8–10-wk-old B6-Ighb 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-Aβ−/− 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-Ighb recipients with μMT BM alone or with a mixture of μMT and B6.Ighb 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 (DS-1), and IgM (AF6-78); PE-conjugated mAbs to B220, CD8α (53-6.7), CD90.1 (OX-7), CD90.2 (53-2.1), and I-Aβ (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 106.8 50% egg infectious doses of the H3N2 influenza A virus HKx31 (30 μl in PBS). Anesthetized mice were exanguinated 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 mAbs 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 (DS-1), IgM (AF6-78), IgG1 (10.9), IgG1 (B6-2), IgG2α (8.3), and IgG2a (5.7) purchased from BD Biosciences, and biotinylated anti-IgA (Hy-16) and anti-IgA (HS-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, 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 mAb or the nonallo-
type-specific anti-IgG2a reagent are referred to as IgG2a, it
should be noted that IgG2a expressed by B6 mice is now recog-
nized as a distinct isotype designated IgG2c (22).

Total AFC numbers were determined using assays specific for
the IgH allotype. Plates were coated with mAbs to IgA\(^\alpha\) (HIS-
M2) or IgG2a\(^\beta\) (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 \(\mu\)g/well of purified, detergent-disrupted influ-
enza HKx31 (Charles River Laboratories). Bound Ab was de-
etected with ALP-conjugated goat anti–mouse Abs specific for
IgM, IgA, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnol-
ogy Associates, Inc.), followed by \(p\)-nitrophenylphosphate sub-
strate (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 Re-
search 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 \(\beta\)-glu-
tamine, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin, 10 \(\mu\)g/ml
gentamicin, and 5% FCS were grown in flat-bottom 96-well tis-
tue culture plates (Sarstedt). Influenza A virus HKx31 was diluted
in MEM supplemented with \(\beta\)-glutamine, antibiotics, 1 \(\mu\)g/ml
TPCK-trypsin (Sigma-Aldrich), and 0.3% BSA to give 4 \(\times\) 10\(^5\)
50% tissue culture infectious dose/ml, and then mixed with equal
volumes of nasal wash concentrates. After incubation for 2 h at
37\(^\circ\)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\(^\circ\)C in a 5% CO\(_2\) atmosphere,
and wells that were positive for virus growth were identified by hem-
agglutination of chicken RBCs.

**Assaying Virus-specific CD4\(^+\) T Cell Activation.** Influenza-specific
CD4\(^+\) T cell frequencies were determined as previously de-
scribed (19). In brief, splenic CD4\(^+\) T cells were enriched by neg-
ative 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-\(\gamma\)-
producing cells measured by ELISPOT assay after 2–3 d incuba-
tion.

**Statistics.** Statistical comparisons of mean values were per-
formed using the nonparametric Mann-Whitney U test for un-
paired samples.

**Results**

**The Influenza-specific B Cell Response Is CD4\(^+\) T Cell De-
dependent.** Influenza A virus infection of the respiratory tract in mice generates a rapid and vigorous humoral re-
response in regional LNs, particularly the MLN that drains the
lower respiratory tract (8). Initial experiments charac-
terized the Ab response elicited in the MLN and estab-
lished 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-spe-
cific 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 par-
allel assays using plates coated with Sendai virus Ags. No
spots representing IgA or IgG AFCs developed on the Sen-
dai-coated plates, and only \(\sim 3\%\) 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.

The depletion of CD4\(^+\) T cells resulted in a dramatic re-
duction in the number of cells producing virus-specific Ab
of each isotype (Fig. 1 B), indicating that the humoral re-
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\(^b\)/–
Characteristics of a CD4^+ T Cell–dependent IgA Response

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^+ donor-derived (Ighb) and host-derived (Igha) cells. Cell assays could be used to distinguish Ig production by donor-derived (Ighb) and host-derived (Igha) populations.

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, IgG^b B cells (potentially of µMT origin) did not contribute to the strong influenza-specific response in the recipients of µMT and B6-Igh^b 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 IgG^b 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).
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.

Virus-specific B cell responses in the MLN were compared after influenza infection of the chimeric mice, I-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-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-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-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...
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).

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 \(\mu\)MT 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)
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, 31, 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. 8A), 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 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-Ighb and B6 plus μMT → B6-Igha as 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-Ighb: P < 0.001 for IgA; P < 0.005 for IgG2a; P < 0.01 for IgG2b. B6 compared with CD40−/− plus μMT → B6-Igha: 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 (Ighb) and recipient (Igha) 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 comparable 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 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 Non-specific 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...

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 WT control mice and B6 plus μMT → B6-Ighb, B6 plus μMT → B6-Igha, and B6 plus μMT → B6-Ighf, as 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 or IgA. 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-Ab−/− plus μMT → B6-Ighf chimeras and 18 ± 7.4 (IgG2a) and 18 ± 7.1 (IgA) for B6 mice and B6 plus μMT → B6-Ighf control chimeras. B6 compared with I-Ab−/− or CD40−/−, and B6 plus μMT → B6-Ighf compared with I-Ab−/− plus μMT → B6-Ighf or CD40−/− plus μMT → B6-Ighf. 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 nonvirus-specific 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-
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dependent on CD4

development of the early, virus-specific IgA response was
dicates a requirement for close cellular interactions. Full
nonspecific B cell activation in the MLN of WT mice, in-
virus-specific B cells even though there was extensive,
surface Ig receptors and other mitogenic signals (40). Our
and other cytokines in combination with cross-linking of

a complex pattern of B cell stimulation involving TGF-β1
in B-1 cells (46). (c) B-1 cells

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, indi-

cates 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 differ-
entiation 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 re-
quires 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 mem-
brane for a prolonged period (42). Such an array of viral
glycoproteins may provide the basis for cognate DC in-

teractions 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 de-

pendent 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 contrib-
ute significantly to secreted IgA in the intestine and gener-
ate specific, T cell–independent IgA responses to intestinal
bacteria (44). Furthermore, we show that at least a com-
ponent 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 produc-
tion by a distinct B cell lineage with different activation re-
quirements, 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 depen-
dent, 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-Bkleid/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 recon-
stituted 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 pro-
ducing IgA in response to influenza infection. However,
comprehensive control experiments (Fig. 2) clearly estab-
lished that Ab responses in chimeric mice were not gener-
ated 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 pro-
duction of IgA by μMT mice is T cell independent and ap-
pearance 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 ab-
sence of signaling to B cells via MHC II establishes that the
process is driven by non-Ag–specific bystander help. It fol-

ows 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 spe-
cific for heterologous Ags. Indeed, an activated CD4+ T
cell population might be a feature of the lung in non-
specific pathogen-free hosts because the airways are continu-
ously 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 poten-
tially 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 in-
teractions. In this way, memory CD4+ T cells may con-
tribute to the so-called heterosubtypic immunity that is
broadly protective against multiple influenza A subtypes
(28, 53).

We thank Jennifer Hoffrage and Suzette Wingo for assistance with
flow cytometry.

This work was supported by U.S. Public Health Service grants
AI29579 and CA21765, and by the American Lebanese Syrian As-

sociated Charities (ALSAC).

Submitted: 2 October 2002
Revised: 25 July 2003
Accepted: 25 July 2003

References

1. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the
control of immunity. Nature. 392:245–252.

2. Jenkins, M.K., A. Khoruts, E. Ingulli, D.L. Mueller, S.J. Mc-
Sorley, R.L. Reinhardt, A. Itano, and K.A. Pape. 2001. In
vivo activation of antigen-specific CD4 T cells. Annu. Rev.
3. Bishop, G.A., and B.S. Hostager. 2001. B lymphocyte activation by contact-mediated interactions with T lymphocytes. *Curr. Opin. Immunol.* 13:278–285.

4. Clark, E.A., and J.A. Ledbetter. 1994. How B and T cells talk to each other. *Nature.* 367:425–428.

5. Grammer, A.C., and P.E. Lipsky. 2001. CD40-mediated regulation of immune responses by TRAF-dependent and TRAF-independent signaling mechanisms. *Adv. Immunol.* 76:61–178.

6. Stavnezer, J. 2000. Immunology - a touch of antibody class. *Science.* 288:984–985.

7. Baumgarth, N., O.C. Herman, G.C. Jager, L.A. Herzenberg, and J.Z. Chen. 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J. Exp. Med.* 192:271–280.

8. Marshall, D., R. Sealy, M. Sangster, and C. Coleclough. 1999. Th1 cells primed during influenza virus infection provide help for qualitatively distinct antibody responses to subsequent immunization. *J. Immunol.* 163:4673–4682.

9. Sangster, M.Y., X.Y. Mo, R. Sealy, and C. Coleclough. 1997. Matching antibody class with pathogenic type and portal of entry: cognate mechanisms regulate local isotype expression patterns in lymph nodes draining the respiratory tract of mice inoculated with respiratory viruses, according to virus replication competence and site of inoculation. *J. Immunol.* 159:1893–1902.

10. Baumgarth, N. 2000. A two-phase model of B-cell activation. *Immuno. Rev.* 176:171–180.

11. Snapper, C.M., and J.J. Mond. 1993. Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today.* 14:15–17.

12. Wykes, M., A. Pombo, C. Jenkins, and G.G. MacPherson. 1998. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J. Immunol.* 161:1313–1319.

13. Fayette, J., B. Dubois, S. Vandenabeele, J.M. Bridon, B. Vanbervliet, I. Durand, J. Banchereau, C. Caux, and F. Brière. 1997. Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2. *J. Exp. Med.* 185:1909–1918.

14. MacPherson, G., N. Kashmir, and M. Wykes. 1999. Dendritic cells, B cells and the regulation of antibody synthesis. *Immuno. Rev.* 172:325–334.

15. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kikutani, and H. Itohara. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunol.* 1:167–178.

16. Mombaerts, P., A.R. Clarke, M.A. Rudnicki, J. Iacomini, S. Itohara, J.J. Lafaille, L.L. Wang, Y. Ichikawa, R. Jaenisch, M.L. Hooper, et al. 1992. Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature.* 360:225–231.

17. Grusby, M.J., R.S. Johnson, V.E. Papaiouannou, and L.H. Glimcher. 1991. Depletion of CD4 + T cells in major histocompatibility complex class-II-deficient mice. *Science.* 253:1417–1420.

18. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature.* 350:423–426.

19. Ribeder, J.M., K.J. Flynn, J. Stech, R.G. Webster, J.D. Altman, and P.C. Doherty. 1999. Protection against a lethal avian influenza A virus in a mammalian system. *J. Virol.* 73:1453–1459.

20. Nedrud, J.G., X.P. Liang, N. Hague, and M.E. Lamm. 1987. Combined oral/nasal immunization protects mice from Sendai virus infection. *J. Immunol.* 139:3484–3492.

21. Sangster, M.Y., D.J. Topham, S. D’Costa, R.D. Cardin, T.N. Marion, L.K. Myers, and P.C. Doherty. 2000. Analysis of the virus-specific and nonspecific B cell response to a persistent B-lymphotropic gammaherpesvirus. *J. Immunol.* 164:1820–1828.

22. Morgado, M.G., P. Cam, C. Gris-Liebe, P.A. Cazenave, and E. Jouvin-Marche. 1989. Further evidence that BALB/c and C57BL/6 γ2a genes originate from two distinct isotypes. *EMBO J.* 8:3245–3251.

23. Williams, G.S., A. Oxeniou, H. Hengartner, C. Benoist, and D. Mathis. 1998. CD4+ T cell responses in mice lacking MHC class II molecules specifically on B cells. *Eur. J. Immunol.* 28:3763–3772.

24. Topham, D.J., R.A. Tripp, A.M. Hamilton-Easton, S.R. Sarawar, and P.C. Doherty. 1996. Quantitative analysis of the influenza virus-specific CD4+ T cell memory in the absence of B cells and Ig. *J. Immunol.* 157:2947–2952.

25. Renegar, K.B., and P.A. Small, Jr. 1991. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. *J. Virol.* 65:2146–2148.

26. Renegar, K.B., and P.A. Small, Jr. 1991. Passive transfer of local immunity to influenza virus infection by IgA antibody. *J. Immunol.* 146:1972–1978.

27. Tamura, S., T. Iwasaki, A.H. Thompson, H. Asanuma, Z. Chen, Y. Suzuki, C. Aizawa, and T. Kurata. 1998. Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. *J. Gen. Virol.* 79:291–299.

28. Liang, S.H., K. Mozdzanowska, G. Palladino, and W. Gerhard. 1994. Heterosubtypic immunity to influenza type A virus in mice: effector mechanisms and their longevity. *J. Immunol.* 152:1653–1661.

29. Oxeniou, A., K.A. Campbell, C.R. Maliszewski, T. Kishimoto, H. Kikutani, H. Hengartner, R.M. Zinkernagel, and M.F. Bachmann. 1996. CD40–CD40 ligand interactions are critical in T–B cooperation but not for other anti-viral CD4+ T cell functions. *J. Exp. Med.* 183:2209–2218.

30. Grewal, I.S., and R.A. Flavell. 1996. A central role of CD40 ligand in the regulation of CD4+ T-cell responses. *Immuno. Today.* 17:410–414.

31. Coutelier, J.P., J.T.M. van der Logt, F.W.A. Heessen, A. Vink, and J. Van Snick. 1988. Virally induced modulation of murine IgG antibody subclasses. *J. Exp. Med.* 168:2373–2378.

32. Coutelier, J.P., P.G. Coulie, P. Wauters, H. Heremans, and J.T.M. van der Logt. 1990. In vivo polyclonal B-lymphocyte activation elicited by murine viruses, *J. Virol.* 64:5383–5388.

33. Stevenson, P.G., and P.C. Doherty. 1999. Non-antigen-specific B-cell activation following murine gammaherpesvirus infection is CD4 independent in vitro but CD4 dependent in vivo. *J. Virol.* 73:1075–1079.

34. Lumsden, J.M., J.A. Williams, and R.J. Hodes. 2003. Differential requirements for expression of CD80/86 and CD40 on B cells for T-dependent antibody responses in vivo. *J. Immunol.* 170:781–787.

35. McHeyzer-Williams, L.J., D.J. Driver, and M.G. McHeyzer-
36. Chirmule, N., J. Tazelaar, and J.M. Wilson. 2000. Th2-dependent B cell responses in the absence of CD40-CD40 ligand interactions. *J. Immunol.* 164:248–255.

37. Sealy, R., S. Surman, J.L. Hurwitz, and C. Coleclough. 2003. Antibody response to influenza infection of mice: different patterns for glycoprotein and nucleocapsid antigens. *Immunochemistry.* 108:431–439.

38. Cazac, B.B., and J. Roes. 2000. TGF-β receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity.* 13:443–451.

39. van Ginkel, F.W., S.M. Wahl, J.F. Kearney, M.N. Kweon, K. Fujihashi, P.D. Burrows, H. Kiyono, and J.R. McGhee. 1999. Partial IgA-deficiency with increased Th2-type cytokines in TGF-β1 knockout mice. *J. Immunol.* 163:1951–1957.

40. McIntyre, T.M., M.R. Kehry, and C.M. Snapper. 1995. Novel in vitro model for high-rate IgA class switching. *J. Immunol.* 154:3156–3161.

41. Litinskiy, M.B., B. Nardelli, D.M. Hilbert, B. He, A. Schaffer, P. Casali, and A. Cerutti. 2002. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat. Immunol.* 3:822–829.

42. Bender, B.S., W.E. Bell, S. Taylor, and P.A. Small, Jr. 1994. Class I major histocompatibility complex–restricted cytotoxic T lymphocytes are not necessary for heterotypic immunity to influenza. *J. Infect. Dis.* 170:1195–1200.