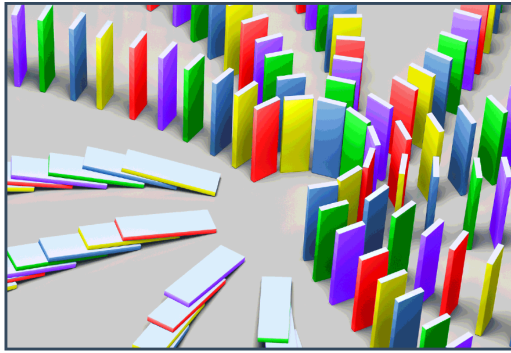

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Primary T Cell Expansion and Differentiation In Vivo Requires Antigen Presentation by B Cells[1]

Alison Crawford,* Megan MacLeod,* Ton Schumacher,† Louise Corlett,* and David Gray[2]*

B cells are well documented as APC; however, their role in supporting and programming the T cell response in vivo is still unclear. Studies using B cell-deficient mice have given rise to contradictory results. We have used mixed BM chimeric mice to define the contribution that B cells make as APC. When the B cell compartment is deficient in MHC class II, while other APC are largely normal, T cell clonal expansion is significantly reduced and the differentiation of T cells into cytokine-secreting effector cells is impaired (in particular, Th2 cells). The development of the memory T cell populations is also decreased. Although MHC class II-mediated presentation by B cells was crucial for an optimal T cell response, neither a B cell-specific lack of CD40 (influencing costimulation) nor lymphotoxin α (influencing lymphoid tissue architecture) had any effect on the T cell response. We conclude that in vivo B cells provide extra and essential Ag presentation capacity over and above that provided by dendritic cells, optimizing expansion and allowing the generation of memory and effector T cells. *The Journal of Immunology,* **2006, 176: 3498–3506.**

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The capacity of B cells to present Ag to CD4 T cells is well established; however, when and where they perform this function in vivo and its consequences for $CD4^+$ T cell responses are still unresolved. Early experiments addressed this question by removing B cells in vivo with anti-mouse μ-chain Abs and examining T cell priming (1, 2). These studies suggested that B cells are required for T cell priming with protein Ag, especially in lymph nodes. However, when mice with a genetic deficiency in B cells were produced, conflicting results arose; in some studies CD4 T cell priming seemed unaffected by the absence of B cells (3–5), whereas in others impairment was observed (6–8).

Despite data demonstrating that under a variety of artificial experimental conditions B cells can activate naive T cells (9–11), the currently popular view is that naive $CD4^+$ T cells are primed in association with dendritic cells (DC)[3] and only interact subsequently with B cells (12). The main argument supporting this position is that the frequency of Ag-specific B cells is too low for them to take part significantly in T cell priming (only B cells with Ag-specific BCR efficiently take up Ag (13)). How early then does B cell presentation come into play? Some studies (14, 15) suggest that B cells can present Ag to T cells very early after immunization. For example, B cells purified from immunized BCR-transgenic mice could stimulate T cells as early as 4–6 h after Ag administration (14). However, this does not address the contribution of B cells when the number of efficient, Ag-specific B cell APC is many orders of magnitude lower (i.e., in normal mice). Presentation of peptide-MHC complexes on the surface of the B cells in vivo (in non-BCR-transgenic mice) has been observed within hours of systemic Ag immunization using Abs against the peptide-MHC complex (16, 17). Again, the physiological relevance of this is unclear (the capacity to stimulate T cells was not examined) and other similar studies document far fewer ($<1\%$) B cells bearing the peptide MHC complex when lower doses of Ag were used (18).

In addition to a role as presenters of Ag, B cells can influence T cells in a number of other ways. They can provide costimulatory signals, such as OX40 ligand (OX40L) (8), secrete cytokines such as IL-10 (19, 20), produce Abs, and affect lymphoid tissue structure (21). In relation to most, if not all, of these functions the maturation signal delivered by CD40 is crucial for B cells (22–24). One consequence of CD40 ligation on B cells is the up-regulation of OX40L, which is a critical regulator of T cell survival during and after the expansion phase in vivo (25). Linton et al. (8) recently suggested that OX40L expression by B cells is important for supporting the CD4 T cell response. In addition to their potential contribution to T cell expansion, B cells also promote CD4 T cell differentiation: OT-II T cells (from OVA peptide-specific TCR-transgenic mice) activated in μMT mice produce less IL-4 than those activated in wild-type (WT) mice (8) and Ag-specific B cells drive IL-4 production in vitro and in vivo (26, 27), suggesting that B cells promote Th2 responses.

Finally, B cells are also essential as organizers of splenic architecture. Spleens of B cell-deficient (μMT) mice are ~6-fold smaller than those of WT mice (28) and display a multitude of architectural defects in the spleen, including the absence of follicular DC, marginal zone macrophages (MZM), and metallophilic macrophages (MM) (29), decreased expression of chemokines such as CCL21 (30), and, importantly, differences in DC function (31). This is the result of an absence of lymphotoxin (LT) β normally expressed by B cells (membrane form, $LT\alpha_1\beta_2$). Thus, mice in which a LTβ deficiency is genetically restricted to B cells exhibit a splenic phenotype very similar to μMT, with greatly reduced FDC, MZM, and MM (21). Moreover, Kabashima et al. (32) recently showed that B cells are a crucial source of $LT\alpha_1\beta_2$ controlling homeostasis of DC populations in the spleen (32). For this and all the other reasons described above, it is imperative that the

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[3] Abbreviations used in this paper: DC, dendritic cell; OX40L, OX40 ligand; FDC, follicular DC; MM, marginal metallophil; MZM, marginal zone macrophage; LT, lymphotoxin; MHC-II, MHC class II; WT, wild type; BM, bone marrow; DNP, dinitrophenyl; TD, T dependent.

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role of B cells as APC in vivo is addressed in a manner as close to the physiological as possible and certainly in a model that does not involve gross B cell deficiency.

In this study, we have transferred mixtures of BM to create chimeric mice in which the B cell compartment was deficient in MHC class II (MHC-II), CD40, or LTα. This allowed us to assess the B cell contribution to Ag presentation, costimulation, and lymphoid architecture in relation to the expansion and differentiation of adoptively transferred TCR-transgenic T cells and of endogenous T cells (with MHC-II tetramers). We show that although CD40 or LTα expression by B cells is dispensable for T cell expansion, a cognate interaction through MHC-II on B cells was essential for both clonal expansion and differentiation of CD4 T cells. In vivo B cells provide essential additional Ag presentation capacity to that provided initially by DC.

Materials and Methods

Mice

C57BL/6, μMT (33), I-A$^{b-/-}$ (34), CD40$^{-/-}$ (22), and OT-II (35) mice were bred and maintained under specific pathogen-free conditions at the School of Biological Sciences animal facility at the University of Edinburgh (Edinburgh, U.K.). LT$\alpha^{-/-}$ mice (36) were provided by Dr. N. Mabbott (Institute for Animal Health, Edinburgh, U.K.). All experiments were covered by a Project License granted by the Home Office under the Animal (Scientific Procedures) Act 1986. Locally, this license was approved by the University of Edinburgh Ethical Review Committee.

BM chimeras

Chimeras were made as described in the study by Fillatreau et al. (37). Recipient mice were lethally irradiated with 1150 cGy gamma radiation from a ^{137}Cs source. The following day BM cells were removed from donor mice (femur, tibia, and humerus) and depleted of T cells using anti-Thy1 microbeads (Miltenyi Biotec) and separated on a MACS magnetic column (Miltenyi Biotec) according to the manufacturer's instructions. The BM cells were resuspended in PBS and mixed at the ratio of 80% μMT and 20% WT or KO BM. Between 3×10^6 and 5×10^6 cells were injected into the irradiated recipients. The chimeric hosts were then left for 8 wk to allow repopulation of the hemopoietic system from the transferred BM. Reconstitution was confirmed by staining for T cells and B cells by flow cytometry. In some experiments, WT B cells were added back to these chimeric mice: Thus, B cells from C57BL/6 mice were purified using anti-CD19 microbeads (Miltenyi Biotec) and then incubated overnight with 1 μg LPS, 10 μg anti-CD40 Ab, and 100 μg/ml OVA. An inoculum of 10^7 activated B cells was transferred i.v. the next day and the mice were immunized with 200 μg dinitrophenyl (DNP)-OVA/alum (i.p.)

Preparation of cell suspensions for adoptive transfer and immunizations

Spleens and peripheral (popliteal, inguinal, auxiliary, brachial, superficial cervical, and iliac) lymph nodes and mesenteric lymph nodes were dispersed using Nytex mesh (Wm Ritchie) and forceps in IMDM (Sigma-Aldrich) with 2% FCS (Labtech International), 2 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies), and 50 μM 2-ME (BDH). Lymph node and spleen preparations were depleted of B cells, other MHC-II positive cells, and CD8 cells before being transferred. Cells were incubated with biotinylated anti-κ (187.1, in-house), anti-MHC-II (M5114 (38)), anti-IgM (Southern Biotechnology Associates), and anti-CD8 (53.6.72, in-house) followed by streptavidin beads (Miltenyi Biotec). They were then purified on MACS magnets according to the manufacturer's instructions. Cells were washed and resuspended in PBS (Sigma-Aldrich) before being injected i.v. into mice at 1–1.5 $\times 10^6$ cells/mouse in 200 μl. Mice were immunized the next day with 200 μg DNP-OVA/alum i.p. For experiments using class II tetramers, mice were immunized with H19-Env (EPLTSLT PRCNTAWNRLKL) of OVA$_{323-339}$ peptide (ISQAVHAAHAEINEAGR) (both supplied by Advanced Biotechnology Centre, Imperial College, London, U.K.). The peptides were either emulsified in CFA (Sigma-Aldrich) and injected s.c. at 100 μg/mouse. In some experiments, peptide-pulsed BM-derived DC were injected s.c.

Flow cytometry

Single-cell suspensions of spleens and lymph nodes were prepared and RBC were lysed from spleens. Stained samples were analyzed using a four-color FACSCalibur flow cytometer (BD Biosciences) running CellQuest software. Further analysis was completed using FlowJo (Tree Star). For OT-II adoptive transfer experiments, up to 10^6 cells were stained in 50 μl of FACS buffer (PBS with 2% FCS) for 15 min on ice, then washed in FACS buffer. The following mAbs were used: Vα-2 PE (BD Pharmingen), Vβ-5 biotin (BD Pharmingen), CD44 FITC (142.5 clone), and CD4$^-$ allophycocyanin (BD Pharmingen). The secondary Ab streptavidin-PerCP (BD Pharmingen) was incubated for an additional 15 min on ice and then the cells were washed twice. For MHC-II tetramer experiments, 1–2 $\times 10^6$ erythrocyte-depleted splenocytes were plated in a 96-well plate, washed in 10% IMDM (Sigma-Aldrich), and then PE-labeled class II tetramers were added. The cells were incubated for 3 h at 37°C with gentle agitation every 20 min before additional Abs were added (anti-CD4 allophycocyanin; BD Pharmingen), anti-F4/80-R-PE-Cy5 (Serotec), and anti-CD44 FITC (142.5, in-house) and left at room temperature for 15 min. Cells were washed three times in FACS buffer (PBS with 2% FCS) and propidium iodide (BD Pharmingen) was added before acquisition. Two hundred thousand live events were collected on the flow cytometer. Tetramer-positive cells were identified as CD4 positive, F4/80 negative, propidium iodide negative that bound the H19-Env-MHC-II tetramer.

For CFSE labeling, cells were washed in PBS then resuspended at 1 $\times 10^7$/ml in 5 μM CFSE in serum-free IMDM for 8 min at 37°C. The reaction was quenched with an equal volume of FCS and the cells were washed several times in serum-free IMDM then resuspended in PBS before injection.

BM-derived DC

BM DC were prepared according to the procedure developed by Inaba et al. (39). Femurs and tibias were removed and a single-cell suspension of BM cells was prepared. Erythrocytes were depleted and cells were plated in RPMI medium (Sigma-Aldrich) with 10% FCS, 2 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies), and 5% GM-CSF containing supernatant (from the X-63Ag8-GM-CSF-transfected cell line (26)) at a concentration of 3.75×10^5 cells/well in a 24-well plate. Cells were washed on days 3 and 6, with RPMI 1640 containing 1% mouse serum (Harlan Sera-Lab). The DC were harvested on day 7 and incubated overnight with LPS (0.1 μg/ml; Sigma-Aldrich) at 1×10^6 cells/ml then washed and peptide pulsed (50 μg/ml) for 90 min before being washed and resuspended in PBS before being transferred into the mice.

Immunohistochemistry

Spleens were frozen in OCT-embedding medium (BDH) in cryomoulds (BDH) on dry ice and stored at −80°C. Tissue sections (5 μm thick) were cut onto glass multiwell slides (Hendley-Essex) using a Leica CM1510 cryostat and left overnight to dry before fixation in acetone for 10 min. Sections were stained with T24 (anti-Thy.1), anti-IgM tetramethylrhodamine (Serotec), FDC-M2 biotin (Immunokontact), or ERTR-9 supernatant (a gift from G. Kraal, Department of Cell Biology and Immunology, Amsterdam, The Netherlands). Primary stains were left for 2 h and the secondary stain Alexa Fluor 350 (Molecular Probes) was left for 1 h. After staining, sections were mounted with the embedding medium moviol (Hoechst) and analyzed using an Olympus BX50 microscope. Images were captured with a Hamamatsu digital camera and Openlab imaging software (Improvision).

Cell-based ELISAs

ELISAs were conducted as described previously (20). T cells (APC- and CD8-depleted splenocytes) were plated at 2×10^5 cells/well with 2×10^6 irradiated APC in a 96-well plate. OVA peptide was added in log-fold dilutions at a starting concentration of 100 μM. After 48 h, 100 μl of cells was transferred to microtiter plates coated 24 h previously with anti-cytokine Abs (IFN-γ (clone R4-6A2), IL-2 (clone JESE-1A12), or IL-4 (clone 11B11; BD Pharmingen). Recombinant cytokine standards (R&D Systems) were added to the top rows. Eighteen hours later, plates were washed and biotinylated Abs (IFN-γ (clone XMG1-2), IL-2 (clone JES6-5H4), or IL-4 (clone BVD6-24G2; BD Pharmingen) were added for 2 h at room temperature. Plates were then washed and Extravidin-alkaline phosphatase (Sigma-Aldrich) was added at 1/10,000 for 1 h at room temperature. Readings were made at an OD of 405 nm. The calculations of cytokine concentrations were extrapolated from standards using GraphPad Prism. The cytokine concentrations secreted by Ag-specific TCR-transgenic OT-II T cells were "normalized" for the number of OT-II T cells

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present in the cultures (assessed by FACS staining); thus, cytokine production is expressed as the amount per 10^4 OT-II T cells.

Results

CD4 T cell expansion is reduced in μMT mice

To examine T cell responses in the complete absence of B cells, we adoptively transferred APC-depleted spleen and lymph node cells from OT-II (OVA peptide-specific, H-2A^b-restricted TCR-transgenic) mice into WT (C57BL/6) or μMT mice. The mice were immunized with DNP-OVA/alum. In Fig. 1*A*, we show the number of the transferred OT-II cells at the peak of clonal expansion (day 4). The expansion of the OT-II T cells is decreased in μMT mice compared with normal mice. Thus, in the absence of B cells, although the OT-II T cells proliferate, their expansion is reduced. It should be noted that we were unable to detect significant levels of serum IgA or IgA-bearing B cells in the spleen or lymph nodes of the μMT mice from our colony, as reported by MacPherson et al. (40).

To establish whether this reduction was due to a block in division or due to fewer cells entering division, OT-II cells were labeled with CFSE. Although the number of divisions the cells undergo is similar, the number of cells entering division is slightly lower in μMT mice (Fig. 1*B*).

CD4$^+$ T cell expansion is reduced in MHC-II$^{B-/-}$ chimeras

The reduced expansion of OT-II T cells in μMT mice, might be due to factors other than B cell Ag presentation. To assess specifically the role of Ag presentation by B cells, we created BM chimeras in which the T cells are unable to interact with B cells in a cognate Ag-specific manner because of a genetic deficiency in MHC-II on the B cells (37). To do this, we created mixed BM chimeras by transferring an inoculum of 20% MHC-II$^{-/-}$ BM and 80%μMT BM into lethally irradiated recipients (see *Materials and Methods*). Two months after reconstitution, most (>80%) of the non-B cell lineage cells were WT while all of the B cells were MHC II deficient (Fig. 2*B*). As controls, we created BM chimeras containing a normal B cell compartment by combining μMT and C57BL/6 BM.

The cellular compartments of these chimeras were compared by flow cytometry and immunohistochemistry to ensure full reconstitution and that the cellular proportions and architecture of the spleen were comparable: The chimeras had similar total splenocyte cell numbers and proportions of CD4$^+$ T cells and B cells (Fig. 2*A*), and essentially all of the B cells were MHC-II negative. Splenic architecture was examined by staining for T and B cell areas as well as follicular DC and marginal zone macrophages. The normality of lymphocyte subsets and microenvironments in the chimeras contrasts dramatically to μMT mice that, as well as grossly different architecture, have increased proportions of CD4$^+$ cells compared with WT mice (29, 36).

To assess T cell expansion in the absence of B cell Ag presentation, APC-depleted OT-II splenocytes were transferred into the MHC-II$^{B-/-}$ chimeras or WT chimeras. The percentages of CD4$^+$ T cells that were OT-II in the spleen was examined on days 3, 4, 6, 8, and 10 after immunization with DNP-OVA. Although both MHC-II$^{B-/-}$ and WT chimeras showed similar kinetics of T cell expansion, the absence of MHC-II on B cells resulted in reduced numbers of OT-II T cells throughout the primary response (Fig. 3). Thus, Ag presentation by B cells is required early in the T cell response and in its absence the clone size of responding T cells is compromised throughout the response.

We next asked whether the impaired T cell expansion in MHC-II$^{B-/-}$ chimeras could be rectified by adding back WT B cells at the time of immunization (10 million B cells achieved a chimerism of between 1 and 5%). When this was done, the clonal expansion of OT-II T cells transferred into MHC-II$^{B-/-}$ chimeras was the same as in WT chimeras (Fig. 4). Supplementing BM-derived DC gave variable reconstitution of T cell expansion but was much less effective than B cells (data not shown). These data confirm that B cells are required as APC for optimal CD4 T cell clonal expansion. They also show that the 20% reduction in other APC expressing MHC-II in the chimeras did not impair the T cell response.

There are two possible explanations for detecting fewer Ag-specific OT-II T cells in the spleens of MHC-II$^{B-/-}$ chimeras: either they have divided less (or fewer cells have divided) or they have left the spleen and migrated to peripheral tissues. To determine whether the T cells had preferentially migrated to peripheral tissues in MHC-II$^{B-/-}$ chimeras, lungs and livers from these and control chimeras were examined for the presence of OT-II T cells. OT-II T cells were present in equal or lower numbers in the peripheral tissues of MHC-II$^{B-/-}$ chimeras, compared with WT chimeras (data not shown). Thus, migration out of the spleen is not the explanation for reduced numbers of T cells in the spleens of MHC-II$^{-/-}$ B cell chimeras. When OT-II T cell division in vivo

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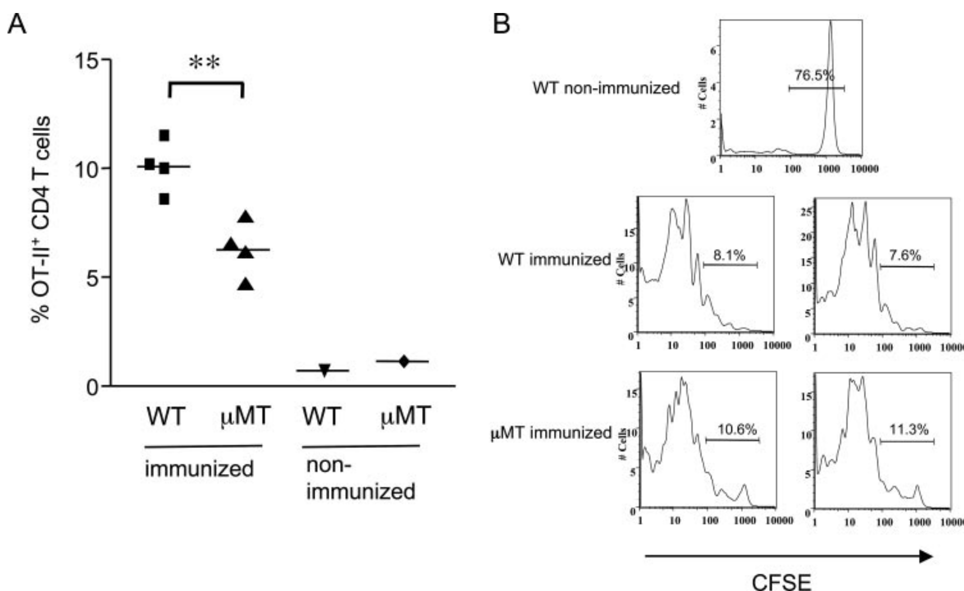

FIGURE 1. OT-II T cell expansion in B cell-deficient mice. OT-II CD4$^+$ cells transferred into μMT or C57BL/6 mice and immunized i.p. with DNP-OVA/alum. *A,* Clonal expansion of OT-II cells on day 4 after immunization is expressed as the percentage of CD4$^+$ cells positive for Vα2 and Vβ5. Each symbol represents the data from an individual mouse and lines show the mean of each group. Statistical comparisons were made using the unpaired *t* test. **, $p < 0.01$. *B,* CFSE labeling of transferred OT-II cells on day 4 after immunization; data from two representative WT and μMT mice are shown. These experiments were performed three times with similar results.

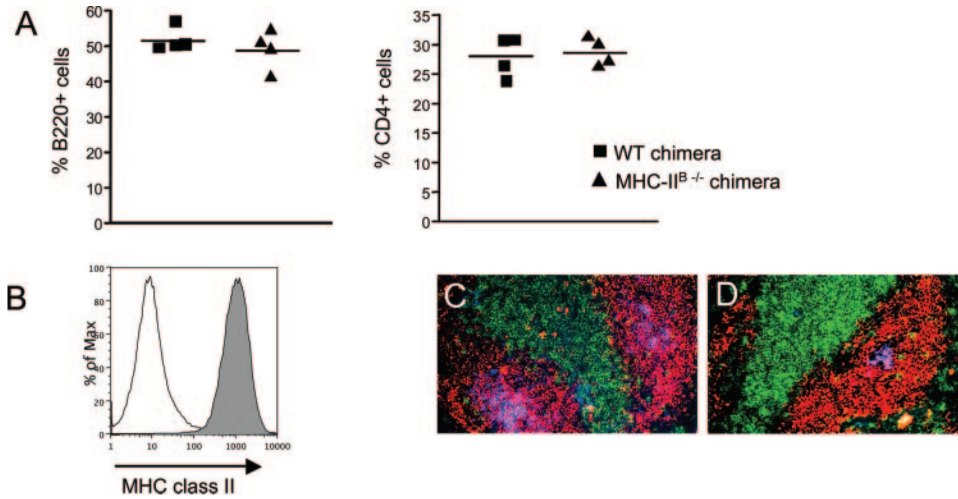

FIGURE 2. Reconstitution of MHC-II$^{B-/-}$ chimeras. *A,* The percentage of splenocytes from WT (■) or MHC-II$^{B-/-}$ (▲) chimeras for B220 and CD4. *B,* Expression of MHC-II on B220$^+$ cells in WT (shaded) or MHC-II$^{B-/-}$ (open) chimeras. All chimeras used in these experiments were analyzed in this manner. *C* and *D,* Staining of spleen sections from WT (*C*) MHC-II $^{B-/-}$ (*D*) chimeras to show T cells (green; anti-Thy1), B cells (red; anti-IgM), and MZM (blue; ERTR-9). FDC were also present in these chimeras (data not shown).

was examined using CFSE, more of the T cells had failed to enter division or were in the early rounds of division in MHC-II$^{-/-}$ B cell chimeras than in WT chimeras (Fig. 5). This suggests that Ag-specific cognate interactions with B cells are important for the optimal induction and maintenance of T cell division.

Impaired CD4 T cell memory generation in the absence of B cell-derived MHC class II

In our hands, the transferred OT-II T cells returned to background levels ~20 twenty days after immunization, preventing us from examining CD4$^+$ T cell memory development in this system. Thus, MHC-II tetramers were used to examine memory responses in these chimeras. The tetramers contain a peptide from the envelope protein (H19-Env) of Moloney murine leukemia virus (41, 42). WT and MHC-II$^{B-/-}$ chimeras were immunized with H19-Env peptide in CFA and the primary response, long-term survival of the Ag-specific memory T cells, and secondary responses were examined. The primary T cell response to H19-Env in CFA is significantly reduced in MHC-II$^{B-/-}$ chimeras compared with WT chimeras (Fig. 6*A*). In fact, although priming had occurred (as evidenced by the enhanced recall response, see below; Fig. 6*B*), the primary clonal expansion of tetramer-positive T cells was not readily and statistically detectable in MHC-II$^{B-/-}$ chimeras. Therefore, in both the OT-II transfer system and by following endogenous Ag-specific T cells using MHC-II tetramers, the CD4$^+$ T cell response was reduced in the absence of MHC-II on B cells.

To assess memory responses, mice were immunized and left for a minimum of 10 wk and either examined for the tetramer-positive memory cells remaining or some of the mice were boosted with H19env-pulsed BM-derived DC to examine the memory recall response in vivo.

The number of memory cells remaining in MHC-II$^{B-/-}$ chimeras was significantly lower than that in WT chimeras, suggesting either that fewer memory cells were generated or that fewer memory cells survived in the absence of MHC-II on B cells. When the mice were reimmunized, the memory recall response was also significantly lower in the MHC-II$^{B-/-}$ chimeras (Fig. 6). To establish whether the recall response in the MHC-II$^{B-/-}$ chimeras represented expansion of surviving memory cells, we compared this recall response to the primary responses in naive age-matched chimeras. It is clear from Fig. 6*B* that the percentage of tetramer-positive cells after reimmunization was much higher than in primary response in the MHC-II$^{B-/-}$ chimeras. Thus, a population of Ag-specific memory T cells had survived in the MHC-II$^{B-/-}$ chimeras and could make an enhanced response.

Cytokine production is impaired in the absence of MHC-II on B cells

To see whether the effector T cell differentiation was impaired in the absence of MHC-II on B cells, we examined the secretion of effector cytokines by OT-II T cells from MHC-II$^{B-/-}$ chimeras.

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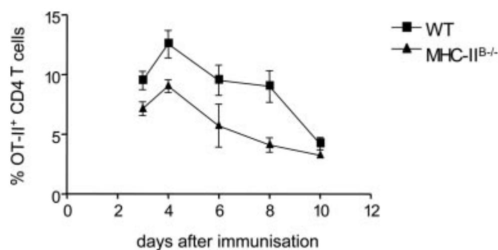

FIGURE 3. OT-II T cell expansion in the absence of MHC-II on B cells. OT-II CD4$^+$ cells transferred into WT and MHC-II$^{B-/-}$ chimeras and immunized i.p. with DNP-OVA/alum. Number of OT-II T cells is expressed as the percentage of CD4$^+$ cells positive for Vα2 and Vβ5. The mean of each group (three to five mice) and the SEM are shown. Statistical comparisons are by unpaired *t* test. The data points at days 3, 4, 8, and 10 are significant at $p < 0.05$ between the WT and MHC-II$^{B-/-}$ chimeras. This experiment was performed three times with similar results.

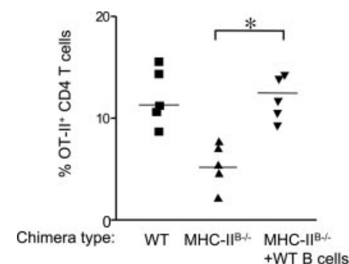

FIGURE 4. Rescue of OT-II T cell expansion in MHC-II$^{B-/-}$ chimeras by transfer of WT B cells at the time immunization. OT-II CD4 T cells transferred into WT and MHC-II$^{B-/-}$ chimeras and immunized i.p. with DNP-OVA/alum, either with or without transfer of 10^7 WT B cells i.v. The number of OT-II T cells on day 4 after immunization is expressed as the percentage of CD4$^+$ cells positive for Vα2 and Vβ5. Each symbol represents the data from an individual mouse and lines show the mean of each group. Statistical comparisons are by unpaired *t* test. *, $p < 0.05$. This is representative of three similar experiments.

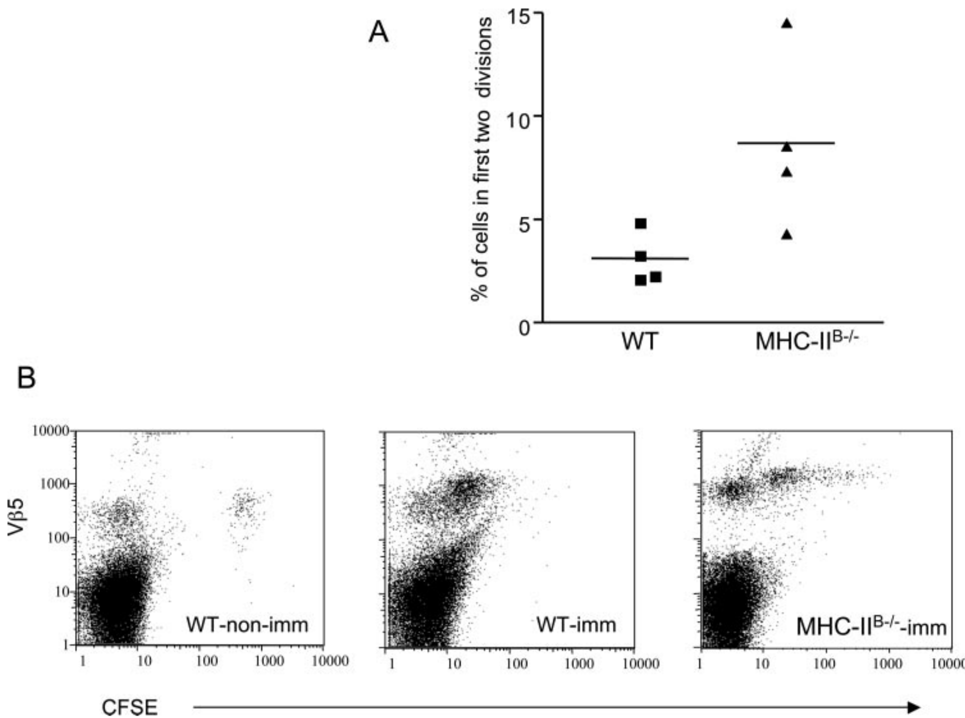

FIGURE 5. OT-II T cells divide less in MHC-II$^{B-/-}$ chimeras. CD4$^+$ cells from OT-II mice were CFSE labeled and transferred into WT (■) or MHC-II$^{B-/-}$ chimeras (▲). CD4$^+$ T cells bearing Vα2/Vβ5 were examined for CFSE labeling on day 3 after immunization. *A,* Plot of the number of OT-II cells in the early rounds of division. Symbols show the result of an individual mouse and lines show the mean of each group. By unpaired *t* test, the two groups differed statistically ($p < 0.05$). *B,* Representative FACS plots of CFSE staining of OT-II T cells in each type of chimera. The Vβ5$^+$ population to the far left (CFSE negative) is endogenous Vβ5$^+$ T cells that are unlabeled and not part of the response. This experiment was performed three times with a minimum of four mice per group, with similar results.

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Splenocytes (APC and CD8 depleted) from chimeras containing the responding OT-II T cells were stimulated with OVA peptide plus irradiated APC in vitro for 3–4 days. Since OT-II expansion differed in the two types of chimeras, the numbers of OT-II T cells within the 10^5 T cells placed in the assay wells differed. Thus, cytokine production is shown as the amount of cytokine produced per 10^4 OT-II T cells (Fig. 7). Consistent with reduced proliferation, IL-2 production was reduced in the absence of MHC-II on B cells (Fig. 7*A*). IFN-γ production was also reduced in the MHC-II$^{B-/-}$ chimeras (Fig. 7*B*) and IL-4 production was completely abolished (Fig. 7*C*).

CD40 expression by B cells is not required for CD4$^+$ T cell expansion

CD40 is an important maturation factor for B cells and other APC and therefore we examined the effect of the CD40 deficiency in APC in general on T cell expansion in vivo. We transferred OT-II T cells into WT (C57BL/6) and CD40$^{-/-}$ mice and followed OT-II expansion after immunization. The OT-II cells expanded in both groups of mice but by day 4 after immunization the numbers of OT-II T cells in WT mice was significantly greater than in CD40$^{-/-}$ mice (Fig. 8*A*). Thus, CD40 is required in vivo for efficient expansion of OT-II T cells.

Was the expression CD40 on B cells required for optimal CD4 Ag-specific T cell expansion to occur? Given the data from MHC-II$^{B-/-}$ chimeras, we predicted that it would. To test this, mixed BM chimeras were made in which the B cell compartment was deficient in CD40, while the other APC were largely sufficient. As shown in Fig. 8*B*, the expansion of the transferred OT-II cells was identical in CD40$^{B-/-}$ and WT chimeras, indicating no obligatory role for B cell-expressed CD40 in T cell priming and proliferation.

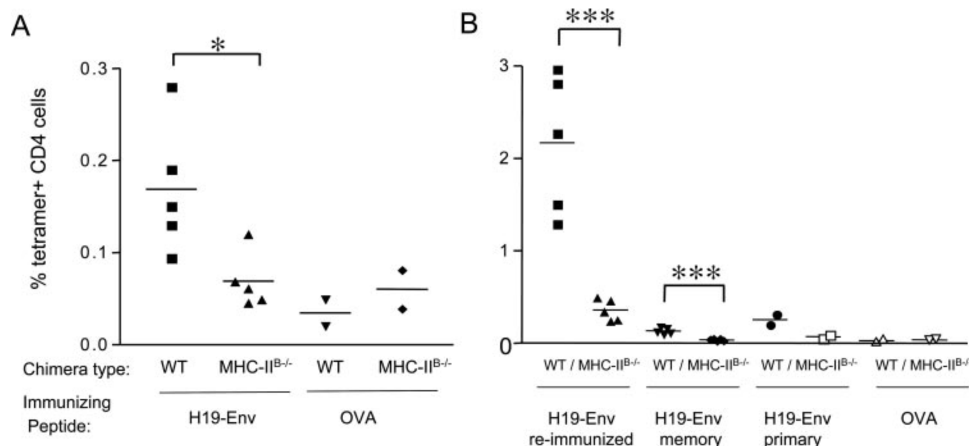

FIGURE 6. Memory responses are impaired in MHC-II$^{B-/-}$ chimeras. *A,* Primary response of WT and MHC-II$^{B-/-}$ chimeras immunized with H19-Env peptide or OVA peptide (negative control) in CFA. Ag-specific CD4 T cells detected using IA^b-H19-Env tetramers. The number of responding CD4 T cells on day 9 is shown. *B,* Chimeras 10 wk after immunization were analyzed directly ex vivo (H19-Env memory) or 5 days after boosting with H19-Env-pulsed BM DC (H19-Env re-immunized). For comparison, primary responses to H19-Env-pulsed BM DC were analyzed (H19-Env primary). Symbols represent the result from an individual mouse and lines show the mean of each group. Statistical comparisons are by unpaired *t* test. *, $p < 0.05$; ***, $p < 0.001$ This experiment was done three times with four mice per group.

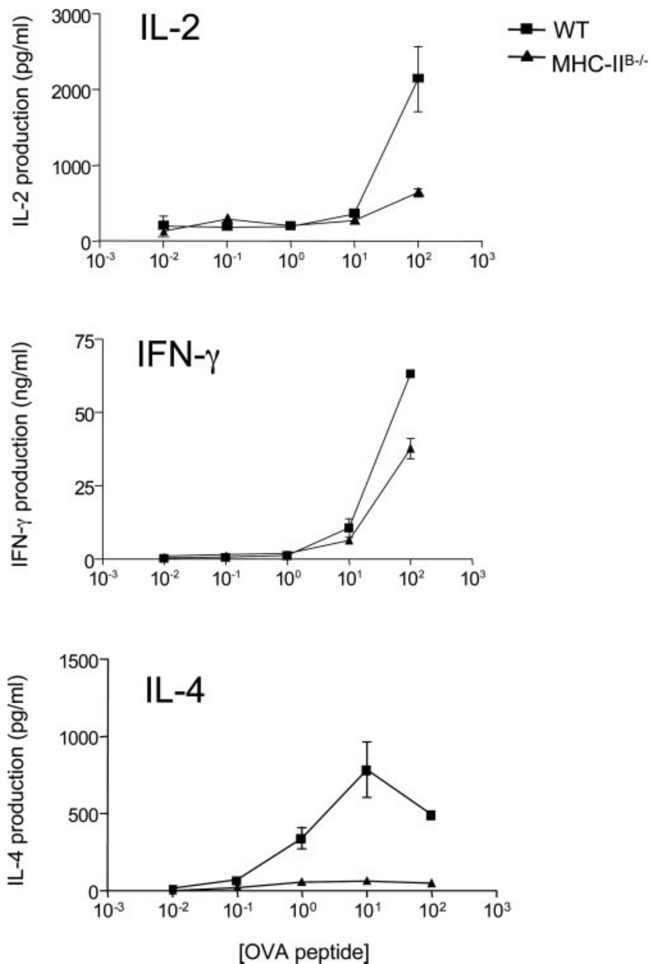

FIGURE 7. MHC class II on B cells is required for CD4 T cell differentiation to cytokine-producing effector cells. Purified CD4 T cells from 6-day immunized WT (■) or MHC-II$^{B-/-}$ chimeras (▲), containing variable numbers of OT-II T cells, were plated with APC and OVA peptide. Cytokines (IL-2, IFN-γ, and IL-4) in the supernatant after 3 days of culture were measured by ELISA. The data are normalized to the number of OT-II T cells in each culture (assessed by FACS staining) and presented as the amount of cytokine produced per 10^4 OT-II T cells. Symbols show the mean of groups of four mice and SEM. This experiment was repeated twice with similar results

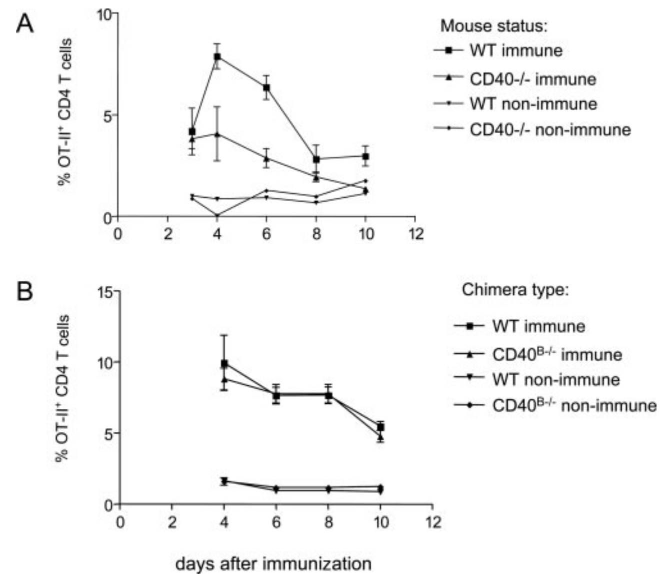

FIGURE 8. T cell expansion is not dependent on CD40 costimulation of B cells. *A*, OT-II T cells transferred into CD40$^{-/-}$ and WT (C57BL/6) mice and then immunized with OVA peptide in CFA. The percentage of Vα2- and Vβ5-bearing CD4 T cells was assessed by FACS over time. The difference in OT-II expansion in CD40$^{-/-}$ mice was statistically significant at days 6 ($p < 0.01$), 4 and 10 ($p < 0.05$). *B*, OT-II T cells transferred into WT or CD40$^{B-/-}$ chimeras and then immunized with OVA peptide in CFA. The percentage of Vα2- and Vβ5-bearing CD4 T cells was assessed by FACS. Symbols show the mean of each group and the error bars show the SEM. Each symbol is representative of at least three mice. Statistical comparisons were made using the unpaired *t* test. These data are representative of two similar experiments.

LTα expression by B cells is not required for complete CD4$^+$ T cell expansion

To investigate whether the B cell contribution to the maintenance of splenic architecture influenced the efficiency of T cell priming and proliferation, we made mixed BM chimeras in which the B cells do not express LTα. Other sources of LTα were normal, allowing lymph node development in these mice. Although there was significantly lower OT-II T cell expansion at day 3 in the absence of B cell-derived LTα, by day 4 expansion was identical to that in control chimeras (Fig. 9). Therefore, T cells are slower to divide but can expand to normal levels by the peak of the response.

Discussion

Clonal expansion of the transferred OT-II CD4 T cells was observed to be subnormal from the earliest stages of the response in the mixed BM chimeras in which B cells bore no MHC-II. We conclude, therefore, that B cells contribute as Ag presenters to the very early stages of the response. The clone size of the responding T cells remained subnormal throughout the course of the response, subsequently giving rise to only small numbers of memory cells. Furthermore, effector T cell differentiation into cytokine-secreting cells was impaired in absence of B cell APC function, a defect particularly severe in the Th2 (IL-4) response.

Some studies (11, 14) have shown that B cells are capable of participating in the priming of naive T cells; however, these relied on an increase in frequency of Ag-specific B cells (by transferring BCR-transgenic cells) to detect such effects. Therefore, despite the demonstration that B cells can prime T cells in vivo, the consensus has been that they play a very minor role in the early stages of responses because the frequency of Ag-specific B cells (the only efficient B cell APC) was too low. Other studies (6–8, 43) have used B cell-deficient mice and have shown that T cell expansion and differentiation (especially Th2) is suboptimal in the absence of B cells. These studies are inconclusive because of the apparent defects in the DC compartment in B cell-deficient mice (21, 32). Our experiments circumvent both of these issues. We constructed mixed BM chimeric mice in which B cells lacked MHC-II and therefore could not present to T cells. The presence of B cells expressing LTβ in these mice should allow normal development of the DC populations and, indeed, we found no defect (data not shown). Moreover, we show that repopulation of the MHC-II$^{B-/-}$ chimeras with WT B cells had the effect of normalizing the T cell expansion. Thus, we are confident that the data presented clearly demonstrate a role for B cells in supporting the T cell response during its initiation. It is, therefore, possible that the presence of peptide-MHC complexes on B cells within hours of immunization (16, 18) could lead to functional Ag presentation and T cell activation. Also, the studies showing movement of Ag-engaged B cells to the boundary between the T and B cell zones within 6 h of

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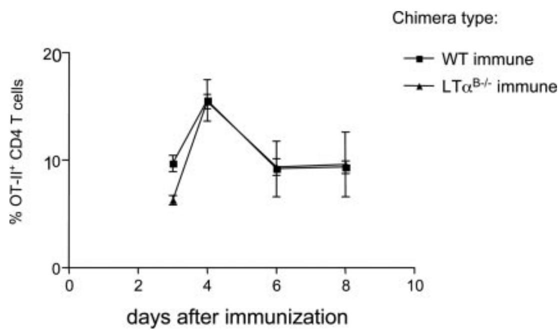

FIGURE 9. LTα expression by B cells is not required for optimal T cell expansion. OT-II T cells transferred into WT or LTα$^{B-/-}$ chimeras and then immunized with OVA peptide in CFA. The percentage of Vα2- and Vβ5-bearing CD4 T cells was assessed by FACS. Symbols show the mean of each group and the error bars show the SEM. This experiment was conducted twice with similar results.

Ag exposure (44, 45), despite using nonphysiologically high frequencies of Ag-specific B and T cells, may well represent the physiological situation. Our data suggest that cognate interactions with B cell APC are necessary for optimal T cell priming and, as such, the migration of the Ag-activated T and B cells to the follicular border driven by changes in chemokine receptors (45) may be crucial for all T cells and not just those destined to become B cell/follicular helpers (46, 47). It should also be noted that Ag-specific B cells start to proliferate in T zones by 2 days after immunization (48, 49) and therefore it may be in these foci that B cells exert their early influence.

What are B cells providing as APC? More Ag presentation events/interactions or something qualitatively different from that delivered by DC? We believe both: the analysis of in vivo cell division shows that in the absence of B cell Ag presentation the population of OT-II T cells divide less. This indicates that DC presentation alone is not sufficient to support and sustain T cell proliferation. This is most easily explained by the insufficient numbers of productive T cell-APC interactions. Lanzavecchia and Sallusto (50) have argued that T cell differentiation is regulated to a large extent by the strength of signal (an integration of the TCR avidity/costimulation, number of signals, and duration of signals). Thus, signal strength can influence differentiation into effector cells or memory cells and into the type of effector T cell (50, 51). Our data highlight the potential for B cells to alter the balance toward a higher signal strength, perhaps explaining the relative lack of effector cytokine secretion from T cells responding in mice in which B cells cannot present Ag. We (26, 37) and others (7, 8) have suggested that B-T cell and DC-T cell interactions are also qualitatively distinct, based mainly on their capacity to bias T cell differentiation toward Th2 (26, 27), an ability dependent on molecules such as OX40L (52) or cytokines (53). However, this might also be explained simply by continued Ag presentation by B cells. Indeed, it is becoming clear that CD4 T cells need sustained access to antigenic stimuli throughout their expansion phase (54), unlike CD8 T cells that require only a short pulse of Ag to drive proliferation and full differentiation (55, 56).

It could be argued that we observe an impaired CD4 T cell response because the frequency of Ag-specific T cells is artificially high in this system and for that reason alone the DC cannot support full clonal expansion. To address this, we looked at the endogenous T cell response in the MHC-II$^{B-/-}$ and control chimeras using MHC-II tetramers. The endogenous T cell primary response was subnormal at day 9 (the peak) and also at day 15 (data not shown) in the MHC-II$^{B-/-}$ chimeras. The WT chimeras exhibit a >5-fold primary increase in Ag-specific T cells, whereas in the MHC II-deficient-B cell chimeras there was barely a 2-fold increase. The actual increases will be greater than this since the background we detect is non-Ag-specific binding and not a measure of Ag-specific cells in the preimmune state, but the fact remains that the MHC-II$^{B-/-}$ chimeras show less expansion. We think the preimmune repertoire/frequency of MHC-II$^{B-/-}$ and WT chimeras is similar since reconstituting them with a cohort of WT B cells restores a normal response.

We also used the endogenous T cell response and the MHC-II tetramers to look at memory formation in the absence of B cell presentation. Memory T cell responses were quite dramatically reduced in the MHC-II$^{B-/-}$ chimeras. Some memory cells had been generated during the primary response as, after boosting, the number of tetramer-positive CD4 T cells was augmented over the numbers in the primary response. We believe that the relative failure to populate the memory pool is directly related to the reduced primary clonal expansion in MHC-II$^{B-/-}$ chimeras. This is in line with observations of memory pool size being directly proportional to clonal burst size for CD8 (57) and CD4 T cells (58). In the WT chimeras, tetramer-positive memory T cells expanded >10-fold after boosting. In the MHC-II$^{B-/-}$ chimeras, this is difficult to measure exactly because the frequency of tetramer-positive memory cells was below detectable limits (0.1%) before reimmunization; however, the expansion in these chimeras is at least 4-fold and possibly much more. This indicates that primary clone size is the limiting factor in forming the memory pool in these mice and not a problem of maintenance.

As a master regulator of costimulatory activity on APC, we expected CD40 to be an important component of the T cell-priming capacity of B cells. However, the CD40-deficient-B cell chimeras showed surprisingly that this was not the case; CD4 T cell clonal expansion was normal whether or not B cells expressed CD40. The expression of CD40 on other APC, such as DC, that initiate the response is absolutely necessary, since T cell responses in CD40ko mice were severely impaired. Thus, the maturation of APC/costimulatory function of DC brought about by CD40 signals is imperative and is sufficient to activate even a large number of (transgenic) T cells. Taking the results from these chimeras and MHC-II$^{B-/-}$ chimeras together, it seems that B cells in sustaining the T cell response do so solely by means of Ag presentation, and CD40-induced costimulatory activity is not required. This lends further weight to the argument that the main role of B cells in driving T cell clonal expansion is a quantitative one. An important consequence of CD40 signaling is the up-regulation of OX40L that is crucial for further differentiation and survival of activated T cells (25); our own previous data (37) and that of others (59) show that DC are sufficient to provide this signal. An alternative explanation for the lack of any role for CD40 activation of B cells in T cell expansion is that B cells' costimulatory activity, although enhanced by CD40, is already sufficiently high due to BCR (and TLR) ligation (60). Some mediators produced by B cells (e.g., OX40L, cytokines) are dependent on CD40 for their expression and therefore it would be interesting to know whether the effector cytokine response and memory generation was normal in the CD40$^{B-/-}$ chimeras. The preliminary data show that the IFN-γ response was unimpaired in the mice that lacked CD40 only on B cells.

Abs because of their ability to capture Ags and then bind to FcR on APC may enhance Ag uptake and presentation by non-B cell APC. In the MHC-II$^{-/-}$ B cell chimeras, no Ag-specific IgG is made (data not shown) and therefore this could contribute to impaired T cell expansion. However, this seems unlikely, since the

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decreased T cell expansion was seen as early as day 3 after immunization, before an Ag-specific IgG response is detectable. Moreover, an Ag-specific IgG response fails to occur in $CD40^{-/-}$ B cell chimeras in which no difference in OT-II T cell expansion was seen. This indicates that T cell expansion is normal in the absence of Ag-specific IgG.

B cells, mainly through their expression of membrane-bound LT ($LT\alpha1\beta2$), profoundly influence the development and architecture of lymphoid tissues. Mice in which a $LT\beta$ deficiency is restricted to B cells lack FDC, MZM, and MM (21). The status of DC in these mice is less clear. However, DC subset insufficiency in B cell-deficient mice has been reported (31) and very recently B cell-derived $LT\beta$ has been shown to control DC turnover in the spleen (32). Despite this, in our experiments we found no functional DC insufficiency or any consequent impairment of T priming resulting from a lack of $LT\beta$ on B cells.

If Ag presentation by B cells is necessary for optimal T cell priming, it is pertinent to ask whether all B cells are equal in their ability to provide this function. Follicular B cells take part in T-dependent (TD) responses, marginal zone B cells are involved in T-independent and possibly TD responses, B1 cells can respond to T-independent Ags, while transitional B cells have not been assigned a clear function. Despite the belief that follicular B cells make TD responses, Kearney and colleagues have recently demonstrated that marginal zone B cells are the most efficient presenters of protein Ag to $CD4^+$ T cells in vitro and in vivo (61). We now need to examine the differential function of these B cell subpopulations in APC/costimulator roles addressed here but also in the potential role of B cells as regulators of T cell responses.

In conclusion, we show that B cells are absolutely required as APC in normal primary responses to Ag. Any role in the CD40-dependent costimulation of the T cell response is either subtle or dispensable, as it was not apparent in these experiments. Our data suggest that B cells provide extra and essential Ag presentation capacity over and above that provided by DC. Since BCR-mediated uptake may make them especially efficient APC at low (physiological) Ag doses, B cells may be crucial when Ag levels are very low. The consequences of deficient B cell presentation on responses to infectious organisms will be of interest.

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Disclosures

The authors have no financial conflict of interest.

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