

# Costimulation via OX40L Expressed by B Cells Is Sufficient to Determine the Extent of Primary CD4 Cell Expansion and Th2 Cytokine Secretion In Vivo

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## Abstract

The development of effector and memory CD4 cell populations depends upon both T cell receptor (TCR) engagement of peptide/major histocompatibility complex (MHC) class II complexes and ligation of costimulatory molecules with counter receptors on antigen-presenting cells (APCs). We showed previously that sustained interactions with APCs could be crucial for optimal expansion of CD4 cells and for development of effectors that secrete cytokines associated with Th2 cells. Using an adoptive transfer model with TCR transgenic CD4 cells, we now show that responses of CD4 cells primed in B cell-deficient mice become aborted, but are fully restored upon the transfer of activated B cells. Although B cells have the capacity to secrete multiple cytokines that could affect CD4 priming, including IL-4, we were unable to distinguish a role for cytokines that are secreted by B cells. However, B cell costimulation via the OX40L/OX40 pathway that has been implicated in CD4 cell expansion, survival, and Th2 development was required. Th2 but not Th1 responses were impaired in OX40L-deficient recipients and normal responses were restored with OX40L sufficient B cells. The results suggest that without engagement of OX40L on B cells, CD4 cell responses to many protein Ag would be dominated by Th1 cytokines. These data have important implications for strategies to achieve optimal priming of CD4 subsets.

Key words: CD4 subsets • B lymphocytes • antigen-presenting cells • cytokines • Th2 cells

## Introduction

The development of immunity depends upon the generation of an expanded population of CD4 effectors which mediate inflammatory processes that lead to clearance of foreign Ag and the persistence of a cohort of primed cells with the capacity to mount a rapid recall response. Initial activation of naive CD4 cells depends upon prolonged signaling through TCR engagement of peptide/MHC complexes as well as ligation of costimulatory molecules on APC. Productive interactions can be achieved by sustained as well as serial encounters of T cells with APC (1). The duration of stimulation can determine the commitment of CD4 cells to division which typically correlates with the development of effector functions (2). Once division commences, effectors can expand and differentiate independently of Ag (3, 4). Cytokine production and optimal induction of costimulation are two major mechanisms that

contribute to the growth, differentiation, and survival of CD4 cells. Through these mechanisms, APCs are integral not only for initiating a CD4 response but also for determining magnitude of expansion, and development of effector populations that secrete cytokines associated with Th1 or Th2 phenotypes (e.g., TNF- $\alpha$  and IFN- $\gamma$ , or IL-4, IL-5, and IL-13, respectively). In this context, APCs can be viewed as orchestrating the outcome of a primary immune response which determines the frequency of persisting memory cells and their cytokine commitment.

Naive CD4 cells initially interact with peptide Ag presented by dendritic cells (DCs)\* which express MHC class II and B7 family molecules that provide the key costimulation for the induction of the T cell response via CD28 (5). As a consequence, additional costimulatory receptors are up-regulated on CD4 cells, including CD40L (CD154),

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\*Abbreviations used in this paper: CFSE, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; LDA, limiting dilution analysis.

OX40 (CD134), and ICOS (6, 7), and become engaged with counter-receptors on DC to promote optimal T cell responses. Once activated, CD4 cells also form conjugates with B cells (8) which become competent to act as APC and provide costimulation after activation by uptake of Ag, ligation of costimulatory/adhesion molecules, or interaction with cytokines. Our previous studies and others have shown that B cells can be necessary *in vivo* to support T cell expansion (9) and in this capacity can play a crucial role in the development of effector responses and memory (4, 10). We also found that sustained CD4 responses are necessary for the generation of high frequencies of effectors that secrete the Th2 cytokines, IL-4, IL-5, and IL-13, and that B cells can function to regulate the processes of differentiation and expansion *in vivo* (10). Although subsets of DCs can also promote development of type I or type II CD4 cells (11), recruitment of DCs diminishes after the initial phase of a response (1), and DCs retain the capacity to present Ag and produce cytokines that affect T cell differentiation for only a limited duration before further maturing and exiting lymphoid sites (12). Thus, a switch in APC usage from DCs to B cells can be favored as a response proceeds, and in many instances may be necessary to sustain progression to Th2 cytokines.

In this study, we investigated mechanisms that underlie the capacity of B cells to support CD4 effector development *in vivo*. As our previous work pointed to Ag-independent processes, we focused on the involvement of cytokines and costimulation in an adoptive transfer model where naive TCR transgenic CD4 cells are dependent upon provision of B cells as APCs for optimal expansion and differentiation to a Th2 response. Although several studies suggest that activated B cells produce cytokines that can regulate DC function as well as T cell survival and effector development (13, 14), we were unable to distinguish roles for key cytokines that could participate in this response, *i.e.*, IL-4, IL-6, and IL-10. However, costimulation via OX40/OX40L, a TNF/TNFR-family pathway which regulates CD4 cells (6, 7) was an essential contribution of B cells.

## Materials and Methods

**Mice.** Normal and splenectomized C57BL/6, C57BL/6 Igh-6  $\mu$ MT (immunoglobulin  $\mu$  chain knock out mice); and B6.PL Thy1<sup>+</sup>/Cy, IL-4, -6, and -10 deficient mice were from The Jackson Laboratory. OX40L-deficient mice were provided by Dr. B. Paigen (The Jackson Laboratory, Bar Harbor, ME) with kind permission from Dr. K. Sugamura (Tohoku University, Sendai, Japan; reference 15) and bred in our vivarium. OT-II TCR ( $\alpha$ 2,  $\nu$  $\beta$ 5) CD4 transgenic mice that respond to peptide 323–339 of OVA were obtained from W. Heath (Walter and Eliza Hall, Melbourne, Australia; reference 16) and bred in our colony to B6.PL-Thy1<sup>+</sup>/Cy mice to facilitate tracking by Thy 1.1 in C57BL/6 and  $\mu$ MT recipients that express Thy 1.2.

**T and B Cell Isolation and Adoptive Transfer.** CD4 cells were isolated from the spleens and pooled lymph nodes of OT-II Thy 1.1 mice by negative selection using magnetic sorting with an enrichment cocktail from Stem Cell Technologies Inc. according to

the manufacturer's instructions.  $\nu$  $\alpha$ 2,  $\nu$  $\beta$ 5<sup>+</sup> cells were quantitated by flow cytometry and  $5 \times 10^6$  transgenic CD4 cells were injected intravenously into C57BL/6,  $\mu$ MT, or OX40L<sup>-/-</sup> mice. For some experiments, CD4 cells were labeled before injection with the fluorescent dye, CFSE (5-and-6)-carboxyfluorescein diacetate succinimidyl ester; Molecular Probes). Mice were immunized on the day of cell transfer by intraperitoneal injection of 100  $\mu$ g alum precipitated OVA (Sigma-Aldrich) with *B. pertussis* vaccine organisms (4). In some experiments, mice were also given a subcutaneous injection of the same dose of Ag at the base of the tail. B cells were isolated from the spleens of C57BL/6 mice or various knockout mice by magnetic separation with an enrichment cocktail (Stem Cell Technologies Inc.) and incubated overnight in RPMI 1640 media (Irvine Scientific) containing 7% FCS (Irvine Scientific), 200  $\mu$ g/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, 10 mM Hepes, and  $5 \times 10^{-5}$  M 2-ME. The cultures were also supplemented with 10 ng/ml rIL-4, 10  $\mu$ g/ml dextran sulfate (Sigma-Aldrich), 5  $\mu$ g/ml LPS (Difco Laboratories), and 100  $\mu$ g/ml OVA.  $5 \times 10^6$  B cells were injected intravenously into T cell recipient mice 24 h after immunization with OVA.

**Flow Cytometry.** The following reagents from BD Biosciences were used for staining of T and B cells: FITC-conjugated rat IgG2a (R35–95), -anti-MHC class II (AF6–120.1), -CD40 (3/23), and -B7–2 (GL1); PE-conjugated anti- $\nu$  $\alpha$ 2 (B20.1); and biotinyl-anti- $\nu$  $\beta$ 5 TCR (MR9–4). Peridinin chlorophyll protein (PerCP)-conjugated streptavidin was from Becton Dickinson. APC-conjugated-anti-Thy1.1 (HIS51) and FITC-conjugated-anti-rat IgG were from eBioscience. FITC-labeled rat anti-OX40L (RM134L) was a gift from Dr. Michael Croft (La Jolla Institute of Allergy and Immunology, La Jolla, CA). Cell division of transgenic cells was analyzed by CFSE staining after gating on  $\nu$  $\alpha$ 2,  $\nu$  $\beta$ 5, and Thy1.1<sup>+</sup> cells. Samples were analyzed with a FACSCalibur<sup>TM</sup> flow cytometer using CELLQuest software (Becton Dickinson). 10,000 gated events were analyzed for surface marker expression and a minimum of 20,000 gated events was collected for CFSE analysis.

**In Vitro Responses of CD4 Cells.** Donor CD4 cells were enriched from spleens of recipient mice at various times after cell transfer as indicated in the text for individual experiments. Anti-Thy 1.2 labeled magnetic beads (Miltenyi Biotec) were added to CD4 enrichment mAb from Stem Cell Technologies before magnetic sorting. Resulting populations of Thy 1.1<sup>+</sup> cells were exclusively transgenic as indicated by  $\nu$  $\alpha$ 2 and  $\nu$  $\beta$ 5 expression. For *in vitro* restimulation, varying numbers of Thy 1.1<sup>+</sup> cells from individual mice were cultured in triplicate in 250  $\mu$ l in 96-well flat-bottomed plates (Costar) with 8  $\mu$ g/ml OVA peptide, and with  $2 \times 10^5$  APCs that were C57BL/6 spleen cells activated by overnight culture with dextran sulfate and LPS, and treated with mitomycin C (4). For limiting dilution analysis (LDA) of IL-2 and IL-4 secreting cells, serial dilutions of CD4 cells were plated in 36-well replicates in combination with APC and peptide. For ELISPOT analysis to enumerate IFN- $\gamma$ -producing effectors, varying numbers of cells were cultured in duplicate with APCs and peptide for 24 h in Immunospot M200 plates (Cellular Technology Limited) that were coated with 4  $\mu$ g/ml anti-IFN- $\gamma$  capture antibody (R46A2 from BD Biosciences).

**Cytokine Assays.** IL-2 and IL-4 secretion were assessed by bioassay (4). The sensitivity of detection is 1 pg/ml for IL-2 and 0.5 pg/ml for IL-4. For LDA, wells with cpm values greater than three standard deviations above the mean of values from 36 wells containing APCs were considered positive. Frequencies were calculated using maximum likelihood analysis. IL-1, -5, -6, -10,

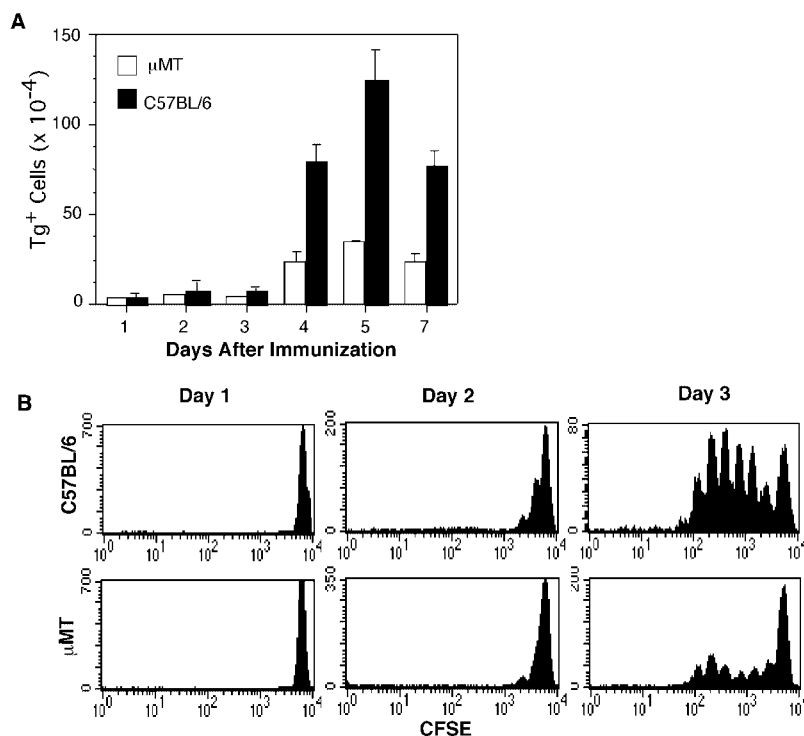
-12, -13, and IFN- $\gamma$  were measured by ELISA as described (10) using capture and detecting mAb pairs from BD Biosciences. The sensitivity of detection for these cytokines is 10–30 pg/ml. Recombinant cytokines from R&D Systems were used for quantitation. IFN- $\gamma$  ELISPOTs were developed by sequential incubation of plates with biotin-anti-IFN- $\gamma$  (XMG1.2; BD Biosciences), streptavidin-alkaline phosphatase, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Kirkegaard & Perry Laboratories) as described (17). ELISPOTs were counted with an ImmunoSpot Analyzer (Cellular Technology Limited).

## Results

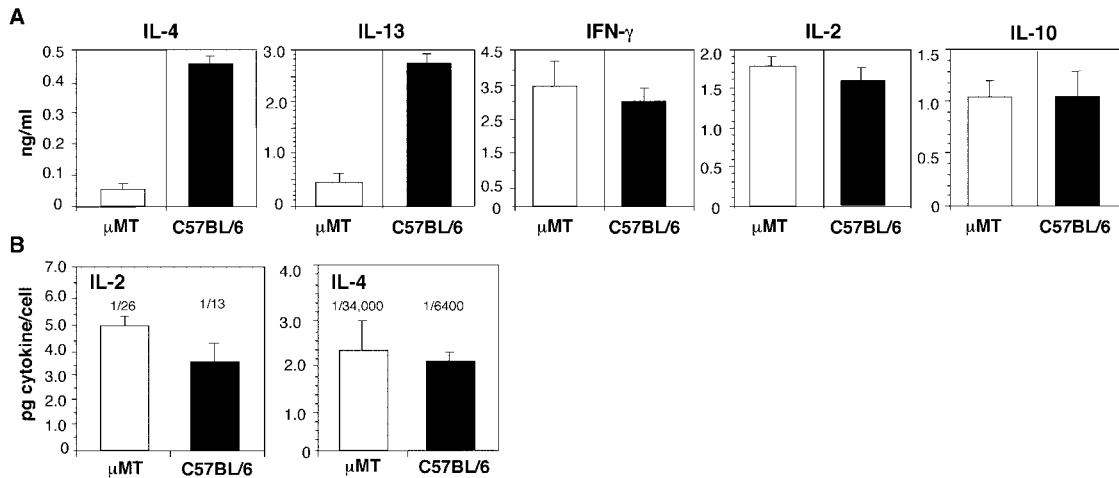
**Deficient CD4 Cell Expansion in B Cell-deficient Mice.** Our previous studies showed that CD4 cell responses are suboptimal in B cell-deficient mice as measured by a low frequency of Ag-specific cells (4) and limited development of effectors that secrete Th2 cytokines (10). To evaluate mechanisms that might affect CD4 priming at the level of Ag-specific CD4 cells, we established an adoptive transfer model using OT-II  $\nu\alpha 2$ ,  $\nu\beta 5$  TCR transgenic CD4 cells that are specific for OVA. The response of naive transgenic CD4 cells was followed in C57BL/6 or B cell deficient ( $\mu$ MT) recipients by flow cytometry after immunization with OVA protein. An increase in the number of transgene<sup>+</sup> CD4 cells in the spleen was observed by d4 after immunization (Fig. 1 A). While similar kinetics were observed with both groups of recipients, a much lower level of donor cell accumulation occurred in the absence of B cells. The data suggest that the response of CD4 cells was not fully supported in  $\mu$ MT mice and was aborted. To determine if the reduced recovery of OT-II cells reflected more limited expansion, cell division of transgenic donor

cells in recipients was analyzed after CFSE-labeling. OT-II cells did not begin to proliferate until d3 after immunization (Fig. 1 B). Substantially fewer cells divided in  $\mu$ MT mice, but division was at the same rate seen in control animals. By d7, the majority of cells in both groups of recipients had undergone several divisions, although a larger proportion of the population remains undivided in  $\mu$ MT mice (14%) than in C57BL/6 mice (3%). Together the results suggest that fewer cells become initially committed to division in the absence of B cells, and that their expansion is not sustained.

**Defective Development of Th2 Cytokine-secreting Effectors in the Absence of B Cells.** Although the capacity of CD4 cells to produce effector cytokines does not depend upon division, several studies indicate DNA synthesis and entry into cell cycle does typically correlate with effector development, particularly for secretion of Th2 cytokines (2, 18). Although our previous study showed limited development of Th2 effectors in the absence of B cells, we could not directly compare Ag-specific populations from the intact animals (10). With the adoptive transfer model we could test whether cytokine production by Ag-specific cells was altered as a consequence of priming in  $\mu$ MT mice. Donor CD4 cells isolated from C57BL/6 and  $\mu$ MT mice on the basis of Thy1.1 expression on d4 after immunization of recipients were compared for their capacity to produce IL-2 and several effector cytokines after restimulation with peptide and APC. As seen in Fig. 2 A, while OT-II cells produced similar quantities of IL-2, IL-10, and IFN- $\gamma$  irrespective of whether they were primed in normal or B cell-deficient mice, those from  $\mu$ MT mice produced markedly lower levels of IL-4 and IL-13. IL-5 was not detectable in this model.



**Figure 1.** Recovery and division of CD4 cells in immunized B cell-deficient mice. (A)  $5 \times 10^6$   $\nu\alpha 2$ ,  $\nu\beta 5$  CD4 cells from OTII-Thy1.1 mice were transferred to Thy1.2  $\mu$ MT or C57BL/6 mice that were then primed by intraperitoneal injection of 100  $\mu$ g OVA/alum and *B. pertussis* organisms. Donor cells in the spleens of recipients were enumerated from the total cell recovery after FACS<sup>®</sup> staining with anti-CD4, -Thy1.1, - $\nu\alpha 2$ , and - $\nu\beta 5$ . Data are the average values from 2 mice/group. B. OT-II Thy 1.1<sup>+</sup> cells were CFSE-labeled before injection into  $\mu$ MT or C57BL/6 mice that were primed as for A. Cell division was analyzed as CFSE fluorescence gating on transgene<sup>+</sup> donor cells. Data are representative of three experiments.



**Figure 2.** Defective development of Th2 cytokines in the absence of B cells. (A) Transgene<sup>+</sup> OT-II Thy 1.1 cells were transferred to  $\mu$ MT or C57BL/6 mice that were immunized as for Fig. 1. On d4, donor cells were enriched from recipient spleens by magnetic sorting and cultured with APCs and OVA peptide. Culture supernatants were tested at 36h by bioassay for IL-2 and IL-4, or by ELISA for IL-10, IL-13, and IFN- $\gamma$ . Data are the average cytokine amounts in cultures of  $5 \times 10^4$  (IL-4 and IL-13) or  $2.5 \times 10^4$  transgene<sup>+</sup> cells/well (other cytokines). (B)  $\alpha\alpha 2$ ,  $\nu\beta 5$ , Thy 1.1 cells that were recovered on d4 from  $\mu$ MT or C57BL/6 OVA-primed mice were serially diluted in 36-well replicate cultures containing APC and OVA peptide. Supernatants were tested for IL-2 and IL-4. At the dilution where IL-2 or IL-4 producers/well is limiting (i.e.,  $\geq 37\%$  negative wells), the average amount of IL-2 or IL-4 per well of replicate cultures that scored positive is shown. Frequencies (shown above each bar) were quantitated using maximum likelihood analysis.

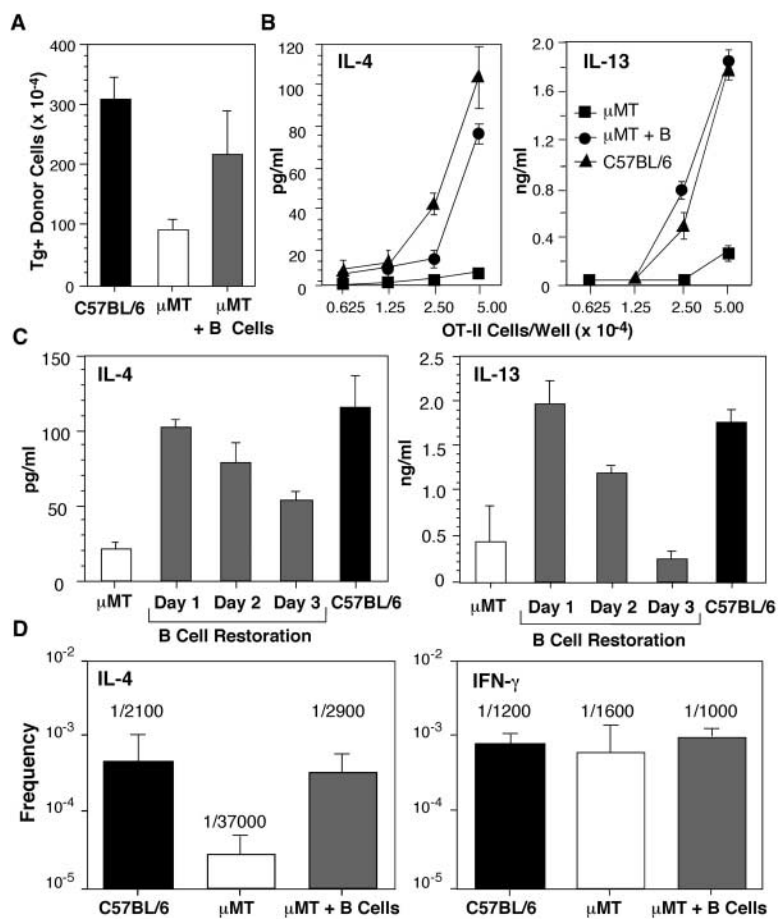
To determine if limited production of Th2 cytokines reflected differences in the levels of secretion by individual cells and/or the frequencies of cytokine producers, we used LDA to evaluate IL-2 and IL-4 production by OT-II cells isolated from recipient mice. As shown in Fig. 2 B, at the single cell level, OT-II cells produced similar levels of IL-2 and IL-4/cell whether primed in normal or  $\mu$ MT mice. The frequencies of IL-2-producing cells were similar in both groups of recipients, but fivefold fewer IL-4 producers were detected among OT-II cells from  $\mu$ MT mice. These data establish that cells with the capacity to produce normal levels of IL-4 do develop in the absence of B cells, but that fewer cells are present in the effector populations from  $\mu$ MT mice.

*Activated B Cells Restore CD4 Cell Responses in  $\mu$ MT Mice.* Our studies showed that splenic DC from  $\mu$ MT mice are not defective in their ability to initiate or support CD4 cell responses but are likely to become limiting (4, 10). To confirm that B cells can be responsible for deficient priming of CD4 effectors, C57BL/6 and  $\mu$ MT mice were reconstituted with activated, Ag-pulsed B cells. Provision of B cells fully restored expansion of OT-II cells in  $\mu$ MT mice (Fig. 3 A) as well as the capacity for Th2 cytokine secretion (Fig. 3 B). We showed previously that transferred B cells persisted throughout CD4 effector development in  $\mu$ MT mice (10) and here we find that the optimal time period for reconstitution of the response was during the initial 48 h (Fig. 3 C). While the numbers of IL-4-producing cells were augmented by the presence of B cells in  $\mu$ MT mice, frequencies of IFN- $\gamma$ -producing effectors were not significantly altered (Fig. 3 D), as we showed previously for innate CD4 cells from  $\mu$ MT mice (10). The results suggest that the IFN- $\gamma$  response which can develop before that of

IL-4 (2, 19) can be optimally primed without a contribution from B cells.

*Limited CD4 Responses in  $\mu$ MT Mice Are Not Restricted to the Spleen.* A previous study showed that B cell-deficient mice have reduced numbers of T cells and DCs in their spleens as well as defective splenic T zone architecture due to the absence of B cells during development (20). Although we find that OT-II cells localize normally in the spleens of  $\mu$ MT mice (unpublished data), their limited accumulation/expansion after priming might reflect not only limited availability of APC, but also defects in the microenvironment that include reduced numbers of stromal cells and expression of the lymphoid tissue chemokine, CCL21. However, T zone development as well as chemokine expression in LN is B cell independent and  $\mu$ MT mice have normal T cell and DC numbers in these tissues. Thus, if B cells directly rather than indirectly contribute to CD4 effector responses, their requirement should also be evident in the LN.

To test this hypothesis, C57BL/6 or  $\mu$ MT recipients were injected with CFSE-labeled OT-II cells and immunized with OVA and adjuvant (subcutaneously and intraperitoneally). An additional group of  $\mu$ MT recipients also received activated B cells. Division of the transgenic donor cells was assessed in LN at 2 1/2 d after priming. Fewer transgene<sup>+</sup> cells were dividing at this time in  $\mu$ MT mice than in C57BL/6 mice, and the presence of B cells improved both expansion (Fig. 4 A) and recovery (Fig. 4 B). The data suggest that, as previously observed in studies mice rendered B cell deficient by antibody treatment (21), B cells are necessary for CD4 cell responses in LN as well as spleen. To determine if migrating cells from the spleen might contribute to the defective response in LN, we also evaluated cytokine secretion of OT-II cells primed in spleen.

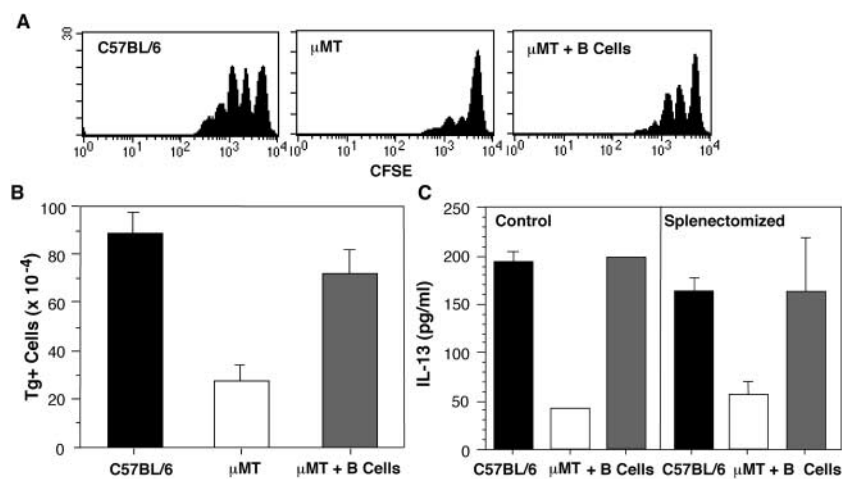


**Figure 3.** Activated B cells restore CD4 cell expansion and Th2 cytokine secretion. (A) Two groups of  $\mu$ MT mice and 1 group of C57BL/6 mice were injected with OT-II Thy 1.1 cells and primed intraperitoneally with OVA/adjuvant as for Fig. 1. After 24 h, 1 group of  $\mu$ MT hosts was injected with  $5 \times 10^6$  B cells that were activated as indicated in the Material and Methods. On d4 after priming, transgene<sup>+</sup> cells were enumerated as for Fig. 1. (B) Using the same groups as in panel A, IL-4 and IL-13 production by  $\alpha$ 2,  $\nu$ 5 CD4 cells isolated 4 d after priming was measured at 36 h after restimulation with APC and OVA peptide. (C) OT-II Thy 1.1 cells were transferred to four groups of  $\mu$ MT mice and one group of C57BL/6 mice that were subsequently primed intraperitoneally with OVA/adjuvant. Activated B cells were injected into each of three groups of  $\mu$ MT mice 1, 2, or 3 d after receiving CD4 cells. On d4 after priming,  $5.0 \times 10^4$  transgene<sup>+</sup> cells/well were tested for IL-4 or IL-13 secretion after restimulation with APC and OVA peptide. (D) IL-4 and IFN- $\gamma$  producing cells in C57BL/6,  $\mu$ MT, and B cell-reconstituted  $\mu$ MT mice were enumerated by LDA and ELISPOT assay, respectively. The data shown for panels A–D are the average responses of three mice/group.

necrotomized recipients. As shown in Fig. 4 C, a more limited Th2 cytokine response was seen in  $\mu$ MT mice unless reconstituted with B cells, and there were no differences relative to the controls.

*B Cell-derived Cytokines Do Not Have an Apparent Role in Sustaining CD4 Responses.* One mechanism by which B cells could contribute to Th2 differentiation is through cy-

tokine production. We found that B cells that were activated in culture produced readily detectable levels of several cytokines that included IL-1, -2, -4, -6, -10, and -13, as well as IFN- $\gamma$  (Fig. 5 A). In view of a major role for IL-4 in CD4 cell differentiation, and a potential in vivo role for B cell-derived IL-4 in Th2 development (13, 22), we isolated B cells from IL-4 knockout mice and assessed their

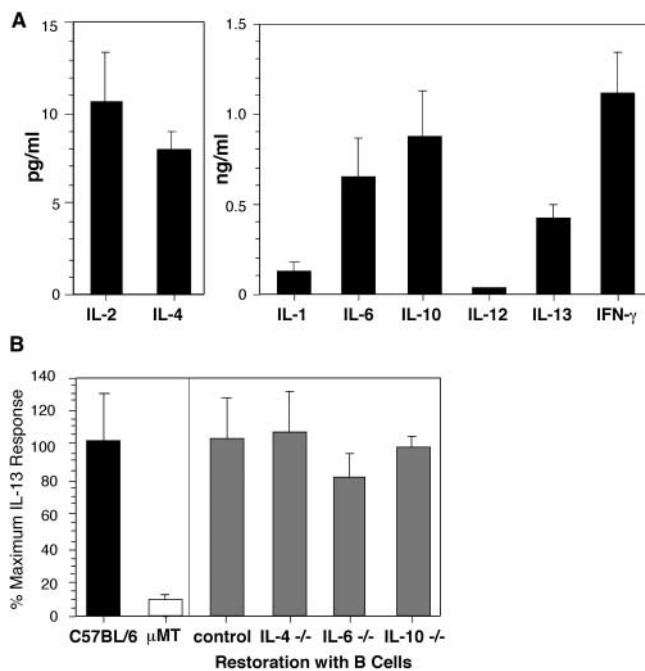


**Figure 4.** Defective CD4 cell response is not limited to the spleen. (A) Transgene<sup>+</sup> OT-II Thy 1.1 cells were CFSE-labeled before injection into one group of C57BL/6 mice or two groups of  $\mu$ MT. At the time of cell transfer, recipients were immunized subcutaneously and intraperitoneally with OVA/adjuvant. One group of  $\mu$ MT mice was injected with B cells on the day after priming as for Fig. 3. At 60 h after cell transfer, cell division of donor cells in draining periaortic and mesenteric lymph nodes was analyzed by CFSE fluorescence of transgene<sup>+</sup> cells. (B) Recovery of transgene<sup>+</sup> cells determined from the total cell counts for the three groups of recipients shown in panel A on d4 after cell transfer. Data are representative of two experiments. (C) Responses of transgene<sup>+</sup> cells from lymph nodes of normal and splenectomized  $\mu$ MT and C57BL/6 recipients on d4 after cell transfer and intraperitoneal and subcutaneous immunization

with OVA/adjuvant as for panel A. Additional control and splenectomized  $\mu$ MT recipients were injected with B cells as for Fig. 3. IL-13 secretion by  $5 \times 10^4$  donor transgene<sup>+</sup> cells was assessed after restimulation with APC and OVA peptide.

capacity to restore responses of  $\mu$ MT mice. We also examined B cells from IL-6-deficient mice, since this cytokine can support differentiation and survival of Th2 cells (23) and IL-10-deficient mice, as IL-10-mediated down-regulation of IL-12 synthesis by DCs can facilitate Th2 responses (24). However, B cells from the cytokine-deficient animals were fully capable of restoring development of IL-13-producing effectors in  $\mu$ MT mice (Fig. 5 B). The levels of IL-4 secretion were also normal when  $\mu$ MT mice were reconstituted by B cells from the cytokine deficient animals (unpublished data). The data suggest either that redundancy of cytokine functions can compensate for a single deficiency, or that B cell production of IL-4, -6, or -10 is not obligatory.

*A Role for Costimulation via B Cell Expression of OX40L in Sustaining CD4 Responses.* Like DC, activated B cells express multiple costimulatory molecules that are involved in the development of CD4 responses as revealed by the dramatic consequences of gene knockout studies (5, 7, 15, 25). However, activated T cells are less dependent upon costimulation. It is unclear whether continued availability is necessary once a response is underway when cytokines that can drive expansion and survival become produced in the local milieu. The OX40L/OX40 pathway that has been



**Figure 5.** Role of cytokine secretion by B cells. (A) Purified B cells were cultured at  $2 \times 10^6$ /ml with 10  $\mu$ g/ml dextran sulfate, 5  $\mu$ g/ml LPS, and 100  $\mu$ g/ml OVA. Supernatants collected at 24 h were tested for IL-2, -4, -1, -6, -10, -12, -13, and IFN- $\gamma$ . The average pg/ml from five separate experiments is shown for each cytokine. (B) B cells purified from C57BL/6 (control), IL-4<sup>-/-</sup>, IL-6<sup>-/-</sup>, and IL-10<sup>-/-</sup> mice were activated and transferred to  $\mu$ MT mice that were injected 24 h previously with OT-II Thy 1.1 cells and immunized intraperitoneally with OVA/adjuvant as for Fig. 1. On d4 after priming,  $\alpha$ 2,  $\nu$ 5, Thy 1.1 cells were isolated and  $5 \times 10^4$  tested for IL-13 secretion by comparison to transgene<sup>+</sup> cells recovered from C57BL/6 mice and from  $\mu$ MT mice that did not receive B cells. Shown are the average responses of 3–5 mice/group normalized to the response by OT-II cells immunized in C57BL/6 mice.

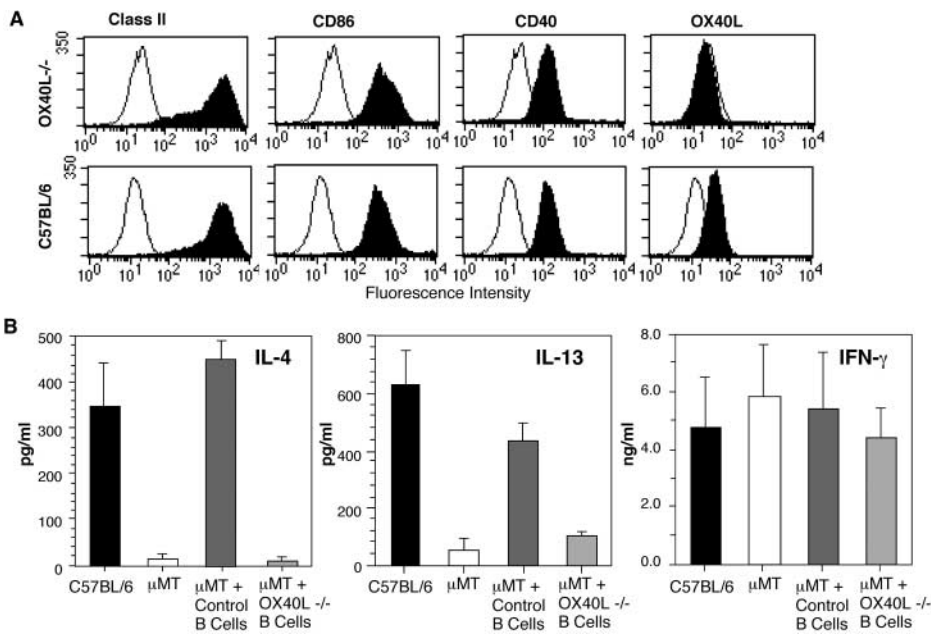
implicated in both T cell priming and T–B interactions (26–28) appears to also have the potential to contribute to effector responses in vivo (15, 29). We therefore determined if this pathway might play a role in B cell regulation of CD4 effector development in our model. We used B cells from the OX40L gene knockout animals (15) to reconstitute OT-II responses in  $\mu$ MT mice. These B cells expressed normal levels of class II MHC, as well as B7-2, and CD40 (Fig. 6 A). However, activated B cells that lacked OX40L were incapable of restoring the IL-4 and IL-13 responses whereas IFN- $\gamma$  responses were normal in the absence of B cells and not further augmented by their addition (Fig. 6 B).

It is noteworthy that CD4 cell proliferation is defective in mice that lack OX40 (30), suggesting that a major contribution of costimulation through this pathway is to promote T cell expansion. OX40 deficient CD4 cells have been shown to become initially activated to secrete IL-2 and divide normally, but they are unable to sustain proliferation (29). As OX40L deficient mice have normal numbers of T and B cells, we asked whether OX40L signals from B cells affect the development of Th2 responses. OT-II cells were transferred into C57BL/6 or OX40L<sup>-/-</sup> mice and primed with OVA protein/adjuvant. An additional group of OX40L<sup>-/-</sup> recipients were then given C57BL/6 B cells as in the experiments with  $\mu$ MT mice. On d4 after immunization, the cytokine responses of reisolated transgenic donor cells were measured. As shown in Fig. 7, OT-II cells primed in OX40L<sup>-/-</sup> mice were defective in their production of IL-4 and IL-13 but not of IFN- $\gamma$  or IL-2 (unpublished data). Importantly, B cells from normal mice fully restored defective Th2 priming in OX40L<sup>-/-</sup> recipients. Together with the experiments showing that OX40L<sup>-/-</sup> B cells do not restore the responses of  $\mu$ MT mice, the data reveal that OX40L expression on B cells can be crucial for the expansion/survival of Th2 cells.

## Discussion

Many studies indicate that B cell and T cell responses are coordinated to orchestrate cross regulation of expansion, survival, and differentiation via cooperative interactions. Although the essential role of CD4 cell-derived cytokines in B cell differentiation and responses has been widely studied, mechanisms that underlie the regulation of CD4 cell responses by B cells have not yet been fully explored. In this study, we determined that B cells can directly affect CD4 effector responses through costimulation by OX40L. We show that in the absence of B cells that express OX40L, Ag-induced expansion of CD4 cells and development of effectors that secrete Th2 cytokines are impaired.

Several studies have identified a link between B cell Ag-presentation and the induction of IL-4 secretion by CD4 cells (31, 32) but did not reveal whether additional signaling pathways were required. Although a recent report suggests that B cells can provide a source of IL-4 for differentiation of Th2 cells (13), this does not appear to be the sole



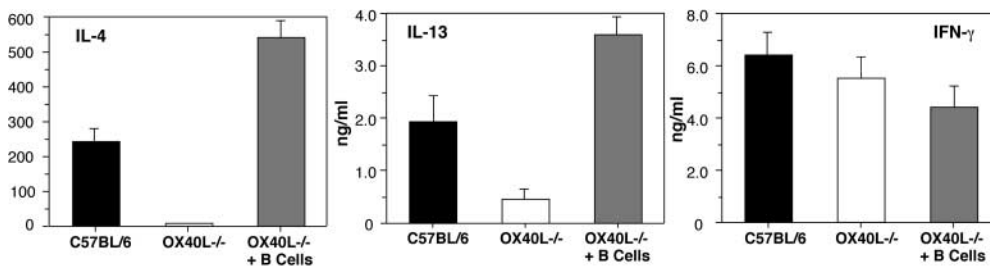
**Figure 6.** A role for OX40L expression on B cells in sustaining CD4 responses. (A) Purified B cells from groups of two OX40L<sup>-/-</sup> or C57BL/6 mice were cultured 64 h with 10 ng/ml rIL-4, 10 μg/ml dextran sulfate, 5 μg/ml LPS, and 100 μg/ml OVA before staining and FACS<sup>®</sup> analysis. Shown as solid histograms is fluorescence staining for class II MHC, CD40, CD86 (B7-2), and OX40L compared with isotype controls (open histograms). Data are representative of 2 experiments. B. B cells purified from C57BL/6 (control B cells) or OX40L<sup>-/-</sup> mice were activated and transferred to μMT mice that were previously injected with OT-II Thy 1.1 cells and immunized with OVA/adjuvant as for Fig. 1. Control C57BL/6 and μMT mice did not receive B cells. On d4 after priming, α2, vβ5 cells were isolated and tested for IL-4, IL-13, and IFN-γ secretion as for Fig. 2. Shown are cytokine levels detected from 2–6 mice per group. The data are representative of four experiments.

mechanism to support Th2 responses since our results show that IL-4-deficient B cells were fully capable of eliciting Th2 cytokines. However, it is striking that several costimulation molecules have profound effects on the development of CD4 cells. It has been proposed that three major costimulatory pathways, CD28/B7, CD40L/CD40, and OX40/OX40L, act sequentially and synergistically to regulate CD4 cell responses from initial activation to optimal expansion, survival, and migration into B cell follicles (6). Although CD4 cell signaling through CD28, CD40L, or OX40 are associated with IL-4 induction (6), *in vivo* studies suggest that IL-4 production and responses that require IL-4 may be particularly dependent upon the OX40/OX40L pathway (27, 33, 34). Our data reveal that B cells can be an essential population to provide signals via OX40L to CD4 cells.

We envision that following CD4 cell activation by TCR-interactions with MHC/peptide complexes and CD28 engagement of B7 molecules, receptors such as CD40L and OX40 are induced and serve to amplify TCR signaling, as well as enable responding T cells to interact with B cells which constitutively express CD40 and up-

regulate OX40L in response to activation. Temporally, OX40 expression on T cells coincides with induction of OX40L on B cells (27). Although interactions with DCs can initially support the response, they become limiting as T cells proliferate, and without further recruitment of DC, usage of B cells can become necessary to sustain the response. Our data suggest that CD4 cells can be maintained in this physiologic context by OX40 interactions with OX40L on B cells. Although this pathway can also contribute to the early activation phase of CD4 responses via DCs (15), our study of the knockout animals as recipients of Ag-specific CD4 cells shows that although priming occurs, Th2 responses are limited. Importantly, normal B cells are sufficient to restore Th2 effector development.

Our previous studies suggest that other APC can fulfill the role of B cells in CD4 cell expansion and Th2 development if made available in sufficient numbers during effector priming (4, 10). In addition, provision of costimulation to T cells via signaling with an agonist antibody to OX40 (29) is associated with increased frequencies of Ag-specific CD4 cells. Recent studies also implicate OX40 in survival of activated CD4 cells (35), suggesting that expansion together



**Figure 7.** OX40L deficiency in B cells is sufficient to limit Th2 development. C57BL/6 mice and two groups of OX40L<sup>-/-</sup> mice were injected with OT-II Thy 1.1 cells and immunized intraperitoneally with OVA/adjuvant. B cells from C57BL/6 mice were activated and transferred to one group of OX40L<sup>-/-</sup> recipients

on the day after T cell transfer. Control C57BL/6 and OX40L<sup>-/-</sup> mice did not receive B cells. On d4 after priming, transgene<sup>+</sup> donor cells were isolated and tested for IL-4, IL-13, and IFN-γ secretion as for Fig. 2. Shown are cytokine levels detected from 2–4 mice per group. The data are representative of two experiments.

with increased lifespan may allow effectors to participate in a response for an extended period. Our results where a high number of Ag-specific naive CD4 cells are present at the time of immunization show that the capacity to secrete several effector cytokines, with the notable exception of Th2 cytokines, can develop independently of expansion that is sustained via OX40/OX40L interactions.

In view of our finding that IL-4 producing effectors, though reduced in number appear to be primed normally, and previous reports that OX40/OX40L interactions maintain later rather than initial CD4 cell expansion, we envision an indirect role of costimulation in the development of Th2 responses through effects on survival (35) and division (29). The data support the concept that sustained responses are necessary for optimal Th2 development (19). As the OX40 pathway has been associated with CD4 cell accumulation in germinal centers (36), it is attractive to speculate that B cell regulation of CD4 cell survival and response by OX40 in turn facilitates a sustained B cell response (37). Although cytokines produced by APC including IL-13, IL-1, and IL-6 can support Th2 differentiation, IL-4 is the key mediator to function in this capacity (38). The lag in optimal priming for IL-4 synthesis in our model may in part be due to dependence of responding CD4 cells upon autocrine secretion and usage of IL-4 to direct Th2 development (39) through induction of transcription factors that include Stat6, Gata3, and Gfl-1 (38, 40). Conditions of strong TCR stimulation and costimulation may be necessary to achieve sufficient endogenous IL-4 levels for commitment to a Th2 phenotype and achieve stable secretion after extended chromatin remodeling that accompanies cell division (41).

Our study shows that *in vivo*, OX40L expressed on B cells can play a crucial role in determining the outcome of a response in terms of the frequency and cytokine commitment of effectors and, from our previous work and that of others, in turn on persisting memory. In the absence of B cell involvement, CD4 cell responses, at least to many protein Ag would most typically be biased toward Th1 cytokines, as is often observed in responses where Ag is limiting or of low affinity. The data indicate that modulation of costimulation through OX40 can provide a key checkpoint in the regulation of CD4 cell responses.

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