

Anergy in Memory CD4⁺ T Cells Is Induced by B Cells^{1,2}

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Induction of tolerance in memory T cells has profound implications in the treatment of autoimmune diseases and transplant rejection. Previously, we reported that the presentation of low densities of agonist peptide/MHC class II complexes induced anergy in memory CD4⁺ T cells. In the present study, we address the specific interaction of different types of APCs with memory CD4⁺ T cells. A novel ex vivo anergy assay first suggested that B cells induce anergy in memory T cells, and an in vivo cell transfer assay further confirmed those observations. We demonstrated that B cells pulsed with defined doses of Ag anergize memory CD4 cells in vivo. We established that CD11c⁺ dendritic cells do not contribute to anergy induction to CD4 memory T cells, because diphtheria toxin receptor-transgenic mice that were conditionally depleted of dendritic cells optimally induced anergy in memory CD4⁺ T cells. Moreover, B cell-deficient muMT mice did not induce anergy in memory T cells. We showed that B2 follicular B cells are the specific subpopulation of B cells that render memory T cells anergic. Furthermore, we present data showing that anergy in this system is mediated by CTLA-4 up-regulation on T cells. This is the first study to demonstrate formally that B cells are the APCs that induce anergy in memory CD4⁺ T cells. *The Journal of Immunology*, 2008, 181: 3221–3231.

Memory T cells are a small subpopulation of a pool of Ag-experienced lymphocytes that have undergone proliferation and morphological changes and survived Ag-induced cell death (1–4). Memory T cells are believed to play crucial roles in mounting fast and efficient recall responses against Ags (5, 6). For naive T cells residing in lymph nodes (LNs),⁶ it is documented that dendritic cells (DCs) that have captured Ag from the peripheral tissues are the primary APCs for initiation of the immune responses. There is also evidence that DCs can tolerize Ag-specific naive T cells (7). Although recent reports document Ag presentation as a contributing factor to the development of memory T cells (8, 9), evidence for specific interaction of different types of APCs with memory CD4⁺ T cells remains elusive. It is not clear whether DCs are also potent activators of memory T cells. Even less information is available on the types of APCs that interact with memory T cells and render them tolerant. In the present study, we address this specific question using our well-

characterized system. By studying human T cell clones, we reported that the presentation of 1–10 peptide/MHC II complexes per APC induce anergy (10). Extending these observations to in vivo systems, we found that memory, but not activated or naïve, T cells were anergized upon the presentation of low densities of specific Ags (11). Induction of anergy in these reports demonstrated that unresponsiveness was not due to deletion or ignorance and that IL-2 plus peptide could reverse the state of unresponsiveness. In another report, we examined differences in the levels of activation of TCR-mediated signaling molecules triggered by the presentation of stimulatory vs anergy-inducing ligands to T cells (12). Those studies provided clear evidence that stimulation of T cells with ligands of different avidity dictated the nature of T cell response: a high avidity stimulation induced T cell activation, whereas a low avidity stimulation by the same agonist peptide caused T cell anergy. TCR engagement below the tolerogenic threshold had no inhibitory effects on recall responses. These observations highlight the significance of APCs that encounter T cells. Because the level of expression of MHC II and other molecules that contribute to T cell-APC membrane interaction can vary on different subpopulations of APCs, it is important to investigate whether certain APCs preferably interact with memory T cells.

Induction of tolerance in memory T cells has profound implications for the treatment of autoimmune diseases and for controlling transplant rejection. It would be of clinical benefit to identify APCs that can present Ag to memory T cells in a tolerogenic fashion. Mature DCs express high levels of MHC II on their surface (13), making them potential candidates for capture and presentation of low amounts of the peptide Ags necessary for the induction of anergy in our system. However, B cells bearing specific receptors for Ag might be suitable APCs to anergize memory T cells when the antigenic load becomes sparse at the end of an infection and a contraction of the T cell response is desired.

To test different populations of APCs for their abilities to trigger anergy ex vivo, we designed an assay that incorporates the cocubation of memory DO11.10 transgenic (Tg) T cells (chicken OVA (cOVA)_{323–339}/I-A^d specific) with sets of APCs devoid of T cells isolated from groups of mice that had previously received

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⁶ Abbreviations used in this paper: LN, lymph node; B6, C57BL/6; cOVA, chicken OVA; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; Tg, transgenic; Treg, regulatory T cell.

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different doses of cOVA_{323–339}. We demonstrate that anergy is induced in memory T cells *ex vivo* when B cells presented the Ag at a certain range of low peptide doses. Further, we validated this finding *in vivo* by demonstrating that transferring resting B cells pulsed with low doses of peptide to mice bearing memory CD4⁺ T cells induced anergy. We ruled out a role for CD11c⁺ or other DCs in the induction of anergy in memory T cells. Consistent with the role for resting B cells expressing low levels of B7-1 in the induction of anergy, we showed that anergy in this system is regulated by CTLA-4.

Materials and Methods

Mice

TCR Tg mice (DO11.10) that express α/β TCR recognizing an I-A^d-restricted cOVA_{323–339} on BALB/c background were used as the source of T cells. Non-Tg female BALB/c mice and C57BL/6 (B6) mice at 5–6 wk of age, Tg female DO11.10 and OTII mice, and B cell-deficient muMT mice were all purchased from The Jackson Laboratory. Diphtheria toxin receptor (DTR) Tg mice on BALB/c background were bred with non-Tg BALB/c mice, and the heterozygous offspring for DTR were used for the study. All mice were housed in the Johns Hopkins University animal facilities (Baltimore, MD) under virus-free conditions. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Peptides and Abs

The peptide cOVA_{323–339} (ISQAVHAHAHAEINEAGR) was synthesized by Global-Peptide Services. The peptide was >90% pure as analyzed by reverse-phase HPLC. Fluorescently labeled Abs to mouse CD4, CD25, CD44, CD45RB, CD62L, CD69, CD45R (B220), CD19, CD11c, IL-2, IFN- γ , anti-mouse CTLA-4, CD80 (B7.1), and CD86 (B7.2) were purchased from BD Pharmingen; the Ab to clonotypic TCR (KJ1.26) specific for DO11.10 CD4⁺ T cells was purchased from Caltag Laboratories; and anti-mouse CTLA-4 Ab was purified from the culture supernatant of hybridoma (UC10-4F10-11; American Type Culture Collection (ATCC)).

Adoptive transfer and immunizations

KJ1.26⁺ Tg CD4⁺ T cells (2.0×10^5 cells per mouse), prepared from pooled LNs and spleens of DO11.10 mice, were resuspended in 100 μ l of sterile PBS (Invitrogen) and transferred *i.v.* into BALB/c recipients. Two days later, mice were immunized *s.c.* (at the base of the tail) with 15 nmol of cOVA_{323–339} peptide emulsified in CFA.

Induction of anergy in cOVA_{323–339}-specific memory CD4⁺ T cells *in vivo* by administration of low doses of peptide in IFA

Five weeks postimmunization, to allow for the generation of memory T cells (verified by staining for memory T cell markers; data not shown), mice were injected *s.c.* with increasing concentrations of cOVA_{323–339} peptide (0.005–50,000 pmol) mixed with IFA. Ten days later, inguinal LNs were removed and T cells were challenged *in vitro* with cOVA_{323–339} peptide. Proliferation was measured by CFSE dilution assay. Briefly, LN cells were labeled with 1 μ M CFSE in PBS for 80 s at room temperature and washed twice with RPMI 1640 containing 10% FCS. Cells were cultured with or without peptide at 37°C for 72 h. Following the culture, T cells were stained with fluorescently labeled anti-mouse CD4 Ab and KJ1.26 anticolonotypic TCR Ab and analyzed by flow cytometry. T cell division in response to *in vitro* peptide challenge was calculated as described (14) using the following formula: number of events (x) at each cell division n divided by 2^n . The percentage of total dividing cells = (total number of cohorts undergoing 1–6 divisions/total number of cohorts undergoing 0–6 divisions) \times 100.

Isolation of T cells

Spleens from 5-wk postimmunized mice were removed and a single-cell suspension was made after lysis of RBCs and used as the source of T cells. The mice sacrificed for this purpose had received a single immunization after adoptive transfer of T cells. By using an R&D Systems CD3 enrichment column, T cells were isolated from splenocytes. T cell purity was >95%.

Isolation of *in vivo* pulsed APCs

Forty-eight hours after *s.c.* injection of peptide emulsified in IFA, mice were sacrificed and inguinal LNs were removed. To separate whole APCs,

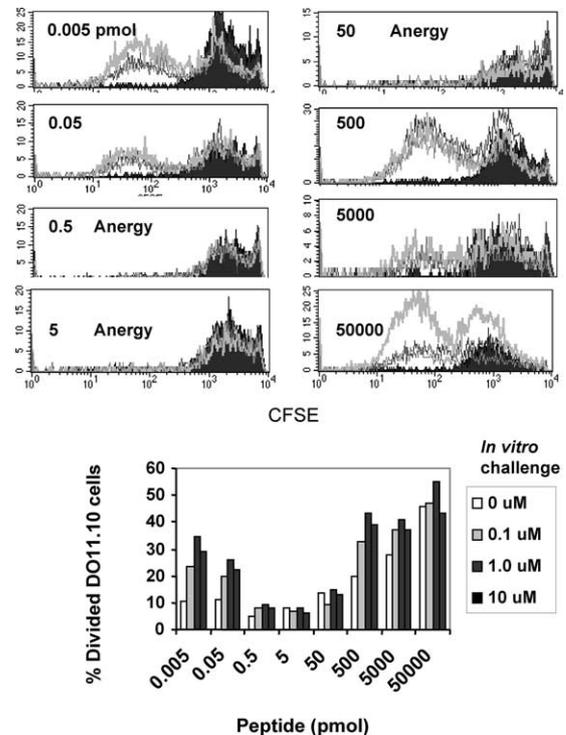


FIGURE 1. Administration of suboptimal doses of cOVA_{323–339} peptide in IFA induces anergy in specific memory CD4⁺ T cells *in vivo*. Eight groups of mice (three mice per group) bearing memory CD4⁺ T cells were challenged with increasing doses (0.005–50,000 pmol) of cOVA_{323–339} peptide in IFA. Ten days later, cells from draining LNs were harvested and anergy was assayed by peptide rechallenge for 72 h *in vitro*. T cell proliferation was measured by CFSE dilution assay of Ag-specific KJ1–26⁺ CD4⁺ T cells. The doses for the *in vitro* challenge with cOVA_{323–339} peptide are represented as follows: filled black histogram, 0 μ M; thin gray line, 0.1 μ M; black line, 1 μ M; thick gray line, 10 μ M. Data shown represent one of three independent experiments. The lower panel represents the percentage of Ag-specific cells (DO11.10) divided in response to *in vitro* challenge.

LN cells were depleted of T cells by using either Dynal-Thy1.2 beads or MACS-Thy1.2 microbeads (Miltenyi Biotec) (~95% depletion). To isolate B cells, a MACS B220 microbead was used (~95% purity). To obtain APCs devoid of B cells, LN cells were depleted of T and B cells by using a combination of Thy1.2 and B220 microbeads (MACS). MACS CD43 microbeads were used to purify B2 B cells. To obtain APCs devoid of B2 B cells, LN cells were depleted of T cells by Thy1.2 microbeads followed by positive selection with CD4 microbeads (15). These APCs are termed *in vivo* pulsed APCs in this study.

Induction of anergy in memory CD4⁺ T cells *ex vivo* by *in vivo* pulsed APCs

To induce anergy in memory CD4⁺ T cells, enriched T cells (1×10^5 /well) were incubated with *in vivo* pulsed APCs (irradiated, 2000 rad) at a ratio of 1:2 for 48 h in a 96-well plate. To test for anergy, splenocytes from normal mice were pulsed *in vitro* with cOVA_{323–339} peptide, irradiated, and added (2×10^5 per well) to triplicate culture wells and incubated further for 72 h before adding [³H]thymidine or for 10 h before assaying for intracellular cytokine synthesis. To determine the percentage of cells making IL-2 or IFN- γ , cells were stained with Abs to CD4, clonotypic TCR (KJ1.26), and IL-2 or IFN- γ and analyzed by flow cytometry. During data analysis, the quadrants were drawn based on the matched isotype Ab controls. The percentage of DO11.10 cells making IL-2 or IFN- γ was calculated after subtracting the percentage of DO11.10 cells stained positive for the matched isotypes.

Induction of anergy in memory CD4⁺ T cells *ex vivo* by *in vitro* pulsed B cells, DCs, or activated B cells

We followed the same anergy protocol as above except that B cells or DCs were purified from the spleens of unimmunized mice by using MACS-B220

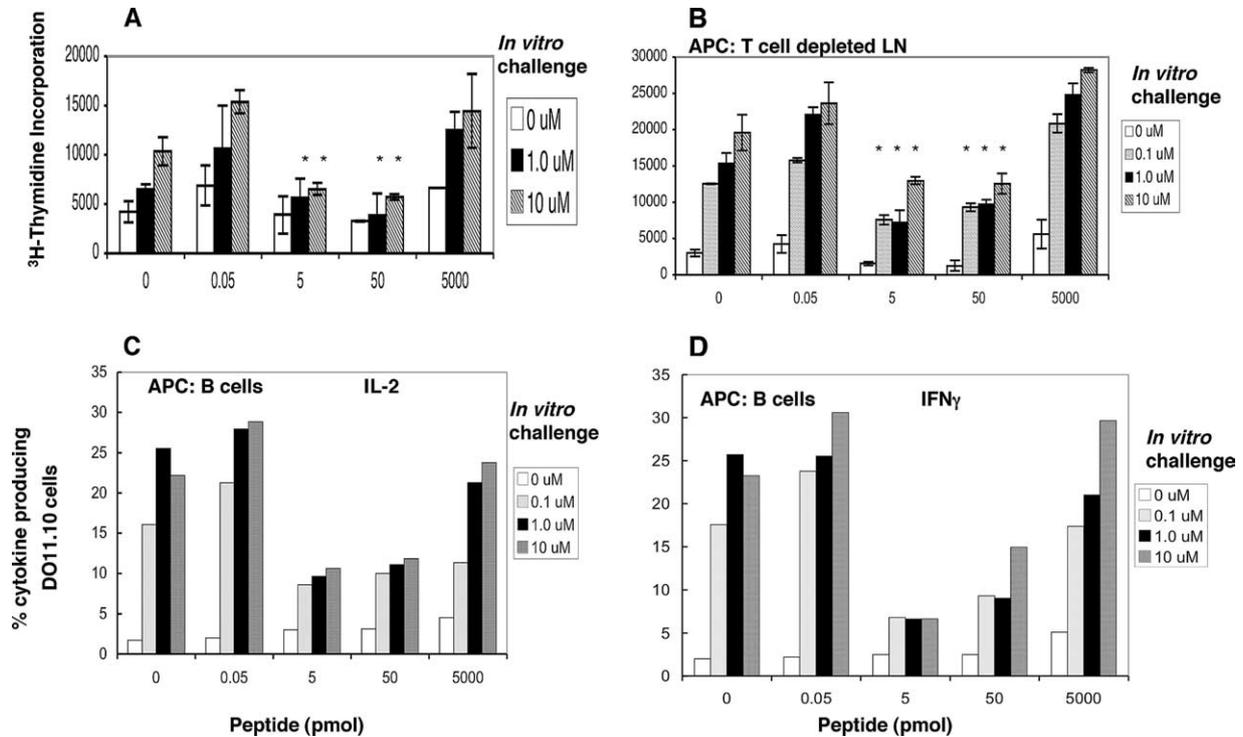


FIGURE 2. B cells loaded with low doses of Ag induce anergy in memory CD4⁺ T cells ex vivo. *A*, Anergy was induced in vivo (as described in *Materials and Methods*). Mice bearing memory CD4⁺ T cells were given cOVA_{323–339} in IFA for the induction of anergy and 48 h later draining LNs were harvested and tested for anergy in vitro using [³H]thymidine incorporation. *B–D*, Anergy was induced ex vivo. Memory T cells isolated (APC-depleted T cells) from mice immunized 5 wk or longer were incubated with APC populations (whole LN cells depleted of T cells (*B*) or enriched for B cells (*C* and *D*)) isolated from BALB/c mice given cOVA_{323–339} in IFA 48 h before the cell harvest. Isolated APCs (depleted of T cells by Thy1.2 microbeads) were pulsed with different doses of peptide as shown on the *x*-axis. Memory T cells and pulsed APCs were incubated for 48 h. Cultured cells were assayed for the induction of anergy during the first 48 h of ex vivo culture by the addition of freshly pulsed splenocytes at 0, 0.1, 1.0, and 10 μ M cOVA_{323–339} peptide in vitro and cell proliferation (*B*) and IL-2 (*C*) and IFN- γ production (*D*) were measured. The data shown represent one of three independent experiments. Bars represent mean \pm SD of triplicate cultures. *, $p < 0.005$, 5, or 50 pmol vs 0.05 or 5000 pmol of peptide doses (Student's *t* test). There were five mice per group and cells from triplicate wells were pooled before measuring IL-2 and IFN- γ synthesis. Cytokine synthesis was analyzed on KJ1.26⁺IL-2⁺ or KJ1.26⁺IFN- γ ⁺ cells gated on CD4⁺ T cells.

for B cells (purity >95%) or MACs-CD11c microbeads for DCs (purity >80%) and pulsed in vitro for 3 h at 37°C with the cOVA_{323–339} peptide before incubating with memory T cells. For activated B cells, purified B cells were stimulated with CpG (6 μ g/ml) for 24 h (16) and washed twice before pulsing with peptide. Note that the amount of peptide for pulsing APCs in vitro was calculated based on molar peptide concentration, whereas the peptides given to mice were in mole units.

Induction of anergy in memory T cells in vivo in the absence of CD11c⁺ DCs

BALB/c mice expressing DTR under the CD11c promoter (17) were adoptively transferred with DO11.10 Tg T cells. Two days later, mice were immunized s.c. with cOVA_{323–339} peptide in CFA. After 5 wk, DCs were depleted by multiple injections of diphtheria toxin (DT) (Sigma-Aldrich) during the induction of anergy in vivo in these mice. Briefly, mice were injected i.p. with 100 ng of DT per mouse 12 h before the injection of peptide emulsified in IFA. Then, mice were injected with the same dose of DT twice more, at 10 and 36 h after Ag challenge. Sampling of spleen and LN tissues during the treatment in a parallel set of mice confirmed the depletion of DCs in DT-injected mice as detected by selection on CD11c and GFP (expressed under the CD11c promoter), indicating <0.2% DCs in the LNs of those mice. On day 3 (60 h after Ag injection), mice were sacrificed and cells harvested from the draining LNs were challenged ex vivo with cOVA_{323–339} peptide for 72 h. Induction of anergy in Ag-specific CD4⁺ T cells was determined by CFSE dilution assay. The percentage of Ag-specific (DO11.10) CD4⁺ T cell divisions in response to in vitro peptide challenge was calculated as described above.

Induction of anergy in vivo in memory T cells by in vitro pulsed B cells

B cells were purified from the spleens of unimmunized mice and pulsed in vitro with increasing amounts of cOVA_{323–339} peptide (0.00001–1000 nM)

for 3 h at 37°C. Following incubation, B cells (not irradiated) were washed twice to remove excess peptide and 10⁷ cells/mouse were i.v. transferred to BALB/c mice bearing cOVA_{323–339}-specific memory CD4⁺ T cells. Ten days later, mice were sacrificed, and cells from draining LNs and spleens were harvested. Induction of anergy in Ag-specific T cells was determined by the CFSE dilution assay after in vitro challenge with the cOVA_{323–339} peptide for 72 h. The percentage of Ag-specific (DO11.10) CD4⁺ T cell division in response to in vitro peptide challenge was calculated as described above.

Induction of anergy in memory CD4⁺ T cells in vitro by in vivo pulsed B2 B cells

All B cells, B2 B cells, or APCs devoid of B2 B cells were obtained from the draining LNs of mice immunized 48 h earlier with peptide in IFA and incubated with memory CD4⁺ T cells for 48 h. Induction of anergy was tested by restimulating T cells with cOVA_{323–339} peptide-pulsed splenocytes for 72 h, followed by [³H]thymidine incorporation.

Induction of anergy in memory CD4⁺ T cells in B cell-deficient muMT mice

Because muMT mice are on B6 background, only for this experiment B6 mice were used as recipients and OT-II Tg CD4 T cells specific for cOVA_{323–339}/I-A^b were used for adoptive transfer and the generation of memory T cells. Recipient mice were immunized with cOVA_{323–339} in CFA for the induction of memory T cells a day after T cell transfer as described previously. In the experiments shown, memory cells were isolated 4–6 mo after immunization. CD4 T cells (7×10^6 to include $\sim 4.2 \times 10^4$ OT-II T cells) were then purified and transferred to muMT mice before the injection of Ag for anergy induction. B6 mice bearing memory OT-II cells were called B cell-sufficient mice. All groups of mice were immunized s.c. with cOVA_{323–339} in IFA. Nine days later, cells from draining LNs were harvested and assayed for anergy by their ability to proliferate

and to make IL-2 and IFN- γ using thymidine incorporation and intracellular cytokine assays. For thymidine incorporation, cells were stimulated with peptide for 72 h followed by the addition of [³H]thymidine and incubated for 18 h before harvest. For intracellular cytokine synthesis, cells were stimulated with peptide for 5.5 h in the presence of GolgiStop. OT-II cells were then stained with Abs to CD4, V α 2, V β 5.1, V β 5.2, and IL-2 or IFN- γ . During data analysis, the quadrants were drawn based on the matched isotype Ab controls. The percentage of OT-II cells making IL-2 or IFN- γ was calculated after subtracting the percentage of OT-II cells stained positive for the matched isotypes.

Expression of CTLA-4 in memory CD4⁺ T cells

Memory CD4⁺ T cells were incubated with in vivo pulsed APCs (B cells or APCs depleted of B cells) for 48 h. Surface expression of CTLA-4 on CD4⁺ T cells was determined by flow cytometry. The level of CTLA-4 was expressed as CTLA-4 index: mean fluorescence intensity \times fraction of cells positive for CTLA-4.

Reversal of anergy in memory CD4⁺ T cells by anti-CTLA-4

Memory CD4⁺ T cells were incubated with in vivo pulsed B cells for 48 h in the presence of 10 μ g/ml anti-mouse CTLA-4 Ab (UC10-4F10-11; ATCC). Anergy or reversal was tested by restimulating T cells with cOVA₃₂₃₋₃₃₉ peptide-pulsed splenocytes for 72 h, followed by [³H]thymidine incorporation.

Statistical analyses

Level of significance (*p* value) was determined by using unpaired Student's *t* test. *p* < 0.02 was considered significant.

Results

cOVA₃₂₃₋₃₃₉ specific CD4⁺ T cells acquire memory phenotype 35 days postpriming

We have previously shown the induction of anergy in memory CD4 T cells in response to low dose antigenic challenge in two antigenic systems (11). To extend those findings to the DO11.10 Tg system (cOVA₃₂₃₋₃₃₉/IA^d specific), DO11.10 Tg T cells were transferred into wild-type BALB/c mice (18) and 2 days later, which allowed the homing of transferred cells to the lymphoid organs, recipients were immunized with 15 nmol of cOVA₃₂₃₋₃₃₉ in CFA. Thirty-five days after immunization, the time required for the generation of memory T cells (11, 19), splenocytes were harvested and DO11.10 CD4⁺ T cells were stained for memory markers. Levels of CD25, CD44, CD45RB, CD62L, and CD69 expression were analyzed on CD4⁺ and KJ1.26⁺ double positive cells and compared with the levels on naive T cells from control mice treated with IFA or CFA alone. By day 35 postimmunization, >80% of the CD4⁺/KJ1-26⁺ T cells acquired the memory phenotype as evidenced by an increased level of CD44 and decreased levels of CD45RB and CD62L expression on their cell surfaces (20).

In vivo administration of low doses of peptide in IFA induces anergy in cOVA₃₂₃₋₃₃₉-specific memory CD4⁺ T cells

Mice previously immunized with cOVA₃₂₃₋₃₃₉ for the generation of memory T cells were administered different amounts of peptide in IFA. After 10 days, cells from draining LNs were labeled with CFSE and CD4⁺ T cells were stimulated in vitro by fresh APCs pulsed with increasing doses of peptide for 72 h. To determine Ag-specific cell proliferation, CFSE dilution analysis was done on CD4⁺/KJ1-26⁺ double positive cells by flow cytometry. We found that Ag-specific T cells that had encountered 0.5–50 pmol of peptide in vivo remained undivided, whereas T cells from the mice immunized with doses below or above that range divided several cycles (Fig. 1). The starting numbers of CD4⁺/KJ1.26⁺ T cells were comparable in all of the groups of mice immunized with peptide in IFA, indicating that T cells are not deleted but rather are anergized. These results extend the applicability of our previous findings to an entirely new antigenic system in which memory

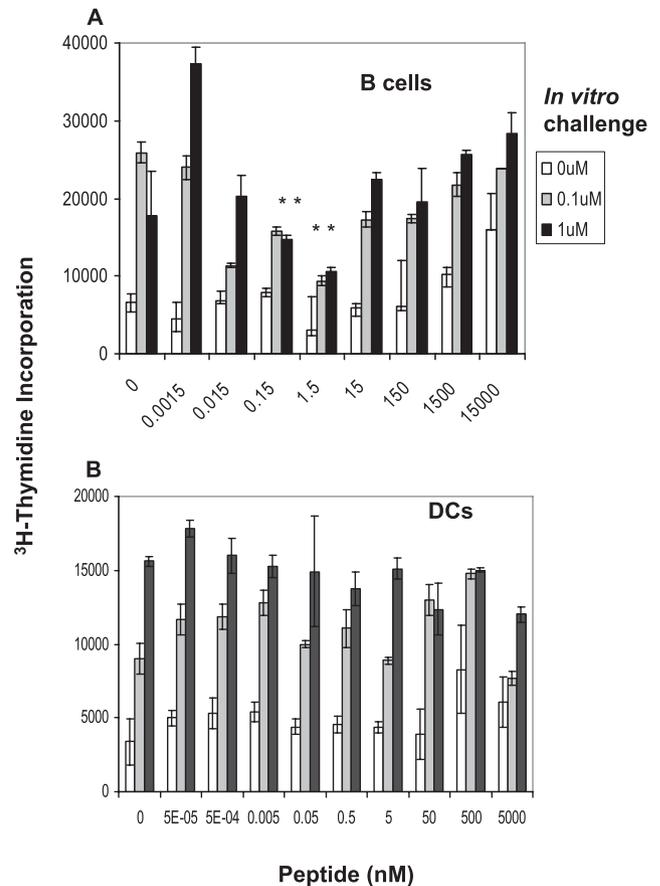


FIGURE 3. In vitro pulsed B cells induce anergy in memory CD4⁺ T cells ex vivo. Enriched B220⁺ B cells (A) or CD11c⁺ enriched DCs (B) from spleens of five normal BALB/c mice were pulsed with the indicated doses of cOVA₃₂₃₋₃₃₉ peptide for 3 h, incubated with memory CD4⁺ T cells for 48 h, and tested for anergy as described in the legend of Fig. 2 using [³H]thymidine incorporation. Data shown represent one of four independent experiments. Bars represent mean \pm SD of triplicate cultures. For A, *, *p* < 0.001, 0.15, or 1.5 nM vs 0.0015 or 15,000 nM peptide (Student's *t* test).

CD4⁺ T cells can be anergized by a range of suboptimal doses of Ags (11, 12).

Ex vivo anergy assay to test roles for different APCs in the induction of anergy in memory CD4⁺ T cells

Determining which APCs uniquely interact with memory T cells in vivo is experimentally challenging. To simplify the problem, an ex vivo assay was designed to evaluate the contribution of different APCs to the induction of anergy in CD4⁺ memory T cells. We hypothesized that APCs loaded in vivo with low doses of peptide could anergize memory T cells ex vivo in a similar manner as they do in vivo. To test this, memory T cells were isolated from mice 35 days postimmunization and coincubated with APCs from the draining LNs of mice that had been immunized with increasing doses of peptide (range 0–5,000 pmol) in IFA 48 h earlier. Cells were cultured for 48 h and then tested for anergy induction upon stimulation with splenocytes pulsed with 0, 0.1, 1, and 10 μ M peptide. Fig. 2A is a control proliferation experiment indicating that anergy is established within 48 h in vivo. Fig. 2, B–D, depict results of the ex vivo assay indicating the proliferation of T cells exposed to APCs from mice injected with different doses of peptide in IFA. These experiments clearly show that during the 48-h exposure to APCs, memory T cells became anergic in response to

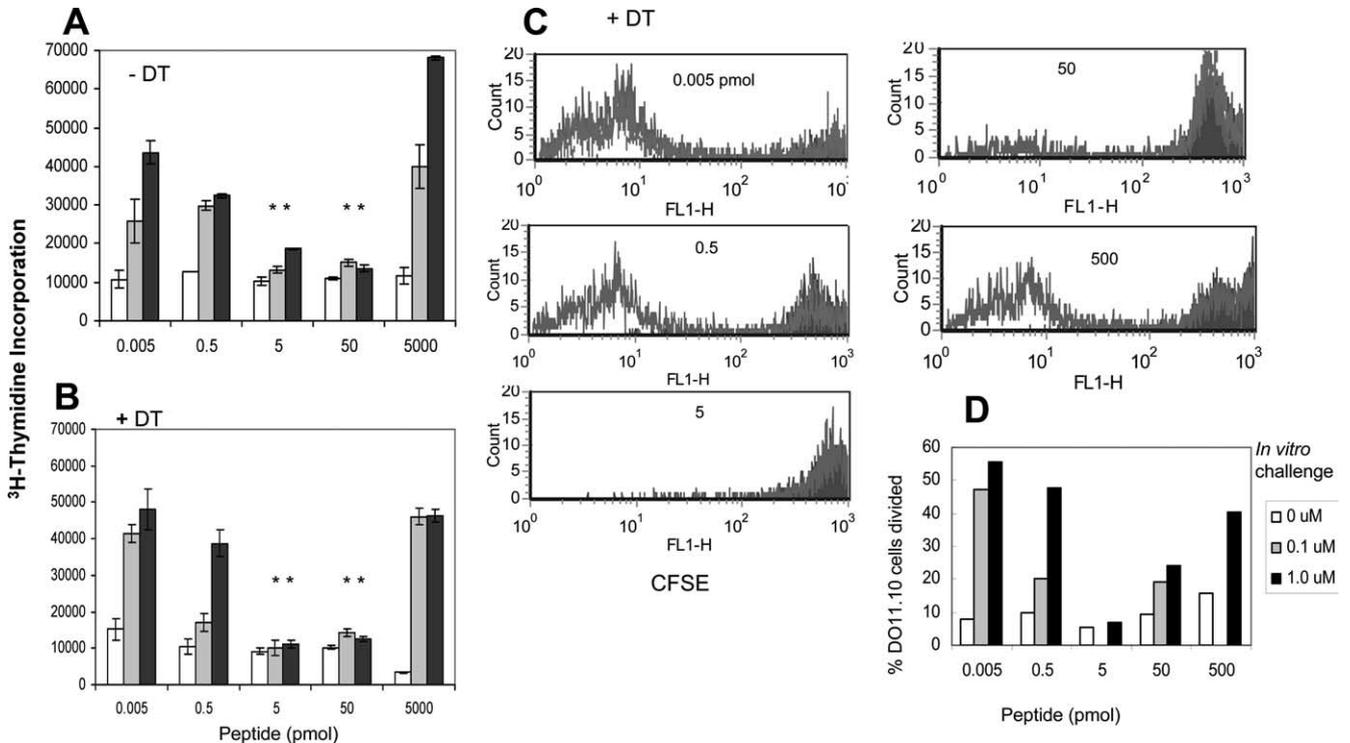


FIGURE 4. Absence of CD11c⁺ DCs does not affect the induction of anergy in memory CD4⁺ T cells in vivo. DTR Tg mice expressing DTR under the CD11c promoter (BALB/c background) were used as the source of memory CD4⁺ T cells. Mice were given daily i.p. injections of DT for 3 days starting 12 h before Ag challenge in IFA. Mice were sacrificed 60 h after Ag challenge, and cells from draining LNs were harvested. Induction of anergy was measured by [³H]thymidine incorporation (A and B) as well as by the CFSE dilution assay (C). For thymidine incorporation, cells were challenged ex vivo with cOVA_{323–339} peptide for 54 h and pulsed with [³H]thymidine for 18 h. For CFSE dilution assay, cells were labeled with CFSE and similarly challenged ex vivo with cOVA_{323–339} peptide for 72 h. Ag-specific T cell proliferation was measured by CFSE dilution assay using flow cytometry on CD4⁺ KJ1.26⁺ double positive cells. Doses for in vitro challenge with cOVA_{323–339} peptide are represented as follows: filled black histogram, 0 μM; black line, 0.1 μM; gray line, 1 μM. Data shown represent one of three independent experiments. Data in C are summarized as the percentage of dividing cells and shown in D. There were three mice per group. Bars represent mean ± SD of triplicate cultures. *, *p* < 0.001, 5, or 50 pmol vs 0.005 or 5000 pmol of peptide doses (Student's *t* test).

whole APCs (LN cells free of T cells) loaded with tolerogenic doses of peptide, while APCs loaded with nontolerogenic doses of peptide did not prevent the subsequent proliferation of T cells (Fig. 2B). The proliferation of memory T cells encountering tolerogenic doses of Ag, as accessed by [³H]thymidine incorporation, was significantly reduced (*p* < 0.005) compared with the proliferation of T cells encountering nontolerogenic doses of Ag, although not completely blocked. This background proliferation was likely because of nonspecific thymidine uptake by bystander lymphocytes. This might be due to the distribution of the MHC II molecules/MHC II-peptide complexes on APCs that most likely follow the Poisson distribution and, thus, those APCs with higher numbers of MHC II-peptide complexes induce activation in T cells rather than anergy.

B cells as the APCs that induce anergy in memory T cells

Next, we tested the ability of purified B220⁺-enriched B cells (to be called purified B cells throughout the article) loaded with peptide in vivo to induce anergy in Ag-specific memory T cells ex vivo (Fig. 2, C and D) by measuring IL-2 and IFN-γ synthesis. We found that B cells exposed to 5–50 pmol of peptide in vivo anergized memory T cells as assessed by their reduced ability to produce intracellular IL-2 and IFN-γ. Encouraged by these results, we investigated whether B cells pulsed with Ag in vitro could induce anergy ex vivo. Purified splenic B cells from unimmunized mice were pulsed in vitro with different amounts of peptide and tested for the induction of anergy in memory T cells ex vivo. We found

that splenic B cells pulsed with 0.1–1 nM peptide in vitro induced anergy (Fig. 3A). Note that we have used mole (mol) units for absolute amounts of peptide injected per mouse and concentrations of peptide used to pulse APCs ex vivo are in molar (M) units.

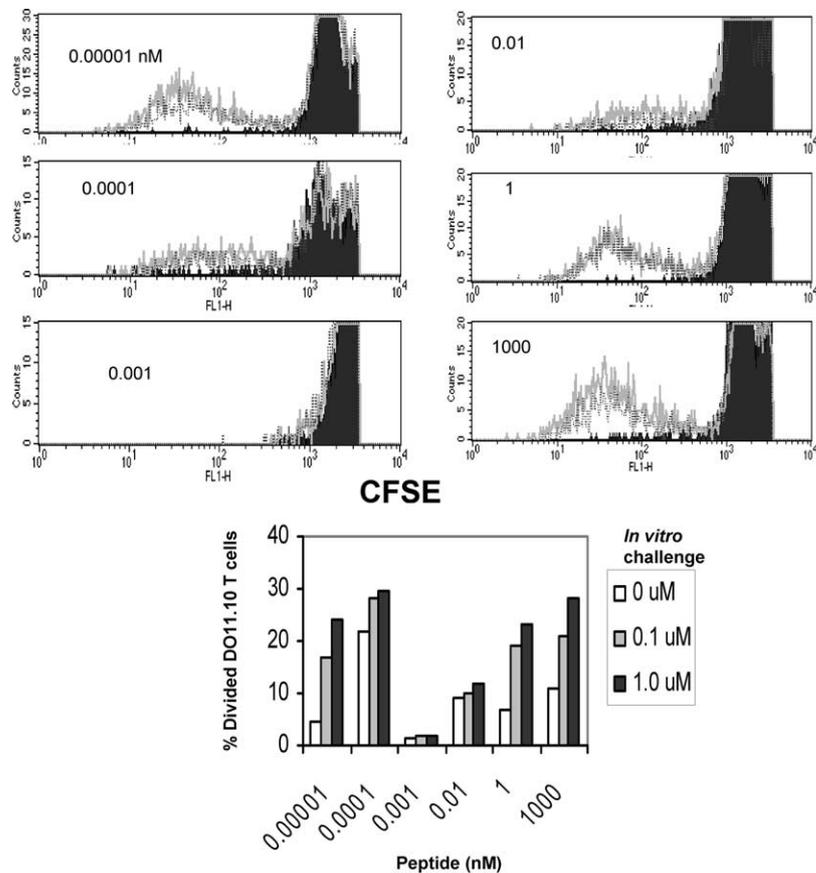
DCs do not induce anergy in memory CD4⁺ T cells ex vivo

We next examined the contribution of DCs to the induction of anergy. Purified DCs from the splenocytes of unimmunized BALB/c mice were pulsed with different concentrations of cOVA_{323–339} peptide and incubated with memory T cells. Because the levels of MHC II expression on DCs were three times higher than those on B cells, the concentration of peptide used for pulsing was adjusted 3-fold to keep the absolute numbers of peptide/MHC complexes between B and DCs the same. Fig. 3B depicts one representative experiment of four showing that DCs did not anergize memory CD4⁺ T cells at any of the peptide concentrations tested.

DC depletion does not affect the induction of anergy in vivo

Although we found that B cells but not DCs induce anergy, because of the broad immunological significance of DCs, it is possible that our in vitro conditions were not ideal and that DCs might contribute to anergy induction in memory T cells in vivo. To address this issue, we used DTR Tg mice as DO11.10 Tg T cell recipients and generated memory T cells by OVA peptide in CFA immunization as described above. DTR Tg mice express a fusion protein of DTR and enhanced green fluorescence protein (EGFP)

FIGURE 5. Induction of anergy in memory CD4⁺ T cells in vivo by in vitro pulsed B cells. Splenic B cells were pulsed in vitro with doses of cOVA_{323–339} peptide (as shown on the x-axis) for 3 h, transferred to mice bearing memory T cells, and 10 days later the mice were sacrificed. T cell proliferation was measured in draining LNs by the CFSE dilution assay after in vitro challenge with cOVA_{323–339} peptide. Doses for in vitro challenge with cOVA_{323–339} peptide are represented as follows: filled black histogram, 0 nM; black line, 0.1 μM; gray line, 1 μM. Data shown represent one of two independent experiments. There were three mice per group. Lower panel represents the percentage of Ag-specific cells (DO11.10) divided in response to in vitro challenge.



under the control of the CD11c promoter (17). This promoter is constitutively active in nearly all conventional DC subsets. Mice do not possess a native DTR and their cells are thus highly resistant to DT. Transgenic DCs express sufficient amounts of DTR-EGFP and thus are depleted following an injection of DT. Because DCs are replenished from precursors, depletion persists only for 24 h after treatment and new DCs are generated 48 h after DT injection (17). Of note, anergy in our system was optimally developed during the first 48 h of Ag encounter in vivo (11). To avoid the presence of DCs during anergy induction, three injections of DT were given: once 12 h before, and twice at 10 h, and + 36 h after peptide injection in IFA. The percentage of DCs, as judged by GFP fluorescence, did not exceed 0.15% of the total leukocytes in draining LNs, i.e., ~85–90% of DCs remained depleted 24 h after the last DT injection.

Fig. 4 shows results from two sets of experiments. In Fig. 4A, experiments were done in the presence of CD11c⁺ DCs, i.e., DTR Tg mice without DT injection, and in B, with multiple DT injections. We observed that T cells from the mice injected with 5–50 pmol of peptide were anergized in the absence of DCs (Fig. 4, A and B) as tested by [³H]thymidine uptake. Similar results were obtained in another set of experiments in Fig. 4C by CFSE dilution assay, suggesting that the contribution of DCs to the induction of anergy in memory T cells is not essential.

In vitro pulsed B cells induce anergy in memory CD4⁺ T cells in vivo

To clarify further the role of B cells in the induction of anergy, we investigated whether memory T cells could be rendered anergic by in vitro pulsed B cells in vivo. To test this, we transferred 10⁷ B cells per mouse (pulsed in vitro with peptide concentrations ranging 0.00001–1000 nM for 3 h) into different groups of mice bear-

ing memory T cells. Ten days later, we used a CFSE dilution assay to determine whether anergy was induced in those memory T cells. We found that Ag-specific T cells ceased to divide beyond 1–2 divisions in groups of mice that were injected with purified B cells loaded with 0.001–0.01 nM peptide, whereas T cells in mice that received B cells pulsed with peptide below or above those concentrations proliferated multiple times (Fig. 5).

B2 B cells are major contributors for induction of anergy in memory CD4⁺ T cells

To dissect the population of B cells that induce anergy in memory CD4⁺ T cells, we purified B2 B cells (conventional B cells residing in lymphoid follicles; Refs. 21 and 22) from draining LNs of mice immunized with peptide in IFA 48 h earlier, and incubated them with CD4⁺ T cells for the induction of anergy ex vivo. In a parallel experiment, we also used all APCs depleted of B2 B cells and tested their ability to induce anergy. We found that both purified B cells and B2 B cells induced anergy in memory T cells whereas APCs devoid of B2 B cells failed to do so, indicating that B2 B cells are the primary B cells that induce anergy in memory T cells (Fig. 6).

B cell-deficient mice do not support memory T cell anergy

To establish that only B cells and not any other APCs induce anergy in memory T cells in vivo, we used B cell-deficient muMT mice (23). Because muMT mice do not support efficient development of CD4 memory T cells (24), we first generated memory T cells using OT II Tg cells specific for OVA_{323–339}/I-A^b (25, 26) by transferring OT II Tg cells to B6 recipients and immunizing them. Memory cells were then transferred to muMT mice. All groups of mice were given different doses of peptide in IFA as before and assayed for anergy 9 days later. We found that while 5 or 50 pmol

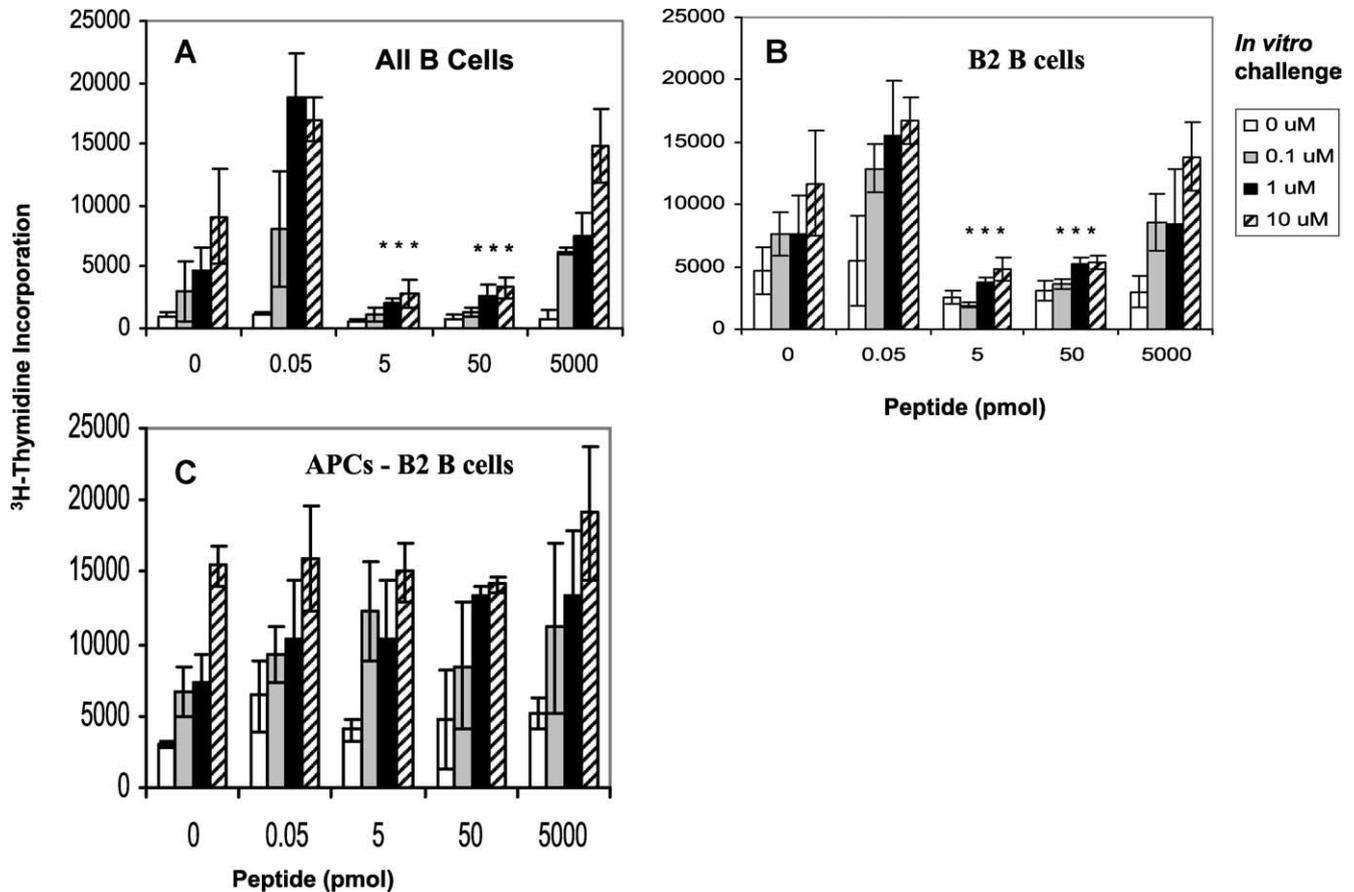


FIGURE 6. B2 B cells loaded with low doses of Ag induce anergy in memory CD4⁺ T cells ex vivo. Draining LN cells were harvested from the mice that had been immunized 48 h earlier with cOVA_{323–339} peptide emulsified in IFA. All B cells (A) were purified by B220 microbeads, B2 B cells (B) were purified by CD43 microbeads, and APCs devoid of B2 B cells (C) were obtained by depleting T cells from LN cells by Thy1.2 microbeads followed by positive selection with CD43 microbeads. All three different types of APCs were incubated with memory T cells for 48 h. Induction of anergy was tested by restimulating the T cells with cOVA_{323–339} peptide-pulsed splenocytes at 0, 0.1, 1, and 10 μ M followed by [³H]thymidine incorporation. The data shown represent one of three independent experiments. Bars represent mean \pm SD of triplicate cultures. *, $p < 0.005$, 5, or 50 pmol vs 0.05 or 5000 pmol of peptide doses (Student's *t* test).

of peptide induced anergy in B cell-sufficient (B6 wild type) mice, memory OT-II cells from B cell-deficient muMT mice did not show signs of anergy as evidenced by IFN- γ and IL-2 production (Fig. 7). This experiment confirms that B cells are the critical APCs for inducing anergy in memory CD4⁺ T cells.

Induction of anergy in memory T cells by B cells is mediated by CTLA-4

CTLA-4 is a potent negative regulator of T cell activation. To test whether CTLA-4 was mediating B cell-induced anergy in memory CD4⁺ T cells, we incubated memory CD4⁺ T cells for 48 h with APCs (B cells or APCs without B cells) pulsed in vivo and measured CTLA-4 expression on the T cell surface (Fig. 8, A and B). We found a direct correlation between the induction of anergy and the up-regulation of CTLA-4 (Fig. 8C). T cells interacting with B cells pulsed with anergizing doses of Ag expressed higher (>2-fold) levels of CTLA-4 compared with T cells interacting with B cells pulsed with nonanergizing doses of peptide. APCs devoid of B cells pulsed with any dose of Ag did not induce anergy and the levels of CTLA-4 remained unchanged. In one further study of the role of CTLA-4, we included an anti-mouse CTLA-4 blocking Ab during ex vivo anergy assay and found that the presence of anti-CTLA-4 Ab prevented the induction of anergy by B cells (Fig. 8D).

Discussion

Induction of T cell tolerance is critical in protection against self-destructive immune responses. Because memory T cells are key players in autoimmune pathogenesis (27–29), it is important to silence them to prevent autoimmunity. Although tolerance is well documented in naive T cells, it is poorly understood in memory T cells. Our early studies showed that low avidity engagement of the TCR by agonist peptide-MHC II complexes induced anergy in memory CD4⁺ T cells (10–12). Anergy induced in those studies was long lasting and met the definition of anergy offered by Schwartz and colleagues as “a state of T cell unresponsiveness accompanied by a lack of response to proliferation signals including IL-2 production, and its reversibility by exogenous IL-2 (30–32).” In the present study, we have taken a step further in understanding how anergy is induced and asked which APCs trigger anergy in memory CD4⁺ T cells.

The ability to manipulate memory T cell responses has high potential for providing new therapeutic avenues. To accomplish this, a novel ex vivo approach was designed in which memory T cells were exposed to different purified APC populations pulsed with different doses of antigenic peptide. We found that resting, but not activated, B cells are the primary APCs that induce anergy in memory CD4⁺ T cells. Our experiments also strongly suggested

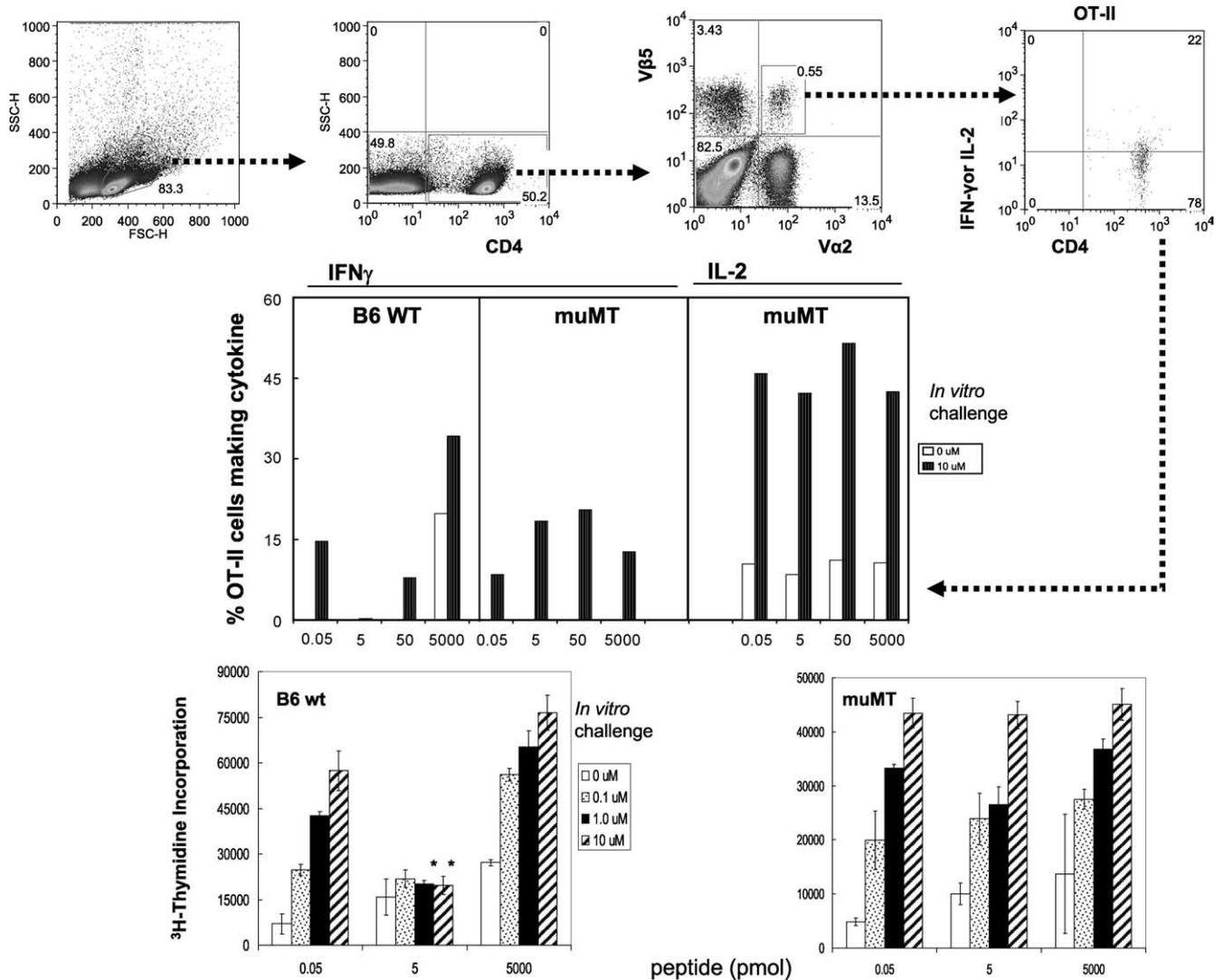


FIGURE 7. B cell-deficient mice do not support memory T cell anergy. Groups of B6 and B cell-deficient muMT mice bearing memory OT-II T cells (18 h after adoptive transfer of purified CD4⁺ T cells containing memory T cells) were challenged with increasing doses (0.05–5,000 pmol) of cOVA_{323–339} peptide in IFA. Nine days later, cells from draining LNs were harvested and tested for anergy *in vitro*. T cell responses were measured by intracellular IFN- γ and IL-2 production (*upper* and *middle* panels) and [³H]thymidine incorporation (*lower* panels). The quadrants for IL-2- or IFN- γ -producing cells (*upper* panels) were drawn based on the matched isotype controls. There were three mice per group and were assayed individually with similar results. The data shown represent one of two independent experiments. Bars represent mean \pm SD of triplicate cultures. *, $p < 0.001$ or 5 pmol vs 0.05 or 5000 pmol of peptide doses (Student's *t* test).

that CD11c⁺ DCs did not play a significant role in the induction of anergy in memory T cells. This was confirmed in both *ex vivo* and *in vivo* settings. Several reports indicate that resting B cells may be the predominant APCs that induce tolerance. The tolerogenic nature of resting B cells may be due to the low abundance of costimulatory molecules such as B7, which is highly expressed in DCs and activated B cells (33–35). A seemingly contrary study reports that resting B cells failed to induce anergy in memory CD8⁺ T cells specific for HY Ag (36). Failure to induce anergy in that study might be because of one or more of the following reasons: 1) the ligand density presented by B cells is not regulated; 2) Ag presentation by MHC-I to CD8⁺ T cells might be different from the presentation by MHC-II to CD4⁺ T cells; and 3) the activation status of CD8⁺ T cells in response to low density of ligand is likely different from that of CD4⁺ T cells.

Our study reveals the dual relationship of B cells with memory T cells: B cells were tolerogenic when presenting limited numbers of peptide/MHC complexes and stimulatory when presenting

higher numbers of complexes. The latter is consistent with the report that all APCs, including resting B cells, can activate memory T cells, which may be due to a less stringent requirement of memory T cells for costimulatory molecules (34, 37). The data presented here provide strong evidence in favor of the former, in that B cells induce anergy in memory CD4⁺ T cells. In this study, memory CD4⁺ T cells were anergized both *ex vivo* by B cells that had captured peptide *in vivo*, and *in vivo* by B cells that were loaded with peptide *in vitro*.

Dendritic cells are potent initiators of immune responses, although they are also reported to be involved in the induction of tolerance *in vivo* (38–40). When the availability of peptide Ag is limited, as was the case here, mature DCs with high MHC class II expression on their surfaces might be the likely APCs to capture low levels of Ag and render T cells anergic. Nevertheless, we clearly demonstrate that DCs were not responsible for inducing anergy in memory CD4⁺ T cells by using several separate experimental designs. In one, APCs depleted of B cells or enriched for

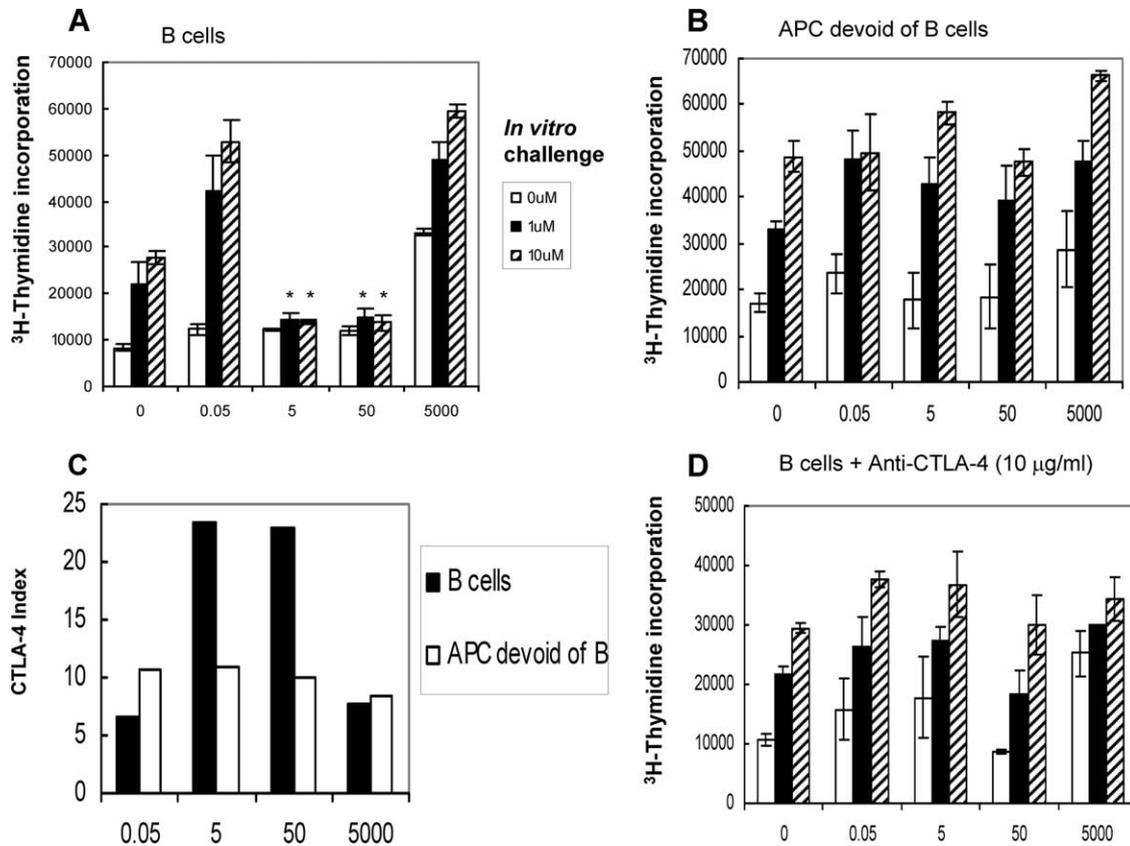


FIGURE 8. Induction of anergy in memory T cells by B cells is CTLA-4 mediated. Four groups of mice were immunized with increasing doses (0.05–5000 pmol) of cOVA_{323–339} peptide in IFA. After 48 h, mice were sacrificed; B cells and APCs devoid of B cells were purified from the draining LNs and incubated with memory CD4⁺ T cells for 48 h. Ex vivo induction of anergy by B cells (A) or APCs devoid of B cells (B) was tested by restimulating T cells with cOVA_{323–339} peptide-pulsed splenocytes (at concentrations shown) for 72 h followed by [³H]thymidine incorporation. CTLA-4 expression on Ag-specific (CD4⁺ KJ1.26⁺) T cells was determined by flow-cytometry (C). Reversal of anergy by B cells was tested similarly, except that anti-mouse CTLA-4 Ab was added to the culture in the beginning of the coincubation of B cells and memory CD4⁺ T cells (D). There were five mice per group. Data shown in A and D represent one of two independent experiments; those in B and C have been repeated four times. Bars represent mean ± SD of triplicate cultures. *, *p* < 0.001, 5, or 50 pmol vs 0.05 or 5000 pmol of peptide doses (Student's *t* test).

DCs failed to induce anergy in our ex vivo anergy assay. In the other, anergy was induced by APCs in the absence of DCs in vivo. Moreover, B cells that were loaded with peptide in vitro successfully induced anergy in memory CD4⁺ T cells when transferred in vivo. Finally, B cell-deficient mice, while presumably sufficient in all other APC types, failed to induce anergy in CD4 memory T cells. Therefore, we feel convinced that B cells are the critical APCs to induce anergy in memory CD4⁺ T cells.

A role for CTLA-4 in the negative regulation of T cell activation is well documented (41–44). Interestingly, we found that B cells presenting tolerogenic doses of peptide induced higher levels of CTLA-4 expression on T cells. Upon the addition of anti-CTLA-4 Ab to the culture, anergy was prevented, strongly suggesting that anergy induced by B cells is mediated by CTLA-4. The expression of low levels of B7.1 on B cells, which does not change at different doses of antigenic stimulation (data not shown), is in agreement with the notion that B cells are the APCs involved in the induction of anergy in a system that is regulated by CTLA-4. Because CTLA-4/B7.1 avidity is higher than CD28/B7.1 interaction, it is certainly feasible that under limiting B7.1 expression the inhibitory effects of CTLA-4 will be dominant (45). In agreement with this notion, CpG-stimulated B cells failed to induce anergy in memory T cells in all concentrations of the peptide (0–10,000 nM) tested, whereas the control B cells pulsed with 0.1–1.0 nM peptide were successful in inducing anergy (data not shown).

Recent reports have indicated that programmed death (PD)-1/PD-L1, and not CTLA-4/B7, interactions dictate the state of exhaustion generated in CD8⁺ T cells in chronic viral infections (46, 47). Based on our data, we suggest that anergy might be regulated differently in CD4⁺ vs CD8⁺ T cells. Indeed, it is reported that anergy in CD8⁺ T cells was induced in the absence of CTLA-4/B7 interaction (48). Thus, our study opens new avenues for investigating differences in molecular mechanisms underlying CD4 vs CD8 anergy.

Because of the involvement of CTLA-4 in the induction of anergy in memory CD4⁺ T cells and in light of expression of CTLA-4 and CD25 on regulatory T cells (Tregs), one possible explanation for our observations might be that the hyporesponsiveness shown here is induced by T cells that have Treg function. Interestingly, a recent report documented that Ag presentation by B cells caused an increase in FoxP3 expression in Treg cells (49). Despite those findings, in our system, Tregs do not seem to be the inducer of the observed hyporesponsiveness. We had previously reported that induction of anergy is not due to regulatory T cells (11). In those experiments, cells from in vivo anergized groups were mixed with the nonanergized responding groups at different ratios to find out whether potential Tregs present in the anergized groups would render the responding cells anergic ex vivo. The results indicated no reduction in response in the cell-mixed culture wells. Additionally, a microarray gene chip assay comparing in

vivo anergized cells to nonanergized cells demonstrated no significant increase of FoxP3 in anergized T cells. In the same experiment, the levels of expression of CD25, a marker of Tregs, remained lower in the anergized cells compared with the nonanergized cells (data not shown). We find these data to argue against a role for Tregs in the induction of anergy in our system.

One might argue that the induction of anergy in memory T cells might not be beneficial to the maintenance of a healthy immune response. We propose that a reduced level of Ag/MHC expression on the surface of B cells might be a mechanism that has evolved to signal memory T cells of the “end of an infection” so that cells would stop proliferating and secreting inflammatory cytokines (50). Undoubtedly, excess cytokine secretion is harmful and not needed once the infection is terminated. Following the termination of infection, Ag load is gradually diminished with inflammation. B cells bearing specific high affinity Ag receptors, such as B2 B cells, at a certain threshold of Ag load would preferentially capture Ag and present it to memory CD4⁺ T cells. Our study, therefore, suggests a new role for B cells as the APCs of choice when Ag falls to nonthreatening low levels (51). Thus, it is logical to hypothesize that B cells signal memory T cells to undergo anergy. In contrast, at the beginning of an infection, when the Ag load might also be low, the induction of anergy in memory T cells would not be desirable. A feature of anergy as described in this article is its reversibility upon an encounter with IL-2 and Ag, a condition that is met during the onset of an infection when a surge of inflammatory responses from the innate immune system coincides with the release of multiple cytokines including IL-2 (52). In support of this hypothesis are our previous reports showing that an encounter with low densities of Ag in the presence of IL-2 does not lead to the induction of anergy and that anergized cells, when incubated with IL-2 and Ag, are no longer anergized (10–12).

The points described above suggest that the induction of anergy in memory T cells might be an evolutionarily beneficial mechanism. A better understanding of this phenomenon could help in revealing the underlying mechanisms for viral and tumor surveillance. Many viruses and several tumors are known to decrease the expression of cell surface MHC class II (53). Also, some tumor-associated peptides bind poorly to the MHC molecules (54). The reduced surface expression of MHC and/or low affinity peptide/MHC complexes leads to the presentation of low densities of specific peptide/MHC, which would induce anergy to the viral or tumor-derived Ags. Thus, our findings suggest new strategies for overcoming viral infections and treating tumors. Rather than repeated immunizations, it might be more productive to focus on the reversal of anergy. In the case of autoimmunity, self-reactive memory T cells might be kept under check from effector function by encountering low densities of peptide-MHC presented by B cells, which could be an important process in the prevention of organ-specific autoimmune diseases. Thus, memory T cells in autoimmune conditions could be targeted specifically for induction of anergy by transferring syngeneic B cells pulsed *ex vivo* with the specific peptides. Controversial observations have been reported on the roles that B cells might play in autoimmune disease models (55–57). The identification of follicular B cells (B2 B cells) as the critical APCs for induction of anergy in memory CD4⁺ T cells leads the way to further studies on the unique characteristics of the membrane of memory CD4⁺ T cells and/or molecules expressed on the surface of memory T cells and B cells that contribute specifically to the induction of T cell anergy.

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Disclosures

The authors have no financial conflict of interest.

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