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CUTTING EDGE

Cutting Edge: CD4$^+$ T Cell Help Can Be Essential for Primary CD8$^+$ T Cell Responses In Vivo[1]

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Recent studies have shown that CD4$^+$ T cell help is required for the generation of memory CD8$^+$ T cells that can proliferate and differentiate into effector cells on Ag restimulation. The importance of help for primary CD8$^+$ T cell responses remains controversial. It has been suggested that help is not required for the initial proliferation and differentiation of CD8$^+$ T cells in vivo and that classical models of helper-dependent responses describe impaired secondary responses to Ag in vitro. We have measured primary CD8$^+$ T cell responses to peptide-pulsed dendritic cells in mice by cytokine ELISPOT and tetramer staining. No responses were detected in the absence of help, either when normal dendritic cells were injected into MHC II-deficient mice or when MHC II-deficient dendritic cells were injected into normal mice. Thus, the primary in vivo CD8$^+$ T cell response depends absolutely on help from CD4$^+$ T cells in our experimental system. The Journal of Immunology,* 2003, 171: 0000–0000.

The importance of CD4$^+$ T cell help for priming CD8$^+$ T cell responses in vivo was first described for responses to noninflammatory Ags such as the MHC class I alloantigen Qa-1 and the male minor histocompatibility Ag HY. Cytotoxicity and skin graft rejection (both effected by CD8$^+$ T cells) were observed only if the immunizing tissue (lymphoid cells or skin grafts) also expressed Ags that could stimulate CD4$^+$ T cells (1–3). Help was originally thought to be mediated by CD4$^+$ T cell secretion of cytokines such as IL-2, which acted at short range to promote CD8$^+$ T cell proliferation in vivo (1). An alternative model (4) suggested that in the absence of inflammation or infection, APC had to be activated by CD4$^+$ T cells to prime CD8$^+$ T cell responses. Subsequent experiments confirmed this, and demonstrated that help required CD40-CD40L interactions between APC and CD4$^+$ T cells (5–7).

Both models proposed that CD4$^+$ T cell help was required for the primary in vivo expansion and differentiation of CD8$^+$ T cells (1, 4). This has now been questioned, after one model of apparently helper-dependent CD8$^+$ T cell cross-priming (8) was re-evaluated using tetramer staining and IFN-γ ELISPOT/intracellular staining to measure CD8$^+$ T cell expansion and function directly ex vivo (9). These more sensitive assays showed that the primary expansion and differentiation of CD8$^+$ T cells into IFN-γ-secreting, cytotoxic effector cells were not helper dependent after all; however, CD8$^+$ T cells primed in the absence of help were unable to proliferate into expanded effector cell populations when restimulated with Ag in vitro (9). The authors therefore suggested that many models of helper-dependent responses to noninflammatory Ags might in fact describe helper-independent primary proliferation and differentiation of CD8$^+$ T cells in vivo, but defective secondary in vitro responses if CD4$^+$ T cell help was absent during priming (1, 3–5, 7, 10, 11). The importance of help for the development of CD8$^+$ T cell memory in apparently helper-independent responses has recently been described by several groups, who found that proliferation and effector function (12–14) and protection against pathogen challenge (13, 14) were severely compromised if memory CD8$^+$ T cells were primed in the absence of CD4$^+$ T cells.

We have now tested the helper dependence of the primary CD8$^+$ T cell response using an experimental system in which CD8$^+$ T cell memory responses are primed by injection with dendritic cells (DC)[3] pulsed with MHC I-restricted peptides (15). Our previous experiments showed that CD8$^+$ T cell responses measured by cytotoxicity after in vitro restimulation were dependent on CD4$^+$ T cell help (16). Here we have used tetramer staining and cytokine ELISPOT assays to measure CD8$^+$ T cell responses directly ex vivo. In contrast to the general model proposed in Ref. 9, we find that the primary expansion and differentiation of peptide-specific CD8$^+$ T cells in vivo is absolutely helper dependent. Thus, under different circumstances, CD8$^+$ T cell responses may require CD4$^+$ T cell help for proliferation and differentiation into effector/memory cells, or only for maturation into long term memory cells that can develop effector functions and mediate protective immunity.

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[3] Abbreviations used in this paper: DC, dendritic cells; NMS, normal mouse serum; spDC, splenic DC; pOVA, OVA peptide aa 257–264; pTRP2, tyrosinase-related protein 2 peptide aa 180–188.

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Materials and Methods

Mice

C57BL/6 (B6) and MHC class II-deficient mice were purchased from Taconic Farms (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME). Male mice (6–12 wk old) were injected with male DC prepared from donors 6 wk to 7 mo old. All mice were maintained in the specific pathogen-free animal facility at the University of Rochester Medical Center (Rochester, NY).

Media

Cell suspensions were prepared and washed in HBSS. Cells were cultured in IMDM with 2 mM L-glutamine, 5×10^{-5} M 2-ME, plus 50 U/ml penicillin G and 50 μg/ml streptomycin. This medium was supplemented with 7% FCS (BioWhittaker, Walkersville, MD) or 0.5% syngeneic normal mouse serum (NMS) for DC preparations, or with 1% NMS for ELISPOT assays.

DC injections

DC were prepared with modifications as described (16). Spleen cells were prepared in HBSS with 5 mM EDTA, washed, resuspended in culture medium with 7% FCS, and incubated at 37°C for 2 h. Nonadherent cells were removed by gentle pipetting with warm serum-free culture medium. Adherent cells were cultured overnight in medium with 0.5% NMS and 1 ng/ml GM-CSF. The MHC I K^b-restricted peptide Ags OVA (pOVA$_{257–264}$; ref. 17) or tyrosinase-related protein 2 (pTRP2$_{180–188}$; Ref. 18) were added at 1 μM during the overnight culture. Nonadherent splenic DC (spDC) were harvested after overnight incubation, washed in HBSS, and treated for 15 min at 4°C with washed sheep anti-mouse Ig-coated Dynabeads (Dynal Biotech, Oslo, Norway) to remove B cells. This routinely yielded populations containing 60–75% DC, determined by flow cytometry using mAb specific for MHC class II (clone 1D9), CD11c (clone N418), and CD86 (clone GL1), gating out dead cells with propidium iodide. Mice were injected i.p. with 1×10^5 DC in 0.2 ml of HBSS. Control mice were left unimmunized.

ELISPOT assays

Filtration plates with 96 wells (MAIP N4550; Millipore, Bedford, MA) were coated with purified anti-IFN-γ (clone AN-18) or anti-IL-2 (clone JES6-1A12) at 5 μg/ml in PBS for 2 h at room temperature or overnight at 4°C. Plates were washed three times with PBS. Medium (100 μl) plus 2% NMS (or 14% FCS in Fig. 1*B*), with or without 2 μM pOVA, was added to each well. Spleen cells from injected and naive mice were diluted to 5×10^6/ml in medium without serum. Several 2-fold dilutions of responder cells were made using naive B6 spleen cells as diluent, keeping the cell concentration constant at 5×10^6/ml. Cells (100 μl) were added to each well. After 12–24 h of incubation at 37°C, plates were washed with PBS plus 0.1% Tween 20, and 50 μl of biotinylated anti-IFN-γ (clone XMG-1.2) or anti-IL-2 (clone JES6-5H4) at 0.5 μg/ml in PBS-Tween plus 3% FCS were added to each well. Plates were incubated for 2 h at room temperature and washed, then 50 μl of streptavidin-alkaline phosphatase conjugate were added, and plates were incubated for 30 min at room temperature. After further washes, the alkaline phosphatase substrate Vector Blue (Vector Laboratories, Burlingame, CA) was added and developed according to the manufacturer's instructions. Spots were counted using an automated ELISPOT counting system (CTL ImmunoSpot Analyzer and ImmunoSpot software version 2.08) from Cellular Technology (Cleveland, OH). Counts are shown as the mean ± SD of triplicate wells. Background counts in the absence of Ag averaged ≤8 to ≤12 spots per 500,000 cells.

Tetramer staining

Spleen cells (5×10^5) were resuspended in 100 μl of tetramer staining buffer (RPMI 1640, 10% FCS, and 3 mM sodium azide) and incubated with 10 μg/ml anti-FcγIII/IIR (clone 2.4G2) at 4°C for 15 min. Cells were spun down and incubated for 1 h at room temperature in 50 μl of tetramer/Ab cocktail containing PE-conjugated K^b/pOVA$_{257–264}$ tetramer (a gift from Dr. David Topham, University of Rochester Medical Center) and APC-conjugated anti-CD8α (clone CT8a). Cells were washed three times and resuspended in staining buffer (PBS, 2% FCS, and 0.1% sodium azide) plus propidium iodide; at least 200,000 live cells per sample were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, Mountain View, CA).

Results

A time course experiment was set up to determine the peak of the primary CD8$^+$ T cell response measured ex vivo by IFN-γ ELISPOT. B6 spDC were pulsed for 2 h with FCS to provide Ags that could stimulate CD4$^+$ T cell help (19) and then cultured overnight with 1 μM pOVA before injection into B6 mice. Spleen cells were assayed 5–14 days later for pOVA-specific IFN-γ secretion. The culture medium in the ELISPOT assay was supplemented with NMS to avoid restimulating FCS-specific T cells. The number of pOVA-specific IFN-γ-secreting spleen cells peaked at days 7–8, falling to ~10–30% of the maximum response by day 14 (Fig. 1*A*). The peak of the response thus corresponded well with the time point when others have observed helper-independent CD8$^+$ T cell priming (9), facilitating our comparison of the two experimental models.

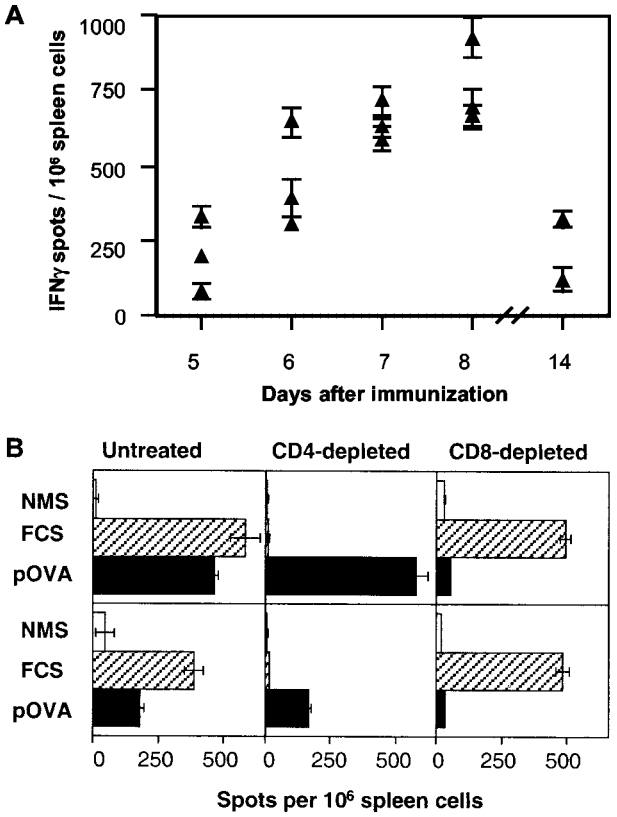

FIGURE 1. Time course and specificity of T cell response to pOVA/FCS-pulsed spDC. B6 mice were immunized with B6 spDC pulsed with 7% FCS plus 1 μM pOVA. *A,* Spleen cells were prepared from groups of three mice on the days indicated and assayed by IFN-γ ELISPOT for pOVA-specific responses. Each point represents the response of one mouse. *B,* Pooled spleen cells from day 8 were treated with Ab-coated magnetic beads to deplete CD4$^+$ and CD8$^+$ cells and cultured in medium with 1% NMS alone, 1% NMS plus 1 μM pOVA, or 7% FCS to measure IFN-γ (*top panels*) and IL-2 (*bottom panels*) ELISPOTs specific for pOVA and FCS Ags, respectively.

To confirm the specificity of CD4$^+$ and CD8$^+$ T cell responses primed by pOVA/FCS-pulsed spDC, pooled spleen cells from day 8 were treated with magnetic beads precoated with anti-CD4 or anti-CD8 Abs to deplete these T cell subpopulations and then cultured in medium with NMS, FCS, or NMS plus pOVA (Fig. 1*B*). Untreated spleen cells made strong IFN-γ and IL-2 elispot responses to both FCS and pOVA; depletion of CD8$^+$ cells abrogated the pOVA-specific response almost entirely, without reducing the FCS-specific response; and depletion of CD4$^+$ cells completely abolished the FCS-specific response but did not affect the pOVA-specific response. This experiment showed that immunization with pOVA/FCS-pulsed spDC primed CD4$^+$ and CD8$^+$ T cell responses to FCS and pOVA, respectively, and confirmed that ELISPOT assays to measure pOVA-specific IL-2/IFN-γ responses did not require CD4$^+$ T cells during the assay.

To analyze $CD8^+$ T cell responses in the absence of help, wild-type and MHC II-deficient B6 mice were injected with B6 spDC pulsed with pOVA and FCS. B6 mice made strong IFN-γ responses to pOVA, but MHC II-deficient mice (which lack $CD4^+$ T cells) made no detectable response (Fig. 2*A*), showing that the response primed by these DC was helper dependent. Although MHC II-deficient mice can make a $CD8^+$ cytotoxic T cell response to $I\text{-}A^b$ when stimulated with B6 APC in vitro (Ref. 20 and our unpublished results), MHC II-deficient mice injected with pOVA-pulsed B6 DC made no IFN-γ response to B6 APC (Fig. 2*A*), suggesting that the lack of priming was not due to rapid killing of the injected B6 DC by $I\text{-}A^b$-specific $CD8^+$ T cells. To confirm our conclusions using fully immunocompetent mice, MHC II-deficient DC pulsed with the same Ags were injected into wild-type B6 mice. Again, these DC (which could not stimulate a $CD4^+$ T cell response) did not prime any detectable IFN-γ response (Fig. 2*A*). Similar results were observed in other experiments for IL-2 as well as IFN-γ responses (data not shown). Thus, help was essential for the generation of primary $CD8^+$ T cell effector cytokine responses in our experimental system.

To test whether the concentration of pOVA was limiting in the ELISPOT assay, pooled spleen cells from B6 mice immunized with pOVA/FCS-pulsed B6 spDC were cultured with 10-fold dilutions of pOVA (Fig. 2*B*). The number of pOVA-specific spots did not decrease until the peptide was diluted to $<10^{-8}$ M, with the half-maximal response at $\sim 10^{-9}$ M. Thus peptide was at least 100-fold in excess for the pOVA-specific responses in this study.

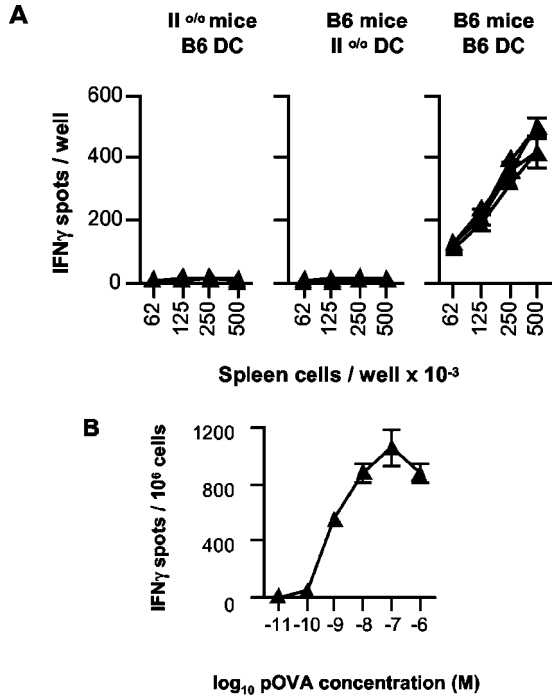

FIGURE 2. $CD4^+$ T cell help is required to prime IFN-γ responses to pOVA. B6 or MHC II-deficient ($II^{0/0}$) mice were injected with B6 or MHC II-deficient pOVA/FCS-pulsed spDC as shown. Spleen cells were assayed 8 days later for peptide-specific IFN-γ responses by ELISPOT, using B6 spleen cells as APC. Each line represents the response of one mouse. Data are representative of three independent experiments. *B,* 250,000 pooled spleen cells from B6 mice immunized with pOVA/FCS-pulsed B6 spDC plus 250,000 naive B6 cells were cultured with 10-fold dilutions of pOVA.

The absence of any response in B6 mice injected with pOVA/FCS-pulsed MHC II-deficient spDC demonstrated that help was stimulated by the injected spDC, and could not be effected by cross-presentation of FCS Ags on host APC. This allowed us to test whether both Ags had to be on the same APC in our experimental model, as described for both direct and cross-primed helper-dependent $CD8^+$ T cell responses (1, 10). B6 mice were injected with pOVA/FCS-pulsed MHC II-deficient spDC (presenting pOVA on DC that could not stimulate help) mixed with equal numbers of FCS-pulsed B6 spDC (to stimulate help). Additional mice were injected with each population separately or with pOVA/FCS-pulsed B6 spDC as a positive control. Fig. 3 shows that mice injected with the mixed DC population responded no better to pOVA than mice injected with MHC II-deficient DC alone, even though mice injected with the mixed DC population responded as strongly to FCS Ags as did mice injected with pOVA/FCS-pulsed B6 spDC (data not shown). We observed no FCS-specific response (i.e., no cross-priming) in mice injected with pOVA/FCS-pulsed MHC II-deficient spDC. Thus, in our experimental system, $CD8^+$ T cells made a significant IFN-γ response only if $CD4^+$ and $CD8^+$ T cells recognized Ag on the same DC.

Finally, we asked whether $CD8^+$ T cells might nevertheless proliferate substantially in vivo without help from $CD4^+$ T cells, but become functionally impaired and unable to secrete cytokines such as IL-2 or IFN-γ when challenged with Ag in vitro (9, 12–14, 21). To test this, $CD8^+$ T cells from B6 mice injected with pOVA/FCS-pulsed B6 or MHC II-deficient spDC were stained with K^b/pOVA tetramers. Cells from naive mice and from B6 mice injected with B6 spDC pulsed with FCS plus an unrelated K^b-restricted peptide (pTRP2), were included as negative controls. Significant K^b-pOVA tetramer staining was observed only with $CD8^+$ T cells from B6 mice injected with pOVA/FCS-pulsed B6 spDC (Fig. 4); the percentage of K^b/pOVA tetramer-positive $CD8^+$ T cells from B6 mice injected with pOVA/FCS-pulsed MHC II-deficient spDC was no higher than that of naive cells or cells from B6 mice injected with pTRP2/FCS-pulsed spDC. Thus, in the absence of help, any expansion of pOVA-specific $CD8^+$ T cells was below the limits of detection by tetramer staining.

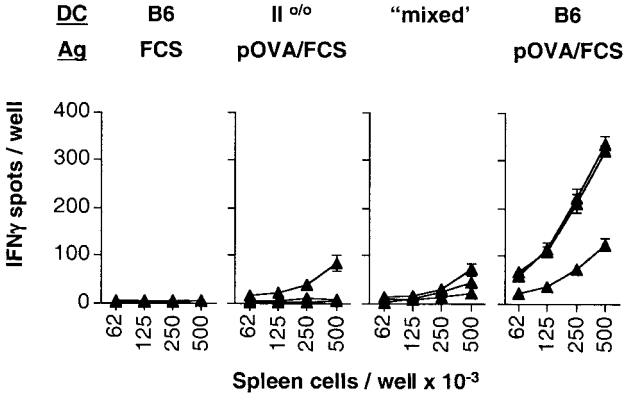

FIGURE 3. $CD4^+$ and $CD8^+$ T cells must be stimulated by the same DC to prime pOVA-specific responses. B6 mice were injected with FCS-pulsed B6 spDC; pOVA/FCS-pulsed MHC II-deficient ($II^{0/0}$) spDC; with a mixture of the two spDC populations; or with pOVA/FCS-pulsed B6 spDC. IFN-γ ELISPOTs were measured 8 days after injection. Each line represents the response of one mouse.

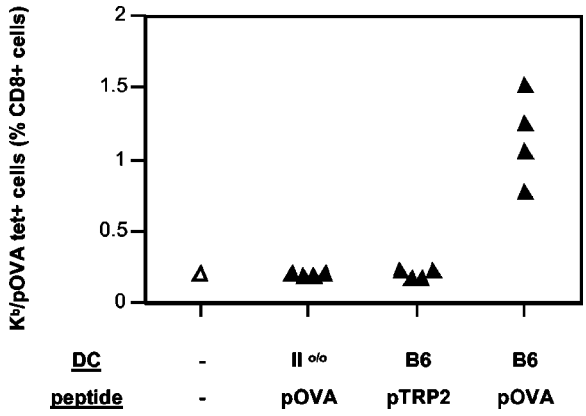

FIGURE 4. CD8+ T cells from B6 mice injected with pOVA/FCS-pulsed MHC II-deficient spDC show no detectable expansion measured by tetramer staining. Spleen cells from B6 mice injected with pOVA/FCS-pulsed B6 or MHC II-deficient spDC were prepared 8 days after injection and stained with anti-CD8 mAb and K^b/pOVA tetramers. Cells from naive B6 mice, and B6 mice injected with pTRP2/FCS-pulsed B6 spDC were included as negative controls. Values are the percentage of live CD8+ cells that were tetramer positive. Each point represents the response of one mouse.

Discussion

This study shows that CD4+ T cell help can be essential for the generation of primary in vivo CD8+ T cell responses. Wild-type mice injected with DC expressing both MHC I- and MHC II-restricted Ags made excellent CD8+ T cell responses, measured ex vivo by IL-2/IFN-γ ELISPOT and tetramer staining. In contrast, mice injected with DC that were unable to stimulate help, either because the mice lacked CD4+ T cells (wild-type DC injected into MHC II-deficient mice) or because the DC did not express MHC II-restricted Ags (MHC II-deficient DC injected into wild-type mice), made no detectable CD8+ T cell response. We therefore conclude that CD4+ T cell help is essential for the primary in vivo expansion and differentiation of CD8+ T cells in our experimental model.

The apparent contradiction between our results and those of Janssen et al. (9) could have several possible explanations, including differences in the frequency of Ag-specific CD8+ T cells, help from NK cells and help from other inflammatory signals resulting from activation of the innate immune system. We injected mice with small numbers of unirradiated splenic DC pulsed with a single MHC I-restricted peptide. In contrast, Janssen et al. injected much larger numbers of irradiated, TAP-deficient mouse embryonal cells transfected with the human adenovirus 5 early region 1, which encodes at least two MHC I-restricted epitopes (9, 22). Increasing the frequency of Ag-specific naive CD8+ T cells, either by immunization with peptides defining multiple epitopes (23) or by injection of transgenic CD8+ T cells (11, 23), can result in helper-independent CD8+ T cell responses, measured by cytotoxicity after in vitro restimulation. One possibility is that the frequency of naive CD8+ T cells stimulated by the transfected mouse embryonal cells was much higher than that of cells activated by the pOVA peptide, resulting in helper-independent expansion. The primary CD8+ T cell expansion and differentiation described by Janssen and colleagues may also have been driven by help from NK cells (24). Transfection of mouse embryonal cells with the adenovirus type 5 *E1A* gene confers susceptibility to NK cell lysis (22), and NK cell activation would also be enhanced by the use of TAP-deficient, MHC I-negative cells. Finally, injection of large numbers of irradiated, damaged, and dying cells may have triggered additional inflammatory signals resulting from the stimulation of the innate immune system, stimulating proliferation and differentiation of CD8+ T cells in the absence of help, without supporting their further development into memory cells.

Our experimental model (helper-dependent primary/secondary responses) thus represents one end of a continuum that extends through helper-independent primary/helper-dependent secondary responses (9, 12) to infectious disease models in which CD8+ T cells primed in the absence of help differentiate into long-lived memory cells that require sensitive techniques to detect impaired secondary responses (13, 14). Models of helper-dependent CD8+ T cell responses must be re-examined to determine where they lie on this continuum.

Do CD4+ T cells use a common mechanism to help CD8+ T cell proliferation and differentiation during the primary response and to program CD8+ T cells to develop into memory cells? In support of this, both helper-dependent primary CD8+ T cell responses and helper cell enhancement of memory require CD4+ and CD8+ T cells to interact with the same APC (this study and Ref. 12). However, helper-dependent CD8+ T cell responses required CD40L-CD40 interactions between CD4+ T cells and DC (6), whereas in one experimental model, CD4+ T cell enhancement of memory CD8+ T cell responses required CD40 expression on the CD8+ T cells but not on the APC (12), which argues for two separate mechanisms (25). Additional experiments are needed to resolve this important question.

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