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Multiple Paths for Activation of Naive CD8⁺ T Cells: CD4-Independent Help

Bo Wang,² Christopher C. Norbury,† Roberta Greenwood,* Jack R. Bennink,† Jonathan W. Yewdell,† and Jeffrey A. Frelinger³,4&lt;sup&gt;†&lt;/sup&gt;

CD8⁺ CTLs play a pivotal role in immune responses against many viruses and tumors. Two models have been proposed. The "three-cell" model focuses on the role of CD4⁺ T cells, proposing that help is only provided to CTLs by CD4⁺ T cells that recognize Ag on the same APC. The sequential "two-cell" model proposes that CD4⁺ T cells can first interact with APCs, which in turn activate naive CTLs. Although these models provide a general framework for the role of CD4⁺ T cells in mediating help for CTLs, a number of issues are unresolved. We have investigated the induction of CTL responses using dendritic cells (DCs) to immunize mice against defined peptide Ags. We find that help is required for activation of naive CTLs when DCs are used as APCs, regardless of the origin or MHC class I restriction of the peptides we studied in this system. However, CD8⁺ T cells can provide self-help if they are present at a sufficiently high precursor frequency. The important variable is the total number of T cells responding, because class II-knockout DCs pulsed with two noncompeting peptides are effective in priming. The Journal of Immunology, 2001, 167: 1283–1289.

The CD8⁺ T cell plays an important role in eradicating virus-infected cells and tumor cells (1, 2). There is increasing interest in vaccine development to induce potent CD8⁺ T cell-mediated cytotoxic responses to viruses and tumor cells. This requires a detailed understanding of how naive CD8⁺ T cells are activated and differentiate into effector cells. Numerous studies have demonstrated that activation and differentiation of naive CD8⁺ T cells into cytotoxic effector cells requires help from CD4⁺ Th cells (3–8). Two models have been proposed to explain how CD4⁺ Th cells might participate in the activation of naive CD8⁺ T cells. The three-cell model suggests that both CD4⁺ T cells and naive CD8⁺ T cells must interact simultaneously with a common APC (9–11). In this model, CD4⁺ Th cells are activated and provide help via production of cytokines such as IL-2. The second model, named the two-cell model, proposes that CD4⁺ T cells first interact with dendritic cells (DCs), resulting in activation of DCs with up-regulation of costimulatory molecules. Subsequently, the preactivated DCs can efficiently activate naive CD8⁺ T cells (11–13). These models provide a general framework for understanding the requirement of CD4⁺ Th cells for activation of naive CD8⁺ T cells. In contrast, diverse numbers of studies have found little or no requirement for CD4⁺ Th cells in induction of anti-viral CTLs (14–19). Furthermore, naive CD8⁺ T cells expressing a transgenic (tg) TCR can be activated in vitro and in vivo with peptide epitope, apparently without involvement of CD4⁺ T cells (20, 21). These observations cannot be easily explained by the above models.

To understand the mechanism of the development of CTL response, it is crucial to consider DCs as APCs. DCs appear to be the principal APC in the initiation of CD8⁺ T cell responses to many Ags (22–25). DCs present Ags to both MHC class II-restricted CD4⁺ and class I-restricted CD8⁺ T cells by classical Ag-presenting pathways (26, 27). DCs can actively acquire exogenous Ags and generate MHC class I-restricted peptides via both TAP-dependent and -independent pathways (28–32). Immunization of animals with DCs pulsed with various Ags has been shown to induce protective cytotoxic responses against viruses (33, 34) and tumor cells (35–37). In this study, we have further dissected the mechanism of help in the induction of CTL response in vivo using DCs generated from spleen. Our data in the present study extend the previous finding from early studies and indicates that CD4⁺ T cells can still provide the help essential for activation of naive CD8⁺ T cells without interacting with the same APC. More important, our data show that CD8⁺ T cells can also provide help for their own activation.

Materials and Methods

**Mice**

Six-week-old C57BL/6 mice (B6) were obtained from The Jackson Laboratory (Bar Harbor, ME), Taconic Farms ( Germantown, NY), or the National Institutes of Health Genetic Stock Center (Taconic Farms). B6 mice carrying a mutation in either the IAβ(B6.129-Abb<sup>tm1 ; I Ab<sup>β</sup>) (38, 39) or β₂-microglobulin (β₂m) gene (B6.129P2(B6)-B2 M<sup>tm1Uscc ; β₂m<sup>tm1Uscc</sup>) (40, 41) have previously been described and were further backcrossed to B6 mice a minimum of nine times. P14 mice (B6.D2-Tfn(TcrLCMV)327Sdz) expressing the H2-D<sup>b</sup>-restricted tg TCR specific for the determinant (amino acid 33–41) derived from the lymphocytic choriomeningitis virus (LCMV) gp33 (42) were obtained from The Jackson Laboratory (at N4) and further backcrossed to B6 mice a minimum of five times. B6 mice tg for green fluorescent protein (GFP) expressed under the

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[6] Abbreviations used in this paper: DC, dendritic cell; β₂m, β₂-microglobulin; LCMV, lymphocytic choriomeningitis virus; GFP, green fluorescent protein; VSV, vesicular stomatitis virus; tg, transgenic.
control of the H2-K\(^b\) promoter (B6-TgN(K\(^b\)-EGFP)) were generated in our laboratory (43). As expected for a gene under the control of a MHC class I promoter, GFP expression is not down-regulated in T cells following activation and is a reliable marker for T cells derived from GFP-expressing mice. All animals used were maintained under specific pathogen-free conditions in the American Association of Laboratory Animal Care-accredited University of North Carolina Department of Laboratory Animal Medicine Facilities (Chapel Hill, NC).

**Peptides**

The following antigenic peptides were synthesized by the University of North Carolina Microchemical Facility and purified by HPLC: LCMV gp33–41 (restricted by H2-D\(^\alpha\)), vesicular stomatitis virus (VSV) N\(_{23-29}\), and OVA\(_{257-264}\) (restricted by H2-K\(^b\)), and peptide 324–332 derived from the nuclear protein of Sendai virus (restricted by both K\(^b\) and D\(^\alpha\)). All the peptides were dissolved in 50% (v/v) DMSO in water at a concentration of 10 mg/ml. The amino acid sequences of the peptides were confirmed by mass spectroscopy and shown in Table I.

**Establishment of DC cultures**

DCs derived from the spleen were used in the experiments for this study. Spleen cell suspensions were prepared from B6 mice, MHC class II-knockout, or β2m-knockout mice and RBCs lysed. Cells were cultured in 5% CO\(_2\) at 37°C in six-well low adherence plates (Costar 3471; Corning Glass, Corning, NY) at 2 × 10\(^5\) in a volume of 3 ml RPMI 1640 medium supplemented with 50 μM 2-ME, 10% FBS, glutamine, 10 mg/ml mouse GM-CSF (BD Pharmingen, La Jolla CA), and 1 ng/ml human TGF-β1 (R&D Systems, Minneapolis, MN). Following culture for 7 days, cells were harvested, washed, and plated into culture in fresh medium prepared as described above. Cultures were typically split every 2–3 days based on the density of cells in each well. To assay for cell surface phenotype, DCs were stained with fluorochrome-conjugated mAbs specific for MHC class I (KH95), class II (25-9-17), CD11b (M1/70), CD11c (HL3), CD80, and CD40 (dotted line). Surface expression was measured by flow cytometry. Solid line depicts unstained cells.

**CTL in vivo priming**

To prime mice for a CD8\(^+\) CTL response, DCs were harvested, washed twice with PBS, and incubated with 10 μM peptide in RPMI 1640 containing 10% FCS. After incubation in 5% CO\(_2\) at 37°C for 2–3 h, cells were washed three times and resuspended in PBS. The 6–10-wk-old mice were primed by i.v. injection (tail vein) of 2–5 × 10\(^7\) peptide-pulsed DCs in 200 μl PBS. For observation, β2m-deficient DCs were treated as described above but without peptide and were then mixed together with the same number (2–5 × 10\(^7\)) of peptide-pulsed MHC class II-knockout DCs in 200 μl PBS. For the adoptive transfer experiments, splenocytes from P14 TCR-tg mice were prepared in PBS. B6 mice, 6–8 wk old, were injected i.v. with varying numbers of P14 splenocytes in 200 μl PBS and, 24 h later, received class II-knockout DCs pulsed with a peptide as described above. Mice that were injected with P14 splenocytes but not primed with peptide-pulsed DCs were used as controls.

**In vitro stimulation and CTL assay**

Seven days following immunization with peptide-pulsed DCs, splenocytes were isolated from immunized mice and RBCs lysed with ACK lysis buffer and resuspended in RPMI 1640 with 10% FCS, antibiotics, glutamine, and 50 μM 2-ME. Cells (1 × 10\(^6\) cells/ml) were stimulated with 1 μM peptide in 75-cm\(^2\) flasks (Falcon; BD Biosciences, Franklin Lakes, NJ) in 5% CO\(_2\) at 37°C for 5 days.

Cells were then harvested, washed with PBS, resuspended in RPMI 1640 with 10% FCS, and used as effector cells in a 51Cr-release assay. Syngeneic EL4 cells were labeled with 51Cr, pulsed with peptides, and used as target cells. Standard 4-h 51Cr-release assays were performed as previously described (44). The percentage of specific lysis was calculated as previously described (45), and each data point represents the mean 51Cr release from triplicate assays.

**Results**

DCs generated from spleen can efficiently prime naive mice for cytotoxic responses specific for viral Ags

DCs are potent APCs and have recently been used in a number of studies to induce protective immune responses against viruses and tumors (33, 46). We have used DCs generated in vitro to prime CTL responses in vivo so that the function of the components in the induction of a CTL response can be analyzed. In the present study, our culture conditions were established to generate large numbers of DCs from mouse spleen. Splenocytes were cultured in vitro with mouse GM-CSF and human TGF-β1 (see Materials and Methods). After culture for 7 days, most B cells and T cells as well as other cell types had died, whereas the remaining cells divided and formed clusters. After expansion for ~2 wk, 3–6 × 10\(^7\) cells could be harvested from a single spleen. These cells exhibited typical DC morphology and expressed MHC class I and II, CD11b, and CD11c, but low levels of CD80 and CD40 on the cell surface were also observed (Fig. 1). No expression of T cell (CD3)- or B cell (IgM)-specific surface molecules was detected (data not shown). Upon overnight culture with LPS, cell surface expression of MHC class II, class I, CD11b, CD11c and costimulatory molecules CD80, CD86, and CD40 was up-regulated and the rate of pinocytosis decreased (data not shown), consistent with maturation of the DCs. DCs derived from class II-knockout or β2m-knockout mice expressed a similar phenotype but lacked expression of class II or class I, respectively.

To determine whether the DCs established from such cultures were able to induce CTL responses, B6 mice were primed by i.v. injection of 2–5 × 10\(^7\) DCs pulsed with the D\(^\alpha\)-restricted gp33–41 peptide derived from the LCMV glycoprotein. As a control, animals were injected with the same number of DCs that were not pulsed with peptide. Seven days following the priming, splenocytes were prepared and restimulated in vitro with the same peptide for 5 days, and peptide-specific CTL activity assessed.

Consistent with the data from other studies, CTL responses specific for LCMV gp33 were induced in B6 mice immunized with LCMV gp33-pulsed DCs (Fig. 2A, ○). In contrast, no CTL activity specific for LCMV gp33 was detected when mice were immunized with DCs alone (Fig. 2A, ●). These results demonstrate that DCs

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**Table I. Peptides used in immunization**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MHC Restriction</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>LCMV gp33</td>
<td>D(^\alpha)</td>
<td>KVANFANTM</td>
</tr>
<tr>
<td>VSV N(_{23-29})</td>
<td>K(^\beta)</td>
<td>RGYVYQGL</td>
</tr>
<tr>
<td>OVA(_{257-264})</td>
<td>K(^\beta)</td>
<td>SINFEKL</td>
</tr>
<tr>
<td>Sendai N(_{324-332})</td>
<td>K(^\beta/D(^\alpha)</td>
<td>FAPGNYPAL</td>
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**FIGURE 1.** Phenotype of DCs grown from spleen. DCs were grown from the spleens of B6 mice under the conditions described in Material and Methods and stained with fluorochrome-conjugated mAbs specific for MHC class I (D\(^\alpha\)), class II (A\(^\beta\)), CD11b, CD11c, CD80, and CD40 (dotted line). Surface expression was measured by flow cytometry. Solid line depicts unstained cells.
FIGURE 2. A. Peptide-pulsed DCs from B6 mice induce Ag-specific cytotoxic responses in B6 mice. The 6- to 8-wk-old B6 mice were injected i.v. with either $5 \times 10^5$ DCs pulsed with 10 $\mu$M LCMV gp33 peptide (●) or B6 DCs alone (○) as a control. Seven days after immunization, splenocytes were isolated and stimulated in vitro with 1 $\mu$M LCMV gp33 peptide for 5 days. Following stimulation, CTL activity was assessed in a standard 4-h $^{51}$Cr-release assay. EL4 cells were pulsed with different concentrations of LCMV gp33 peptide and used as targets. Each data point represents the average specific lysis of EL4 cells in triplicates. B. MHC class II-deficient DCs fail to induce a cytotoxic response in B6 mice. B6 mice were immunized by injection of $5 \times 10^5$ LCMV gp33 peptide-pulsed B6 DCs or MHC class II-deficient DCs. The CTL assay was conducted as described in Fig. 2A. Percentage of specific lysis of target cells by spleen cells from mice immunized with LCMV gp33-pulsed B6 DCs (filled symbols) or LCMV gp33-pulsed MHC class II-deficient DCs (open symbols) is shown. Circles and squares represent individual mice. C. DCs deficient for MHC class II can stimulate the proliferation of spleen cells from P14 TCR-tg mice in vitro. A total of $2 \times 10^5$ splenocytes from P14 TCR-tg mice was cultured with different numbers of either LCMV gp33 peptide-pulsed B6 DCs (●) or class II-deficient DCs (■). As a control, spleen cells were stimulated with either B6 (○) or class II-deficient DCs (□) pulsed with an irrelevant peptide. Proliferation was evaluated by incorporation of $[^3]$H]thymidine. Each data point represents an average of triplicates. When error bars are not visible, the error bars are smaller than the symbol.

Expression of MHC class II molecules by DCs is essential for the induction of CTL responses in vivo

Several groups have reported that activation of naive CD8$^+$ T cells by DCs is independent of CD4$^+$ T cells (47–49). To determine whether naive CD8$^+$ T cells can be activated and differentiate into cytotoxic effector cells upon specific interaction with DCs but without CD4$^+$ T cells, MHC class II-deficient DCs were used to prime CTL responses in naive B6 mice. The lack of MHC class II molecules on the surface prevents these DCs from interacting with CD4$^+$ T cells in recipient B6 mice.

Seven days after immunization with LCMV gp33-pulsed class II-negative DCs, the B6 splenocytes were isolated and restimulated in vitro with the same peptide. CTL activity was measured by a standard 4-h $^{51}$Cr-release assay. Cytotoxic activity was also examined in mice receiving wild-type DCs pulsed with LCMV gp33 peptide. Wild-type B6 DCs pulsed with LCMV gp33 peptide were capable of priming naive B6 mice (Fig. 2B, filled symbols). In stark contrast, no CTL response was detected in cultures prepared from mice receiving DCs lacking MHC class II expression (Fig. 2B, open symbols), even after three injections of peptide-pulsed DCs (data not shown). CD8$^+$ T cells were purified and restimulated with irradiated syngeneic spleen cells pulsed with peptide. CTL activity was detected from mice immunized with peptide-pulsed B6 DCs, but not from mice primed with peptide-pulsed class II-deficient DCs (data not shown). This indicates that the failure to induce a CTL response with class II-deficient DCs is due to the absence of CD4$^+$ T cells during priming in vivo.

In addition to CTL activity, restimulation in vitro with LCMV gp33 peptide resulted in proliferation, expansion, and activation (measured by flow cytometry) of CD8$^+$ T cells from mice primed with normal DCs. After culture, there was a significant expansion of CD8$^+$ T cells and increased numbers of CD25$^+$ and CD69$^+$ cells. >70% of CD8$^+$ T cells in mice primed with B6 DCs were CD69 positive. In contrast, only 18% of the CD8$^+$ T cells were CD69$^+$ in culture prepared from mice primed with MHC class II-deficient DCs.

To ensure that the failure to prime CTLs was not due to some other defect than MHC class II deficiency, we stimulated TCR-tg CD8$^+$ T cells with MHC class II-deficient DCs pulsed with LCMV gp33 peptide in vitro. Both B6 DCs (Fig. 2C, ●) and MHC class II-deficient DCs (Fig. 2C, □) could stimulate proliferation of P14 TCR-tg T cells in vitro to the same extent and with identical kinetics, suggesting that the latter is not defective in activating CD8$^+$ T cells. We have also used B6 DCs to immunize mice lacking CD4$^+$ T cells, due to a targeted disruption of the class II $\beta$ gene. Transfer of B6 DCs pulsed with LCMV gp33 peptide into class II-knockout mice failed to prime a CD8$^+$ T cell response (Fig. 3B), whereas the same DCs induced an easily detected CTL response in B6 mice (Fig. 3A, ●).

Taken together, these data clearly demonstrate that the interaction between CD4$^+$ T cells and APCs is essential for the activation of naive CD8$^+$ T cells in vivo. It is likely that efficient induction of CTL immunity against virus or tumor cells with DCs in early studies may reflect the activation of CD4$^+$ T cells specific for peptides generated during the in vitro culture of DCs in medium containing FBS.

CD4$^+$ and CD8$^+$ T cells are not required to interact with the same APC

The two-cell model proposes two sequential events during activation of CD8$^+$ T cells. Accordingly, CD4$^+$ Th cells first interact with DCs, and the “conditioned” DCs then encounter and activate naive CD8$^+$ T cells. Indeed, treatment of APCs with anti-CD40 Ab in vitro or in vivo can overcome the requirement of CD4$^+$ Th cells for the activation of cytotoxic T cells (11–13). These data can explain the role of DCs in the activation of CD8$^+$ T cells in vivo. Indeed, several studies have found that CTL immunity was induced in MHC class II-deficient mice by virus infection (16, 17), presumably because virus infection resulted in activation of APCs such as DCs. However, these studies lack evidence for how, in the
two-cell model, CD4\(^+\) T cells condition DCs. We reasoned that the DCs used in our experiments would have been conditioned because they were cultured in the presence of CD4\(^+\) T cells, which should be able to interact with DCs carrying Ag derived from culture medium. Therefore, once conditioned, these DCs should be able to prime naive CD8\(^+\) T cells in vivo without the need for CD4\(^+\) T cells. However, the results presented in Fig. 3 show that this was not the case.

To gain insight into how CD4\(^+\) T cells provide help, B6 mice were primed by coinjection of LCMV gp33 peptide-pulsed DCs from MHC class II-knockout mice and β\(_m\)-m-deficient DCs that were not incubated with any specific peptides. Two immune reactions could take place in vivo. First, MHC class II-deficient DCs pulsed with peptide interact with CD8\(^+\) T cells, but not CD4\(^+\) T cells. In contrast, the β\(_m\)-knockout DCs carrying MHC class II-restricted Ags from the culture medium could interact with CD8\(^+\) T cells. However, these DCs could not present Ags to CD8\(^+\) T cells because they lack MHC class I molecules. As described above, LCMV gp33 peptide-pulsed-B6 DCs induced a strong CTL response (Fig. 4, □), whereas MHC class II-knockout DCs failed to do so (Fig. 4, ○). Interestingly, coinjection of β\(_m\)-m-knockout DCs with MHC class II-knockout DCs induced a modest CTL response (Fig. 4, △).

To examine the extent that the requirement for CD4\(^+\) Th cells varies with determinants used to pulse DCs, we immunized mice with DCs pulsed with CTL epitopes derived from different Ags. VSV N\(_{52-59}\) and OVA\(_{257-264}\) are restricted by K\(^b\); and Sendai virus N\(_{324-332}\) is restricted by K\(^b\) and D\(^b\) (Table I). As described above, B6 mice were primed with DCs prepared from B6 or class II-knockout mice or by coinjection of peptide-pulsed DCs deficient for class II and β\(_m\)-deficient DCs. Regardless of MHC restriction or origin of the peptides used, CD8\(^+\) T cell responses specific for each of the determinants were induced in mice primed with peptide-pulsed B6 DCs (Fig. 5, □). The activation of CD8\(^+\) T cells specific for each of the CTL epitopes is CD4\(^+\) Th cell-dependent because peptide-pulsed class II-knockout DCs were not able to prime B6 mice for CTL response (Fig. 5, ○). However, coinjection of β\(_m\)-m-knockout DCs with peptide-pulsed class II-knockout DCs restored at least partial CTL reactivity to all the tested peptides (Fig. 5, △).

These results demonstrate that, in this system, CD4\(^+\) Th cells are generally required for the activation of naive CD8\(^+\) T cells in vivo. Our data indicate that CD4\(^+\) Th cells need not interact with a common APC to provide help to CD8\(^+\) T cells. This is consistent with the idea that help can be provided by cytokine secretion in the general locale of naive CD8\(^+\) T cells. The data also suggest that optimal help is provided when both CD4\(^+\) and CD8\(^+\) T cells interact with the same DC, which is consistent with the findings of Bennett et al. (10).

The help essential for CD8\(^+\) T cell activation can also be provided by CD8\(^+\) T cells themselves

We next wanted to determine whether CD8\(^+\) T cells in high numbers could substitute for CD4\(^+\) Th cells. We studied P14 TCR-tg mice bred onto the recombination-activating gene-deficient background, which nearly exclusively produce CD8\(^+\) T cells expressing a TCR specific for the LCMV gp33 determinant and have very few CD4\(^+\) T cells. Despite the absence of CD4\(^+\) T cells, the tg CD8\(^+\) T cells can be activated by LCMV infection class I tetramers (44) or by LCMV gp33 peptide-pulsed DCs, respectively (data not shown). Consistent with these findings, peptide-pulsed DCs prepared from class II-knockout mice are equally as competent as B6 DCs in activating naive tg CD8\(^+\) T cells (Fig. 2C).

These findings suggest either that 1) TCR-tg CD8\(^+\) T cells from P14 mice differ intrinsically from wild-type B6 mice in their requirement for CD4\(^+\) Th cell-mediated help, or 2) higher numbers of CD8\(^+\) T cells expressing the Ag-specific TCR in P14 mice generate the help required for their own activation. To distinguish between these possibilities, we injected increasing numbers of splenocytes from P14 TCR-tg mice into B6 mice, and 1 day later, the recipient mice were either injected with class II-knockout DCs pulsed with LCMV gp33 or left untreated. Following immunization, spleen cells were restimulated in vitro for 5 days, and the CTL response was measured as described above. As shown in Fig. 6, peptide-pulsed DCs prepared from class II-knockout mice did not induce CTL activity in mice without the transfer of P14 TCR-tg T cells, consistent with the previous results (Fig. 6, far right). CTL activity was not detected in mice that were injected with TCR-tg spleen cells but not primed (○), indicating that activation of CD8\(^+\) T cells requires immunization with DCs. Ag-specific CTL responses were elicited in B6 mice that had received higher doses of P14 TCR-tg spleen cells, and the CTL induction was dependent on the numbers of TCR-tg spleen cells transferred. For example, transfer of 6×10\(^3\) tg spleen cells (which contained 2×10\(^4\) TCR-tg CD8\(^+\) T cells) resulted in CTL activity. However, no CTL activity was induced in mice that received 3×10\(^3\) TCR-tg splenocytes.

To determine the contribution of TCR-tg donor cells vs host CD8\(^+\) T cells to the CTL activity, B6 mice were injected i.v. with various numbers of P14 TCR-tg spleen cells expressing GFP and then immunized with peptide-pulsed class II-knockout DCs. This enables the simple identification of donor CD8\(^+\) T cells by flow cytometry using GFP fluorescence. After restimulation, cells were stained with mAbs specific for CD69 and CD8 and analyzed by flow cytometry. Following transfer of 2×10\(^3\) or 5×10\(^3\) GFP\(^+\) splenocytes, there was an expansion of CD8\(^+\) cells, and the vast majority (>97%) of activated CD8\(^+\) T cells were derived from the recipient (CD8\(^-\) CD69\(^+\) GFP\(^+\) ) (Fig. 7, □). Because CD69 is considered an early activation marker and might have been downregulated, we also performed the identical experiment using the cell surface markers CD25\(^+\), CD44\(^high\), and CD62L\(^low\) with similar results (data not shown). In addition, when we tested these cells for expression of IFN-γ by intracellular cytokine staining, all of the IFN-γ-producing cells were CD69\(^+\). Several studies have demonstrated that TCR-tg T cells were activated and then deleted in vivo following peptide immunization (20, 21). It is possible that, upon DC priming, the transferred TCR-tg T cells were activated in

![FIGURE 4.](https://www.jimmunol.org/content/167/4/1286/F4.large.jpg)
The requirement for help is a general rule for the activation of naive cytotoxic CD8\(^+\) T cells. Sendai virus N\(_{324-332}\) (left panel), VSV N\(_{52-59}\) (middle panel), and OVA 257-264 (right panel) peptides were used to pulse DCs for immunization. The 6- to 8-wk-old B6 mice were injected with peptide-pulsed B6 DCs, peptide-pulsed class II-deficient DCs, or peptide-pulsed class II-deficient DCs together with the same numbers of \(\beta_2\)m-deficient DCs. Restimulation and CTL assay were performed with the same procedure as described before. Cytotoxic activities in mice immunized with B6 DCs (○), with class II-deficient DCs (□), or with both class II-deficient DCs and \(\beta_2\)m-deficient DCs together (■) are presented in the figure.

FIGURE 6. CD8\(^+\) cytotoxic T cells can provide help for their own activation. The 8-wk-old mice were transferred i.v. with different numbers of GFP-expressing splenocytes from P14 TCR-tg mice as indicated on the top of each graph. After 24 h, mice were immunized with 5 \(\times\) 10\(^3\) class II-deficient DCs pulsed with 10 \(\mu\)M LCMV gp33 (○ and ■, each representing individual mice) or left untreated (□). Mice that did not receive P14 TCR-tg spleen cells but were immunized with peptide-pulsed class II-deficient DCs were used as a control (far right panel). Seven days after immunization, spleen cells were prepared and stimulated in vitro with the same peptide for 5 days. CTL activity against EL4 cells pulsed with 10 \(\mu\)M LCMV gp33 peptide was measured at different E:T ratios in a standard \(^{51}\)Cr-release assay.

FIGURE 7. Endogenous CD8\(^+\) T cells are activated by MHC class II-deficient DCs in the presence of transferred P14 TCR-tg T cells. B6 mice injected with different numbers of splenocytes (as indicated at the bottom) from P14 TCR-tg mice expressing GFP were primed 24 h later either with peptide-pulsed class II-deficient DCs (■) or left untreated (□). Seven days after spleen cells were prepared and stimulated as described in Fig. 6. Following the in vitro stimulation, cells were stained with anti-CD8 (Red-670) and anti-CD69 (PE) mAbs and analyzed by flow cytometry. The histograms show the percentage of donor cells (CD8\(^+\)CD69\(^+\)GFP\(^+\)) of the total CD8\(^+\)CD69\(^+\) cells.

To further determine the impact of CD8\(^+\) T cell frequency on the induction of CTL response, we reasoned that using two class I-restricted peptides at the same time would increase the frequency of cells able to respond to any given DC. Therefore, we immunized B6 mice with MHC class II-deficient DCs pulsed with two peptides, LCMV gp33 and VSV N\(_{52-59}\). Because the defined peptides added to the cells were not known to be recognized by CD4\(^+\) T cells, they should not compete for class I loading. CD8\(^+\) T cell precursors specific for either LCMV gp33 or VSV N\(_{52-59}\) will interact with the same MHC class II-deficient DCs pulsed with the peptides. B6 mice were immunized with MHC class II-deficient DCs pulsed with LCMV gp33 and VSV N\(_{52-59}\). Seven days after immunization, splenocytes were restimulated in vitro with LCMV gp33, and a CTL assay was performed. A CTL response specific for LCMV gp33 was induced in mice primed with class II-knockout DCs pulsed with both LCMV gp33 and VSV N\(_{52-59}\) (Fig. 8R, filled symbols). In contrast, when mice were immunized with the same DCs but pulsed with only LCMV gp33 peptide, no CTL activity was detected (Fig. 8A, ○). As a control, B6 DCs pulsed with both LCMV and VSV peptides induced a LCMV gp33-specific CTL response (Fig. 8A, □). Our data suggest that increased numbers of specific CD8\(^+\) T cell precursors can help their own activation into effector cells. The identical experiment has been performed using OVA peptide in place of VSV with similar results.

Discussion
There is intense interest in using DCs propagated in vivo to elicit CTL responses, particularly in the immunotherapy of cancer (50). In this study, we show that CD4\(^+\) Th cells are generally required for the generation of CD8\(^+\) T cell-mediated responses to peptide-pulsed DCs. Given that the defined peptides added to the cells were chosen on the basis of binding MHC class I molecules and have not been studied, it is extremely likely that the requirement

restricted by different MHC class I molecules, they should not
The requirement for CD4⁺ Th cells in generating CD8⁺ CTL responses varies considerably among systems (4–8, 15–18). In our study, activation of CD8⁺ T cells was assessed in the absence of preactivated APCs or inflammatory signals normally associated with viral or bacterial infections, which may inhibit help required for activation of CD8⁺ T cells (51, 52). Indeed, we show that CD8⁺ T cells themselves can provide help for other responding CD8⁺ T cells if present in sufficient numbers. This provides one mechanism for CD4⁺ Th cell-independent CD8⁺ T cell responses. Possibly, other cells (particularly NK cells) can also substitute for CD4⁺ Th cells under the appropriate conditions. In retrospect, it is not surprising that the dependence of CD8⁺ T cell responses on CD4⁺ Th cells varies considerably with the exact conditions of the experimental system, given the permutations for activating different cell types in different anatomical locations in different ways.

Our findings provide insight into how CD4⁺ Th cells (and by inference, other cell types) help the activation of naive CD8⁺ T cells. The observation that β₂m-knockout DCs are required for the partial induction of determinant-specific CTL by peptide-pulsed MHC class II-knockout DCs raises three important points. First, because CD4⁺ T cells can interact with APCs distinct from those encountering naive CD8⁺ T cells, the help is likely being provided by cytokine secretion. Second, this cytokine-based help is inefficient relative to both CD4⁺ T cells and CD8⁺ cytokotoxic T cells interacting with the same DC, either because of a higher concentration of cytokines at the site of CD4⁺ Th cell activation or the modification of DCs by their interaction with CD4⁺ T cells. Third, activation of CD8⁺ T cells in these experiments is not due to the interaction between CD8⁺ T cells and DCs activated by CD4⁺ T cells, because the DCs that can interact with CD4⁺ T cells lack MHC class I molecules (β₂m-knockout DCs). These results do not support the two-cell model, which suggests that DCs first activated by CD4⁺ T cells in turn interact with CD8⁺ T cells, leading to the activation of CD8⁺ T cells.

Importantly, we found that CD8⁺ T cells can also provide help when tg CD8⁺ T cells were adoptively transferred into mice receiving peptide-pulsed MHC class II-knockout DCs. In support of this, a CTL response specific to LCMV gp33 was also induced when mice were immunized with class II-deficient DCs pulsed with both LCMV gp33 and VSV N52. But class II-knockout DCs pulsed with only LCMV gp33 could not prime CTL in B6 mice. Two major possibilities exist for how CD8⁺ T cells mediate help under these conditions. First, the simultaneous interaction and activation of a critical number of CD8⁺ T cells establishes a microenvironment in which cytokine concentration exceeds the threshold necessary for activating naive CD8⁺ T cells. This could account for the priming of LCMV gp33-specific T cells in P14 TCR-tg recombination-activating gene-knockout mice by peptide-pulsed DCs in the absence of CD4⁺ T cells (our unpublished data). This interpretation is supported by our previous data demonstrating that MHC class I tetramers alone were sufficient to activate naive CD8⁺ T cells from P14 TCR-tg mice (44), accompanied by production of large amounts of both IL-2 and IFN-γ. Our intracellular staining demonstrates that CD8⁺ T cells made more IFN-γ (data not shown).

Second, the interaction of a large number of CD8⁺ T cells with DCs may activate the DCs and lead to up-regulation of costimulatory molecules or secretion of cytokines by DCs. There is increasing evidence for this type of mechanism. It has been reported that both influenza virus and LCMV induce maturation and activation of DCs, which can then directly activate CD8⁺ T cells (51, 53). Recent data from three groups indicates that DCs activated by anti-CD40 mAb either in vitro or in vivo can induce a CTL response in MHC class II-deficient mice, and MHC class II-deficient DCs activated by anti-CD40 mAb prime CTL responses in the absence of CD4⁺ T cells (11–13). Similarly, mice treated with anti-CD40 mAb can generate CD8⁺ T cell-mediated CTL responses against tumors in the absence of CD4⁺ T cells (54).

One explanation that could explain part of our data is that the lack of CD4 help has no effect in vivo, but, simply, the lack of CD4 priming only impacts the expansion of the CD8⁺ T cells in vitro. Although this explanation could explain some of the results, it cannot explain the fact that increasing the number of CD8⁺ responding cells alone, either by transfer of CD8⁺ TCR-tg cells or by mixing two different peptides, now allows CD8⁺ priming. Under these conditions, the number of primed CD4⁺ cells would be equal in all cultures (i.e., low), because the priming occurred with class II knockout DCs.

In conclusion, we have demonstrated that the induction of CD8⁺ CTL responses requires help that can be obtained from multiple sources, including CD4⁺ T cells and CD8⁺ T cells. These findings provide a basis for understanding the variable requirement for CD4⁺ T cells in generating CD8⁺ T cells responses that has been recorded in the literature, because experimental systems are expected to vary in the extent to which non-CD4 cells could provide help. The important practical implication is that the induction of help should be taken into account in the design of vaccines meant to induce CD8⁺ CTL responses (55, 56).

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