IL-2 Is Required for the Activation of Memory CD8+ T Cells via Antigen Cross-Presentation

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Dendritic cells (DCs) are capable of capturing exogenous Ag for the generation of MHC class I/peptide complexes. For efficient activation of memory CD8+ T cells to occur via a cross-presentation pathway, DCs must receive helper signals from CD4+ T cells. Using an in vitro system that reflects physiologic recall memory responses, we have evaluated signals that influence helper-dependent cross-priming, while focusing on the source and cellular target of such effector molecules. Concerning the interaction between CD4+ T cells and DCs, we tested the hypothesis that CD40 engagement on DCs is critical for IL-12p70 (IL-12) production and subsequent stimulation of IFN-γ release by CD8+ T cells. Although CD40 engagement on DCs, or addition of exogenous IL-12 are both sufficient to overcome the lack of helper, neither is essential. We next evaluated cytokines and chemokines produced during CD4+ T cell/DC cross talk and observed high levels of IL-2 produced within the first 18–24 h of Ag-specific T cell engagement. Functional studies using blocking Abs to CD25 completely abrogated IFN-γ production by the CD8+ T cells. Although required, addition of exogenous IL-2 did not itself confer signals sufficient to overcome the lack of CD4+ T cell help. Thus, these data support a combined role for Ag-specific, cognate interactions at the CD4+ T cell/DC as well as the DC/CD8+ T cell interface, with the helper effect mediated by soluble noncognate signals. The Journal of Immunology, 2006, 176: 7288–7300.

ytotoxic T lymphocytes are an important component of the adaptive immune response. They destroy virally infected cells and are considered critical for the eradication of cells on their way toward malignant transformation. To become an effector cell and thus perform these tasks, CTLs must first be of cells on their way toward malignant transformation. To become an effector cell and thus perform these tasks, CTLs must first be

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CD40 engagement on DCs results in the production of IL-12, this model fits well with our previous studies and that of Mescher and colleagues (17), who report that activation of CD8+ T cells requires three signals: Ag (signal 1), costimulation (signal 2), and IL-12 or adjuvant (signal 3). In contrast to this DC-centric view, Bourgeois et al. (18) suggest that injected CD40 agonists are acting on the CD8+ T cell and that, during helper-dependent priming, CD4+ and CD8+ T cells may directly interact. Further extending this idea, a distinct sequential two-cell model has been proposed, predicated on CD4+ T cells acquiring molecules from the APC at the immunologic synapse (along with bystander MHC I/peptide complexes and costimulatory molecules); accordingly, engagement of CD4+ T cells by the DCs is followed by engagement of CD8+ T cells by CD4+ T cells that had acquired cognate Ag (19).

Recent work has further modified our understanding of helper-dependent CD8+ T cell activation. Based on the studies of the Schoenberger and Bevan laboratories (20, 21), the requirement for help may also act at the level of generating CD8+ T cells competent in responding during secondary restimulation. The mechanism for this helper effect may act via the APC, resulting in the programming of naive T cells as supported by Janssen et al. (22). Alternatively, other models support a role for Ag-nonspecific CD4+ helper cells, which provide survival signals for the maintenance of a high precursor frequency of memory CD8+ T cells (23). What is clear from all of these studies is the need to differentiate the signals involved in CD8+ T cell proliferation from those that govern the acquisition of effector function. Additionally, it points out that much of our understanding about the priming of naive T cells was contingent on assays that evaluated secondary restimulation.

To gain better insight into the coordination of this three-cell interaction, we established a defined, physiologically relevant in vitro mouse model using polyclonal T cells to test candidate signals that influence the activation of CD8+ T cells. Using a population of expanded memory CD4+ and CD8+ T cells present after recovery from influenza infection, we characterized how a naturally generated T cell repertoire responds to re-exposure to Ag. Importantly, the use of CD4+ T cells isolated from IFN-γ-deficient mice allowed us to evaluate the full repertoire of signals at the CD4/DC or CD4/CD8 interface while remaining focused on the effector activity/IFN-γ production of the CD8+ T cells. Reported herein, we find redundancies in the defined helper-mediated pathways because neither CD40 engagement nor IL-12 production is critical at the respective cellular interfaces when the full repertoire of signals is available during CD4+ T cell/DCs cross talk. Using a screen for cytokines or chemokines involved in CD4 licensing of DCs, we also discovered a surprising role for IL-2 in the differentiation of IFN-γ-producing effector CD8+ T cells. Our results support a re-evaluation of the three-cell model, mediated by Ag-specific cognate interactions at the CD4/DC and the DC/CD8 interfaces, with the helper effect mediated by soluble noncognate signals.

**Materials and Methods**

**Mice, cell line, Abs, and reagents**

C57BL/6, IFN-γ−/− (stock no. 002287), CD40−/− (stock no. 002928), IL-12Rβ−/− (stock no. 002984), and IL-2−/− (stock no. 002252) mice were obtained from The Jackson Laboratory. CD122−/− mice were obtained from CLEA. In all experiments, 4- to 6-week-old mice were used. BALB/3T3 cells, clone A31, was obtained from the American Type Culture Collection and grown in DMEM containing 10% FBS, supplemented with nonessential amino acids, sodium pyruvate, glutamine, 22-ME, and gentamicin (D-10). Influenza A/PR8/34 was provided as allantoic fluid from Charles River Laboratories and used to infect 3T3 (1000 hemaglutinin units (HAU)/106 cells) or DCs (300 HAU/106 cells). Pansorbin cells (SAC) were obtained from Calbiochem and LPS 055:B5 was obtained from Sigma-Aldrich. Recombinant mouse TNF-α, IL-12, agonist CD40 Ab (1C10), and rat IgG, were obtained from R&D Systems. All FACS Abs used in this study were obtained from BD Pharmingen; reagents for the ELISPOT assays were obtained from Mabtech.

**Preparation of Ag-loaded DCs**

Bone marrow-derived DCs were prepared as previously described (24). Briefly, bone marrow obtained from tibia and femurs was lysed of RBC and cultured at a density of 3 × 106 cells/well in six-well plates with RPMI 1640 containing 10% FBS, nonessential amino acids, sodium pyruvate, glutamine, 22-ME, gentamicin (R-10), and in the presence of GM-CSF (provided by 3% v/v J558L, a generous gift from A. Lanzavecchia, Research in Biomedicine Division, Bellinzona, Switzerland). Fresh GM-CSF-supplemented medium was added to the wells on days 2, 4, and 6. On day 7, immature DCs were harvested and plated in fresh wells with or without UV-B-irradiated 3T3 cells. In addition, 80 ng/ml recombinant murine TNF-α, 5 μg/ml SAC, or 1 μg/ml LPS was added as a maturation stimulus.

To generate influenza-infected apoptotic cells, living cells were first infected with influenza for 1 h at 37°C in serum-free medium. A total of 3 × 106 infected cells per well of a six-well plate was cultured for 5 h at 37°C, allowing for expression of viral proteins (confirmed by intracellular FACS analysis) (data not shown). Cells were washed three times with 3 ml of PBS and UV-B irradiated (120 mJ/cm2) in 0.5 ml of PBS, and 0.5 ml of R-10 was added. Cells were allowed to undergo apoptosis for 8–10 h before adding 106 immature DCs per well. Nonadherent cells were harvested 36 h later, and mature DCs were purified to >95% purity using anti-CD11c microbeads and LS− columns (Miltenyi Biotec). DCs were monitored by FACS and found to express high levels of I-A and CD40. To generate influenza-infected DCs, day 9 mature DCs were infected with influenza for 1 h at 37°C in serum-free medium. These cells were washed three times in serum-containing medium, counted, and used in T cell stimulation assays.

**In vitro cross-presentation studies**

Four- to 6-wk-old mice were infected i.p. with 200–300 HAU of influenza. After 2–4 wk, CD4+ and CD8+ T cells were isolated using MACS purification (Miltenyi Biotec). These cells served as responders in Ag cross-presentation ELISPOT assays. A total of 2 × 106 T cells was added to 6.6 × 106 DCs to give a ratio of 30 T cells to 1 DC. Where indicated, IL-12 (12 pg/ml) or anti-CD40 Ab (1 μg/ml; clone 1C10) was added to the cocultures. Cultures were incubated in the plates for 36–40 h at 37°C, after which cells were washed out of the ELISPOT plates using a mild detergent followed by incubation with 1 μg/ml biotin-conjugated anti-IFN-γ mAb. Wells were then developed using the Vectastain Elite Kit as per manufacturer’s instructions (Vector Laboratories). Colored spots represent IFN-γ-producing cells and are reported as spot-forming cells (SFCs) per 106 cells. The ELISPOT plate was evaluated using a KS ELISPOT reader (Carl Zeiss) with KS Elispot 4.8 software.

**Flow cytometry and cell sorting**

For surface staining of cells, 106 cells were resuspended in 100 μl of staining buffer (PBS containing 5% FBS and 5% goat serum) and Fc-blocked for 10 min using 1 μg of anti CD16/32 (FcγRIIIb). One monoclonal of each specific Ab was added directly to cells and incubated for 20 min on ice. Cells were then washed twice and either analyzed by flow cytometry using a BD FACS Calibur instrument and CellQuest Pro software (BD Biosciences). When indicated, cell sorting was performed using a MoFlo cell sorter (DakoCytometry). Isolated cells were reanalyzed to determine purity. CD4+ T cells and CD8+ T cells were determined to be >99% pure, whereas CD11c+ cells were contaminated with >20% CD4+ T cells due to inefficient dissociation of DC/T cell conjugates. For intracellular cytokine staining, cultures were treated with BD Golgiplug (brefeldin A) for 4 h before harvesting the cells. Cells were surface stained as described and washed twice with PBS prior to fixation and permeabilization using the BD Cytofix/Cytoperm kit, following the manufacturer’s instructions. A total of 106 cells was intracellularly stained with 1 μg of PE-conjugated Ab.

**Detection of cytokines and chemokines**

CD4:DCs (30:1 ratio) were cultured for 24 or 36 h in a 96-flat-bottom plate in R-10. Culture supernatants were harvested at 36 h and stored at −80°C. Cytokines and chemokines were detected using RayBio Mouse Cytokine Array II. Assay was performed following the manufacturer’s instructions. Briefly, membranes were blocked for 30 min before incubation with 1 ml
of culture supernatant at room temperature for 2 h. Membranes were then washed and incubated overnight in bioin-Abs provided. After washing membranes, revelation of Ag-Ab complexes was performed. To quantify the amount of IL-2 present in the supernatant, BD Cytometric Bead Array (CBA) Mouse Th1/Th2 cytokine kit (BD Biosciences) was used as per manufacturer’s instructions.

Quantitative analysis of IL-2 mRNA expression

RNA was extracted from 1–4 × 10^7 sorted cells using TRizol (Invitrogen Life Technologies), and cDNA was synthesized from 0.5–1 μg of RNA using oligo(dt) (Roche) and Superscript reverse transcriptase (Invitrogen Life Technologies) according to the manufacturers’ instructions. IL-2-specific mRNA was quantified relative to TATA box binding protein (TBP) using the following primers: IL-2 forward, 5′-TTCTCTGACATGTAACGACCT CAAAGTC-3′; IL-2 reverse, 5′-TGACAGAAGGCTATCCATCTCC-3′; TBP forward, 5′-TAAGAGGGCACCGACACTGCT-3′; TBP reverse, 5′-TGCTGCCACTGTAAAAGTGAGT-3′.

Quantitative RT-PCR was performed using the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). The reactions were run on a PTC200 equipped with a Chromo4 detector (MJ Research). The analyses were performed with the Opticon Monitor software, version 2.03. All of the measurements were performed in duplicate and validated when the difference in Ct between the 2 measures was <0.3. The ratio gene of interest/housekeeping genes was calculated according to the formula: ratio = 2^(-ΔΔCt) where ΔCt = mean Ct gene − mean Ct housekeeping.

Results

Activation of influenza-reactive CD8+ T cells via cross-presentation requires CD4+ T cell help

We first established an in vitro mouse model that would allow dissection of the signals required to activate memory CD8+ T cells specific for exogenous Ag. Immature bone marrow-derived DCs were generated from C57BL/6 (H-2b) and cocultured with apoptotic influenza-infected 3T3 cells (H-2b), thus ensuring the need for Ag cross-presentation. As previously shown, DCs cross-presenting Ag were inefficient in activating influenza-reactive CD8+ T cells in the absence of CD4+ T cell help as measured by IFN-γ ELISPOT (8). To focus our analysis on memory CD8+ T cell responses, we used CD4+ T cells purified from influenza-primed IFN-γ−/− mice. Titration of IFN-γ−/− CD4+ cells into the DC/CD8 ELISPOT wells permitted full activation of the CD8+ T cells with an observed response of 1200–2000 SFCs/10^6 CD8+ T cells (the expected precursor frequency present in a primed mouse). Of note, responses plateau between 1:1 and 2:1 CD4+ and CD8+ T cells, which corresponds well to the physiologic 2:1 ratio (Fig. 1a).

In contrast to the requirements for T cell activation via the cross-presentation pathway, direct infection of DCs permits activation of influenza-reactive CD8+ T cells without additional CD4+ helper signals. Here, we demonstrate that the presence of CD4+ helper T cells did not enhance this response, confirming that full activation (recall of the 0.2% of the influenza-reactive CD8 cells) can be achieved in the absence of CD4+ T cell help (Fig. 1b).

To establish Ag specificity, we tested whether memory CD4+ T cells isolated from mice primed with an antigenically distinct viral strain, influenza B/Le/40, were able to provide help to DCs cross-presenting influenza A/Puerto Rico/8/infected apoptotic cells. As expected, influenza B-reactive CD4+ and CD8+ T cells obtained from mice infected with influenza B, but not influenza A (Fig. 1c). When we assessed the specificity of T cell help using IFNγ−/− CD4+ T cells, we found that influenza A-reactive, but not influenza B-reactive CD4+ T cells were able to provide help to DCs cross-presenting influenza A for the activation of influenza A-reactive CD8+ T cells (Fig. 1d).

These results demonstrate that the CD4+ T cell help provided to DCs cross-presenting apoptotic Ag is dependent on paired cognate interactions between the CD4+DC and the CD8+DC.

Engagement of CD4+ T cells does not alter the DC maturation state

We considered whether cognate CD4+DC engagement could simply be acting to enhance the maturation state of the DC. To test this possibility, we assessed the maturation state of our DCs by culturing TNF-α-mature DCs in the presence of Ag-reactive CD4+ T cells or IL-12 for 36 h. (Note that, although there are no reports of IL-12 maturing DCs, they do express both chains of the IL-12R.) The DCs were recovered from the culture wells and assayed for expression levels of MHC II, CD86, and CD40 (phenotypic markers of DC maturation). As shown, we observed no difference in maturation markers when comparing mature TNF-α-DCs alone to those that had received cognate or noncognate helper signals (Fig. 2a and data not shown). To evaluate whether the choice of maturation stimulus was able to impact the observed dependence on CD4+ T cell help, we tested the TLR-agonists LPS and SAC in our experimental system. As seen with TNF-α-induced maturation, LPS- and SAC-matured DCs failed to stimulate CD8+ T cells in the absence of help (Fig. 2b). These data suggest that discrete signals result from CD4+DC engagement compared with those responsible for DC maturation.

CD4+ T cell help does not require CD40 engagement on DCs nor IL-12Rβ signaling in CD8+ T cells

We next explored whether cognate CD4+ T cells might provide help to the memory CD8+ T cells directly, or whether signals are transmitted via the DC. It has been demonstrated in several experimental models that CD40 engagement or the addition of exogenous IL-12 can overcome the lack of CD4+ T cell help, thus allowing for efficient cross-priming of CD8+ T cells (25). To determine whether these signals are indeed critical to direct CD4/DC engagement, we used the approach described above, using relevant knockout mice as the source of DCs and influenza-reactive CD8+ T cells. We first evaluated the ability of an agonist anti-CD40 mAb to license DCs. As previously reported (8), engagement of CD40 on DCs permitted CD8+ T cell activation (Fig. 3a, □). This effect was via signaling on the DC, because DCs generated from CD40−/− mice were not capable of stimulating the CD8+ T cells to produce IFN-γ (Fig. 3a, □).

To determine whether CD4+ T cells act via licensing of DCs, or through direct activation of CD8+ T cells via CD40L/CD40 engagement, we isolated memory CD8+ T cells from influenza-infected CD40−/− mice, and assessed whether CD4 T cells were dependent on CD40 for their ability to help DCs cross-present Ag to activate CD8+ T cells. We found that there was in fact no requirement for CD40 expression on either the DC or the CD8+ T cell (Fig. 3b). As a control for the generation of influenza-specific T cells in CD40-deficient mice, we assayed CD8+ T cells stimulated by influenza-infected DCs (Fig. 3c). These results indicate that CD4+ T cells may provide efficient helper signals via both CD40-induced and CD40-independent pathways.

To evaluate downstream effector cytokines that may result from CD4/DC engagement and act at the DC/CD8 interface, we tested the importance of IL-12. We confirmed the ability of IL-12p70 to overcome the lack of CD4+ T cell help (Fig. 4a). Because both DCs and CD8+ T cells have been shown to express IL-12Rβ, it was important to establish on which cell the IL-12 was acting. By comparing cocultures of wild-type and IL-12Rβ−/− DCs, it was possible to rule out autocrine licensing of DCs by IL-12 (Fig. 4a).

Using memory CD8+ T cells from influenza-infected IL-12Rβ−/− mice, it was evident that the exogenous IL-12 was principally acting on CD8+ T cells, because Ag-specific activation was absent.
FIGURE 1. Activation of influenza-reactive CD8\(^+\) T cells via an indirect pathway requires CD4\(^+\) T cell help. a and b, To evaluate the full repertoire of signals at the CD4\(^+\)/DC interface, while remaining focused on the activation of CD8\(^+\) T cells, we used IFN-\(\gamma\)/CD4\(^+\) T cells as the source of help. DCs were generated from bone marrow precursors. On day 7, immature DCs were cocultured with apoptotic, influenza-infected 3T3 cells (flu-3T3) for 36–48 h in the presence of TNF-\(\alpha\). On day 9, mature, Ag-loaded DCs were purified and cultured with memory CD8\(^+\) T cells (ratio, 1 DC:30 CD8\(^+\) T cells), in the presence of increasing numbers of IFN-\(\gamma\)/CD4\(^+\) T cells. An IFN-\(\gamma\) ELISPOT assay was performed to assess activation of Ag-reactive CD8\(^+\) T cells (a). To load Ag via the endogenous pathway, DCs were matured with TNF-\(\alpha\), directly infected with influenza (flu-DCs) and cultured as described above (b). c, To generate a memory T cell population specific for an irrelevant Ag, wild-type (WT) mice were immunized with live influenza B/Lee/40 (flu B). After 14 days, CD4\(^+\) and CD8\(^+\) T cells were isolated from primed mice and the precursor frequency of influenza B-reactive cells was evaluated using DCs directly infected with flu B (flu-B DCs) as stimulators in an ELISPOT assay. Potential cross-reactivity with influenza A/PR/8 (flu A) Ag was determined using DCs directly infected with flu A (flu-A DCs), d, DCs cross-presenting apoptotic flu A-infected 3T3 cells were tested for their ability to activate influenza A-reactive CD8\(^+\) T cells (CD8 T (A)) via the exogenous pathway. naive IFN-\(\gamma\)/CD4\(^+\) T cells (\(\gamma\)/CD4 T (N)), influenza A-reactive IFN-\(\gamma\)/CD4\(^+\) T cells (\(\gamma\)/CD4 T (A)), or influenza B-reactive IFN-\(\gamma\)/CD4\(^+\) T cells (\(\gamma\)/CD4 T (B)) were used as helper stimuli. In mixed cultures, the ratio of CD4\(^+\) T cells to CD8\(^+\) T cells was 2:1. As above, IFN-\(\gamma\) ELISPOT assay was performed to assess activation of Ag-reactive CD8\(^+\) T cells. Data in Fig. 1 are representative of eight experiments. The assays were performed in triplicate; means are plotted and error bars represent SDs of the mean.
**FIGURE 2.** Mature DCs require CD4⁺ T cell help. *a,* Immature DCs were cultured with apoptotic cells for 36–48 h in the presence of TNF-α. CD11c⁺ cells were purified and incubated in the presence of flu-specific CD4⁺ T cells (pink), IL-12 (green), or medium (black) for an additional 40 h. Cultured cells were harvested, labeled with Abs specific for CD11c-PE, and I-A<sup>a</sup>-FITC, CD40-FITC, or CD86-FITC, and analyzed by flow cytometry. DCs were gated based on CD11c expression, and histograms for activation markers are shown. *b,* Immature DCs were cultured with apoptotic cells in the presence of SAC, LPS, or TNF-α for 40 h. CD11c⁺ cells were isolated and used as stimulators for the activation of CD8⁺ T cells in the presence of absence of IFN-γ/CD4⁺ T cells. IFN-γ production was assessed in an ELISPOT assay.

(Fig. 4b). Again, we reintroduced the CD4⁺ T cells to the coculture, this time to assess the ability for CD4⁺ T cell help to act in an IL-12-independent manner. And again, we observed no requirement for IL-12Rβ expression on either the DC or the CD8⁺ T cell when the full set of helper signals are offered by influenza-reactive memory CD4⁺ T cells (Fig. 4c). To control for the possibility that we could have selected for IL-12-independent memory CD8⁺ T cells by generating them from IL-12R<sub>β</sub>⁻/⁻ mice, we used IL-12p40⁻/⁻ mice as the source of bone marrow DCs (data not shown). From the data presented in Figs. 3 and 4, we conclude that CD40 engagement of DCs or the addition of exogenous rIL-12 is sufficient to active memory CD8⁺ T cells; however, neither is necessary when Ag-specific CD4⁺ T cells are the source of the helper signals.

**IL-2 is required for the activation of memory CD8⁺ T cells**

To gain additional insight into the signals that mediate licensing of DCs by Ag-specific CD4⁺ T cells, we screened for soluble factors that are released into the culture medium. Supernatants were generated from DC/CD4⁺ T cell cocultures and exposed to protein arrays to determine the presence of defined cytokines and chemokines (Fig. 5a). We compared DCs cross-presenting cell-associated viral Ag (Fig. 5b) to those directly infected with influenza (Fig. 5c), assessing Ag-dependent effects in both cases. As suggested by the kinetics of our IFN-γ ELISPOT assays (data not shown), we did not observe production of IFN-γ until 36 h of DC/CD4⁺ T cell coculture (Fig. 5, red circles). Analytes identified in this screen included: MCP-1, MCP-5, and IL-2. Interestingly, the induction of IL-2 was robust, and it preceded the release of IFN-γ by at least 12 h (Fig. 5, blue circles). To determine the concentration of IL-2 in the culture supernatants we used Cytometric Bead Arrays (BD Pharmingen). Similar levels of IL-2 were detected in DC/CD4⁺ T cell coculture in which the Ag had been presented via cross-presentation (Fig. 5d, DCs x/p flu-AC + CD4 T) or direct infection (Fig. 5d, flu-DCs + CD4 T).

We evaluated the importance of IL-2 in the stimulation of CD8⁺ T cells in our experimental model by adding blocking anti-CD25 mAb to the cocultures. This resulted in a striking inhibition of CD8⁺ T cell activation (Fig. 6a). Interestingly, stimulation of effector CD8⁺ T cells by DCs cross-presenting Ag is more sensitive to IL-2 concentration than cultures containing infected DCs (Fig. 6b). At high concentrations of anti-CD25 mAb, we observed >70% inhibition of T cell activation in conditions in which the DC was cross-presenting exogenous Ag vs 25% inhibition for influenza-infected DCs.

Because mature DCs express CD25 (IL-2Rα), CD122 (IL-2Rβ), and CD132 (γ<sub>c</sub>-chain), we tested whether IL-2 is acting to license the DC. IL-2 signals via high-affinity (αβγ<sub>c</sub>) and low-affinity (βγ<sub>c</sub>) receptor; therefore, to test the effect of IL-2 on DCs, we used bone marrow-derived DCs generated from CD122⁻/⁻ mice, thus preventing IL-2 from acting on either receptor complex. As shown, we observed no requirement for CD122 expression on the DC in either the indirect (Fig. 7a) or direct (b) presentation of Ag. We also evaluated IL-2⁻/⁻ DCs, because it has been demonstrated...
that, under some conditions, conventional DCs have the ability to produce high levels of IL-2. Although we do not rule out the possibility that DCs are contributing to the IL-2 produced, there is no effect seen when IL-2/DCs are used as stimulators for CD8 T cell responses (Fig. 7, c and d).

Due to the inability to generate influenza-reactive memory T cells in either IL-2/ or CD25/ mice (our unpublished data), we used an alternative approach to assess which cells were responding to, and which cells were producing the IL-2. To determine the importance of IL-2 on the activation of bulk CD4 T and CD8 T cells, we monitored IFN-γ production by intracellular cytokine staining. Cocultures of DCs (cross-presenting influenza Ag), wild-type CD4 and wild-type CD8 T cells were established in the presence of anti-CD25 mAb or control rat IgG.
note, this assay is sensitive to low levels and nonsecreted IFN-γ, and as a result significant levels of non-Ag-specific IFN-γ-producing cells may be seen. Nonetheless, it was possible to evaluate Ag-specific stimulation, and the data demonstrate that IL-2 principally affects CD8+ T cell activation. We demonstrated a 75% reduction in the number of IFN-γ-producing cells (accounting for

FIGURE 4. IL-12 acts directly on the CD8+ T cell to overcome the lack of CD4+ T cell help but is rendered unnecessary when helper cells are present. a–c, Immature WT DCs (■) or IL-12Rβ2/− DCs (■) were cultured with apoptotic 3T3 cells or flu-infected apoptotic 3T3 cells (flu-3T3) in the presence of TNF-α for 36–40 h. CD11c+ cells were purified and cultured with CD8+ T cells in the presence or absence of mouse rIL-12p70 (rIL-12) (a). To confirm that the IL-12 was acting on the effector CD8+ T cells, influenza-reactive memory IL-12Rβ2/−/− CD8+ T cells were generated and assayed for activation in the presence of rIL-12 (b). Next, using memory 12Rβ2/− CD8+ T cells and 12Rβ2/− DCs, the ability CD4+ T cell to license DCs was evaluated (c). As above, IFN-γ production by CD8+ T cells was assessed by ELISPOT; and data are representative of two experiments. The assay was performed in triplicate; means are plotted, and error bars represent SDs of the mean.
Ag-nonspecific staining), compared with only a 15% decrease in the number of CD4$^+$ T cell activation (Fig. 8). Finally, we assessed which cell type(s) is responsible for the production of IL-2. This was determined by quantitative RT-PCR, monitoring the relative amount of IL-2 mRNA in each cell population. RNA was extracted from unsorted or FACS-sorted cells, and IL-2-specific mRNA was quantified relative to TBP using published primer sets (see Materials and Methods). When cultured alone, no IL-2 mRNA could be detected in any of the three cell types: DCs, CD4$^+$ or CD8$^+$ T cells (data not shown). When DCs were cocultured with CD8$^+$ T cells, the only condition in which IL-2 mRNA was measurable was if the DCs were directly infected with influenza (Fig. 9a). Consistent with published data, we observed >10 times more IL-2 mRNA from cocultures containing DCs and CD4$^+$ T cells; and now we detected IL-2 being produced in situations of both cross-presentation and direct presentation of Ag (Fig. 9b). When the three cell types were in coculture together, the majority of the IL-2 mRNA could be attributed to the CD4$^+$ T

**FIGURE 5.** A screen for cytokines/chemokines produced during cognate DC-CD4$^+$ T cell engagement. a, Layout of the RayBiotec protein array used for the screen is shown. b and c, Protein arrays were incubated with supernatants obtained from cocultures of influenza-reactive memory CD4$^+$ T cells and DCs cross-presenting influenza-infected apoptotic cells (b) or DCs infected with live virus (c). Supernatants were harvested after 24 or 36 h of coculture. Red circles highlight the signal for IFN-γ; and blue circles indicate the signal for IL-2. Spot intensity relative to the positive and negative control offers an indication of the relative amount of chemokine/cytokines present in the supernatants. d, Supernatants obtained after 36 h of coculture were analyzed using Cytometric Bead Array to determine the concentration of IL-2.
Interestingly, we still detected low levels of IL-2 mRNA in the CD8⁺/H11001 T cells when influenza-infected DCs were the APC (Fig. 9c). This was not observed when cross-presenting DCs were used (Fig. 9d), possibly reflecting the increased sensitivity of CD8⁺/H11001 T cells to paracrine IL-2 during activation via the cross-presentation pathway. Regarding IL-2 mRNA in the sorted CD11c⁺CD8⁺/H11001CD4low cells, we interpret this as being a result of DC/CD4⁺/H11001 T cell conjugates that were not excluded by the cytometer; however, it remains possible that DCs are an additional (albeit, not a requisite; see Fig. 7c) source of IL-2.

In summary, we define the helper-mediated activation pathways responsible for differentiation of Ag-specific memory CD8⁺ T cells. These signals can be mediated by CD40 engagement of DCs, which triggers the production of IL-12 that in turn acts on CD8⁺ T cells (Fig. 9e). However, our data indicate that neither of these signals is required because CD4⁺ T cells provide multiple activation signals to the DC. In addition to stimulation of DCs, the CD4⁺ T cells are responsible for producing IL-2, which serves as a non-cognate helper signal, permitting full activation of effector CD8⁺ T cells (Fig. 9e). Importantly, IL-2 must act in concert with Ag-specific signals from the DC, thus avoiding nonspecific activation of bystander cells.

**Discussion**

Although the activation of CD8⁺ T cells is clearly linked to the engagement of the TCR and the presence of costimulatory signals, data support the requirement for a third signal. All three signals act early in the engagement and activation phase, thus differentiating memory cells into effectors. Most of the studies on this subject have focused on the requirements for activating naive TCR transgenic CD8⁺ T cells; however, some reports also indicate that additional signals are critical for the activation of memory T cells.

**FIGURE 6.** Helper-dependent activation of CD8⁺ T cells is highly dependent on IL-2. 

*a*, Immature DCs were cultured with influenza-infected apoptotic 3T3 cells for 36–40 h in the presence of TNF-α. CD11c⁺ cells were purified and cultured with CD8⁺ T and IFN-γ⁺/CD4⁺ T cells obtained from influenza-infected mice ( ), in the presence of 16 μg/ml anti-CD25 mAb ( ), or 16 μg/ml isotype-matched control rat IgG2b mAb ( ). As above, the ratio of γ⁺/CD4⁺ T cells to CD8⁺ T cells was 2:1. 

*b*, To compare the sensitivity when Ag is cross-presented vs directly presented, we titrated the anti-CD25 mAb and compared the activation influenza-reactive CD8⁺ T cells. Data reported are representative of five experiments, and error bars represent SDs of the mean.
DCs neither respond to, nor produce, the IL-2 that is required for activating CD8+ T cells. 

**a.** Immature DCs from wild-type (WT) DCs (■) or CD122−/− DCs (□) were cultured with influenza-infected apoptotic 3T3 cells for 36–40 h in the presence of TNF-α. CD11c+ cells were purified and cocultured with CD8+ and IFN-γ− CD4+ T cells obtained from influenza-infected mice. 

**b.** Alternatively, mature DCs were infected with influenza and assayed as above. 

**c and d.** DCs were isolated from IL-2−/− (□) or IL-2+/− littermate mice and evaluated as above. IFN-γ ELISPOT assay was performed to assess the activation of CD8+ T cells. Data reported are representative of three experiments, and error bars represent SDs of the mean.
FIGURE 8. Activation of CD8⁺ T cells are preferentially dependent on IL-2. a and b, DCs cross-presenting cell-associated Ag were cocultured with WT CD4⁺ and CD8⁺ T cells in the presence of 16 μg/ml anti-CD25 or rat IgG2b mAbs. Four hours before peak IFN-γ production of CD4⁺ T cells or CD8⁺ T cells, cultures were treated with brefeldin A. Cultured cells were harvested, labeled with for anti-CD4-FITC and anti-CD8-allophycocyanin, fixed, and permeabilized, followed by intracellular staining with anti-IFN-γ-PE mAb. Flow cytometry was performed and IFN-γ-producing CD4⁺ T cells (a) and CD8⁺ T cells (b) were assessed. Data shown are representative of three experiments.

IL-12 can provide this necessary third signal both in vitro and in vivo, and is believed to originate from DCs that have received signals from CD4⁺ T cells (17). Belz and colleagues (26) have addressed some of these questions using in vivo models, drawing attention to the cognate help that provides CD40L to DCs, thus rendering them fully competent to stimulate naive CD8⁺ T cells. We extend this work by dissecting the cellular signals involved in the restimulation of polyclonal memory T cells. Our data support a cognate licensing of DCs by CD4⁺ T cells, thus allowing for the production of signal 3 (Fig. 1, c and d); the requisite third signal can be mediated by CD40 engagement of DCs (Fig. 3a) or the addition of exogenous IL-12 (Fig. 4, a and b). However, we demonstrate that neither of these signals is required when the full repertoirio of stimuli are available at the CD4⁺ T cells/DC interface (Figs. 3b and 4c). IL-23, a heterodimeric cytokine that comprises a unique p19 subunit and the p40 subunit of IL-12, may also be considered redundant in our experimental system, because DCs from IL-12p40⁻/⁻ mice (data not shown) or CD8⁺ T cells derived from IL-12Rβ1⁻/⁻ mice (Fig. 4c) showed no defect in helper-dependent responses.

Although these results initially troubled us due to the numerous examples of CD40 or IL-12-dependent mouse models, there exist several examples of helper-mediated signals that act independently of this established pathway (27–29). Moreover, when considering humans with inherited deficiency of CD40L, CD40, or IL-12 receptor, there is no obvious correlation with a defect in cross-priming. Absence of the CD40L/CD40 pathway in humans results in a clinical syndrome termed hyper-IgM type 3 (30) and includes an inability of B cells to undergo isotype switching resulting in susceptibility to bacterial and opportunistic infections such as *Pneumocystis carinii*. The clinical phenotype of IL12RB1 deficiency is more narrowly defined—the only opportunistic infections observed (34 of 41 patients) were of childhood onset and caused by *Salmonella* or weakly virulent *Mycobacteria* (bacillus Calmette-Guérin and environmental mycobacterial strains) (31–33). In fact, many of these cases came to attention only because a relative was discovered to carry an *IL12RB1* mutation. Unexpectedly, this suggests that IL-12 and IL-23 are redundant in protective immunity to most microorganisms, including those presumed to be controlled by the cross-priming of CD8⁺ T cells (e.g., human papilloma virus).

Although CD40 and IL-12 seem to be redundant, we discovered a critical role for IL-2 in the differentiation of resting memory CD8⁺ T cells into IFN-γ producing effector cell. IL-2 was originally characterized as T cell growth factor but is now known to exert an array of pleiotropic effects on numerous cell types. The most prominent effects are on T cells, and one of the most studied consequences of signaling via the IL-2R is the selective expansion of effector T cells that have received antigenic stimulation. It is reported to be required for Th1 and Th2 differentiation; it augments cytokine secretion and cytolytic activity in CD8⁺ T cells; and it is now recognized for its role in regulating autoreactive T cells (34).

The importance of IL-2 for the in vitro growth of CD8⁺ T cells, along with the observation that the main producers of IL-2 are CD4⁺ T cells, led to the assumption that IL-2 is the mediator of help. Yet, to our knowledge, these results offer the first evidence for IL-2 as a helper cytokine, produced by CD4⁺ T cells and required for the production of IFN-γ by memory CD8⁺ T cells (Fig. 9e). Importantly, IL-2 must act in concert with Ag-specific signals from the DC, thus permitting the avoidance of nonspecific activation of bystander cells. Of note, addition of exogenous IL-2 does not overcome the lack of CD4⁺ T cell help (data not shown). These data suggest that the IL-2 effect may be due to local production of low amounts of IL-2. Although we have taken care to establish a physiologically relevant model to evaluate the requirements for CD8⁺ T cell activation, we are aware of the need to validate these findings in vivo. Nonetheless, these findings are instructive in several ways and support a need to reconsider a model for cross-priming that involves clusters of CD4⁺ T cells, DCs, and CD8⁺ T cells.

Although three-cell interactions (CD4/DC/CD8 clusters) have been considered unlikely due to the precursor frequency of naive or memory T cells and the limited number of Ag-bearing DCs, our data suggest that such rare events facilitate efficient cross-priming of CD8⁺ T cells. Using intravital two-photon imaging, such three-cell clusters have in fact been visualized (P. Bousso, unpublished observations). To facilitate such interactions, licensing of DCs by CD4⁺ T cells or, conversely, the activation of CD4⁺ T cells by DCs may in turn provoke release of chemokines such as MCP-1 (Fig. 5), which would attract memory CD8⁺ T cells to the DC/CD4 conjugates. In this way, CD8⁺ T cells would receive cognate...
signals from the DC and noncognate factors such as IL-2 from activated CD4+ T cells. Alternatively, secreted IL-2 may bind to the extracellular matrix, thus defining a niche for licensed DCs to cross-prime CD8+ T cells via sequential two-cell interactions. Such a niche might also be created by IL-2Rα, presenting IL-2 in trans to CD8+ T cells (35). Although there is support for other cytokines acting via trans-presentation by their surface and/or soluble receptor (e.g., IL-6, IL-15), it is unclear whether IL-2Rα has a high enough affinity for IL-2 to facilitate such a mechanism (36).

Our data also suggest a selective requirement for IL-2 when Ag is acquired from an exogenous source (Fig. 6). In contrast to the cross-presentation of viral Ag, infected DCs can stimulate influenza-specific memory CD8+ T cells in the absence of CD4+ T cell help (Fig. 1b). Thus, direct presentation secondary to influenza infection offers a strong enough signal to trigger CD8+ T cells to produce their own IL-2 (Fig. 9, a and c). This finding is supported by the in vivo work of Lefrançois and colleague (37), who observed that IL-2 production by CD8+ T cells may begin as early as 6 h postinfection with vasicular stomatitis virus. If IL-2 acts as an intracrine (38), it would explain the lower sensitivity to anti-CD25 mAb when CD8+ T cells are stimulated by influenza-infected DCs. However, we do not rule out other effector molecules that could account for the efficient helper-independent activation of CD8+ T cells in such conditions.

In summary, this study supports a requirement for IL-2 in the activation of CD8+ T cells when Ag is acquired by DCs from

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**FIGURE 9.** CD4+ T cells are the source of IL-2 for activation of helper-dependent CD8+ T cell responses. a–d, DCs were loaded with Ag by cross-presentation or by direct infection, and cultured with wild-type (WT) CD8+ T cells (a), WT CD4+ T cells (b), or both CD8+ and CD4+ T cells at a ratio of 2 CD4+ T cells to 1 CD8+ T cell (c). After 12 h, cells were harvested and RNA was extracted for analysis by quantitative RT-PCR using primers specific for IL-2 and TBP. In addition, cocultures of DCs, CD4+ T cells, and CD8+ T cells were stained with anti-CD4-FITC, anti-CD11c-PE, and anti-CD8-allophycocyanin. Highly purified (>99%) CD4+ T cells and CD8+ T cells were obtained by FACS sorting cells. The CD11c+ fraction was less pure, containing >20% contaminating CD4+ T cells. Again, IL-2 and TBP mRNA production was assessed. e, Schematic diagram illustrating the cognate and noncognate signals offered by CD4+ T cells for the activation of CD8+ T cells.
internalized apoptotic cells. The principal source for IL-2 are Ag-
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