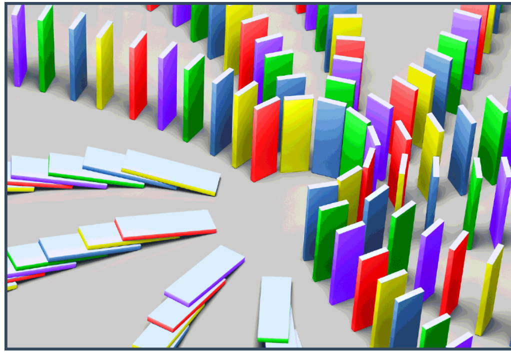
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J Immunol (2005) 174 (2): 710–717.
https://doi.org/10.4049/jimmunol.174.2.710

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Induction of CD70 on Dendritic Cells through CD40 or TLR Stimulation Contributes to the Development of CD8$^+$ T Cell Responses in the Absence of CD4$^+$ T Cells[1]

Timothy N. J. Bullock[2]* and Hideo Yagita[†]

The expansion of CD8$^+$ T cells in response to Ag can be characterized as either dependent or independent of CD4$^+$ T cells. The factors that influence this dichotomy are poorly understood but may be dependent upon the degree of inflammation associated with the Ag. Using dendritic cells derived from MHC class II-deficient mice to avoid interaction with CD4$^+$ T cells in vivo, we have compared the immunogenicity of peptide-pulsed dendritic cells stimulated with molecules associated with infection to those stimulated via CD40. In the absence of CD4$^+$ T cell help, the expansion of primary CD8$^+$ T cells after immunization with TNF-α- or poly(I:C)-stimulated dendritic cells was minimal. In comparison, LPS- or CpG-stimulated dendritic cells elicited substantial primary CD8$^+$ T cell responses, though not to the same magnitude generated by immunization with CD40L-stimulated dendritic cells. Remarkably, mice immunized with any stimulated dendritic cell population generated fully functional recall CD8$^+$ T cells without the aid of CD4$^+$ T cell help. The observed hierarchy of immunogenicity was closely correlated with the expression of CD70 (CD27L) on the stimulated dendritic cells, and Ab-mediated blockade of CD70 substantially prevented the CD4$^+$ T cell-independent expansion of primary CD8$^+$ T cells. These results indicate that the expression of CD70 on dendritic cells is an important determinant for helper-dependence of primary CD8$^+$ T cell expansion and provide an explanation for the ability of a variety of pathogens to stimulate primary CD8$^+$ T cell responses in the absence of CD4$^+$ T cells. *The Journal of Immunology,* **2005, 174: 710–717.**

The CD8$^+$ T cells play a critical role in protection against a variety of intracellular pathogens and tumors. The expansion of robust primary CD8$^+$ T cell populations and the establishment of long-lasting memory CD8$^+$ T cells is the goal of many vaccination protocols. The role of CD4$^+$ T cells in these processes is controversial and is apparently dependent upon the context in which Ag is delivered. The expansion of primary CD8$^+$ T cells after immunization with peptide or dendritic cells (DC)[3] is usually dependent upon CD4$^+$ T cell help (1–7), which can be overcome by cross-linking CD40 (1, 2, 4, 8, 9). Because activated CD4$^+$ T cells transiently express high levels of CD40L (10), it has been proposed that one of the major helper functions of CD4$^+$ T cells is to provide CD40L that would license the complete activation of CD8$^+$ T cells, either directly (11) or via DC (1, 2, 4). In contrast, primary CD8$^+$ T cell responses to pathogens are frequently (12), although not always (13,14), independent from CD4$^+$ T cells. This raises the question whether, analogous to CD40 stimulation, an inflammatory environment associated with infection (e.g., the production of TNF-α) or a by-product of the infection themselves (e.g., TLR ligands) can mitigate the requirement for CD4$^+$ T cell support.

It is also currently unclear whether DC that are sufficiently stimulated by either CD4$^+$ T cells or pathogens possess all of the instructions necessary to stimulate CD8$^+$ T cells not only to undergo expansion into primary effector cells, but also to further differentiate into memory cells. On the one hand, several in vivo studies have shown that the generation of functional memory CD8$^+$ T cells is compromised in the absence of CD4$^+$ T cells (15–20), even for CD8$^+$ T cells that are helper-independent in primary responses. In contrast, in vitro studies have suggested that a short duration of Ag stimulation is sufficient to drive naive CD8$^+$ T cells through a series of programmed developmental steps that ultimately result in the generation of memory CD8$^+$ T cells (21, 22), even in the absence of CD4$^+$ T cells (23). A further study using mice immunized with DC that had been stimulated ex vivo by Ab-mediated cross-linking of CD40 suggested that functional memory CD8$^+$ T cells had developed (1), supporting the notion that an appropriately activated CD8$^+$ T cell can develop into a memory CD8$^+$ T cell in the absence of CD4$^+$ T cell help.

To address some of these questions, we have investigated the ability of DC that have been conditioned with a variety of stimuli associated with infections to expand primary CD8$^+$ T cells that are specific for the immunodominant peptide derived from OVA, OVA_{257} (SIINFEKL), in the presence or absence of CD4$^+$ T cell-mediated help. To achieve this, we have used DC derived from MHC class II$^+$ mice, which present peptides derived from xenogeneic proteins taken up during culture (3, 24), and compared their immunogenicity to DC derived from MHC class II$^-$ mice, which cannot present peptides to CD4$^+$ T cells (25). Furthermore, we have examined the ability of differentially stimulated DC to generate OVA_{257}-specific memory CD8$^+$ T cells and determined the

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Received for publication August 26, 2004. Accepted for publication November 2, 2004.

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[1] This work was supported by the Kimmel Foundation for Cancer Research (SKF-02-160) and a Howard Temin K01 Award from the National Cancer Institute (CA95093).

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[3] Abbreviations used in this paper: DC, dendritic cell; ICS-IFN-γ, intracellular staining for the accumulation of INF-γ; OVA-vac, vaccinia virus expressing full-length OVA.

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functional status of these memory cells. We report a close correlation between the expression of CD70 on the surface of DC and the capacity of DC to expand primary $CD8^+$ T cells in the absence of $CD4^+$ T cells. Additionally, immunization with DC stimulated with various stimuli can lead to the development of fully functional memory $CD8^+$ T cells independently of the presence of $CD4^+$ T cell-mediated help.

Materials and Methods

Animals

MHC class II-deficient mice (ABBN12) and control C57BL/6 mice were obtained from Taconic Farms. Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at the University of Virginia (Charlottesville, VA).

Cell lines

LB15.13 (H-2^bxH-2^d) and EL4 (H-2^b) cells were maintained in RPMI 1640 containing 5% FBS supplemented with SerXtend (Irvine Scientific)

Peptides

Synthetic peptides were made by standard F-moc chemistry using a model AMS422 peptide synthesizer (Gilson) in the Biomolecular Core Facility at the University of Virginia. All peptides were purified to >98% purity by reverse-phase HPLC on a C-8 column (Vydac). Purity and identity were confirmed using a triple quadruple mass spectrometer (Finnigan).

Viruses

Recombinant vaccinia virus expressing OVA (OVA-vac) was a kind gift from Dr. J. Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Dendritic cells

DC were generated from bone marrow cells as described previously (26), with modifications (27). Immature DC were isolated on a StemSep column (StemCell Technologies) after incubation with a mixture of Abs that enrich for DC. For maturation, DC were incubated at a 2:1 ratio overnight with *Mycoplasma*-free CD40L-transduced NIH-3T3 fibroblasts (a kind gift from Dr. R. Lapointe, National Cancer Institute, Bethesda, MD) or with either 100 U/ml TNF-α (Sigma-Aldrich), 8 nmol/ml CpG (sequence: TCCATGA CGTTCCTGATGCT; Operon Technologies), 4 μg/ml poly(I:C) (Sigma-Aldrich), or 10 μg/ml LPS (Sigma-Aldrich). All stocks were diluted in endotoxin-free water (Sigma-Aldrich). To collect DC from CD40L-3T3 cells, culture medium was harvested and then the fibroblasts were gently washed with ice-cold cold PBS to dislodge DC without perturbing the fibroblasts. DC were characterized by costaining with Abs against the following molecules, conjugated as indicated: CD11c-allophycocyanin and H2-D^b-FITC, IA^b-FITC, CD80-PE, CD86-PE, CD40-PE, CD30-PE, CD153-PE, 4-1BB-PE, 4-1BBL-PE, OX40-PE, OX40L-PE, CD27-PE, or CD70-PE; and for intracellular accumulation of IL-12p40 after a 5-h incubation in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich). All Abs were purchased from eBioscience,

Immunization

Mice were immunized i.v. either with 1×10^7 PFU of OVA-vac diluted in PBS or DC that had been pulsed with 10 μg/ml OVA_{257} (SIINFEKL) peptide for 4 h at 37°C in HBSS containing 5% FBS and 5 μg/ml human β_2-microglobulin (β_2m; Calbiochem), washed twice, and resuspended in HBSS. Mice were injected in tail veins with 10^5 DC. In some cases, DC were incubated for 3 h with 10 μg/ml anti-CD70 (28), anti-CD80 and anti-CD86 mAbs (eBioscience), or isotype-matched control Ab RatIgG2b (eBioscience) before washing and immunization. Primary $CD8^+$ T cells were assessed 7 days after immunization. Quiescent memory $CD8^+$ T cells were assessed at least 21 days after immunization, and recall $CD8^+$ T cells were assessed 5 days after secondary Ag challenge.

Intracellular cytokine staining

Cytokine expression was examined ex vivo in freshly isolated spleen cells. Spleens from primed mice were harvested and depleted of erythrocytes and filtered through nylon mesh. $CD8^+$ T cells were then directly assessed for cytokine production after a 5-h incubation with EL4 or LB15.13 stimulator cells that had been pulsed overnight with 10 μg/ml OVA_{257}. To measure the production of intracellular cytokines, peptide-pulsed stimulator cells were incubated with T cells for 5 h at a ratio of 1:1 in medium supplemented with 50 U/ml IL-2 and 10 μg/ml brefeldin A. Stimulated cells were counterstained with anti-CD8-PE (eBioscience), washed, then fixed and permeabilized in PermWash/Fix (BD Pharmingen), followed by staining with anti-IFN-γ-allophycocyanin (eBioscience). Flow cytometry was conducted on a FACSCaliber using CellQuest software (BD Biosciences) and analyzed with FloJo software (Tree Star).

Tetramer staining

H2-K^b tetramers that had been folded around OVA_{257} were kindly provided by Dr. V. Engelhard (University of Virginia) or purchased from Beckman Coulter. Primary and recall $CD8^+$ T cell populations were obtained as described above. For the analysis of quiescent memory populations, $CD8^+$ T cells were isolated from spleens of immunized mice after incubation with a mixture of Abs to enrich $CD8^+$ cells by negative selection, followed by passage over a StemSep column (StemCell). Preparations were consistently 85–95% $CD8^+$ as assessed by flow cytometry. Enriched T cells were coincubated for 45 min at room temperature with tetramer-allophycocyanin, anti-CD8-PerCP, anti-CD44-PE, and anti-CD69-FTIC, washed twice, and fixed in 1% paraformaldehyde. Staining was assessed by flow cytometry as above. Nonspecific staining values of $CD8^+$ T cells from mice immunized with irrelevant Ag were subtracted.

Results

Primary $CD8^+$ T cell expansion without $CD4^+$ T cell help after immunization with DC

We first investigated the ability of DC that had been stimulated with different maturation factors to elicit primary $CD8^+$ T cell responses in the presence or absence of $CD4^+$ T cell help. DC were enriched from bone marrow cultures derived from either MHC class II^+ wild-type or MHC class II^- mice. These cultures contained FCS, which would be a rich source of xenogeneic proteins for presentation on MHC class II molecules to $CD4^+$ T cells. DC were then incubated overnight with TNF-α, poly(I:C), LPS, CpG, or CD40L-expressing 3T3 cells. Stimulated DC were then collected, pulsed with 10 μg/ml OVA_{257}, and used to immunize C57BL/6 mice. The frequency of primary OVA_{257}-specific $CD8^+$ T cells in the spleen was enumerated directly ex vivo 7 days after immunization. The total number of OVA_{257}-specific $CD8^+$ T cells was determined by MHC-tetramer staining, and those with effector function were determined by intracellular staining for the accumulation of IFN-γ (ICS-IFN-γ) after a 5-h coculture with OVA_{257}-pulsed stimulator cells. Mice immunized with MHC class II^+ DC developed strong primary $CD8^+$ T cell responses, regardless of the stimuli used to mature DC. The magnitude of the response induced by each DC population was quite similar, with only that induced by CD40L-treated DC being noticeably greater (Fig. 1*A*). The magnitudes of the primary $CD8^+$ T cell responses after immunization with MHC class II^- DC were comparatively smaller than those obtained with MHC class II^+ DC (Fig. 1, *B–D*), and a hierarchy in immunogenicity was observed. In the absence of $CD4^+$ T cell help, DC stimulated with either TNF-α or poly(I:C) were weakly immunogenic. The magnitude of the primary $CD8^+$ T cell response to LPS- or CpG-stimulated DC was ~2-fold greater. Most strikingly, the $CD8^+$ T cell response to CD40L-stimulated DC was reduced by only ~15% in the absence of $CD4^+$ T cells when compared with the magnitude of the $CD8^+$ T cell response generated in the presence of $CD4^+$ T cell help (cf Fig. 1, *A* and *B*). Similar results were obtained with either wild-type mice that had been depleted of $CD4^+$ T cells (Fig. 1*C*) or when MHC class II^- DC were used to immunize MHC class II^- mice (Fig. 1*D*). DC that were stimulated with soluble CD40L were also capable of inducing primary $CD8^+$ T cell responses in the absence of $CD4^+$ T cell help, while those incubated with untransfected 3T3 cells were not (data not shown). Using higher concentrations of either TNF-α or TLR ligands did not increase the magnitude of the helper-independent primary $CD8^+$ T cell response, but did decrease the DC

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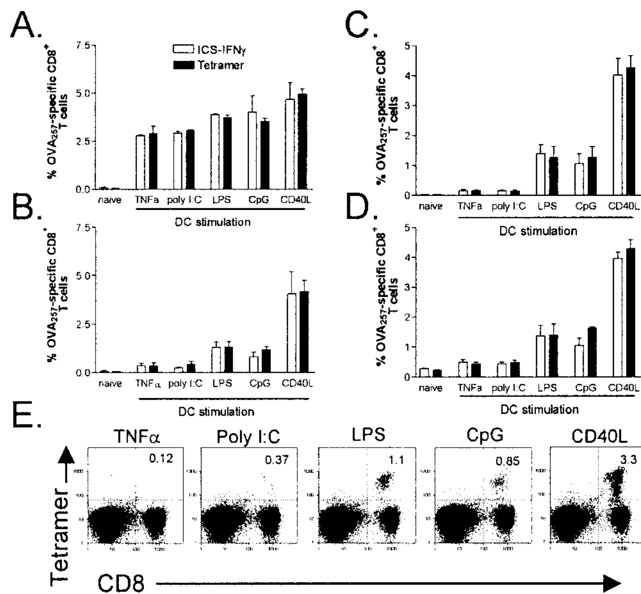

FIGURE 1. CD40- or TLR-stimulated DC elicit the expansion of primary CD8$^+$ T cells in the absence of CD4$^+$ T cells. Primary responses were generated by immunizing mice with 10^5 OVA$_{257}$-pulsed DC that had been incubated with the indicated stimuli. DC were derived from MHC class II$^+$ (*A* and *C*) or MHC class II$^-$ (*B*, *D*, and *E*) mice and injected into MHC class II$^+$ (*A*, *B*, and *E*), CD4$^+$ T cell-depleted MHC class II$^+$ mice (*C*), or MHC class II$^-$ (*D*) mice. The number of peptide-specific CD8$^+$ T cells was enumerated either by ICS-IFN-γ) (□) after a 5-h incubation with OVA$_{257}$-pulsed stimulator cells or by tetramer staining (■). Data are the average and SD of three mice per group. ICS-IFN-γ responses obtained after incubation with unpulsed stimulator cells have been subtracted. Representative FACS profiles (*E*) of OVA$_{257}$ tetramer staining of CD8$^+$ T cells expanded after immunization with MHC class II$^-$ DC are shown. Numbers in plots refer to the percentage of CD8$^+$ T cells that bound tetramer. Data shown are representative of two similar experiments.

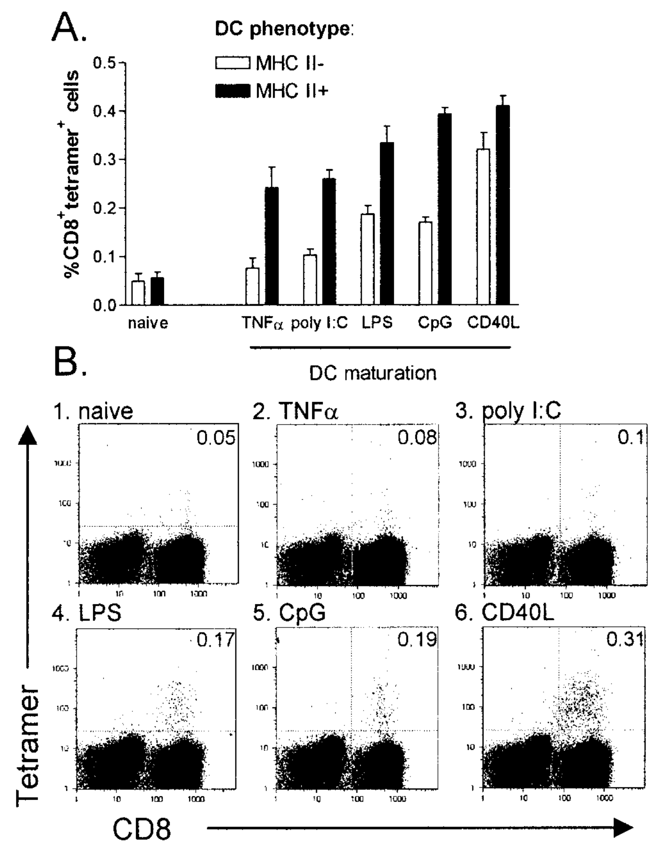

FIGURE 2. Memory CD8$^+$ T cells develop in the absence of CD4$^+$ T cell help. DC were generated from MHC class II$^+$ (■) or class II$^-$ (□) mice and incubated with the indicated stimuli, pulsed with OVA$_{257}$, and injected into mice. Twenty-eight days after immunization, OVA$_{257}$-specific quiescent memory populations were enumerated by OVA$_{257}$ tetramer staining (*A*). Data in graphs indicate the average and SD of three mice per group. Nonspecific responses have been subtracted. Representative FACS profiles (*B*) are from mice immunized with MHC class II$^-$ DC and are gated on CD44^{high}CD69^{low} cells. Numbers in plots refer to the percentage of CD8$^+$ T cells that bound tetramer. Data are representative of four similar experiments.

viability (data not shown). Thus, of the stimuli tested, the ability to induce CD4$^+$ T cell-independent primary CD8$^+$ T cell expansion is most effectively conferred to DC by CD40 ligation. However, this property is not unique to CD40 engagement since ligation of TLR by LPS or CpG also generated DC with the capacity to elicit primary CD8$^+$ T cells without CD4$^+$ T cell help, albeit with considerably less efficiency than CD40 engagement.

Formation of memory CD8$^+$ T cells in the absence of CD4$^+$ T cells

The preceding results have indicated that appropriately stimulated DC can elicit the expansion of primary CD8$^+$ T cells in the absence of CD4$^+$ T cell help. We next asked whether CD8$^+$ T cells elicited in this manner could complete the developmental changes necessary to form memory CD8$^+$ T cells. Therefore, we analyzed the spleens of mice immunized 28 days previously with either MHC class II$^+$ or MHC class II$^-$ DC that had been stimulated with TNF-α, poly(I:C), LPS, CpG, or CD40L. CD8$^+$ T cells were enriched and stained with OVA$_{257}$ tetramers, anti-CD44, and anti-CD69 to identify CD44^{high}CD69^{low} resting, peptide-specific memory cells. In the presence of CD4$^+$ T cell help, memory CD8$^+$ T cells could be detected in mice that had been immunized with DC treated with any of the stimuli. The frequency of memory CD8$^+$ T cells was highest in mice immunized with CD40L-treated DC and lowest in mice immunized with TNF-α-stimulated DC (Fig. 2*A*). In the absence of CD4$^+$ T cell help, memory CD8$^+$ T cells could only be consistently detected in mice immunized with DC that had been treated with LPS, CpG, or CD40L (Fig. 2). These data indicated that DC that had been appropriately stimulated could program primary CD8$^+$ T cells to fully differentiate into memory cells.

Memory CD8$^+$ T cells generated after immunization with stimulated DC in the absence of CD4$^+$ T cell help are functional

Several recent studies have indicated that memory CD8$^+$ T cells generated in the absence of CD4$^+$ T cells are functionally perturbed. Therefore, we next assessed whether memory CD8$^+$ T cells generated in the absence of CD4$^+$ T cell help after immunization with stimulated MHC class II$^-$ DC can expand and perform effector functions. Mice were immunized with MHC class II$^+$ or MHC class II$^-$, OVA$_{257}$-pulsed DC that had been stimulated by TNF-α, poly(I:C), LPS, CpG, or CD40L and rested for at least 3 wk. Recall responses were induced by challenging the primed mice with recombinant OVA-vac and were assessed 5 days after challenge. Naive mice were immunized with OVA-vac to provide an estimate of the magnitude of the primary CD8$^+$ T cell response to OVA-vac. Mice that had been immunized with MHC class II$^+$ DC mounted substantial recall responses as judged by either ICS-IFN-γ or MHC tetramer staining, regardless of the stimulus used to condition the DC (Fig. 3*A*). Recall CD8$^+$ T cells were also readily detected in mice that had been immunized with MHC class

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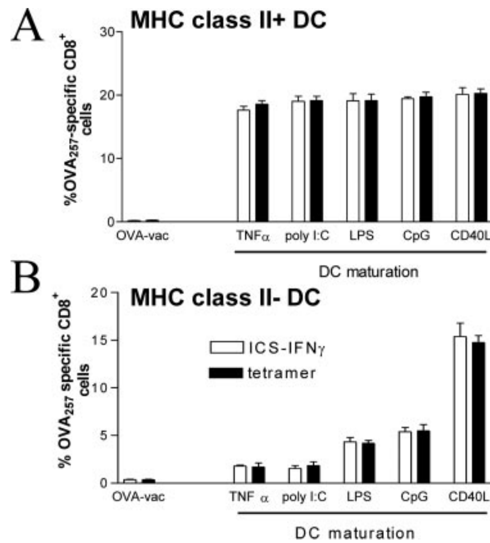

FIGURE 3. Expansion and effector function of memory CD8$^+$ T cells formed in the absence of CD4$^+$ T cell help after DC immunization. Cohorts of MHC class II$^+$ mice were immunized with 10^5 OVA$_{257}$-pulsed DC derived from MHC class II$^+$ (*A*) or MHC class II$^-$ (*B*) mice that had been treated with the indicated stimuli. After 28 days, mice were challenged with 10^7 PFU. OVA-vac and recall CD8$^+$ T cells were enumerated by ICS-IFN-γ (□) or tetramer staining (■) 5 days after challenge. Mice that had not received a previous immunization with DC provide an indication of the magnitude of the primary CD8$^+$ T cell response to OVA-vac. Data represent the average and SD of three mice per group from one of three experiments performed.

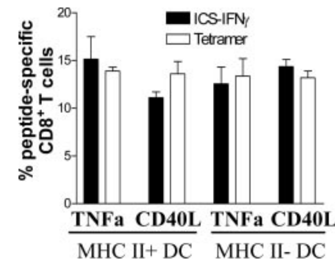

FIGURE 4. Expansion of recall CD8$^+$ T cells is independent of CD4$^+$ T cell help. Cohorts of mice were immunized with 10^7 PFU of OVA-vac rested for 21 days. DC were generated from MHC class II$^+$ or MHC class II$^-$ mice, stimulated with either TNF-α or CD40L-3T3 cells, and pulsed with peptide. Mice were challenged with 10^5 DC and assessed for OVA$_{257}$-specific CD8$^+$ T cells by either ICS-IFN-γ (□) or tetramer staining (■) 5 days after challenge. Data represent the average and SD of three mice per group from one of two similar experiments performed.

II$^-$ DC, indicating that memory CD8$^+$ T cells generated without CD4$^+$ T cell help were capable of proliferation in response to challenge (Fig. 3*B*). The magnitudes of the recall CD8$^+$ T cell responses in the mice immunized with each DC population were proportional to those observed in the primary CD8$^+$ T cell responses and also in the memory CD8$^+$ T cell populations. Importantly, there was a high degree of correlation in the numbers of OVA$_{257}$-specific recall CD8$^+$ T cells detected by either MHC tetramer staining or ICS-IFN-γ, indicating that the majority of recall CD8$^+$ T cells maintained effector functions. Surprisingly, the frequency of OVA$_{257}$-specific CD8$^+$ T cells was substantially higher in mice that had been primed with TNF-α- or poly(I:C)-stimulated DC than in mice undergoing a primary response to OVA-vac. Thus, the apparent absence of quiescent memory CD8$^+$ T cells in mice primed with TNF-α- or poly(I:C)-stimulated MHC class II$^-$ DC was likely due to a limitation in the sensitivity of the detection techniques. In sum, these results indicated that the memory CD8$^+$ T cells that were generated without CD4$^+$ T cell help after immunization with MHC class II$^-$ DC were capable of proliferation and effector functions.

Recall CD8$^+$ T cells can expand equivalently after challenge with either TNF-α-matured or CD40L-activated DC

Because the expansion of memory T cells is thought to be less dependent on costimulation (29, 30), we next asked whether DC that were poor at expanding primary CD8$^+$ T cells in the absence of CD4$^+$ T cell help would also be compromised in their ability to elicit recall CD8$^+$ T cells. MHC class II$^+$ or MHC class II$^-$ DC were stimulated with TNF-α or CD40L, pulsed with OVA$_{257}$, and used to challenge mice that had been immunized with OVA-vac 21 days previously. Analysis of OVA$_{257}$-specific recall CD8$^+$ T cell populations 5 days later showed that TNF-α-stimulated MHC class II$^-$ DC could initiate an equivalent level of expansion of memory CD8$^+$ T cells as MHC class II$^+$ DC (Fig. 4). Therefore, the expansion of recall CD8$^+$ T cells after peptide-pulsed DC challenge was CD4$^+$ T cell independent. Interestingly, there was no significant difference in the magnitude of the recall response in mice challenged with either CD40L- or TNF-α-stimulated DC, indicating that memory CD8$^+$ T cells could invariably respond to DC in differential states of activation.

Differential expression of CD70 on DC

Full activation of T cells requires not only a cognate interaction between the TCR and MHC-peptide complex, but also a costimulation via various cell surface proteins belonging to either TNFR or Ig superfamilies. Since CD40L-stimulated DC were most potent at eliciting a primary CD8$^+$ T cell response in the absence of CD4$^+$ T cell help and TNF-α-stimulated the weakest, we compared the expression of costimulatory molecules on these two DC populations. We found that both TNF-α- and CD40L-stimulated DC expressed very high levels of MHC class I and class II molecules (data not shown). CD80 and CD86 were expressed by a similar percentage of both DC populations (Fig. 5*A*), but with consistently higher levels found on CD40L-stimulated DC (Fig. 5*B*). OX40L was expressed equally by both populations, while CD30L and, surprisingly, 4-1BBL expression was absent. Consistent with the mode of activation, CD40 expression was down-modulated on CD40L-stimulated DC (data not shown). Most strikingly, we found that CD70 was expressed at high levels on CD40L-stimulated DC, while being virtually absent from TNF-α-stimulated DC (Fig. 5, *A* and *D*). Given the differential ability of TLR-stimulated DC to initiate primary CD8$^+$ T cell responses in the absence of CD4$^+$ T cell help, we assessed the expression of CD86 and CD70 on these DC. We found that poly(I:C)-, LPS-, or CpG-stimulated DC up-regulated CD86 expression (Fig. 5*B*), but only LPS- and CpG-stimulated DC consistently expressed CD70 (Fig. 5, *C* and *D*). Interestingly, LPS or CpG stimulation resulted in a slower up-regulation of CD70 than CD40L stimulation, and the levels of expression were also lower. Thus, the CD70 expression on DC appeared to be closely correlated with their ability to expand primary CD8$^+$ T cells in the absence of CD4$^+$ T cell help.

CD70 blockade substantially inhibits expansion of primary CD8$^+$ T cells in the absence of CD4$^+$ T cell help

To directly determine the contribution of CD70 expression to the generation of CD8$^+$ T cell responses in the absence of CD4$^+$ T cell help, CD40L-stimulated MHC class II$^-$ DC were pulsed with OVA$_{257}$ and treated with 1) control Ig, 2) anti-CD80 and anti-CD86 mAbs, 3) anti-CD70 mAb, or 4) anti-CD70, anti-CD80, and

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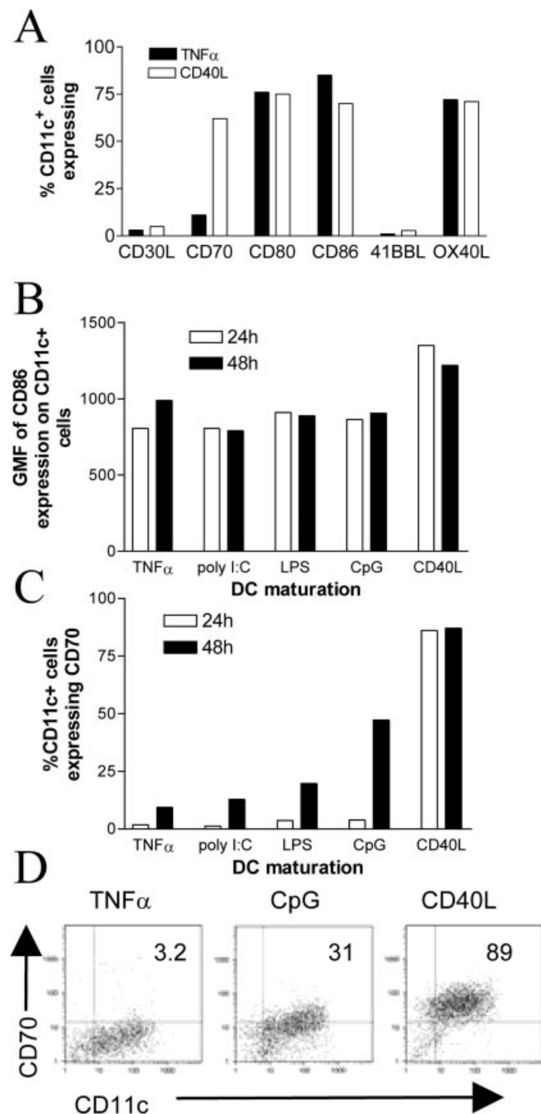

FIGURE 5. Expression of CD70 on DC after stimulation. *A,* Day 7 bone marrow-derived DC were stimulated with either TNF-α or 3T3-CD40L cells. After 16 h, cells were stained for CD11c expression to identify DC and counterstained with Abs specific for the indicated costimulatory molecules. Alternatively, DC were incubated with the indicated stimuli for either 24 or 48 h and costained with Abs for CD11c and either CD86 (*B*) or CD70 (*C* and *D*). Numbers in dot plots indicate the percentage of CD11c$^+$ cells expressing CD70. Data are representative of several similar experiments.

anti-CD86 mAbs and then used to immunize mice. Ex vivo analysis 7 days after immunization confirmed that mice immunized with OVA_{257}-pulsed MHC II$^-$ DC in the presence of control Ig mounted robust primary CD8$^+$ T cell responses to OVA_{257}. The blockade of CD80 and CD86 on the immunizing DC reduced the magnitude of the primary response by 50 ± 3% (Fig. 6). In addition, the blockade of CD70 resulted in a 33 ± 5% reduction in the magnitude of the primary CD8$^+$ T cell population, indicating that the CD70 expressed by DC significantly ($p = 0.016$) contributed to the expansion of primary CD8$^+$ T cells in the absence of CD4$^+$ T cell help. Interestingly, the blockade of both CD80/CD86 and CD70 diminished the primary response by 80 ± 7%, suggesting that the CD70-mediated costimulation works synergistically with the CD80/CD86-mediated costimulation. Blocking CD70 on CpG-stimulated DC also significantly diminished the magnitude of the primary CD8$^+$ T cell response (data not shown), indicating that the

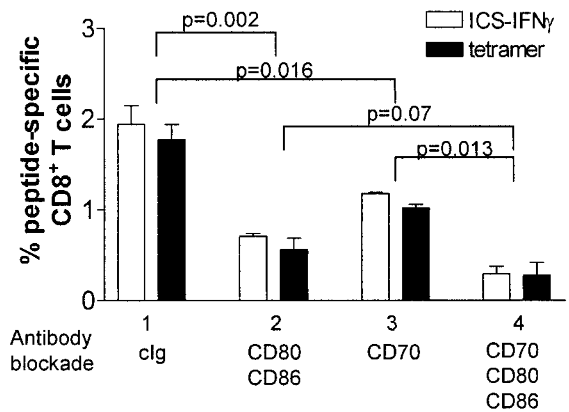

FIGURE 6. Blockade of CD70 on CD40L-activated DC reduces magnitude of primary CD8$^+$ T cell response. DC were generated from MHC class II$^-$ mice and stimulated by 3T3-CD40L. DC were harvested, pulsed with OVA_{257}, incubated with the indicated Abs, and injected into cohorts of MHC class II$^-$ mice. Primary CD8$^+$ T cell responses were assessed by ICS-IFN-γ (□) or tetramer staining (■) directly ex vivo 7 days after immunization. Data represent the average and SD of three mice per group from one of three similar experiments. Values of p were determined by a two-sample Student's t test using Minitab software.

CD70-CD27 costimulation pathway also contributed to the capacity of CpG-stimulated DC to elicit helper-independent primary expansion of CD8$^+$ T cells.

Discussion

We have examined the CD4$^+$ T cell dependence of the primary expansion and subsequent generation of memory CD8$^+$ T cells after immunization with DC that have been exposed to different maturation stimuli. Peptide-pulsed DC stimulated with either TNF-α or poly(I:C) were poor at eliciting primary CD8$^+$ T cell responses in the absence of CD4$^+$ T cell help. In contrast, DC stimulated with either LPS, CpG, or CD40L induced a more extensive expansion of primary CD8$^+$ T cells in the absence of CD4$^+$ T cell help. CD8$^+$ T cells generated after immunization with stimulated DC were capable of attaining memory status in the absence of CD4$^+$ T cell help. The capacity of DC to induce CD4$^+$ T cell-independent expansion of primary CD8$^+$ T cells correlated well with the expression of CD70. These data suggest that one of the consequences of CD4$^+$ T cell help is likely to be the CD40L-mediated up-regulation of CD70 expression by DC, which in turn provides important costimulation for the expansion of CD8$^+$ T cell populations. This process is also achieved, but with less efficiency, by engagement of TLR4 and 9.

We initially examined the necessity of CD4$^+$ T cell help for the expansion of primary CD8$^+$ T cells after immunization with DC and found that the requirement for CD4$^+$ T cell help varies depending upon the activation state of the DC. Our data demonstrating the high immunogenicity of CD40L-activated DC in the absence of CD4$^+$ T cells are consistent with previous observations (1, 2, 4), confirming that one of the primary roles for CD4$^+$ T cells is to provide a source of CD40L. However, the magnitude of the primary CD8$^+$ T cell response in the absence of CD4$^+$ T cell help was always smaller than in its presence, indicating that CD4$^+$ T cells provide additional support to expand CD8$^+$ T cells. Whether this is simply the provision of cytokines or growth factors (e.g., IL-2) (31) or attributable to inter-T cell costimulatory molecule interactions, such as CD40-CD40L (11), remains to be clarified. Although CD40L-mediated activation of DC was clearly potent at circumventing the requirement for CD4$^+$ T cell help of primary

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CD8$^+$ T cell expansion, we found that other factors could also stimulate DC sufficiently. LPS- or CpG-stimulated DC were also capable of initiating substantial CD4$^+$ T cell-independent expansion of CD8$^+$ T cells, whereas responses initiated by TNF-α- or poly(I:C)-stimulated DC were considerably smaller. These data indicate that CD40-mediated activation of DC is not an absolute prerequisite for the primary expansion of CD8$^+$ T cells and that different maturation stimuli have a different capacity to "license" DC, perhaps accounting for the ability of various pathogens to cause the expansion of primary CD8$^+$ T cells in the absence of CD4$^+$ T cells (13, 18, 32, 33). However, TNF-α- or TLR-stimulated DC were considerably less effective than CD40L-stimulated DC at eliciting primary and memory CD8$^+$ T cells in the absence of CD4$^+$ T cell help. This indicates that although innate responses to infection raise the activation state of DC, full licensing of DC requires interaction between DC and CD40L-expressing cells of the adaptive immune response.

The variable ability of stimuli to activate DC to elicit primary CD8$^+$ T cell expansion independently of CD4$^+$ T cell help might be accounted for by the differential expression of cytokines and/or costimulatory molecules. We initially investigated the involvement of IL-12 because its expression has been shown to be induced in DC by CD40L, CpG, and LPS stimulation (34), and it is known to support the development of CD8$^+$ T cell responses (35). However, CD40L-stimulated DC derived from IL-12p40-deficient mice were not impaired in their ability to elicit helper-independent primary CD8$^+$ T cells (T. N. J. Bullock, unpublished observations). Instead, we found a clear correlation between the expression of CD70 on DC and the ability of DC to generate primary CD8$^+$ T cell responses in the absence of CD4$^+$ T cell help.

The close association between CD40 ligation and CD70 expression raises the question whether the expression of CD70 is a critical costimulatory function that licenses primary CD8$^+$ T cells to expand. We have demonstrated that the blockade of CD70 on DC led to a substantial, but not complete, reduction in the CD4$^+$ T cell-independent expansion of primary CD8$^+$ T cells initiated by CD40L- or CpG-activated DC. Although the failure to completely block CD8$^+$ T cell expansion could be attributed to Ab dissociation, it more likely indicates that additional costimulatory molecules such as CD80 and CD86 contribute to the conditioned status of CD40L-stimulated DC (36). The data presented in this study indicate that CD70 provides an important stimulus from the DC to the responding CD8$^+$ T cells. This notion is in accord with the fact that CD70-transgenic mice showed enhanced expansion and survival of primary CD8$^+$ T cells and that T cells from CD27-deficient mice were constrained in their ability to expand after Ag challenge (37, 38). Interestingly, we have observed more complete blocking of primary CD8$^+$ T cell expansion by additional in vivo infusion of anti-CD70 mAbs (T. N. J. Bullock, unpublished observation). This suggests that CD70-CD27 interactions are important beyond the initial activation of T cells by DC, perhaps at an inter-T cell level (39, 40). However, the observation that TNF-α-stimulated DC (which did not express CD70) were equally competent at eliciting recall CD8$^+$ T cells as CD40L-stimulated DC implies that CD70-mediated costimulation may be redundant beyond primary CD8$^+$ T cell expansion. We also found that CD4$^+$ T cell-independent primary CD8$^+$ T cell expansion could be further suppressed by blocking CD80 and CD86 in addition to CD70, indicating that CD27- and CD28-mediated costimulation pathways act cooperatively (38). Thus, the enhanced immunogenicity of conditioned DC is likely to be due to the increased expression of several costimulatory molecules that support CD8$^+$ T cell expansion coordinately.

The mechanisms by which CD27-mediated costimulation enhances CD8$^+$ T cell responses are not well established. In CD27-deficient animals, CD4$^+$ and CD8$^+$ T cell responses to influenza infection were diminished due to an increase in T cell death after activation (37). Thus, CD27-mediated signaling is thought to be important in T cell survival via a TNFR-associated factor-mediated induction of anti-apoptotic signals, as opposed to facilitating the initial activation of T cells or enhancing their proliferative capacity (41). This is reminiscent of the observations indicating that the absence of CD4$^+$ T cells during CD8$^+$ T cell priming can often be compensated for by supplementary IL-2 (1, 15, 31) or CD28 costimulation (37), which also provide anti-apoptotic signals. Thus, it will be of interest to determine whether the relatively weak immunogenicity of TNF-α-stimulated DC will be enhanced by either supplementary ligation of CD27 or addition of IL-2. In vivo administration of an anti-CD27 mAb has been found to result in T cell depletion (J. Borst, unpublished observations); however, two recent reports using either CD70-Ig (42) or secreted CD70 (43) indicated that CD8$^+$ T cell responses can indeed be enhanced by targeting CD27 on T cells.

Several recent studies have suggested that CD4$^+$ T cells can play a critical role in the generation of memory CD8$^+$ T cells (15–17). We have observed that ligation of CD40, TLR4, or TLR9 on DC not only licensed the expansion of primary CD8$^+$ T cell effectors in the absence of CD4$^+$ T cell help, but also resulted in the generation of memory CD8$^+$ T cells that could proliferate and perform effector functions. It should be noted that the numbers of primary CD8$^+$ T cells and memory CD8$^+$ T cells were reduced in the absence of CD4$^+$ T cell help, indicating that CD4$^+$ T cell-mediated help beyond providing CD40L is important in determining the magnitude of the primary CD8$^+$ T cell response, and the number of subsequent memory CD8$^+$ T cells. Importantly, the reduction in the number of CD8$^+$ T cells generated without CD4$^+$ T cell help was consistent between primary and recall responses (Table I), indicating that CD8$^+$ T cells activated by CD40L-,

Table I. *Reduction in number of OVA$_{257}$-specific CD8$^+$ T cell responses in the absence of CD4$^+$ T cell help is maintained in primary and recall responses*

	TNF-α[a]		Poly(I:C)		LPS		CpG		CD40L	
	Primary[b]	Recall[c]	Primary	Recall	Primary	Recall	Primary	Recall	Primary	Recall
MHC II^{+d}	2.9 ± 0.1	18.56 ± 1.3	3.06 ± 0.22	19.16 ± 2.6	3.7 ± 0.56	19.2 ± 3.4	3.53 ± 0.18	19.7 ± 1.4	4.93 ± 1.3	20.3 ± 2.1
MHC II$^-$	0.34 ± 0.11	1.67 ± 0.23	0.42 ± 0.09	1.84 ± 0.21	1.3 ± 0.18	4.17 ± 0.56	1.19 ± 0.14	5.48 ± 0.68	4.17 ± 0.28	14.76 ± 1.14
% Reduction[e]	88	91	86	90	65	78	66	72	16	27

[a] Maturation stimuli used to treat bone marrow-derived DC.
[b] Percent OVA$_{257}$-specific CD8$^+$ T cells 7 days after immunization; average of three experiments ± SD.
[c] Recall responses from mice primed with MHC class II$^+$ or class II$^-$ DC and challenged with OVA-vac.
[d] MHC class II expression on DC.
[e] One hundred − (magnitude of CD8$^+$ T cell response to MHC II$^-$ DC/magnitude of response to MHC class II$^+$ DC) × 100.

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LPS-, or CpG-stimulated DC have received sufficient information to propel them through the developmental processes that culminates in the generation or survival of memory CD8^{+} T cells (21–23). The ability of highly activated DC to induce the generation of memory CD8^{+} T cells in the absence of CD4^{+} T cell help is consistent with earlier studies that used DC that had been activated ex vivo by Ab-mediated CD40 cross-linking (1), but contrasts with data from several recent studies with viral (16), or bacterial (17) infections, or cell line challenge (15), indicating that primary CD8^{+} T cells can expand in the absence of CD4^{+} T cells but subsequent generation of memory CD8^{+} T cells was defective. In these studies the defects in memory CD8^{+} T cells appeared either as a reduced capacity to expand after Ag challenge (11, 15, 16), which clearly differs from our observations, or a degradation in the number of detectable memory CD8^{+} T cells (17). The 21- to 28-day time point at which recall responses were initiated in our study is after the point at which CD25^{-}CD44^{+}CD69^{-} memory CD8^{+} T cell populations form (T. N. J. Bullock, unpublished observations). However, a recent study by Kaech et al. (44) suggests that memory CD8^{+} T cell formation is a more drawn out, dynamic process that might not be complete until 8 wk after initial exposure to Ag. Therefore, it is possible that a long-term study of mice immunized with TLR- or CD40L-activated DC will reveal a degraded ability to mount recall responses. If no degradation in CD8^{+} T cell memory becomes apparent, it will indicate that appropriately stimulated DC present all of the information necessary for a CD8^{+} T cell to differentiate into a memory cell.

What is currently not clear is why DC that have been exposed to TLR ligands are capable of driving the generation of memory CD8^{+} T cells, whereas mice that have been infected with pathogens that likely express such ligands have deficiencies in their memory CD8^{+} T cell population. One possibility is that the dose of TLR ligands used to stimulate DC in the present study was supraphysiological, and infection by pathogens might not have similar affects on local DC populations. Alternatively, it is well known that many pathogens possess immune evasion mechanisms that modulate the magnitude of immune responses and can interfere with the maturation of DC (45, 46). Thus, in an infectious environment TLR-mediated activation of DC could be compromised, and activation of DC via CD40 becomes necessary for the expression of the molecules, such as CD70, that are required for differentiation/survival of CD8^{+} T cells that are destined to become memory cells. Distinguishing between these possibilities will require further understanding of how CD8^{+} T cells become committed to become memory cells.

In conclusion, our data support a model that once DC have been raised to a certain activation state they can stimulate CD8^{+} T cells sufficiently to expand and form fully functional memory cells in the absence of CD4^{+} T cells. Our data suggest that CD70 upregulation after CD40 or TLR ligation contributes to this process, perhaps cooperatively with CD80 and CD86, and that CD70-mediated costimulation may be an attractive target for enhancing vaccination effectiveness.

Acknowledgments

We thank Brian Norris and Lisa Hwang for excellent technical assistance; Dr. Rejean Lapointe for providing CD40L-transduced NIH-3T3 cells; Dr. John Yewdell for supplying OVA-vac; Dr. Vic Engelhard for OVA_{257} tetramers; and Drs. Mike Brown, David Mullins, and Kimberly Chianese-Bullock for critical reading of this manuscript.

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