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Antigenic and costimulatory signals trigger a developmental program by which naive CD8 T cells differentiate into effector and memory cells. However, initial cytokine signals that regulate the generation of effector and memory CD8 T cells are not well understood. In this study, we show that IL-12 priming during in vitro antigenic stimulation results in the significant increase of both primary and memory CD8 T cell population in mice after adoptive transfer of activated cells. The effect of IL-12 priming is closely associated with qualitative changes in CD8 T cells, such as reduced MHC I tetramer binding and CD69 expression, altered distribution of lipid rafts, decreased cytolytic activity, and less susceptibility to apoptosis. Furthermore, exogenous IL-12 priming improved the intrinsic survival properties of memory CD8 T cells, leading to better protective immunity and vaccine-induced memory CD8 T cell responses. However, the experiments with IL-12p40- and IL-12Rβ1-deficient mice showed similar levels of primary and memory CD8 T cell responses compared with wild-type mice, implying that endogenous IL-12 and/or IL-12R signaling in vivo is not critical for CD8 T cell immunity. Together, our results suggest that IL-12 can serve as an important, but dispensable regulatory factor for the development of CD8 T cells, and IL-12 priming could be useful in many medical applications. The Journal of Immunology, 2004, 172: 2818–2826.

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ntigen-specific naive T cells exist at very low frequencies, but undergo massive clonal expansion after infection or immunization, resulting in effector and memory populations of a higher frequency. These quantitative and qualitative changes in T cells eventually establish immunological memory, in which the majority of the effector T cells (90–95%) are eliminated and the remaining memory T cells (~5%) form the basis for protective immunity against pathogenic infection and diseases together with Abs. It has been proposed that after initial Ag encounter, naive CD8 T cells initiate a program for their autonomous clonal expansion and development into functional effectors and memory cells (1–3). However, the other studies showed that the strength and duration of antigenic and costimulatory signal can affect the process and regulate the functional qualities of developing effector and memory T cells (4–7). The experiments with human and mouse CD4 T cells suggest that progressive differentiation is driven by sustained TCR signaling and cytokine stimulation (4, 6). In human CD4 T cells, the strength of antigenic stimulation as determined by various factors including TCR avidity seems to regulate T cell progression through thresholds of proliferation, differentiation, and death, in which T cell death was closely related to the extent of TCR stimulation (5). Also, a prolonged TCR stimulation in the presence of IL-12 or IL-4 drives differentiation into Th effector cells, whereas a short TCR stimulation leads to nonpolarized T cells with different homing properties. Thus, it is likely that initial programming of CD4 and/or CD8 T cells to become effector and memory cells might be different depending on the extent of TCR stimulation and cytokine environment.

Professional APCs not only process and present Ag, but provide costimulatory signal to T cells recognizing the Ag presented by APCs. In addition, activated APCs produce a variety of cytokines that may provide an additional signal required to fully activate T cells (8–11). Of various factors for stimulating T cells, cytokines have been focused by many investigators because accumulating evidences revealed their critical role in the generation and maintenance of memory CD8 T cells (12–16). Several groups have shown that cytokines can provide an important regulatory signal to naive CD8 T cells for the activation and proliferation, as well as for differentiation into memory CD8 T cells (9, 17–19). For example, it was suggested that distinct inflammatory cytokines such as IL-12 act directly on naive CD8 T cells to provide a third signal, along with Ag and IL-2, to optimally activate differentiation and clonal expansion (9), and naive T cells in the absence of IL-12 during in vitro activation fail to develop effector function (8). In contrast, another study reported that IL-4, but not IL-2 or IL-12 exposure for 3 days during in vitro TCR stimulation of naive CD8 T cells induces long-lasting memory (18). Thus, it remains to be determined which and how exactly initial cytokine priming affects development of CD8 T cell immunity quantitatively and qualitatively.

In this study, we show that short-term exposure of IL-12 during initial Ag contact increases primary expansion of effector CD8 T cells mainly due to the reduced cell death, rather than the increased proliferation, which results in a larger memory CD8 T cell population. Particularly, initial IL-12 priming directly caused qualitative changes in CD8 T cells, which are possibly associated with
substantial improvement in the survival of CD8 T cells. Our results also establish the potential application of initial IL-12 priming in CTL-based vaccine technologies for the formation of long-lasting memory CD8 T cells.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). OT-I and OT-II TCR-transgenic mice were a kind gift from W. Heath (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). IL-12p40- and IL-12Rβ1-deficient mice on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). To obtain IL-12Rβ1-deficient OT-I cells, IL-12Rβ1-deficient mice were crossed to OT-I mice, and both TCR transgene and IL-12Rβ1 gene disruption were confirmed by PCR. All mice were housed under specific pathogen-free conditions and were used between 6 and 8 wk of age.

Abs and reagents

All Abs were purchased from BD PharMingen (San Diego, CA), unless specified otherwise. Purified hamster anti-mouse CD3 and CD28 mAbs were purchased from Southern Biotechnology Associates (Birmingham, AL). Anti-CD11c, anti-MHC class II, anti-CD11b, and anti-CD11c microbeads were obtained from Miltenyi Biotec (Auburn, CA). Reconstitut mouse IL-2, IL-4, IL-6, IL-12p40, IL-12p70, and human IL-15 were all purchased from R&D Systems (Minneapolis, MN).

In vitro T cell activation and adoptive transfer

Lymph node (LN) and spleen cells from OT-I or OT-II TCR-transgenic mice were stimulated with 100 nM OVA257–264 peptide (OVAp) for 48 h or with 10 μM OVA253–265 peptide for 72 h, respectively, in the presence or absence of the indicated cytokines in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and streptomycin. In neutralization experiments, these cultures were treated with 10 μg/ml anti-IL-2, anti-IFN-γ, or anti-TNF-α neutralizing mAb to block the potential activities of endogenous sources of these cytokines. At 2 days after in vitro activation, live lymphocytes were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation, and then purified by negative selection using MACS. In brief, cells were incubated with mAb-conjugated magnetic beads for CD4 (for OT-I) or CD8 (for OT-II), MHC II, CD11b, and CD11c, followed by depletion of positive cells using LS+ MACS columns (Miltenyi Biotec), and then adoptively transferred into normal C57BL6 mice (2 × 106 cells/mouse in 200 μl of PBS). For Ab stimulation, MACS-purified cells were stimulated for 2 days with plate-bound anti-CD3 (300 ng/well) and anti-CD28 mAb (100 ng/well). For assessing the cell type dependency of IL-12, MACS-purified CD8 T cells from OT-I mice were mixed with indicated syngenic APCs as stimulator cells purified from LN and spleen of normal C57BL6 or IL-12Rβ1-deficient mice by MACS-positive selection using anti-MHC II, anti-CD20, anti-CD11b, and anti-CD11c microbeads.

Surface staining, intracellular staining, and flow cytometry

For counting a total number of donor T cells, recipient mice were sacrificed, and cells from LN and spleen (in some cases from liver and lung) were resuspended in FACS buffer (1% FCS and 0.02% sodium azide in PBS) at a concentration of 1 × 107 cells/ml. A total of 100 μl of these cells (1 × 106 cells) was stained for CD8, Vα2, and Vβ5, and samples were acquired on FACScalibur (BD Biosciences, San Jose, CA). PE-conjugated OVA-specific MHC I tetramer, Kb/OVA-Ap-Tet, was produced, as described (20). Anti-CD107a was stained with Pe-conjugated mAb. For intracellular cytokines, MACS purified cells were stimulated for 2 days with plate-bound anti-CD3 (300 ng/well) and anti-CD28 mAb (100 ng/well). For detecting the cytokine type dependency of IL-12, MACS-purified naive OT-I or IL-12Rβ1-deficient OT-I cells were mixed with indicated syngenic APCs as stimulator cells purified from LN and spleen of normal C57BL6 or IL-12Rβ1-deficient mice by MACS-positive selection using anti-MHC II, anti-CD20, anti-CD11b, and anti-CD11c microbeads.

CFSE labeling

For assessing cell division during in vitro activation, naïve OT-I cells were negatively purified by MACS, labeled with CFSE, and then incubated with APCs pulsed with 100 nM of OVAp. Remainer cells after purification of naïve OT-I cells were used as APCs. Cell division was assessed on various time points by monitoring the CFSE intensity of these cultures, followed by staining with anti-CD8 and anti-CD9. For detecting in vivo cell division of activated CD8 T cells, purified OT-I cells were labeled with CFSE, and injected i.v. to normal mice. At day 3 after transfer, CFSE intensity of donor OT-I cells from spleen, liver, and PBMC of recipient mice was measured by flow cytometry.

Detection of apoptotic death

Apoposis of in vitro activated T cells was determined by annexin V and propidium iodide (PI) staining, as recommended by the manufacturer (BD PharMingen). In brief, purified CD8 T cells were seeded in 96-well flat-bottom plate in triplicate wells (2 × 105 cells/well) without any stimulants in 200 μl of complete RPMI 1640 medium. At indicated time points, these cells were washed, resuspended in annexin V binding buffer at a concentration of 1 × 105 cells/ml, incubated with FITC-conjugated annexin V and PI (2 μg/ml), and analyzed by flow cytometry. To assess spontaneous apoptosis of memory CD8 T cells, cells were isolated from splenocytes of the recipient mice on day 70 by CD8 MACS, and plated in flat-bottom plate in triplicate wells (2 × 105 cells/well) without any stimulants in 200 μl of complete medium. At indicated time points, cells were stained with anti-Vα2, anti-Vβ5, and annexin V.

Cytolytic activity, tumor protection, and ELISPOT assay

For measuring ex vivo cytotoxic activity, total splenic CD8 T cells were isolated by positive selection with MACS. Purified cells were then incubated with OVA-pulsed EL4 target cells that had been labeled with 111Cr (Amersham Pharmacia Biotech). Cytotoxic activity was determined by standard 4-h 51Cr release assay. Spontaneous lysis of EL4 cells without anti-IL-2, anti-IFN-γ, or anti-IL-10 neutralizing mAb was added as substrate. The tumor-injected mice were regularly checked, and their survival rate against tumor development was monitored for indicated time points. For ELISPOT assay, 96-well filtration plates (Millipore, Billerica, MA) were coated with 50 μl of 3 μg/ml rat anti-mouse IFN-γ Ab. After overnight incubation, plates were blocked with DMEM containing 10% FBS for 1 h. For the depletion of CD4 T cells, 105 of splenocytes were incubated with 10 μg of anti-CD4 (clone RM4-5) Ab for 20 min on ice. The cells (105 or 5 × 104 per well) were applied into plates with E2 peptide pool (1 μg/ml for each peptide). After 24 h, the plates were washed, and 50 μl of 2 μg/ml biotinylated rat anti-mouse IFN-γ Ab was added and incubated for 3 h at room temperature. After washing the plates, 50 μl of 1/2000 diluted streptavidin-alkaline phosphatase (BD PharMingen) was added. The plates were washed and then 50 μl of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Promega) was added for color development. When spot color was sufficiently developed, the reaction was stopped by washing the plates with tap water. The number of spots was counted using AID ELISPOT Reader System (AID, GmbH, Strassberg, Germany).

Recombinant adenosovirus infection

Replication-defective adenosovirus (rAd) was constructed and produced using the AdEasy vector system, as recommended by the manufacturer (Quantum Biotechnologies, Carlsbad, CA). Mice were i.n. injected with 2 × 105 PFU of rAd, or lightly anesthetized with 2:1 mixture of ether and chloroform, followed by intranasal (i.n.) inoculation in 50 μl 24 h after adoptive transfer.

Results

Increased primary and memory CD8 T cells by initial IL-12 priming

To examine whether short-term exposure of various cytokines during initial Ag contact affects the primary expansion and memory development of CD8 T cells, splenic and LN CD8 T cells from OVA-specific TCR-transgenic mice (referred to hereafter as OT-I cells) were stimulated with OVA (257–264) peptide (SIINFEKL; referred to as OVAp) for 48 h in the presence or absence of the indicated cytokines, such as rIL-4, rIL-12, and rIL-15. Purified CD8 T cells were then adoptively transferred into normal C57BL6/6 mice.
mice. At day 3 after adoptive transfer for primary expansion and day 70 for memory phase, the frequency of OT-I cells in the spleen and LN of the recipient mice was analyzed by flow cytometry. Among these cytokines, IL-12 treatment resulted in the significant (~5-fold at day 3 and ~20-fold at day 70) increase of primary and memory OT-I cells, compared with other groups (Fig. 1A). In this analysis, there was 27- to 31-fold reduction in the frequency of OT-I cells primed with OVAp between days 3 and 70 after transfer, but just 2- to 3-fold decrease in those primed with OVAp/IL-12. As a control, naive OT-I cells did not show any sign of primary expansion and memory cell generation. We further examined the kinetics of donor CD8 T cells during expansion, contraction, and maintenance phases. On the average from five independent experiments, the percentages of transferred OT-I cells primed with OVAp/IL-12 among total CD8 T cells were 3.2-fold high at day 3 (73.0 vs 22.8%), 4-fold at day 32 (41.6 vs 10.3%), and 10-fold at day 70 (12.1 vs 1.2%), compared with those primed with OVAp, respectively.

We next evaluated the effect of initial cytokine priming on OVA-specific TCR-transgenic CD4 T cells (OT-II cells) with a similar protocol described in Fig. 1A. In this case, the presence of IL-4, IL-12, and IL-15 during initial Ag contact did not alter the size of primary and memory OT-II CD4 T cell population (Fig. 1B). These results clearly indicate that IL-12 selectively acts on CD8 T cells, but not on CD4 T cells, resulting in the increase of memory CD8 T cells as well as effector CD8 T cells.

However, it was unclear whether IL-12 acts directly on CD8 T cells, or indirectly by enhancing the ability of the APCs. Therefore, we determined which type of cells are affected by initial IL-12 priming. Naive OT-I (OT-I (IL-12R–/–)) and OT-I cells from IL-12Rβ1-deficient mice (OT-I (IL-12Rβ1–/–)) were mixed with either syngenic APC (APC (IL-12Rβ1–/–)) or APC from IL-12Rβ1-deficient mice (APC (IL-12Rβ1–/–)), stimulated for 48 h, and adoptively transferred into normal mice, as described in Materials and Methods. At days 3 and 70 after transfer, increased numbers of donor cells were observed in the spleen and LN from mice that received IL-12-primed OT-I (IL-12Rβ1–/–) cells, but not OT-I (IL-12Rβ1–/–) cells, regardless of the source of APC (Fig. 2A). These results clearly indicate that the effect of initial IL-12 priming needs IL-12/IL-12R interaction on CD8 T cells, but not on APC. In addition, we observed similar results when donor cells were analyzed in the liver and lung, indicating that increased primary expansion and memory cell generation by initial IL-12 priming are not due to differential migration of donor cells into lymphoid and nonlymphoid tissues (Fig. 2A).

Our data argue that IL-12 exerts its effect on CD8 T cells directly. However, it is possible that other cytokines produced from CD8 T cells mediate the effect of initial IL-12 priming. To test this possibility, various neutralizing Abs to IL-2, IL-2Rβ, IFN-γ, or TNF-α were added at the time of stimulation, and the frequencies of OT-I cells were analyzed in the spleen and LN of recipient mice at days 3 and 70 after transfer. The frequency of donor cells primed with IL-12 was invariable even with the addition of the neutralizing Abs, showing that the effect of IL-12 is not mediated by these cytokines (Fig. 2B). In addition, the effect of initial IL-12 priming was independent on the sources of TCR stimulation, because we still observed increased primary expansion and memory generation of donor cells primed with IL-12 by anti-CD3/anti-CD28 stimulation (Fig. 2C).

### FIGURE 1. In vitro priming of exogenous IL-12 during antigen stimulation increases the size of both activated and memory CD8 T cells, but not CD4 T cells.

Mixed spleen and LN cell suspensions from naive OT-I (A) or OT-II (B) mice were cultured with 100 nM of OVAp or 10 μM of OVA (323–339) peptide in the presence or absence of indicated recombinant cytokines at a concentration of 10 ng/mL. After 2-day culture, MACS-purified cells (2 × 10⁶) were adoptively transferred into B6 mice. In each case, purified naive OT-I and OT-II cells were used as a negative control. Donor cell recovery was determined at days 3 and 70 post-transfer from the spleen and LN of recipient mice by flow cytometry. Values represent the absolute number of CD8+Vα2+Vβ5+ or CD4+Vα2+Vβ5+ T cells and are shown as the mean ± SD of three to five mice in one experiment. All data are representative of two independent experiments with similar results.

### Phenotypic and functional changes induced by IL-12 priming

The previous study proposed that initial Ag encounter triggers an instructive developmental program of CD8 T cells that become committed to differentiating into effector and memory cells (3). Thus, it is likely that IL-12 signaling during initial antigen stimulation modified the intrinsic properties of CD8 T cells to develop into effector and memory cells. To determine whether IL-12 priming induces any phenotypic changes on CD8 T cells during in vitro stimulation, we compared TCR structural avidity measured by OVA-specific MHC I tetramer (Kb/OVAp-Tet) staining and expression of surface activation markers between OVAp/IL-12-stimulated and OVAp-stimulated OT-I cells. Surprisingly, Kb/OVAp-Tet binding at the saturating concentration was gradually decreased in IL-12-treated cells compared with nontreated cells, reaching the greatest difference at 48 h after stimulation (Fig. 3A). In contrast, treatment with IL-12 had little effect on the expression level of the T cell surface markers such as CD11a, CD44, and CD3, while there was modest increase (less than 2-fold) in the staining intensity of CD25 and CD43 molecules (Fig. 3A and data not shown). This difference induced by IL-12 was not due to the differences in cell sizes or Kb/OVAp-Tet concentration because the forward scatter profiles were similar, and graded dilutions of the tetramer showed the same percentage of maximum signal between two groups, respectively (data not shown). As a control, we also stimulated splenocytes and LN cells from IL-12Rβ1-deficient...
FIGURE 2. IL-12 directly acts on CD8 T cells independent of both cytokines and TCR stimulants. A, MACS-purified, naive OT-I<sup>IL-12R<sup>–/–</sup></sup> (OT-I) or OT-I<sup>IL-12R<sup>+/+</sup></sup> cells were mixed with indicated MACS-purified syngeneic APC<sup>IL-12R<sup>+/+</sup></sup> (APC) or APC<sup>IL-12R<sup>–/–</sup></sup> and cultured for 2 days with OVAp in the presence or absence of IL-12. B, Mixed spleen and LN cell suspensions from naive OT-I mice were cultured for 2 days, as described in Fig. 1, except for the addition of neutralizing Abs to IL-2, IL-15, IFN-γ, or TNF-α at a concentration of 10 μg/ml. C, MACS-purified naive OT-I cells were cultured for 2 days with precoated mAbs to CD3 and CD28 in the presence or absence of IL-12. A–C, MACS-purified, in vitro activated cells (2 × 10<sup>5</sup>) were adoptively transferred into B6 mice. At days 3 and 7 posttransfer, donor cell recovery was determined from the spleen and LN of recipient mice by staining and flow cytometry. Values represent the absolute number of CD8<sup>+</sup>V<sup>α2</sup>V<sup>β8</sup><sup>+</sup> T cells and are shown as the mean ± SD of three to five mice in one experiment. Data are representative of two independent experiments with similar results.

OT-I mice in the same experimental setting and found no difference in Kb/OVAp-Tet binding, clearly showing that the altered TCR avidity was specifically induced by IL-12-mediated signaling (Fig. 3A).

It has been reported that the ability of TCR on activated CD8 T lymphocytes to bind MHC I tetramer complexes is dependent on the integrity of lipid rafts on the membrane (21). Because the topological arrangement of TCR on the cell surface is presumably influenced by lipid raft integrity, we next examined IL-12-treated or nontreated OT-I cells using cholera toxin β subunit (CTx) staining to identify the ganglioside GM1, which is enriched in lipid raft. On OVAp-stimulated OT-I cells harvested at 48 h after in vitro stimulation, several areas of intense CTx binding were clearly observed, suggesting that lipid raft-associated GM1 is asymmetrically localized to distinct regions of the plasma membrane (Fig. 3B). By contrast, we repeatedly observed that GM1 was distributed on OVAp/IL-12-stimulated OT-I cells more uniformly throughout the plasma membrane in a diffuse, ring-like manner than OVAp-stimulated cells. These results clearly demonstrated that the decrease in MHC I tetramer binding is associated with changes in lipid raft distribution induced by IL-12.

Upon activation, Ag-specific T cells up-regulate several activation marker molecules. We therefore determined the expression of representative activation marker CD69, a very early activation marker, to see whether IL-12 priming alters the expression of this molecule. Notably, the expression of CD69 was dramatically decreased in OVAp/IL-12-stimulated cells during activation period (i.e., 24–72 h), whereas cells in OVAp group maintained substantial CD69 expression until 72 h (Fig. 3C). Our results indicate that IL-12-treated cells started to sequentially down-regulate CD69 expression from 24 h after stimulation, which coincided with the start point of the decrease in MHC I tetramer binding, while initial up-regulation of CD69 at very early stage (i.e., 6 h) was comparable between two groups. However, the division profiles of both groups accessed by CFSE intensity were not significantly different during in vitro stimulation period (Fig. 3C).

Based on our previous observation, it is very likely that IL-12 priming also influences the functional aspects of activated CD8 T cells such as cytolytic activity and cytokine production. As expected, activated OT-I cells were highly cytolytic at 48 h, but this lytic capacity per cell decreased significantly in the presence of IL-12 (Fig. 3D). In contrast, the frequency of IFN-γ-producing cells and the staining intensity for the intracellular cytokine after 3-h restimulation were higher in OVAp/IL-12 group than in OVAp group (Fig. 3E). These results are in part supported by the previous reports that perforin can negatively influence the expansion of Ag-specific CD8 T cells (22–24), while IFN-γ can enhance expansion by up-regulating proteosome-associated molecules (25). Thus, it is likely that decreased cytolytic activity possibly with less expression of perforin molecule and higher IFN-γ production in OVAp/IL-12-stimulated cells resulted in the increased expansion of effector and memory cells. Together, these results indicated that phenotypic and functional properties of activated CD8 T cells have been altered by IL-12 priming during antigenic stimulation, whereas proliferative capacity remains the same, eventually leading to increased primary and memory CD8 T cells.

IL-12 priming decreases death rate of CD8 T cells

After massive expansion of Ag-specific cells and acquisition of effector functions, contraction phase initiates and majority of effector cells undergo apoptosis. Interestingly, it has been recently shown that the contraction of the Ag-specific CD8 T cell response may also be programmed during initial Ag contact (26). Because the proliferation kinetics of OT-I cells was similar during in vitro
Altered characteristics of CD8 T cells by IL-12 priming.

A, In vitro stimulated cells were stained with anti-CD8 and Kb/OVAp-Tet at indicated time points. Cells were gated on CD8$^+$ cells, and then Kb/OVAp-Tet intensity for OVAp-stimulated cells (filled line) was compared with OVAp/IL-12-stimulated cells (gray line) by histogram analysis. The mean fluorescence intensity of Kb/OVAp-Tet binding is shown for each group. For the analysis of activation markers, cells were harvested 48 h after stimulation, stained, and gated for CD8$^+$ and CFSE$^+$ staining.

B, Each group of cells was stained with CTx to localize lipid raft at 48 h after stimulation, and analyzed with confocal microscopy.

C, Naive OT-I cells were negatively purified by MACS, labeled with CFSE, and then mixed with the remainder cells and stimulated with 100 nM of OVAp in the presence or absence of IL-12. The expression of CD69 and CFSE intensity was determined by gating on CD8$^+$ cells at various time points. The numbers in the upper and lower right quadrants reflect the percentage of CD8$^+$ T cells that are CFSE-labeled CD69$^+$ and CD69$^-$, respectively.

D, The cytolytic activity of purified activated OT-I cells was measured at 48 h after stimulation with OVAp-pulsed EL4 target cells. E, IFN-$\gamma$ production at 48 h after stimulation was assessed by intracellular staining following 3-h stimulation with OVAp-pulsed naive splenocytes.
stimulation period, it is very likely that IL-12-primed cells have differential susceptibility to cell death throughout their life cycle. This possibility is further supported by the previous studies that in vitro IL-12 exposure reduces activation-induced cell death of CD4 and CD8 T cells (27–30). To determine whether initial IL-12 priming affects cell death during primary expansion, 48-h-activated OT-I cells were rested without stimulation and monitored for apoptotic cell death at the various time points by PI and annexin V staining. IL-12-treated cells showed similar death rate to non-treated cells by 9 h, but exhibited a gradual decrease of death rate from 21 to 48 h (Fig. 4A). To examine the involvement of intracellular apoptotic molecules in the inhibition of cell death, the level of active caspase-3 was analyzed after 48 h of resting culture period. As expected, significantly lower frequency of active caspase-3-positive cells was observed in OVAp/IL-12-stimulated cells (Fig. 4B). These results are consistent with other studies showing that IL-12-mediated inhibition of cell death is associated with decreased expression of active caspase-3 (29–31). Consistent with in vitro experiments, we observed no difference in the proliferative capacity between IL-12-treated and nontreated donor cells in the spleen, PBMC, and liver of the recipient mice at day 3 after adoptive transfer (Fig. 4C). Taken together, our results indicate that initial IL-12 priming increases primary expansion of CD8 T cells by reducing death rate rather than by promoting cell division.

Given the slower reduction of the percentage of memory OT-I cells primed with OVAp/IL-12 at memory phase, as shown in Fig. 1, it is very likely that initial IL-12 priming also affects survival properties of memory CD8 T cells that are crucial for the long-term persistence in vivo. To assess whether initial IL-12 priming also affects death rate of memory CD8 T cells, purified total CD8 T cells from recipient mice at day 70 were rested for an additional 3 days without stimulation and spontaneous apoptosis was determined at indicated time points. Memory cells established from OVAp/IL-12-stimulated donor cells were much more resistant to spontaneous apoptosis than those from OVAp-stimulated cells (Fig. 4D). Consistent with increased resistance to spontaneous apoptosis, memory OT-I cells in OVAp/IL-12 group showed decreased active caspase-3 expression compared with those primed with OVAp (Fig. 4E). Together, these results indicate that initial IL-12 priming imprints improved survival property upon memory CD8 T cells, which allows them to persist for longer periods of time.

For possible application of IL-12 priming to vaccines, it is of interest to see whether long-lasting memory CD8 T cells generated by initial IL-12 priming contribute to protective immunity. To this end, direct ex vivo cytolytic activity was measured at day 70 post-transfer. As expected, donor cells primed with OVAp/IL-12 showed higher levels of CTL activity than those stimulated with OVAp, whereas transferred naive OT-I cells did not (Fig. 5A).

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Increased expansion of IL-12-primed CD8 T cells is due to their reduced death rather than increased proliferation. Mixed spleen and LN cell suspensions from naive OT-I mice were cultured for 2 days with OVAp in the presence or absence of IL-12. A. In vitro stimulated OT-I cells were purified and rested for additional 2 days without any stimulant. At indicated time points, apoptotic cell death was determined by annexin V and PI double staining. B. Expression of active caspase-3 of 2-day rested cells in A was analyzed by intracellular staining. Histograms show the live cells gated for Vα2+Vβ5+. Values represent the percentage of active caspase-3-positive cells. C, MACS-purified naive or in vitro activated OT-I cells were labeled with CFSE, and 2 × 10⁶ cells were adoptively transferred into B6 mice. After 3 days, cells were collected from the spleen, PBMC, and liver of recipient mice, and analyzed by flow cytometry. Equal numbers of Vα2+Vβ5+ cells were collected for each group by setting the acquisition criteria of flow cytometer. Histograms show the CFSE intensity of CD8+Vα2+Vβ5+ T cells. Filled histogram, naive; filled line, OVAp; dotted line, OVAp/IL-12. All data are representative of two independent experiments with similar results. D, Total CD8 T cells purified at day 70 were rested without any stimulants. At indicated time points, apoptotic cell death was determined by annexin V and PI staining. Values represent the percentage of annexin V+ cells in the same number of gated Vα2+Vβ5+ T cells (1000 event counts) and are shown as the mean ± SD. E, Histograms show the expression of active caspase-3 in the gated Vα2+Vβ5+ T cells (1000 event counts). All data are representative of two independent experiments with similar results.
These data reflect increased number of memory OT-I cells primed with OVAp/IL-12, indicating positive correlation between size of memory CD8 T cells and magnitude of their ex vivo cytolytic responses. However, in vitro cytotoxicity exhibited by both groups was similar when the E:T ratio was adjusted to total numbers of Ag-specific OT-I cells (data not shown). To further determine the protective efficacy of long-lasting memory OT-I cells, recipient mice were injected with E.G7 tumor cells expressing OVA i.p. at day 70. Consistent with the increased level of ex vivo CTL activity, mice that received OVAp/IL-12-primed OT-I cells exhibited significantly higher survival rate than mice that received OVAp-primed cells after E.G7 tumor challenge (Fig. 5B).

The effect of exogenous and endogenous IL-12 on efficient generation of memory CD8 T cell immunity

Next, we examined the effect of in vivo IL-12 priming at the time of immunization using rAd infection model. Mice were injected i.m. with rAd expressing the structural genes of HCV as surrogate Ag (rAd-ST) and rAd-IL-12 or rAd expressing enhanced green fluorescent protein (rAd-EGFP), and Ag-specific CD8 T cell responses were measured using IFN-γ ELISPOT assay at days 9 and 30 after immunization (Fig. 6A). We observed 4-fold increased frequency of E2 Ag-specific CD8 T cells both at days 9 and 30 in rAd-ST + rAd-IL-12 group compared with rAd-ST + rAd-EGFP group, indicating that in vivo IL-12 priming by rAd delivery has a similar effect to the expansion of both primary and memory CD8 T cells. Because the expression of IL-12 by rAd-IL-12 was transient (i.e., peaks within 24 h and becomes undetectable after 48 h in blood; data not shown), this regimen is likely to mimic the condition of transient in vitro IL-12 priming. In addition, the Ag-specific CD8 T cell responses were further increased when IL-12 was replaced with the previously reported IL-12 mutant, which has better activity on long-lasting memory CD8 T cells (32) (Fig. 6A). These results strongly suggest that initial IL-12 priming could be applied to in vivo system through active immunization.

Next, we investigated the role of endogenous IL-12 on the generation of memory CD8 T cell responses. For this analysis, naive OT-I cells were adoptively transferred to IL-12p40-deficient (p40−/−) recipient mice or IL-12Rβ1-deficient OT-I cells to C57BL/6 wild-type mice, and then recipient mice were immunized i.n. with rAd-expressing OVA (rAd-OVA). At days 30 and 42 after infection, lungs and spleens were recovered, and donor cells were identified by tetramer staining (Fig. 6B). The frequencies of donor OT-I cells from wild-type or p40−/− mice were similar, indicating that the absence of endogenous IL-12 (and possibly IL-23) may not influence the efficient generation of memory CD8 T cell immunity. In the experiments with IL-12Rβ1-deficient OT-I cells, we observed similar expansion of donor cells in the lung and spleen during primary and recall responses, and the relative cytolytic activity during recall responses against rAd-OVA challenge was similar (Fig. 6C). We also obtained the same results with intracellular IFN-γ staining after 6-h stimulation (data not shown). These results suggest that IL-12R-mediated signaling by endogenous IL-12...
is not critical for the efficient generation of primary and secondary CD8 T cell responses.

**Discussion**

CD4 and CD8 T cells initiate a program for their autonomous clonal expansion and development into functional effector and memory cells after brief interaction with stimulatory APC (1–3, 33), implying that effector cells are programmed during their first encounter with Ag and can develop in the absence of additional TCR signaling. However, the strength and duration of antigenic plus costimulatory stimulation can affect the development and differentiation process and regulate the functional quantities of the effector and memory cells (7, 34). Compared with CD8 T cells, CD4 T cells appear to require different costimulatory molecules and cytokine environment with regard to the activation of naive CD4 T cells and maintenance of memory CD4 T cells (35, 36). Indeed, our results demonstrated that exogenous IL-12 treatment did not affect the size of effector and memory CD4 population. In contrast, the same IL-12 priming significantly reduces overall TCR avidity of CD8 T cells and changes lipid raft distribution during initial antigenic stimulation, resulting in the increased survival of effector and memory CD8 T cells. A possible explanation is that these phenotypic changes along with antiapoptotic effects of IL-12 and increased memory development. Notably, recent data support the existence of a perforin-dependent mechanism to regulate the magnitude of CD8 T cell expansion in various models (39), which are consistent with our results that cells stimulated with OVAp/IL-12 showed decreased cytolytic activity, but eventually increased expansion of primary and memory cells. In addition, it is of interest to note that exogenous IL-12 priming affects intrinsic properties of memory CD8 T cells as well as the size of primary expansion, mainly contributing to the decreased cell death and the increased memory formation. This suggests that initial IL-12 priming is able to change the survival property of memory CD8 T cells.

We have observed contradictory results regarding the effects of exogenous and endogenous IL-12 priming. This might be due to different experimental settings such as the local concentrations of exogenous vs endogenous IL-12 and the levels of antigenic stimulation (i.e., in vitro concentration of OVAp vs infection of defective rAd-OVA). In our in vivo immunization experiments shown in Fig. 6A, local codelivery of recombinant adenoviruses expressing HCV structural proteins and IL-12 increased primary and memory CD8 T cell responses to E2 protein, indicating that ectopic expression of IL-12 is helpful for the efficient generation of Ag-specific CD8 T cell response. However, comparable CD8 T cell responses were elicited by i.n. infection of rAd-OVA among wild-type and IL-12p40- and IL-12R-deficient mice after adoptive transfer of OT-I cells, indicating that endogenous IL-12- and/or IL-12R-mediated signaling is not critical for the generation of CD8 T cell response. Thus, it is possible that the ample amount of exogenous IL-12 might provide an additional advantage for CD8 T cells to survive during massive contraction phase.

To determine the size of CD8 T cell population after activation and differentiation are likely to be the exponential rate of cell death and division rate. In a series of our experiments, we carefully measured both the rate of cell death in vitro (Fig. 4A) and division rate of proliferating cells after activation (Figs. 3C and 4C). Primarily, we could not observe any significant differences in division rate accessed by CFSE diffusion between OVAp-primed and OVAp/IL-12-primed cells both in vitro (Fig. 3C) and in vivo (Fig. 4C), but there were relatively big differences in the rate of cell death when cells were cultivated in vitro (Fig. 4, A and D). These observations strongly suggest that IL-12 priming increases generation of effector and memory CD8 T cells primarily by increasing survival of CD8 T cells rather than by increasing the rate of cell division. Our results are consistent with the previous report showing that IL-12 may provide a survival advantage to the responding CD8 T cells to allow continued clonal expansion (8). It seems that small changes in kinetic parameters during T cell proliferation can lead to large differences in total cell number (37). Thus, it is very likely that IL-12 priming primarily changes the kinetic parameter during T cell proliferation by providing survival advantage and contributes to the increased expansion of effector and memory CD8 T cell population. Several studies have demonstrated that exogenously treated IL-12 can prevent apoptotic cell death of activated T cells (27–31), presumably due to the decreased expression of active caspase-3. These studies are consistent with our data that active caspase-3 expression in resting OT-I cells primed with OVAp/IL-12 is significantly reduced. In general, clonal burst size is thought to be positively correlated with the size of memory pools (38). Thus, it is likely that increased primary expansion is a predominant mechanism responsible for increased memory development. Notably, recent data support the
stimulants and initial exposure of IL-12 for 3 days (Fig. 2 and data not shown). It is worth noting that CD8 T cells were stimulated for 2 days without additional resting period to avoid further in vitro division and differentiation during resting culture period in our experimental system. Although IL-2 appears to function as T cell growth and survival factors, it could be also involved in the down-regulation of T cell responses mediated through mechanisms such as activation-induced cell death under certain circumstances (13). Thus, one possibility is that the discrepancy may be caused by the presence of additional resting period with exogenous IL-2, which might have changed the properties of developing effector cells to become long-lasting memory CD8 T cells in initial IL-4, but not IL-12, priming-dependent manner. A further careful study would be necessary to prove this possibility.

In conclusion, our findings provide an important insight into the mechanisms in which initial cytokine priming influences the size of memory CD8 T cells and the long-term survival of the established memory cells. In particular, the role of initial exogenous IL-12 priming in improving survival property of memory CD8 T cells may partly account for the potent adjuvant effect of IL-12, as previously documented in various in vivo model systems (41). However, our results showed that endogenous IL-12 production and/or IL-12R-mediated signaling on T cells in vivo were dispensable for the generation of primary and memory CD8 T cell responses. The advantage of exogenous IL-12 priming for the generation of long-term memory T cells could be applied in medical areas, such as ex vivo expansion of autologous Ag-specific CTLs and T cell infusion, and for improving the efficacy of the preventive and therapeutic CTL vaccines for cancers and infectious diseases.

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References