

Generation of Polarized Antigen-specific CD8 Effector Populations: Reciprocal Action of Interleukin (IL)-4 and IL-12 in Promoting Type 2 versus Type 1 Cytokine Profiles

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Summary

We have generated primary effector populations from naive CD8 T cells in response to antigen and determined their patterns of cytokine secretion upon restimulation. The effect of exogenous factors on the effector generation was examined and compared with responses of antigen-specific CD4 effectors generated under comparable conditions. CD8 cells from bm1 mice were stimulated with C57BL/6 (B6) antigen presenting cells (APCs) bearing allogeneic class I and CD8 cells from female severe combined immunodeficiency (SCID) B6 mice, transgenic for a T cell receptor α/β (TCR- α/β) that recognizes H-Y on D^b, were stimulated with APCs from male mice. In parallel, CD4 cells from bm12 mice were stimulated with alloantigen and CD4 cells from V β 3/V α 11 TCR transgenics were stimulated with a peptide of pigeon cytochrome c on IE^k. T cells from both transgenic mice were of naive phenotype whereas normal mice contained 10–20% memory cells. Effector CD8 populations generated were L-selectin low, CD45RB high, and CD44 high. Naive CD8 cells from SCID anti-H-Y mice made little or no cytokine immediately upon stimulation in contrast to naive CD4 which produced large amounts of interleukin 2 (IL-2). Both populations, however, generated primary effectors over 4–5 d that made substantial quantities of many cytokines upon restimulation. Both CD8 and CD4 effectors produced similar patterns of cytokines with alloantigen or specific antigen. Cytokines present during naive CD8 stimulation influenced the cytokine secretion profile of the effectors, as previously shown for CD4 cells, although secretion by CD8 effectors was generally lower than that of CD4 effectors. CD8 cells cultured with IL-2 alone made predominantly interferon γ (IFN- γ) and no IL-4 or IL-5, similar to CD4 cells. Priming with IFN- γ increased IFN- γ secretion from CD4 effectors, but had little if any effect on CD8 cells. In contrast, priming with IL-12 generated CD8 effectors, as well as CD4 effectors, producing elevated quantities of IFN- γ , with similar levels from both the CD4 and CD8 populations. The presence of IL-4 during effector cell generation promoted synthesis of IL-4 and IL-5 from both CD8 and CD4 cells while downregulating IFN- γ secretion. CD8 cells made only small amounts of IL-4, more than 100-fold less than CD4 cells, whereas significant levels of IL-5 were induced, only 3–10-fold lower than from CD4. Thus there are strong similarities between CD8 and CD4 T cells in the development of primary effector populations, but some key differences. We predict that the polarized subsets of CD8 effector T cells will be found to play different roles in the immune response.

Classical studies have defined an essential dichotomy between CD4 helper T cells and CD8 cytotoxic precursors. As a consequence, most studies of cytokine production and of T cell–B cell contact-mediated regulatory signals have focused on the activities of CD4 T cells. CD8 T cells have, however, been shown to make a number of cytokines and there is a considerable literature concerning the activities of CD8 suppressor cells. Recent studies have clearly established that CD8 T cells can have a regulatory role in certain cir-

cumstances and may not necessarily function purely as cytotoxic killers and that differential cytokine production may be largely responsible for such immune regulation.

The earliest observation (in retrospect) of cytokine production by CD8 T cells was by Granger and Williams (1) who discovered lymphotoxin (TNF- β). Later, Klein et al. (2) and Morris et al. (3) showed that CTLs produced IFN- γ when incubated with target cells. IFN- γ can be directly cytotoxic, has an important role in downregulating certain B cell re-

sponses, and can also modulate CD4 T cell responses. In classical studies, CD8 T cells were seen as being deficient in the production of IL-2 relying on IL-2 from CD4 T cells for growth and differentiation and it is clear that in most situations the bulk of the IL-2 produced does indeed come from CD4 cells. However, certain CD8 cells are able to make some IL-2 and can develop into CTLs in the absence of CD4 cells (4, 5).

CD8 T cells have, historically, been associated with downregulation or suppression rather than help. After the demise of the antigen-specific suppressor factors, a number of investigators have sought to explain T cell-mediated suppression in terms of inhibitory cytokines. IL-4 (6), IFN- γ (7), IL-10 (8), TGF- β (9), and IL-8 (10) have all been shown to reduce the effector function of certain target cells in various experimental models, suggesting that CD8 cells, if secreting one or several of such cytokines, could profoundly modulate many immune responses, in addition to providing killer function.

Significantly, recent studies have shown that the cytokine production of CD8 T cells can sometimes overlap with that of CD4 T cells. Kelso et al. (11) polyclonally activated CD4 and CD8 cells and cultured them at limiting dilution. Analyses of the clones that arose demonstrated that CD8 cells could synthesize and secrete every conceivable cytokine, singly or in combination, and that there was enormous heterogeneity between clones. Surprisingly, there was no obvious qualitative difference between the collective profiles of cytokine production between CD4 and CD8 populations. More recently, evidence has been obtained that the cytokines produced by CD8 T cells can be influenced by the culture conditions under which they are generated. Seder et al. (12) showed that anti-CD3-stimulated CD8 cells can be primed *in vitro* to secrete IL-4, with the production of this cytokine being dependent on prior exposure to IL-4 in a manner analogous to that shown in studies of CD4 T cells (13, 14). Moreover, stimulation with PMA and ionomycin in the presence of IL-4 induced CD8 T cells to downregulate CD8 and become double negative cells (15), but secrete type 2 cytokines, including IL-4 and IL-5. In addition, functionally different populations of CD8 T cells have also been identified *in vivo*. For example, Salgame et al. (16) have isolated separate CD8 T cell clones secreting IFN- γ vs. IL-4 from patients with leprosy.

Overall, it is clear that a more comprehensive knowledge of the properties of CD8 effector T cells generated under a variety of conditions would be of value in further efforts to interpret the regulatory role of these class I MHC-restricted T cells in the intact animal. The availability of TCR transgenic mice has made it possible to study the response of homogeneous populations of T cells to antigen. Previously we (17, 18), and others (19, 20), have made use of mice transgenic for TCR- α/β recognizing peptide antigens on class II to study the responses and cytokine secretion patterns of CD4 T cells. The CD4 T cells from these mice are all of naive phenotype, respond vigorously to peptide antigen on certain APCs, and efficiently differentiate into potent effector populations *in vitro* that can secrete a range of cytokines

(17–22). Such studies have shown that the cytokine secretion profile of the effector cells is highly regulated by exposure of naive CD4 to cytokines during activation, and that IFN- γ and IL-12, compared with IL-4, have distinct roles in promoting Th1 and Th2-type responses, respectively.

We employ here a similar strategy using naive T cells from female anti-H-Y transgenic mice (23) for the study of the response of CD8 effector populations. The H-Y antigen was first defined by skin graft rejection studies (24) in which female C57BL/6 mice were shown to reject male skin from the same strain. Further analyses suggested that there is a protein coded for on the Y chromosome that supplies a peptide that binds the class I allele, D^b, and that this ligand is recognized by CD8 T cells that do not undergo negative selection in female mice. In parallel studies, we have examined the allogeneic response of CD8 T cells from bm1 mice stimulated by APCs from C57BL/6 mice.

We found that freshly isolated naive CD8 T cells made little or no cytokines, including IL-2, upon initial stimulation. CD8 T cell effectors could be generated by 4-d culture *in vitro* and release substantial quantities of cytokines after restimulation. Effectors generated in IL-2 alone made predominantly IFN- γ . The amount of IFN- γ produced could be significantly increased if effectors were generated in the presence of IL-12, but not IFN- γ . In contrast, effector generation in the presence of IL-4 downregulated IFN- γ secretion while promoting secretion of IL-4 and particularly IL-5. Comparison with CD4 effectors showed that the ratio of IL-5 to IL-4 was much higher in IL-4-primed CD8 effector populations than in CD4 effector populations. In this model, the CD8 effectors retained the expression of CD8 and displayed the classic effector phenotype.

Thus, CD8 T cells can be divided into functionally distinct subsets that secrete unique patterns of cytokines and that can be expected to play unique regulatory roles.

Materials and Methods

Mice. Male and female homozygous H-2^b SCID anti-H-Y V β 8.2/V α 3 TCR transgenic mice were obtained from Dr. Drew Pardoll (Johns Hopkins University School of Medicine, Baltimore, MD) and were bred in the animal facilities at UCSD. For some experiments, these mice were crossed with C57BL/6. In both cases only female mice were used in the experiments detailed. H-2^{b/k} anti-PCCF V β 3/V α 11 TCR transgenic mice, (B10.BR \times C57BL/6)F₁, and C57BL/6 mice were also bred at UCSD, and B6.C-H-2^{bm1} (bm1)¹ and B6.C-H-2^{bm12} (bm12) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were generally used at 2–4 mo of age.

T Cells and APCs. Purified splenic CD8 T cells were isolated from anti-H-Y TCR transgenic or bm1 mice by passing through nylon wool and treating with anti-CD4 (RL172.4), anti-heat stable antigen (HSA) (J11D), anti-IA^{b/d} (D3.137), and complement. Splenic CD4 T cells were isolated as described previously from anti-

¹ Abbreviations used in this paper: bm1, B6.C-H-2^{bm1}; bm12, B6.C-H-2^{bm12}; DXS, dextran sulphate; PCCF, pigeon cytochrome c fragment 88-104.

pigeon cytochrome c fragment 88-104 (PCCF) TCR transgenic or bm12 mice (18) again by passing over nylon columns, followed by treatment with anti-CD8 (HO2.2 and AD4), anti-HSA, anti-IA^{b/d} and complement. Splenic APCs were enriched from C57BL/6 and (B10.BR × BL/6)F₁ mice by T cell depletion using anti-Thy1.2 (F7D5 and HO.13.14), anti-CD4 (RL172.4), and anti-CD8 (HO.2.2 and AD4) plus complement as previously described (17). In some cases, APCs were activated overnight with 25 µg/ml each of LPS and dextran sulphate (DXS; Sigma Chemical Co., St. Louis, MO). Dendritic cells were prepared by isolating low density cells followed by two rounds of plastic adherence as previously described (18). All APC populations were treated with mitomycin (50 µg/ml; Sigma Chemical Co.) for 30 min at 37°C before use.

Cell Cultures. Cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with penicillin, streptomycin, glutamine, 2-ME, sodium pyruvate, Hepes, and 10% FCS (Hyclone Laboratories, Logan, UT). For primary cultures, effector generation or effector restimulation, cells were set up in 1–2-ml volumes in 24- or 48-well plates (Costar Corp., Cambridge, MA). For alloantigen stimulation, CD8 cells from bm1, or CD4 cells from bm12 mice, were generally cultured at 10⁶/ml with APCs from B6 mice at 2 × 10⁶/ml. For antigen stimulation, CD8⁺ cells from female anti-H-Y TCR transgenic mice were cultured at 0.5 × 10⁶/ml with 2 × 10⁶/ml LPS/DXS-activated APCs from male B6 mice, and CD4⁺ cells from anti-PCCF TCR transgenics were cultured with LPS/DXS-APC from (B10.BR × B6)F₁ mice with 5 µM PCCF (peptide 88–104, purified by HPLC from whole pigeon cytochrome c [Sigma Chemical Co.]) at the same concentrations. In some experiments dendritic cell populations were prepared from B6 mice (as described in reference 18) and used to stimulate CD8 T cells from the anti-H-Y transgenic mice. Dendritic cell numbers (0.1 × 10⁶/ml) were chosen, based on previous results, to give equivalent levels of activation as T-depleted APCs (data not shown). T cells were stimulated for 4–5 d in the presence of cytokines added at the initiation. All cultures received 20 U/ml IL-2 (supernatant from the X63Ag.IL-2 murine cell line) with or without varying concentrations of recombinant IL-4 (X63Ag.IL-4 supernatant) or IFN-γ (recombinant murine; Genzyme Corp., Cambridge, MA) similar to previous studies (17), or IL-12 (specific activity 8 U/ng, kindly provided by Dr. Stanley Wolf, Genetics Institute Inc., Cambridge, MA and Hoffman-LaRoche, Inc., Nutley, NJ). An additional 20 U/ml IL-2 was added to the cultures on day 2 to promote CD8 expansion. In some cases Abs to IFN-γ (XMG1.2) and IL-12 (Genetics Institute) were also added. T cells were washed and restimulated at different numbers with 2–4 × 10⁶/ml LPS/DXS-activated APCs. The number of cells recovered after a 4-d stimulation of either CD4 or CD8 cells with alloantigen was 50–80% of the initial T cell number, whereas two to three times the starting number of CD8 cells were recovered with H-Y stimulation and three to four times the starting number of CD4 cells were recovered with PCCF stimulation. Addition of IL-4, IFN-γ, or IL-12 did not significantly alter the number of effectors generated compared with IL-2 alone.

Cytokine Secretion. Cytokine secretion was assessed in supernatants collected from T cell cultures 40–48 h after primary stimulation and 24 h after secondary stimulation. IL-2 and IL-4 secretion were measured using the NK bioassay, in the presence of anti-IL-4 (11B11) or anti-IL-2 receptor mAbs (PC61 and 7D4) respectively, as previously described (25). IL-5 and IFN-γ were detected by ELISA using the anti-IL-5 Abs TRFK4 and TRFK5, and the anti-IFN-γ Abs R46A2 and XMG1.2, as also described (25). Standard curves were constructed with purified murine cytokines, IL-2 (X63. IL-2

supernatant), IL-4 (X63.IL-4 supernatant), IL-5 (X63.IL-5 supernatant), and IFN-γ (Genzyme Corp.). 1 U of IL-2 or IL-4 is defined as the quantity required to induce half maximal proliferation in their respective assays. 1 U of IFN-γ as defined by Genzyme Corp. is the quantity required to neutralize viral destruction of L cells by 50%. 1 U of X63.Ag.IL-5 is equivalent to 1 U of IL-5 as defined by Genzyme Corp. as the amount required to induce half maximal proliferation of dextran sulphate stimulated B cells. 1 U of IL-2 is ~14 pg, 1 U of IL-4 is ~0.7 pg, 1 U of IL-5 is ~150 pg, and 1 U of IFN-γ is ~100 pg.

Flow Cytometry. T cells were stained as previously described (13). CD8 T cells were analyzed immediately out of the animal or after 4 d of in vitro stimulation. CD8 cells were visualized with PE-anti-CD8 (Caltag Laboratories, San Francisco, CA) and various surface markers analyzed with rat anti-mouse Abs to L-selectin (Mel-14), CD45RB (23G2), and CD44 (Pgp-1) followed by FITC-labeled mouse anti-rat κ (RG7). Expression of the transgene on CD8 cells from anti-H-Y TCR mice was examined with biotinylated anti-Vβ8.1/2 followed by FITC-streptavidin (PharMingen, San Diego, CA). Isotype-matched rat Abs were used as negative controls for staining, and dead cells were gated out by excluding those which stained with propidium iodide. Cell populations were examined on a FACScan[®] analyzer Lysis II software (Becton Dickinson & Co., Mountain View, CA).

Results

The Phenotype of Resting and Activated CD8 T Cells.

Resting T cells can be subdivided into naive and memory subsets based on their prior exposure to antigen. In the case of CD4 cells, and most likely CD8 cells, antigen stimulation results in up- or downregulation of various cell surface markers which often can be used to isolate and characterize the cell populations (26). Naive CD4 cells that have not encountered antigen since exit from the thymus express high levels of both CD45RB and L-selectin and low levels of CD44, whereas memory CD4 cells have the reciprocal phenotype. Effector CD4 cells that are still activated, IL-2 receptor⁺ blast cells, derived from either naive or memory cells after several days stimulation, can have mixed phenotypes but generally express high levels of CD44, low levels of L-selectin and are CD45RB high under most circumstances (17, 18, 26).

To study CD8 T cell responses, we chose two systems. First, we used CD8 cells from transgenic mice expressing a TCR-α/β recognizing the male antigen H-Y in association with D^b. Either SCID or (SCID × B6)F₁ mice were used. Second, we used CD8 cells from bm1 mice responding to allogeneic B6 APCs. Here the response is dependent on the recognition of K^b by T cells bearing the mutant K^{bm1}. With the anti-HY transgenics one would expect only antigen-inexperienced (naive) cells, whereas with the bm1 mice both naive and memory cells should exist in the T cell population. The phenotype of the CD8 cells taken immediately from the animals was examined and compared with effectors derived over 4–5 d by stimulation with antigen (Fig. 1) or alloantigen, not shown.

It can be seen, as expected, that >95% of the CD8 T cells from the female SCID anti-H-Y transgenic mice were resting (as shown by forward scatter, Fig. 1, top) and had a pheno-

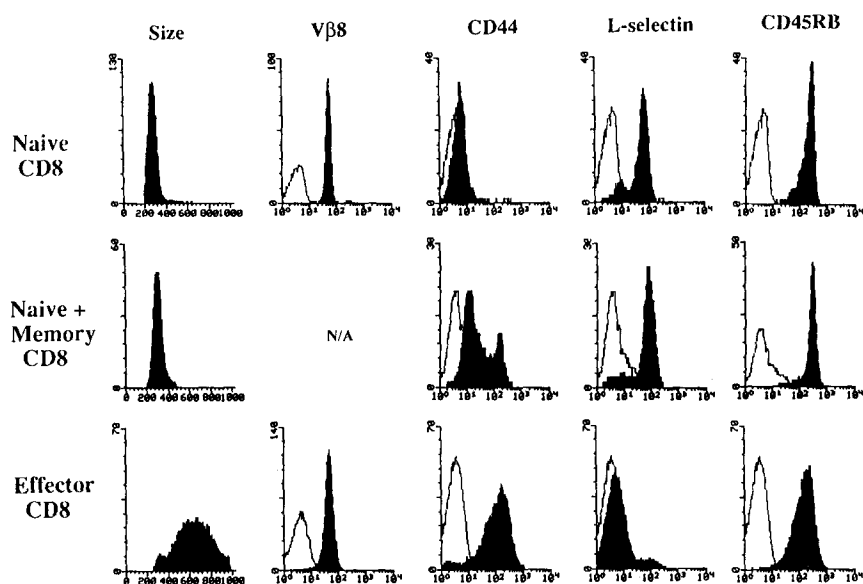


Figure 1. Phenotype of naive and effector CD8 T cells. CD8 cells were isolated from anti-H-Y TCR transgenic mice or bm1 mice. T cells were examined for surface phenotype before stimulation (*top line*, naive from H-Y; *middle line*, naive and memory from bm1) or after 4-d in vitro culture with APCs from male BL/6 mice in the presence of IL-2 (*bottom line*; effectors). Cells were dual stained for CD8 and either V β 8, CD44, L-selectin, or CD45RB. FACS[®] profiles of gated CD8⁺ cells are shown, together with the forward scatter characteristics of each population.

type that has previously been shown to be characteristic of naive CD4 T cells. They were CD45RB high, L-selectin high, and CD44 low. Virtually all of the cells stained for the transgenic TCR V β 8. This is compatible with other studies examining the phenotype of CD8 T cells that have suggested that while there may be a variation in expression of these markers between CD4 and CD8 particularly after antigen encounter, the antigen-inexperienced CD8 cell (naive) has essentially the same phenotype as an antigen-inexperienced CD4 cell. CD8 T cells from an F1 cross of the SCID anti-H-Y with C57BL/6 also contained a major population (95%) of resting cells with the CD45RB high, L-selectin high, CD44 low phenotype. Again, virtually all of the CD8 cells stained positive for the V β 8 transgene (data not shown).

Resting CD8 T cells from young bm1 mice showed the expected phenotype of cells from unimmunized mice (27) with the heterogeneity normally seen for a mix of naive and memory T cells (Fig. 1, *center*). These cells were CD45RB high, L-selectin high but about 20% also expressed high levels of CD44. In older mice, which have previously been shown to bear elevated numbers of memory cells presumably due to environmental antigen stimulation (27), a subpopulation of resting cells with low levels of CD45RB and L-selectin emerged with 30–50% of the cells being CD44 high (not shown). By comparison, the majority (90%) of resting CD8 T cells from mice that had undergone adult thymectomy, a process that selectively depletes naive cells over time, still retained high levels of CD45RB and L-selectin, but up to 80% also expressed high levels of CD44 (not shown).

Based on these phenotypic analyses, we conclude that antigen inexperienced naive CD8 express the same markers as naive CD4 cells: CD45RB high, L-selectin high, CD44 low. Memory CD8 T cells in contrast to memory CD4 T cells may only be distinguishable from naive cells based on high expression of CD44, a suggestion that is in agreement with previous studies by other investigators (28, 29).

In some mice, a minor population of activated cells were

present (activated in vivo) that were predominantly CD45RB high, L-selectin high but that contained cells expressing both high and low levels of CD44 (data not shown). We assume that these cells had encountered environmental antigens that they could recognize. In vivo activated cells predominantly expressed high levels of all markers but subpopulations existed with various complex phenotypes.

In contrast to in vivo activated cells, effector cells derived by stimulating resting CD8 T cells for 4–5 d in vitro exhibited a very uniform phenotype (Fig. 1, *bottom*). Effectors from anti-H-Y SCID mice or bm1 mice were identical and the phenotype did not change when experiments were performed in the presence of priming cytokines (not shown). The live effector cell populations were better than 95% CD8⁺ with no contaminating CD4 cells. In contrast to other studies (15) CD4⁻CD8⁻TCR⁺ cells did not grow out and represented no more than 2–3% of the population (not shown). Almost all of the cells had high forward scatter and were CD45RB high, L-selectin low, and CD44 high (Fig. 1), and this phenotype appeared to be very stable being retained 4–5 d after a secondary stimulation (not shown).

Cytokine Production from Resting Naive CD8 T Cells. CD8 T cells were obtained from spleens immediately after killing and were stimulated with antigen. In the case of the bm1 cells the antigen was presented on allogeneic T-depleted spleen cells from C57BL/6 mice. For stimulation of the CD8 T cells from anti-H-Y mice it was necessary to use 24-h LPS/DXS-activated T-depleted cells (mainly B cells but also macrophages and dendritic cells) since the antigen is poorly expressed on resting APCs. Cells were stimulated for 40–48 h at varying cell densities and supernatants assayed for IL-2, IL-4, IL-5, and IFN- γ . Little cytokine production was seen from alloantigen-stimulated cells with only small amounts of IL-2 detected in some experiments (Table 1). This is not surprising considering the low frequency of T cells that respond. It is interesting to note that even with the H-Y-specific populations, in which all the T cells bear the transgenic TCR

and can potentially respond, only small amounts of IL-2 were produced. Mitogen stimulation of CD8 T cells also generates very little IL-2 (data not shown). Small amounts of IFN- γ was also detected in these cultures, which was surprising considering the T cells are all naive, and it is possible that this cytokine may have originated from contaminating non-T cells such as NK cells or the APCs. By comparison, CD4 T cells from bm12 mice stimulated with class II MHC disparate BL/6 cells also only made relatively small amounts of IL-2, but when the frequency of responding T cells was maximized using PCCF-specific cells from V β 3/V α 11 TCR transgenics, it was clear that naive CD4 cells could produce up to 100-fold more IL-2 than naive CD8 cells (Table 1).

Cytokine Production by CD8 Effector T Cells Generated in the Presence of IL-4 or IFN- γ . CD8 T cells, stimulated for 4–5 d and then restimulated, produced large amounts of cytokines, and the presence of additional factors in the initial culture period, influenced the cytokine profile of the effector populations (Table 2). CD8 cells were either stimulated with alloantigen (bm1) or specific antigen (H-Y) as above in the presence of IL-2 alone (IL-2 effectors), IL-2 plus IL-4 (IL-4 effectors), or IL-2 plus IFN- γ (IFN- γ effectors). The small

number of alloreactive cells from the bm1 mice expanded markedly to a level that was 50–80% of the initial total cell population by day 4 and the cells from the anti-H-Y transgenic mice expanded to three to four times the original starting number. Effector cells were restimulated at 4–5 d and supernatants assayed for cytokines after 24 h. As previously shown for CD4 cells, CD8 effectors did not produce cytokines unless restimulated. APCs, cultured alone, did not produce detectable levels of any of the cytokines measured (not shown). As can be seen in Table 2, CD8 populations generated with alloantigen or antigen produced similar levels and patterns of cytokines, with one exception (see below). IL-2 secretion by CD8 effectors was generally not seen although small amounts were detected in some experiments. Effectors generated in IL-2 alone produced high levels of IFN- γ , whereas IL-4 and IL-5 were not detected. Cells cultured with IL-2 and IFN- γ produced slightly more IFN- γ with alloantigen and slightly less IFN- γ with antigen in the experiment shown, but still no detectable IL-4 or IL-5. Any priming effect of IFN- γ on CD8 cells was marginal over several experiments (see also Figs. 2–4) and in general we conclude it has little enhancing effect on subsequent IFN- γ secretion. Cells cul-

Table 1. Cytokine Secretion from Resting CD8 T Cells on Initial Stimulation

		T cell/ml	Cytokine Secretion			
			IL-2	IL-4	IL-5	IFN- γ
<u>Alloantigen Stimulation:</u>			<i>U/ml</i>			
CD8 T cells	Expt. 1.	20 \times 10 ⁵	20	<5	<10	<10
		5 \times 10 ⁵	<5	<5	<10	<10
	Expt. 2.	5 \times 10 ⁵	<5	<5	<10	<10
		2.5 \times 10 ⁵	<5	<5	<10	<10
	Expt. 3.	5 \times 10 ⁵	14	<5	<10	<10
		2.5 \times 10 ⁵	10	<5	<10	<10
CD4 T cells	Expt. 1.	5 \times 10 ⁵	22	<5	<10	<10
		2.5 \times 10 ⁵	10	<5	<10	<10
<u>Antigen Stimulation:</u>						
CD8 T cells	Expt. 1.	5 \times 10 ⁵	29	<5	<10	31
		2.5 \times 10 ⁵	15	<5	<10	<10
	Expt. 2.	5 \times 10 ⁵	35	<5	<10	<10
		2.5 \times 10 ⁵	26	<5	<10	<10
	Expt. 3.*	5 \times 10 ⁵	55	<5	<10	29
		2.5 \times 10 ⁵	44	<5	<10	16
CD4 T cells	Expt. 1.	5 \times 10 ⁵	2,625	<5	<10	<10
		2.5 \times 10 ⁵	2,050	<5	<10	<10

Splenic CD8 T cells were isolated from either bm1 mice (alloantigen) or female H2^b anti-H-Y TCR transgenic mice (antigen) and stimulated with fresh or 1-d LPS/DXS-activated T-depleted APCs, respectively, from male BL/6 mice. Splenic CD4 T cells were also isolated from either bm12 mice (alloantigen) or H2^{b/k} anti-PCCF TCR transgenic mice (antigen) and stimulated with fresh T-depleted BL/6 APCs, or LPS/DXS-activated T-depleted (B10.Br \times BL/6)F₁ APC plus 5 μ M PCCF, respectively. T cells were cultured with 2–4 \times 10⁶/ml APC and supernatants collected for cytokine assay after 40–48 h.

* High density percoll purified T cells.

Table 2. Cytokine Secretion from CD8 Effector Cells Primed with IL-4 and IFN- γ

Effectors primed with	T cell/ml	Cytokine Secretion			
		IL-2	IL-4	IL-5	IFN- γ
<i>U/ml</i>					
Alloantigen:					
IL-2	10 \times 10 ⁵	<5	<5	<10	1,690
	5 \times 10 ⁵	<5	<5	<10	784
	2.5 \times 10 ⁵	<5	<5	<10	566
IL-2 + IL-4	10 \times 10 ⁵	<5	49	339	1,468
	5 \times 10 ⁵	<5	42	343	1,428
	2.5 \times 10 ⁵	<5	35	250	988
IL-2 + IFN- γ	10 \times 10 ⁵	<5	<5	<10	2,123
	5 \times 10 ⁵	<5	<5	<10	1,517
	2.5 \times 10 ⁵	<5	<5	<10	555
Antigen:					
IL-2	10 \times 10 ⁵	<5	<5	<10	1,316
	5 \times 10 ⁵	<5	<5	<10	1,108
	2.5 \times 10 ⁵	<5	<5	<10	627
IL-2 + IL-4	10 \times 10 ⁵	<5	<5	220	72
	5 \times 10 ⁵	<5	<5	265	88
	2.5 \times 10 ⁵	<5	<5	205	88
IL-2 + IFN- γ	10 \times 10 ⁵	<5	<5	<10	638
	5 \times 10 ⁵	<5	<5	<10	609
	2.5 \times 10 ⁵	<5	<5	<10	406

CD8 T cells were stimulated with alloantigen or antigen, as described in Table 1, and cultured for 4 d in the presence of either IL-2 alone (20 U/ml), IL-2 plus IL-4 (400 U/ml), or IL-2 plus IFN- γ (2,000 U/ml). T cells were harvested, washed, and restimulated at the indicated concentrations with LPS/DXS-activated T-depleted APC (2×10^6 /ml) and cytokines assessed in supernatants taken 24 h later.

ured with IL-2 plus IL-4, however, made low levels of IL-4, but substantial amounts of IL-5. The amount of IL-4 produced under these conditions was always low, somewhat variable, and not just restricted to alloantigen stimulation (see Fig. 4), whereas the higher levels of IL-5 were seen in all experiments. With antigen stimulation, priming of effectors with IL-2 plus IL-4 also resulted in greatly reduced levels of IFN- γ , whereas with alloantigen stimulation this did not occur (Table 2). This was consistent over many experiments, but the reason for the difference is not clear.

Comparison of Cytokine Production from CD8 and CD4 Effector T Cells Primed with IL-4 or IFN- γ . Cytokine secretion from alloantigen-specific CD4 effector cells from bm12 mice was compared with that of CD8 effector cells from bm1 mice (Fig. 2) and secretion from antigen-specific CD4 effectors from anti-PCCF TCR transgenics was compared with that of CD8 effectors from anti-HY TCR transgenics (Fig. 3). Both CD4 and CD8 effectors were generated under identical conditions. In almost all cases CD4 T cells made more cytokines than CD8 T cells, and patterns of cytokines were in general similar between antigen and alloantigen systems. CD8 and CD4 cells

made comparable levels of IFN- γ if generated in IL-2 alone. However in contrast to CD8 cells, IFN- γ secretion was increased substantially from CD4 effectors (three- to eightfold in several experiments) when cells were primed with IFN- γ . As shown previously (25, 27), IL-4 priming of CD4 effectors induced secretion of massive quantities of IL-4 and high levels of IL-5. In contrast, IL-4-generated CD8 effectors produced only low levels of IL-4 (>100-fold less than CD4) but substantial levels of IL-5 (3–10-fold less than CD4 over several experiments). In all experiments the ratio of IL-5/IL-4 was high for IL-4-primed CD8 effectors (3–15:1) and reversed for IL-4-primed CD4 effectors (1:3–12). Again there was a dichotomy in the effect of IL-4 priming on IFN- γ synthesis between systems. Downregulation of IFN- γ secretion for both CD8 and CD4 effectors occurred in the antigen systems, whereas little to no effect on IFN- γ was seen for both populations in the alloantigen systems.

Effect of IL-12 on Cytokine Secretion from CD8 Effectors. Because IFN- γ had little to no effect on promoting IFN- γ secretion from CD8 cells, we investigated the effect of IL-12, which has recently been shown to substantially increase IFN- γ

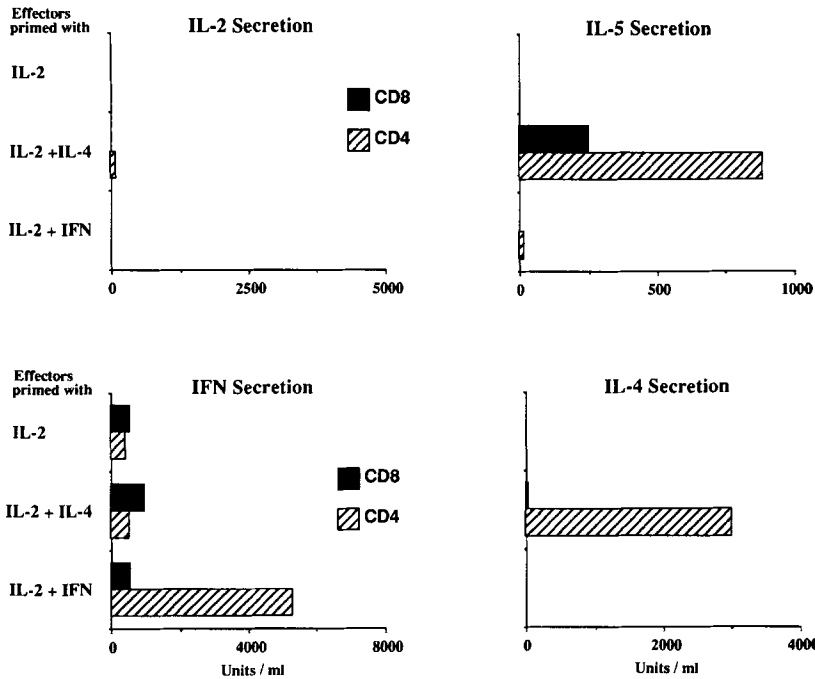


Figure 2. Comparison of cytokine secretion by CD8 and CD4 effector T cells generated with alloantigen. CD8 (solid bars) and CD4 (hatched bars) effector T cells were generated during 4 d in culture. They were stimulated with the same alloantigen preparations as described in Table 1, added at time zero, and were primed in the presence of IL-2 alone (20 U/ml), IL-2 plus IL-4 (400 U/ml), and IL-2 plus IFN- γ (2,000 U/ml). The effectors populations were harvested after four days, adjusted to 2.5×10^5 T cells/ml and restimulated with 2×10^6 /ml allogeneic LPS/DXS-APC. Supernatants generated in the restimulated cultures were harvested at 24 h and the levels of IL-2 (top left), IFN- γ (bottom left), IL-5 (top right), and IL-4 (bottom right) were determined. Similar patterns of cytokines were also produced at other T cell concentrations.

production from CD4 cells (22, 30, 31). Experiments were set up as before using CD8 or CD4 cells stimulated with alloantigen or antigen in the presence or absence of IL-12. In this case both CD4 and CD8 effectors secreted substantially more IFN- γ when generated with IL-12 (Table 3). From several experiments, increases in IFN- γ were two- to threefold for IL-12-primed CD8 effectors, and three- to sixfold for IL-12-primed CD4 effectors over that produced by effectors grown in IL-2. Little effect was seen on IL-2 secretion from

CD8 cells, whereas IL-2 was downregulated to some extent from CD4 cells, and no IL-4 and IL-5 secretion was induced.

Concentration Dependent Effects of IL-4 and IL-12 in the Generation of Effector T Cells. The concentrations of cytokines used above for generating CD8 effectors were chosen because these were previously found to produce optimal effects on CD4 cells. We therefore wanted to determine whether the cytokine effects seen with CD8 cells exhibited a similar concentration dependency as CD4 cells. Results of adding increasing amounts

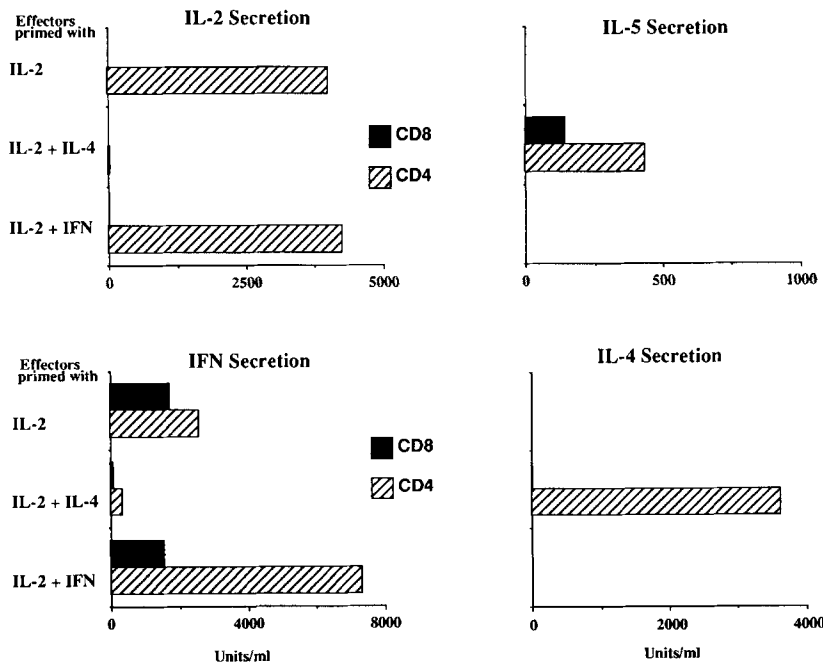


Figure 3. Comparison of cytokine secretion by CD8 and CD4 effector T cells generated with antigen. CD8 (solid bars) and CD4 (hatched bars) T cells were stimulated with antigen as in Table 1 and effector cells were generated over 4 d of culture, primed in the presence of IL-2, IL-2 plus IL-4, or IL-2 plus IFN- γ . Effectors were harvested and restimulated and cytokine secretion measured at 24 h as in Fig. 2, using 2.5×10^5 effector T cells/ml restimulated with 2×10^6 /ml antigen-presenting LPS/DXS cells. (Top left panel, IL-2; bottom left, IFN- γ ; top right, IL-5; and bottom right, IL-4). Cytokine secretion at higher or lower T cell numbers also displayed similar profiles.

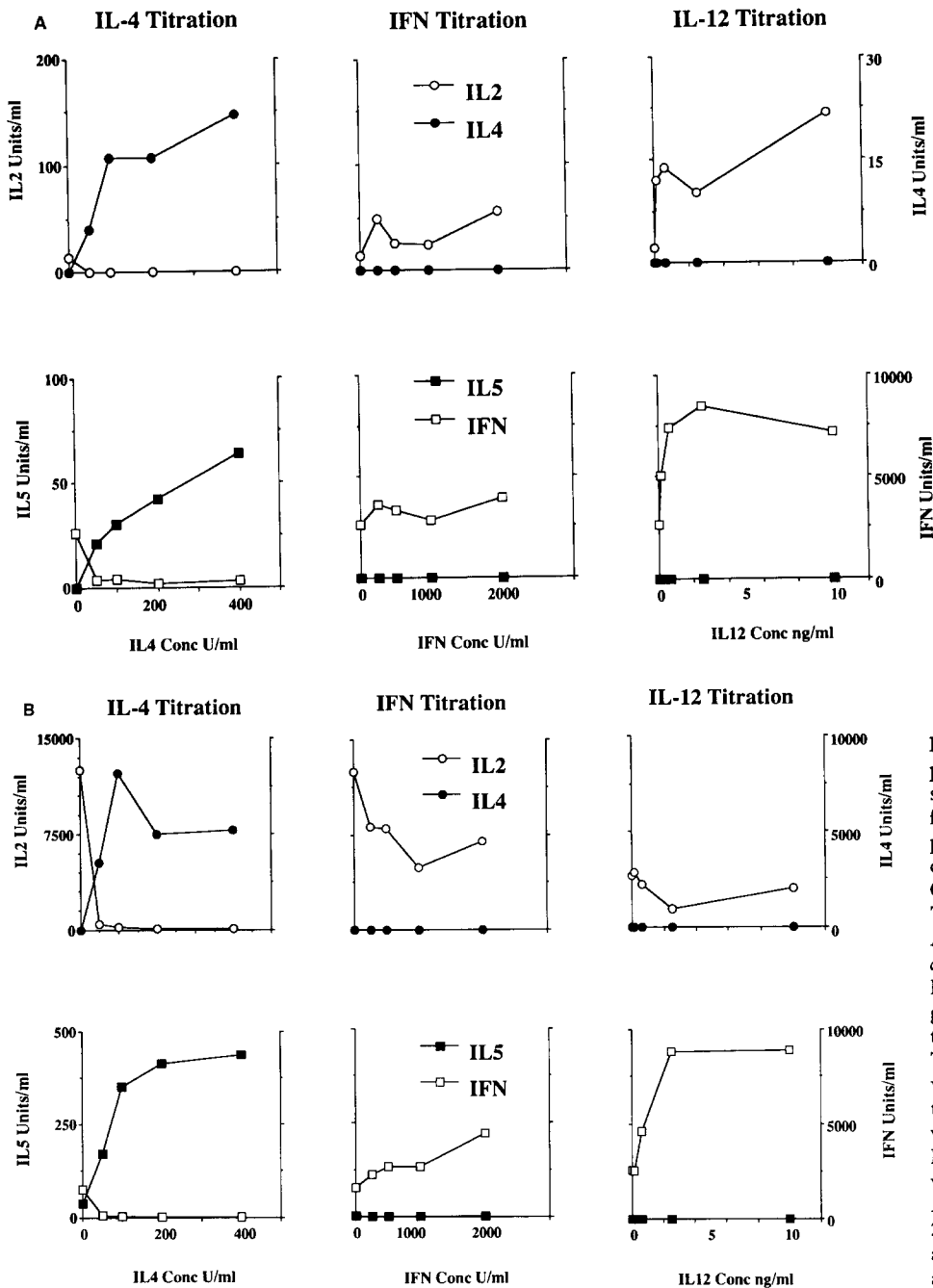


Figure 4. (A) Titration of cytokines for priming effector CD8 T cells. Antigen-stimulated CD8 effectors were generated from anti-H-Y TCR transgenic mice in the presence of IL-2 and varying concentrations of IL-4 (left), IFN- γ (middle), or IL-12 (right). Cells were harvested at 4 d and 2.5×10^5 T cells/ml restimulated with the appropriate APC, as before, and the cytokines (top: IL-2, open circles, and IL-4, solid circles; and bottom: IFN- γ , open squares, and IL-5, solid squares) generated were assessed at 24 h. (B) Titration of cytokines for priming effector CD4 T cells. Antigen-stimulated CD4 effectors were generated from anti-PCCF TCR transgenic mice in the presence of IL-2 with varying concentrations of IL-4, IFN- γ , or IL-12. At 4 d, the effector cells were harvested and restimulated with the appropriate APC, as before. Cytokine secretion from 2.5×10^5 T cells/ml was measured after a 24-h restimulation. Additional details are as for CD8 in A above.

of IL-4, IFN- γ , and IL-12 while generating H-Y-specific CD8 and PCCF-specific CD4 effectors are shown in Fig. 4, A and B (CD8 and CD4, respectively). Dose-dependent effects of IL-4 and IL-12 on IL-5/IL-4 and IFN- γ secretion, respectively, were seen with CD8 cells that strongly paralleled those seen with CD4 cells. Although IL-4 was produced by CD8 effectors primed with IL-4, and the amount increased with greater levels of priming cytokine, secretion was still very low (max at 20 U/ml) compared with CD4 cells (>7,000 U/ml). IL-5 secretion from CD8 effectors over the range of IL-4 priming concentrations was always 5–10-fold less than from CD4 effectors and downregulation of IFN- γ secretion

was seen for both effector populations. IL-12 enhanced IFN- γ production over the same concentration range and similar levels of secretion (8,000 U/ml) were observed from both CD8 and CD4 populations.

Endogenously Produced IFN- γ or IL-12 Has Little Effect on the Cytokine Profiles of CD8 Effectors. Because IFN- γ and IL-12 can inhibit or downregulate many activities of IL-4, we investigated whether endogenously produced IFN- γ or IL-12 was limiting the generation of CD8 effectors secreting type 2 cytokines. The inclusion of anti-IFN- γ or anti-IL-12 during the generation of effectors with IL-4 either had no effect or slightly enhanced the development of IL-5- and IL-

Table 3. Cytokine Secretion from CD8 effector cells primed with IL-12

	Effectors primed with	T cells/ml	Cytokine Secretion			
			IL-2	IL-4	IL-5	IFN- γ
Alloantigen:			<i>U/ml</i>			
CD8 T cells	IL-2	2.5×10^5	<5	<5	<10	2,788
		1.2×10^5	<5	<5	<10	1,116
	IL-2 + IL-12	2.5×10^5	<5	<5	<10	5,210
		1.2×10^5	<5	<5	<10	2,972
CD4 T cells	IL-2	2.5×10^5	145	<5	149	5,436
		1.2×10^5	105	<5	59	2,848
	IL-2 + IL-12	2.5×10^5	<5	<5	9	19,730
		1.2×10^5	<5	<5	8	9,890
Antigen:						
CD8 T cells	IL-2	2.5×10^5	14	<5	<10	2,620
		1.2×10^5	32	<5	<10	2,446
	IL-2 + IL-12	2.5×10^5	68	<5	<10	8,454
		1.2×10^5	42	<5	<10	5,871
CD4 T cells	IL-2	2.5×10^5	4,000	<5	<10	2,581
		1.2×10^5	2,400	<5	<10	1,208
	IL-2 + IL-12	2.5×10^5	1,375	<5	<10	8,808
		1.2×10^5	925	<5	<10	9,419

CD8 or CD4 T cells were stimulated with alloantigen or antigen, as described in Table 1, and cultured for 4 d in the presence of IL-2 (20 U/ml) or IL-2 and IL-12 (2.5 ng/ml = 20 U/ml). T cells were washed and restimulated at varying numbers with 2×10^6 /ml LPS/DXS-APC and cytokines measured after 24 h.

Table 4. Cytokine Secretion by IL-4-primed CD8 Effectors Generated in the Presence of anti-IFN- γ or anti-IL-12

	Effectors primed with	Cytokine Secretion			
		IL-2	IL-4	IL-5	IFN- γ
Alloantigen:		<i>U/ml</i>			
	IL-4	<5	36	178	900
	IL-4 + anti-IFN- γ	<5	49	225	1,155
	IL-4 + anti-IL-12	<5	23	137	988
Antigen:					
Expt. 1.	IL-4	<5	<5	145	89
	IL-4 + anti-IFN- γ	<5	<5	554	95
Expt. 2.	IL-4	<5	22	64	326
	IL-4 + anti-IL-12	<5	16	39	272

Effector CD8 T cells were generated with alloantigen or antigen and IL-2 plus IL-4 in the presence of neutralizing antibodies to IFN- γ or IL-12 (10 μ g/ml). T cells were washed and restimulated after 4 d and cytokine secretion from 2.5×10^5 /ml measured at 24 h.

4-secreting cells in both alloantigen and antigen systems (Table 4). In other experiments we added anti-IL-4 in cultures primed with IL-12 and as naive CD8 cells do not make IL-4, not surprisingly this had no effect (data not shown).

CD8 Effectors Cells Generated by Stimulation with Dendritic Cells Secrete Similar Patterns of Cytokines to Those Generated on T-depleted APCs. We also determined the ability of dendritic cell populations to present H-Y antigen to resting CD8 T cells and generate CD8 effector populations. It can be seen (Fig. 5) that dendritic cells were comparable with unstimulated or LPS/DXS stimulated T-depleted APCs in their ability to generate polarized effectors when primed with IL-4 and IL-12. In the experiment shown in Fig. 5, anti-CD3 was used for the restimulus and induced quite high levels of IL-4 as well as IL-5, suggesting that the magnitude of response may be affected by the nature of the secondary stimulus.

Discussion

These studies reveal a number of important facets of CD8 T cell heterogeneity. First, they confirm that naive CD8 T cells have a phenotype similar to naive CD4 T cells and like them can be stimulated *in vitro* to develop into a potent cytokine-secreting effector population. Second, they show that the effector population can be polarized into type 1 and type 2 subsets and that cytokines IL-12 and IL-4 can determine this pattern. Third, the studies emphasize the differences in naive and effector CD8 and CD4 T cells, revealing that CD4 T cells produce much greater quantities of IL-2 and IL-4 than CD8, but only 3–10-fold more IL-5 and comparable amounts of IFN- γ . The relative amounts of the various cytokines made by CD8 and CD4 effector cells and the polarizing effects of the cytokines during their generation are summarized in Fig. 6.

Four features of the experimental model that we have used

require emphasis. First, we chose a stimulus. In these studies we have used an antigen (H-Y or alloantigen) rather than anti-CD3 to stimulate the CD8 T cells. In the case of the CD8 cells from bm1 mice the antigen is the K^b class I MHC which is seen as foreign by the T cells bearing the bm1 mutation of H-2 K^b. We presume that the K^b molecule displays a number of different peptides that are recognized by a spectrum of antigen specific T cells in the CD8 population from the bm1 mice. It is generally believed that on the order of 1% of the entire population may recognize the alloantigen. The T cell recoveries that we observed at day 5 would represent a 50-fold expansion of the original population of responding cells. In the case of the CD8 T cells from the anti-H-Y transgenic mice, the range of peptides derived from the male antigen protein may be much more restricted but is as yet unidentified. The transgenic T cells recognize the peptides from the male antigen bound to the D^b allele and potentially all of the CD8 population can recognize that antigen. Second, we have focused on the response of naive precursors. The CD8 T cells in the female anti-H-Y transgenic SCID mice are all naive and contain no memory T cells (Fig. 1). All of the T cells bear the anti-H-Y receptor and only the CD8 T cells are positively selected in the thymus on the D^b allele (23). We are thus, in this model, able to monitor the development of primary CD8 effector T cells in the absence of memory precursors. Third, we have measured the responses of effector T cell populations. In our experimental model, we generate effector cells by 4-d culture in the presence of various cytokines. The effectors are then restimulated with fresh APCs and the cytokines secreted in a 24-h period are harvested and assayed. This model has been extensively exploited for the study of CD4 T cells and has led to the discovery that the cytokines present in the initial 4-d culture period determine the cytokine profile of the effector

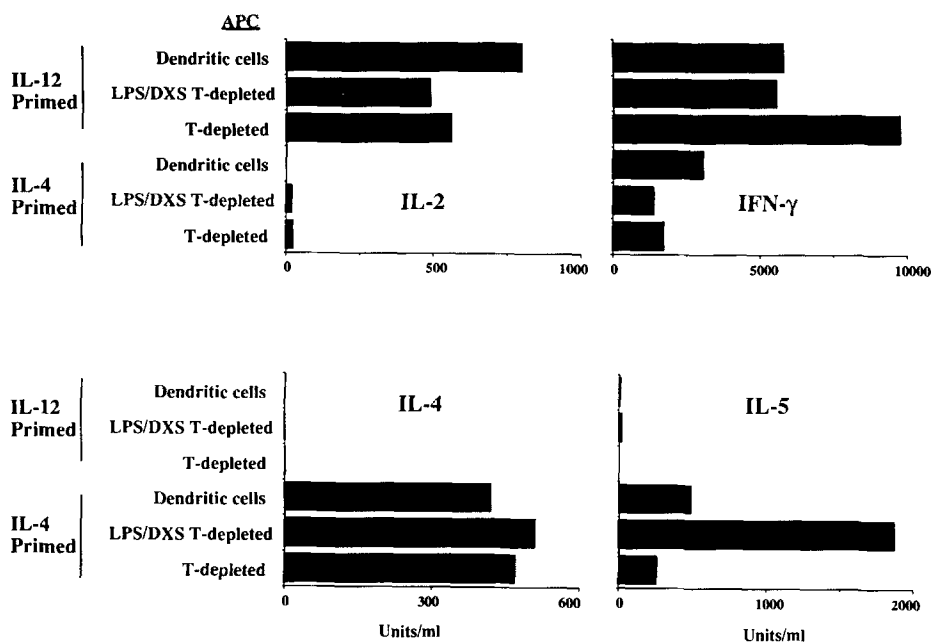


Figure 5. Generation of CD8 effectors on H-Y expressing dendritic cells. Naive CD8 cells (2×10^5 /ml) from anti-H-Y transgenics were stimulated with either unstimulated T-depleted APC (10×10^5 /ml), LPS/DXS T-depleted APC (5×10^5 /ml), or dendritic cells (10^5 /ml) from male B6 mice. Effectors were generated as before in either IL-2 and IL-4, or IL-2 and IL-12 and restimulated with plate-bound anti-CD3 ($10 \mu\text{g}/\text{ml}$) at 2×10^5 /ml. Supernatants were harvested at 24 h and assayed for IL-2 (top left), IFN- γ (top right), IL-4 (bottom left), and IL-5 (bottom right).

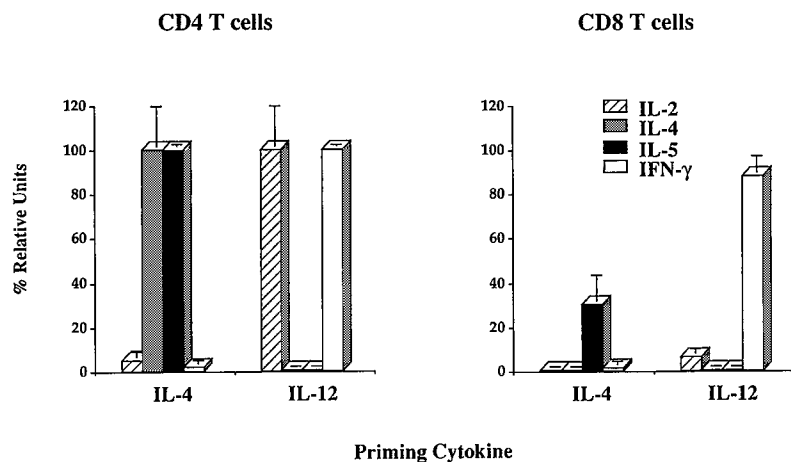


Figure 6. Polarized secretion of cytokines by CD8 and CD4 effector T cells. The figure shows relative amounts of cytokine secreted by CD8 and CD4 effectors and the polarizing effects of IL-4 or IL-12 during the generation of the effectors in the initial 4 d of culture. The actual values for each cytokine are normalized, the highest amount of a particular cytokine (under any of the conditions tested) being referred to as 100% and all other values expressed as the percent of this value. Data are the means of two to four separate determinations.

populations that develop (21). In further studies, we have shown that these changes are stable, suggesting that different subsets of CD4 T cells have been generated (21). Fourth, we have made direct comparisons of the performance of CD8 and CD4 T cells under comparable conditions.

The phenotypes of the CD8 naive and effector populations were similar to, but not identical to, those of the corresponding CD4 populations. The naive cells (which we can be confident predominate in the SCID H-Y TCR transgenic mice) were CD45RB high, L-selectin high, and CD44 low. The cells from the bm1 mice contained small to medium numbers of activated and memory T cells with a more varied phenotype, the only apparently distinguished feature between antigen-inexperienced and -experienced cells being low vs. high expression of CD44. This conclusion is in agreement with previous studies (28, 29). After 4 d in culture, most of the effector CD8 T cells were CD45RB high, L-selectin low, and CD44 high, a phenotype common to similarly derived *in vitro* CD4 effector populations (17, 26).

Freshly isolated CD8 T cells made barely detectable amounts of the cytokines measured. This was true of the cells from bm1 mice, which from the cell surface marker profile represent an 80:20 ratio of naive to memory T cells. The cells from the anti-H-Y transgenic mice that appeared totally naive, made slightly more cytokines with detectable quantities of IL-2 and IFN- γ (Table 1). We attribute this to the fact that a much higher percentage of the cells responded in the transgenic cell population (see cell recoveries, Materials and Methods) rather than to any difference in the ratio of naive to memory T cells. This finding is in accordance with the general belief that most CD8 cells are dependent on CD4 cells for their supply of IL-2, CD4 cells being able to make copious quantities (see Table 1 and Figs. 2-4). It is possible that helper independent responses may be obtained under certain conditions, perhaps when the TCR has high affinity for the ligand (Sprent, J., personal communication), and certainly easily detectable levels of IL-2 (100-200 U/ml) are produced if dendritic cells are used as the stimulus, although still significantly below that produced by dendritic cell stimulated CD4 cells (>1,000 U/ml) (data not shown). It has been suggested that only antigen-experienced CD8 cells can produce

IFN- γ based on anti-CD3 stimulation of isolated CD44 high-expressing cells (29). We do not necessarily disagree with this. The amount of IFN- γ produced from the CD8 cells from the H-Y transgenics was extremely low and variable. It is unlikely this came from memory cells based on the phenotype data, and did not come from *in vivo*-preactivated CD8 cells as percoll separated resting cells also gave IFN- γ . A distinct possibility is that this cytokine came from contaminating NK cells, although we have failed to detect these in our CD8 preparations or from the APC, although these made no cytokines when cultured alone.

CD8 effector populations only produce cytokine when re-stimulated, a phenomenon previously observed with comparable CD4 T cell populations (Tables 2-4). Thus the model of a naive precursor population that must first develop into an effector population before being capable of significant cytokine secretion, which we proposed for the CD4 helper T cell (21), also applies to the CD8 T cell. Again, as seen in the generation of CD4 effector populations, the presence of cytokines during development can determine the profile of cytokines that effector CD8 populations make upon re-stimulation. The level of cytokine secretion in CD8 cells was generally less than that seen for CD4 cells, although this did vary depending on the cytokine examined and the way the effectors were generated. IL-2 and IL-4 were generally produced in much lower quantities than by CD4 cells, IL-5 when secreted was produced somewhat less by CD8 cells, whereas IFN- γ under most conditions was secreted to a similar extent by both CD8 and CD4 cells.

CD8 cells cultured in the presence of IL-2 alone (IL-2 effectors) made high amounts of IFN- γ and no IL-2, IL-4, or IL-5. Effectors generated with IL-12 (IL-12 effectors) made substantially more IFN- γ while those generated with IL-4 (IL-4 effectors), at least in the antigen-specific model, dramatically downregulated IFN- γ secretion. The patterns of IFN- γ secretion by CD8 cells are thus similar to previous findings with CD4 cells (e.g., 21, 22, 27, 31, 32), shown again here in direct comparison. Thus IFN- γ may be regulated by similar factors in the two T cell subsets. One exception to this was the finding that priming with IFN- γ had little to no enhancing effect on production of IFN- γ in CD8

effectors whereas previous studies, reproduced here, have consistently shown IFN- γ to be a strong regulator of its own secretion in CD4 effectors. It has been suggested that IL-12 action in promoting IFN- γ secretion by CD4 effectors may be either via NK cells by inducing IFN- γ which can then act on the naive CD4 cells (22), or may be directly on the T cells (30, 31). The direct action may or may not involve the autocrine production of IFN- γ . Our own preliminary studies of CD4 cells suggests that IL-12 may act directly on the T cells, but also partly through autocrine induction of IFN- γ during effector generation. Conversely, IFN- γ priming of effectors may also be partly due to a direct effect and partly due to induction of IL-12 (Croft, M., and L. Carter, unpublished data). In contrast, as IFN- γ had little priming effect on IFN- γ secretion by CD8 effectors, it is likely that IL-12 action may be a direct effect on the naive CD8 cells. The mechanism of action of IL-12 vs. IFN- γ during effector generation of both T cell subsets deserves further study.

IL-2 and IL-4 may be regulated differently in CD8 cells vs. CD4 cells. Both naive and effector CD4 cells generally secrete high levels of IL-2, unless cultured with IL-4, whereas CD8 cells make very little IL-2, at least in the experiments here. One possibility could be that CD8 cells have adapted to generate effectors without requiring large quantities of IL-2, and unlike CD4 cells they rarely are involved in recruiting other T cells for response, making the production of IL-2 unnecessary. Alternatively the culture conditions we use in these experiments may bias our results. As mentioned above, there is evidence of subsets of CD8 cells being IL-2/helper independent (4, 5), which may suggest they synthesize sufficient quantities of their own IL-2. Because we culture CD8 cells in IL-2 from their outset, this may result in preferential outgrowth of the IL-2-dependent, non-IL-2-producing cells, resulting in a population of effectors that are geared to secrete only other cytokines. We are currently investigating this possibility.

In most experiments using alloantigen, T-depleted spleens were the APCs, however in experiments using T cells from the anti-H-Y mice, we stimulated the T-depleted populations with LPS and DXS overnight to enhance the presentation of the H-Y antigen. The nature of the H-Y peptide remains unknown and its presence is only revealed by its ability to stimulate a T cell response. It was therefore important to show that dendritic cells, a more physiological APC population, were equally effective in activating the T cells and generating effectors. Fig. 5 shows that IL-4 and IL-12 promoted generation of polarized CD8 effectors regardless of the initiating APC population.

Although exogenous IL-4 was able to promote generation of CD8 effectors secreting some IL-4, the amount produced was minimal compared with IL-4-primed CD4 effectors generated under identical conditions. The finding of IL-4 cytokine secretion in the CD8 IL-4-effector populations is in agreement with the prior observation of Seder et al. (12), although in their hands, IL-4 secretion was only a few orders of magnitude less than that produced by CD4 cells. The reason for this discrepancy may again lie in culture conditions. Seder

used anti-CD3 as the stimulus with few APC, whereas we used antigen with high numbers of APCs. It is therefore possible that endogenous factors were present which somehow suppressed generation of IL-4-producing cells. Blocking studies with anti-IFN- γ and anti-IL-12 however failed to significantly alter IL-4 induction (Table 4), suggesting that at the very least these factors were not involved. In contrast, restimulation of effectors with anti-CD3 promoted significant levels of IL-4 as well as IL-5 (Fig. 5) suggesting even greater complexity in regulation of type 2 cytokine secretion. Further studies addressing APC-associated factors for the primary and secondary stimulation will no doubt shed light on regulation of IL-4 secretion by CD8 cells. In contrast to IL-4, it was the secretion of IL-5 that appeared to be the hallmark of the IL-4-primed effectors. Unlike comparable populations of CD4 cells, the CD8 effectors made consistently more IL-5 relative to IL-4 and this was only several orders of magnitude less than that produced by CD4 effectors. These cells therefore represent a very unique, novel population secreting predominantly IL-5 with only low levels of IL-4, IFN- γ , and IL-2. It is interesting to note that generation of IL-5-producing cells seemed to be suppressed slightly by endogenous IFN- γ (Table 4), suggesting that this phenotype could be even more pronounced.

Whether such contrasting cytokine secretion profiles for CD8 effectors shown in this paper has any bearing on effector function is not known. Interestingly, it has recently been demonstrated that IL-12 can increase CTL activity of CD8 effectors (33) although under the conditions in which we generate effectors, we do not know if this effect of IL-12 will be seen. Preliminary investigation of cytotoxicity of IL-4-primed effectors has not shown any significant difference compared with effectors grown in IL-2 which are very efficient killers (data not shown). This finding is not particularly surprising as several studies have previously implicated IL-4 as being active in promoting CTL generation (34–36). Efficient CTL activity of a population of IL-5-secreting CD8 effectors however does not mean that these cells cannot have profound bystander activities on other cells during immune responses, which do not involve direct cell–cell contact. Certainly IL-5 is a very efficient promoter of Ig secretion from previously activated B cells, an action that does not require interaction between the B cell and the IL-5-secreting cell (25, 37), and IL-5 is also one of the main factors inducing recruitment, activation and differentiation of eosinophils (38), a phenomenon that also does not necessarily involve cell recognition. We plan additional experiments to address these possibilities. In contrast to our studies, Erard et al. (15) and LeGros and Erard (39), primarily using mitogen stimulation of CD8 cells in the presence of IL-4, demonstrated that they could induce cells that had downregulated CD8 expression (CD8⁻CD4⁻TCR⁺) and secreted IL-4 and IL-5, but lacked CTL activity. The CD8⁻ effectors were shown to display “helper activity” but this was measured in a mitogen induced model and may have merely been a measure of the effects of the cytokines that were released rather than an indication that cognate B cell help could occur in a physiological situation.

These investigators were also able to generate the double negative cells with alloantigen, although only 20–30% of the effector population had this phenotype, the other cells apparently being cytotoxic CD8⁺ cells. This suggests that IL-4 may be able to promote the generation of two distinct populations of cells which could have different functional activities, one that expresses CD8, secretes type 2 cytokines but has CTL activity, and another that does not express CD8, also secretes type 2 cytokines, but has no CTL activity. In our experiments we did not generate any CD8⁻ cells suggesting that minor differences in stimulation conditions, not readily apparent at the moment, may affect which population predominates. Obviously, if these do represent distinct effector subsets it will be important to fully understand how to generate one vs. the other.

In conclusion, the data presented here complement previous studies (11, 12, 16, and others) that suggest that CD8 T cells

are capable of producing a very wide range of cytokines. They additionally suggest that naive CD8 cells can differentiate into distinct effector populations secreting type 1 vs. type 2 cytokines, similar to CD4 cells, in response to antigen, and that exogenous factors, particularly IL-4 and IL-12, encountered during the differentiation phase can profoundly influence the cytokine phenotype of the effectors generated. IFN- γ can be secreted equivalently by both subsets, whereas there can be large differences in secretion of the other cytokines.

Thus, there are substantial similarities but also substantial differences between CD8 and CD4 populations. A knowledge of the biological functions of the different CD8 T cell subsets should provide crucial insight into their role in vivo. Further studies in which CD8 effectors are generated in the presence of other cytokines and in which a wider range of cytokines and other functions are assayed are currently in progress.

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