

# Interleukin (IL) 4, in the Absence of Antigen Stimulation, Induces an Anergy-like State in Differentiated CD8<sup>+</sup> TC1 Cells: Loss of IL-2 Synthesis and Autonomous Proliferation but Retention of Cytotoxicity and Synthesis of Other Cytokines

By Subash Sad and Tim R. Mosmann

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*From the Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Canada T6G 2H7*

## Summary

Naive T cells in the periphery mainly secrete interleukin (IL) 2 upon activation. After stimulation in the presence of appropriate costimulators, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells differentiate into effector cells secreting distinct T helper (Th) 1- and Th2-like cytokine patterns. Subsequent to differentiation, both CD4<sup>+</sup> (Th1 and Th2) and CD8<sup>+</sup> (TC1 and TC2) cells are stable and cannot be induced to differentiate into the opposite pattern or revert to the naive cytokine secretion pattern. We now show that IL-4 caused committed TC1 bulk populations or clones to lose the ability to synthesize IL-2. The cells retained the ability to secrete interferon (IFN)  $\gamma$ , granulocyte/macrophage colony-stimulating factor, and tumor necrosis factor, did not synthesize any Th2 cytokines, and did not alter cell surface marker expression. IL-4 rapidly inhibited IL-2-synthesizing ability in the absence or presence of antigen-presenting cells, thus demonstrating that IL-4 acted directly on TC1 cells. The defect in IL-2 synthesis could not be reversed by subsequent stimulation with potent antigen-presenting cells in the presence of IL-2 and anti-IL-4, or with anti-CD3 plus anti-CD28 antibodies. Both IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells were strongly cytotoxic toward allogeneic but not syngeneic targets. However, IL-2<sup>-</sup> TC1 cells were unable to proliferate unless exogenous IL-2 was provided. TC1 cells that lose IL-2 synthesis but retain IFN- $\gamma$  synthesis and cytotoxicity may be similar to the "anergic" cells induced by stimulation of CD4<sup>+</sup> or CD8<sup>+</sup> cells in the absence of costimulators. These results suggest that during a mixed type 1/type 2 response *in vivo*, IL-4 may induce the IL-2<sup>+</sup> TC1  $\rightarrow$  IL-2<sup>-</sup> TC1 conversion, and thus curtail the expansion of the TC1 response without impairing short-term effector function.

Functionally different subsets of CD4<sup>+</sup> and CD8<sup>+</sup> cells have important regulatory and effector roles in immune responses. Based on the cytokine synthesis patterns of long-term clones, CD4<sup>+</sup> T cells were classified into Th1 (IL-2, IFN- $\gamma$  and TNF- $\beta$ ) and Th2 (IL-4, IL-5, IL-6, IL-10, and IL-13) (1-3). Many of the functions of T cells are mediated by cytokines that are secreted upon their stimulation. Th1 cells, through their production of IFN- $\gamma$  and TNF- $\beta$ , induce delayed-type hypersensitivity and microbicidal activity in macrophages (cellular immunity), whereas Th2 cells secrete cytokines that help B cells become antibody-producing cells (humoral immunity) and enhance allergy (3). Distinct cytokine-secreting subsets of CD8<sup>+</sup> T cells have also been identified (4-7). Cytokine patterns of CD8<sup>+</sup> subsets are similar to their CD4<sup>+</sup> counterparts: TC1 cells secrete IL-2 and IFN- $\gamma$ , and TC2 cells secrete IL-4, IL-5, IL-6, and IL-10. The existence of subsets of both CD4<sup>+</sup> and

CD8<sup>+</sup> T cells *in vivo* has been demonstrated in various infections (8-13).

Cytokines are the major factors that determine the differentiation of precursor T cells. IL-12, TGF- $\beta$ , and IFN- $\gamma$  induce the differentiation of naive CD4<sup>+</sup> T cells into Th1 but not Th2 cells (14-17), whereas IL-4 is essential for differentiation into Th2 cells and inhibits development of Th1 cells (18, 19). IFN- $\gamma$  and IL-4 also induce the differentiation of naive CD8<sup>+</sup> T cells into TC1 and TC2 phenotypes, respectively (4-6), and IL-12 enhances the cytotoxicity and IFN- $\gamma$  production of CD8<sup>+</sup> cells (20). Cytokine-directed differentiation may be due to a combination of inhibitory and enhancing effects on the two arms of the differentiation pathway.

Cytokines secreted by Th1 and Th2 cells also cross-regulate the proliferation or cytokine secretion of committed cells of the opposite subset (3). IL-10 secreted by Th2 cells

inhibits cytokine secretion by Th1 cells (21, 22). Similarly, IFN- $\gamma$  secreted by Th1 cells inhibits the proliferation of Th2 but not Th1 cells (23, 24). Less has been known about the influence of cytokines on the proliferation and cytokine secretion of committed CD8<sup>+</sup> effector cells.

Subsequent to differentiation, effector T cells maintain a relatively stable cytokine secretion pattern, as they rarely, if ever, change into the opposite phenotype or revert to their precursor state. However, some alterations have been described. Th0 cells (secreting Th1 and Th2 cytokines) become more Th1- or Th2-like in response to different cytokines (25, 26). Stimulation of Th0 cells with anti-CD3 in the absence of costimulators induced a shift to a more Th2-like phenotype (25). In the absence of appropriate costimulators, for example, stimulation with antigen on fixed APC, both CD4<sup>+</sup> Th1 and CD8<sup>+</sup> TC1 cells lose the ability to synthesize IL-2 and proliferate (27–29). This “anergic” state was reversed when Th1 cells were subsequently stimulated with allogeneic non-T cells (30), IL-2, or IL-4 (31, 32), although reversal was not observed in another study (33).

We now show that, in addition to the well-established effects of IL-4 on naive T cells, IL-4 inhibits the ability of differentiated CD8<sup>+</sup> TC1 cells to synthesize IL-2. This was not reversed by IL-2 during or after IL-4 treatment. Loss of the ability to synthesize IL-2 did not impair TC1 functional capability, as both the IL-2-secreting (IL-2<sup>+</sup>) and the IL-2-nonsecreting (IL-2<sup>-</sup>) CD8<sup>+</sup> TC1 cells were highly cytolytic and secreted normal amounts of other TC1 cytokines (IFN- $\gamma$ , GM-CSF, and TNF). However, the subsequent proliferation of IL-2<sup>-</sup> TC1 cells became dependent on exogenous IL-2, suggesting that this may be another example of cross-inhibition of the type 1 and type 2 cytokine patterns.

## Materials and Methods

**Mice.** Female C57BL/6 mice, 4–6-wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the Health Sciences Laboratory Animal Services (Edmonton, Alberta, Canada) in accordance with the guidelines of the Canadian Council on Animal Care.

**Cell Lines.** The mouse hybrid B cell line M12.4.1 (34) expressing H-2<sup>d</sup> was obtained from Dr. L. Glimcher. J774A.1 (mouse macrophage cell line expressing H-2<sup>d</sup>), P815, EL4, and WEHI-164-13 cell lines were obtained from American Type Culture Collection (Rockville, MD).

**Cytokines, Antibodies, and Reagents.** Mouse recombinant IL-2 was expressed in *Escherichia coli* (35) and used as a partially purified preparation at 2.5 ng/ml. Recombinant mouse IL-4 and IL-12 were expressed in COS cells and used as a dilution of COS transfection supernatants (36). Hamster anti-mouse CD28 antibody clone 37.51 (37) was obtained from Dr. James P. Allison. Anti-IL-4 and anti-CD28 antibodies were purified on a protein G column from the supernatants of 11B11 (38) and 37.51 cell lines. Con A was obtained from Pharmacia (Uppsala, Sweden). Fetal bovine serum (FBS)<sup>1</sup> was obtained from Hyclone Laboratories

Inc., (Logan, UT). Biotinylated anti-mouse Pgp1 (CD44, clone IM7), Ly3-PE (anti-CD8 $\beta$ , clone 53-5.8), and NK1.1-PE (clone PK136) were obtained from PharMingen (San Diego, CA). Streptavidin-FITC was obtained from Southern Biotechnology Associates (Birmingham, AL). Rabbit anti-hamster IgG-FITC was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rat anti-mouse Ly2-FITC and Ly2-PE (anti-CD8 $\alpha$ , clone YTS 169.4) were obtained from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada).

**Cell Cultures.** C57BL/6 CD8<sup>+</sup> T cells were enriched by removal of B cells, CD4<sup>+</sup> T cells, and macrophages on rat T columns (Biotex, Edmonton, Alberta, Canada). Splenocytes ( $150 \times 10^6$ ) were incubated with a rat anti-mouse CD4 antibody (clone YTS 191.1) and loaded onto glass bead columns coated with sheep anti-rat Ig (cross-reactive with mouse Ig). Cells that passed through the column were 80–90% CD8<sup>+</sup> by FACScan<sup>®</sup> analysis. In some experiments, CD8<sup>+</sup> T cells were further purified by sorting on a cell sorter (EPICS Elite; Coulter Corp., Hialeah, FL) after staining the cells with rat anti-mouse Ly2-FITC (clone YTS 169.4). C57BL/6 CD8<sup>+</sup> T cells (H-2<sup>b</sup>,  $10^3$ /well) were stimulated with  $10^4$  cells/well of irradiated (10,000 rad) allogeneic APC lines M12.4.1 (H-2<sup>d</sup>, B cell lymphoma [34]) and J774 (H-2<sup>d</sup>, macrophage) in 96-well tissue culture plates in 200  $\mu$ l of RPMI plus 8% FBS in the presence of various cytokines. IL-2 was added at 2.5 ng/ml, and, where appropriate, anti-IL-4 (11B11) at 25  $\mu$ g/ml and IL-4 at 10 ng/ml were added unless otherwise mentioned. Media were changed every third day after splitting cultures as necessary.

For purification of CD4<sup>+</sup> T cells, C57BL/6 splenocytes were incubated with a rat anti-mouse CD8 antibody (clone YTS 169.4) and processed as mentioned above for CD8<sup>+</sup> T cell purification.

Cytokine secretion was determined after Con A stimulation (5  $\mu$ g/ml) of effector cells ( $10^6$ /ml) in 200  $\mu$ l of RPMI 1640 plus 8% FBS. Arithmetic means and SD of triplicate cultures were calculated.

**Generation of Long-Term T Cell Clones.** C57BL/6 CD8<sup>+</sup> T cells were cloned by limiting dilution in the presence of cytokine combinations favoring differentiation into the TC1 (IL-2 + IL-12 + anti-IL-4) phenotype as described above. Limiting dilution cloning was carried out by stimulating the C57BL/6 CD8<sup>+</sup> T cells (1,000 cells/well to 0.5 cell/well) with irradiated M12.4.1 cells ( $5 \times 10^4$ /well) in 96-well round-bottomed tissue culture plates in 200  $\mu$ l of RPMI 1640 plus 8% FBS. Every third day, 100  $\mu$ l of media was changed.

**Cytokine Assays.** Cytokines produced in the supernatants were measured by ELISA (39). The following antibodies were used: R4-6A2 (ATCC HB170) and XMG1.2-biotin (2) for IFN- $\gamma$ ; BVD4-1D11 and BVD6-24G2-biotin (PharMingen) for IL-4; TRFK5 (40) and TRFK4-biotin (PharMingen) for IL-5; JES6-1A12 and JES6-5H4-biotin (PharMingen) for IL-2; 8F8 and 43D11-biotin (41) for IL-3; SXC2 and SXC1-biotin (42) for IL-10; 35E10 and 22E9-biotin for GM-CSF; and MP5-20F3 and MP5-32C11-biotin (PharMingen) for IL-6. TNF was assayed on WEHI-164.13 cells as described (43), except that the assay was carried out in the presence of saturating (5 ng/ml) amounts of IFN- $\gamma$ .

**Thymidine Incorporation.** IL-2<sup>-</sup> or IL-2<sup>+</sup> TC1 cells were generated by stimulating effector TC1 cells on day 6 with allogeneic J774A.1 cells in the presence or absence of IL-4 for another 6 d. On day 12, the proliferative capability of allospecific IL-2<sup>+</sup> and IL-2<sup>-</sup> CD8<sup>+</sup> TC1 cells was assessed by analyzing the incorporation of labeled thymidine every day for 4 d. TC1 cells ( $1.1 \times 10^4$

<sup>1</sup> Abbreviation used in this paper: FBS, fetal bovine serum.

cells/ml) were stimulated with irradiated (10,000 rad) J774A.1 cells ( $5 \times 10^4$ /ml) in 96-well flat-bottomed tissue culture plates in 200  $\mu$ l RPMI 1640 plus 8% FBS in the presence or absence of various growth factors. Radiolabeled thymidine was added (1  $\mu$ Ci/well) at the same time or after every 24 h. Cells were then harvested 24 h after the thymidine pulse, and radioactive incorporation was determined by liquid scintillation counting.

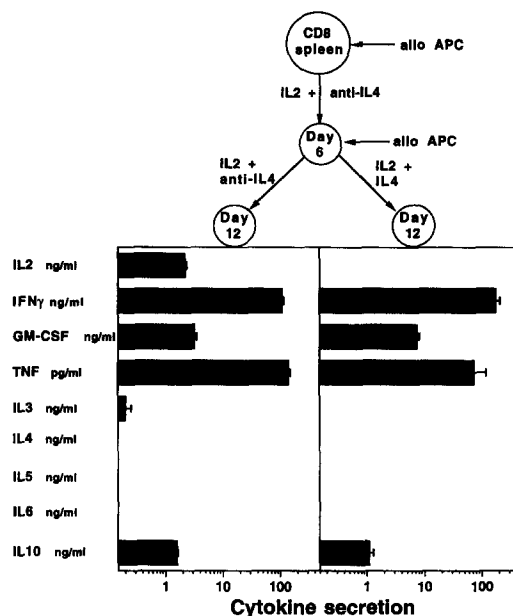
**Cytotoxicity Assays.** 6 d after the stimulation of naive CD8<sup>+</sup> T cells with M12.4.1 cells in the presence of IL-2 plus anti-IL-4, aliquots of the cells were stimulated for another 6 d with M12.4.1 cells in the presence of (a) IL-2 plus anti-IL-4, (b) IL-2 plus IL-12 plus anti-IL-4, or (c) IL-2 plus IL-4. The resulting IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells were then analyzed for their cytolytic activity toward labeled P815 (allogeneic) and EL4 (syngeneic) tumor cell lines. Target cells were radiolabeled by incubation of  $10^7$  cells with 100  $\mu$ Ci of <sup>51</sup>Cr in 50  $\mu$ l medium for 45 min. After thorough washing, various ratios of effectors to targets were cocultured for 4 h in 96-well round-bottomed tissue culture plates. Radioactivity was detected in the supernatants by gamma counting. The percentage of cytotoxicity was calculated using the following formula:

$$\frac{(\text{cpm experimental} - \text{cpm spontaneous}) / (\text{cpm total} - \text{cpm spontaneous}) \times 100.$$

**Flow Cytometry Analysis.** Normal C57BL/6 splenocytes or effector IL-2<sup>+</sup> or IL-2<sup>-</sup> TC1 cells were washed and incubated on ice ( $10^6$  cells in 50  $\mu$ l RPMI 1640 plus 1% FBS) with the following antibodies: anti-CD8 $\alpha$ -PE, anti-CD8 $\alpha$ -FITC, anti-CD8 $\beta$ -PE, anti-NK1.1-PE, biotin-anti-mouse CD44, and hamster anti-mouse CD28. CD44 and CD28 expression were revealed by second staining with streptavidin-FITC and rabbit anti-hamster IgG-FITC, respectively. Each incubation lasted 30 min, after which cells were thoroughly washed. Cells were fixed in 1% formaldehyde in PBS and analyzed on a FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA).

## Results

**IL-4 Inhibits the Subsequent Ability of CD8<sup>+</sup> TC1 Cells to Secrete IL-2 without Abrogating IFN- $\gamma$ , GM-CSF, and TNF Synthesis or Altering Expression of Cell Surface Markers.** Effector CD8<sup>+</sup> TC1 cells were established by stimulating freshly isolated splenic CD8<sup>+</sup> T cells with allogeneic M12.4.1 cells in the presence of IL-2 and anti-IL-4 antibodies. After 6 d in culture, the resulting TC1 effectors were stimulated with M12.4.1 cells in growth media containing IL-2 and either IL-4 or anti-IL-4. After another 6 d, cells were harvested, washed, and stimulated with Con A for 24 h. Fig. 1 demonstrates that effectors generated in the presence of anti-IL-4 secrete IL-2, IFN- $\gamma$ , GM-CSF, and TNF, whereas an aliquot of the same culture stimulated on day 6 in the presence of IL-4 lost the subsequent ability to secrete IL-2 when stimulated with Con A on day 12. The ability to secrete IFN- $\gamma$ , GM-CSF, and TNF was maintained. In a large number of experiments, IFN- $\gamma$  synthesis was moderately increased or decreased after IL-4 treatment, but never strongly reduced or lost. Stimulation in the presence of IL-4 did not result in a shift toward the TC2 phenotype, as these cells did not secrete IL-4, IL-5, or IL-6. Low levels of IL-10 were synthesized by TC1 cells, consistent with pre-

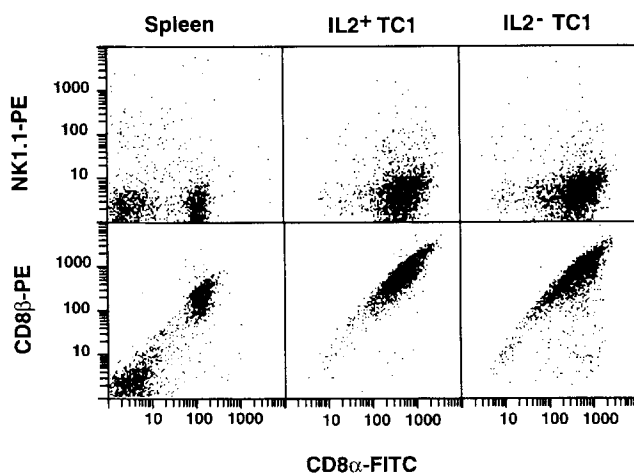
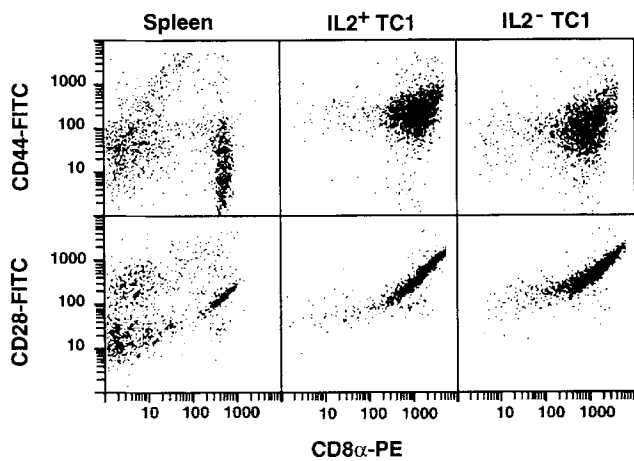


**Figure 1.** IL-4 selectively impairs the subsequent ability of committed CD8<sup>+</sup> TC1 cells to secrete IL-2 on restimulation. C57BL/6 splenic CD8<sup>+</sup> T cells ( $10^3$ /well) were stimulated with allogeneic M12.4.1 cells ( $10^4$ /well) in the presence of IL-2 plus anti-IL-4. On day 6, the resulting effector TC1 cells were then split and restimulated with M12.4.1 cells in the presence of either IL-2 plus anti-IL-4 or IL-2 plus IL-4 for another 6 d. On day 12, cells were thoroughly washed and stimulated ( $10^6$ /ml) with Con A (5  $\mu$ g/ml) for 24 h. Cytokines were measured in the supernatants.

vious findings (4), and IL-4 treatment did not alter these levels. Both IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells expressed similar levels of CD8 $\alpha$ , CD8 $\beta$ , CD28, and CD44 and remained NK1.1<sup>-</sup> (Fig. 2). Thus, IL-4-treated TC1 cells selectively lost the ability to subsequently secrete IL-2, without any other detectable alterations. This occurred even in the presence of exogenous IL-2.

**IL-4 Induced the Loss of IL-2-synthesizing Ability during Stimulation of TC1 Cells by Either B Cell or Macrophage APC Lines.** To determine whether the loss of IL-2-synthesizing ability occurred under different stimulation conditions, we established allospecific TC1 cells by primary allostimulation with M12.4.1 (a B cell line) or J774A1 (a macrophage cell line) in the presence of IL-2, IL-12, and anti-IL-4 antibodies. When these established TC1 cells were restimulated with the same APC, IL-4 induced both TC1 populations to lose the subsequent ability to synthesize IL-2 (Fig. 3). Synthesis of other cytokines (IFN- $\gamma$  and IL-4) was not affected.

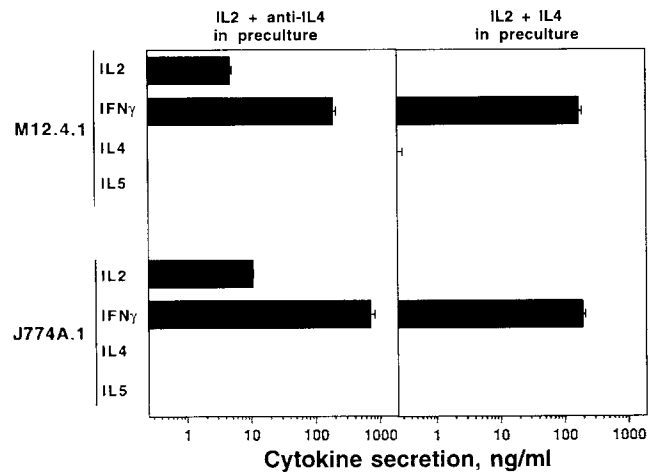
**IL-4-induced Loss of IL-2 Synthesis in Clones of CD8<sup>+</sup> TC1 Cells.** To rule out the possibility that IL-4-induced generation of IL-2<sup>-</sup> cells was a result of selective outgrowth of a minor population of cells within the bulk culture, we generated clones of TC1 cells in the presence of anti-IL-4. After their commitment into a TC1 phenotype, each clone was split into growth media containing either anti-IL-4 or IL-4 and stimulated with M12.4.1 cells for another 6 d. Fig. 4 demonstrates that 8 of the 10 clones generated in the presence of anti-IL-4 secreted IL-2, whereas



**Figure 2.** IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells express similar surface antigens. Normal C57BL/6 splenic CD8<sup>+</sup> T cells were stimulated with allogeneic J774A.1 cells in the presence of IL-2 plus IL-12 plus anti-IL-4. On day 6, the resulting effector TC1 cells were then washed, split, and cultured with either IL-2 plus anti-IL-4 or IL-2 plus IL-4 for another 6 d. On day 12, IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells were washed, stained with various antibodies, and analyzed by flow cytometry.

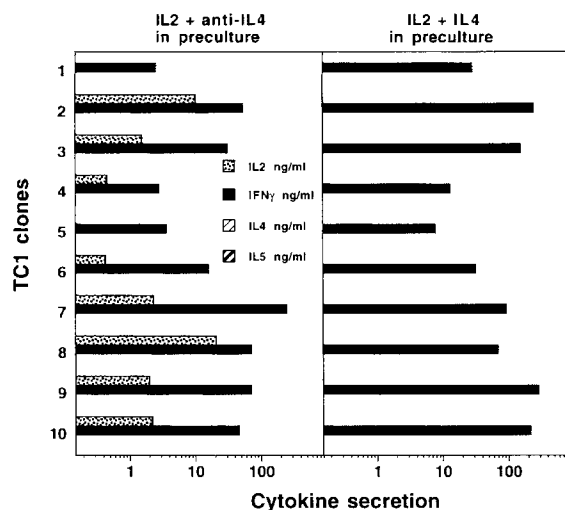
all the clones secreted IFN- $\gamma$  and no IL-4 or IL-5. However, when cultured in the presence of IL-4, all clones continued to secrete IFN- $\gamma$  but lost their ability to synthesize IL-2. Furthermore, neither the IL-2<sup>+</sup> nor IL-2<sup>-</sup> cells secreted IL-4 or IL-5 on subsequent stimulation showing that there was no switch to the TC2 or TC0 phenotypes (Fig. 4). These results demonstrate that the loss of the ability to synthesize IL-2 occurs within clones of TC1 cells, suggesting that there is a direct IL-2<sup>+</sup> TC1  $\rightarrow$  IL-2<sup>-</sup> TC1 transition.

*IL-4 Acts on TC1 Cells to Induce Differentiation into the IL-2<sup>-</sup> Phenotype.* In the experiments shown in Figs. 1 and 2, IL-4 may have acted directly on T cells, or indirectly by altering the antigen-presenting or costimulatory properties of the APC. To distinguish between these possibilities, freshly isolated splenic CD8<sup>+</sup> T cells were stimulated with

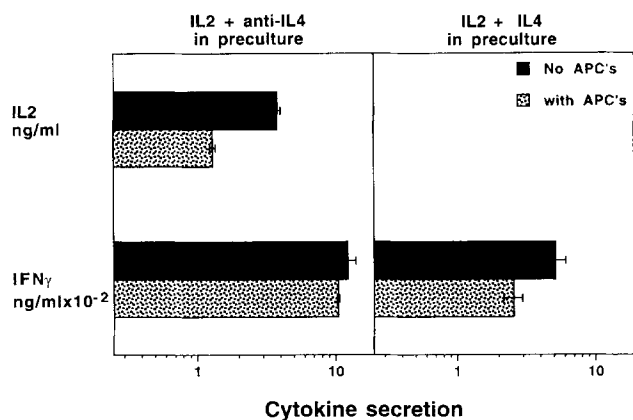


**Figure 3.** Inhibition of IL-2 synthesis on restimulation occurs when TC1 cells are stimulated with either B cell or macrophage APC lines in the presence of IL-4. Allospecific TC1 cells were generated by stimulating splenic CD8<sup>+</sup> T cells with either M12.4.1 (B cell) or J774A.1 (macrophage) cells in the presence of IL-2 plus IL-12 plus anti-IL-4. 6 d later, resulting TC1 cells were split into either IL-2 plus anti-IL-4 or IL-2 plus IL-4 and restimulated with the same APC line. On day 12, TC1 cells were washed and stimulated ( $10^6$ /ml) with Con A (5  $\mu$ g/ml). Supernatants were collected 24 h later, and cytokines were measured.

irradiated allogeneic J774 cells in the presence of IL-2 plus IL-12 plus anti-IL-4 for 7 d. The resulting TC1 cells were then cultured in either IL-2 plus anti-IL-4 or IL-2 plus IL-4 for 6 more days in the presence or absence of fresh irradiated APC. On day 13, cells were washed and restimulated with Con A for 24 h for cytokine secretion. Fig. 5 clearly

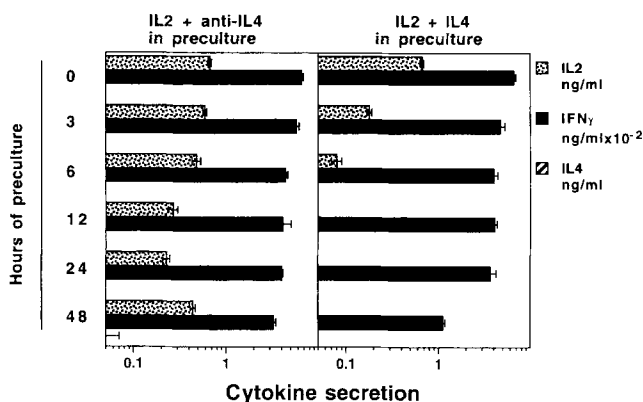


**Figure 4.** Clones of CD8<sup>+</sup> TC1 cells lose the capacity to secrete IL-2 but not IFN- $\gamma$  when stimulated in the presence of IL-4. CD8<sup>+</sup> clones were generated by limiting dilution of purified C57BL/6 CD8<sup>+</sup> T cells in the presence of irradiated M12.4.1 cells under conditions favoring differentiation into TC1 cells (IL-2 plus anti-IL-4). On day 6, each clone was stimulated with M12.4.1 cells for another 6 d in the presence of the same cytokine combinations. Aliquots of each clone were split on day 12 and restimulated with M12.4.1 cells in the presence of either IL-2 plus anti-IL-4 or IL-2 plus IL-4. 6 d later, cells were thoroughly washed and stimulated with Con A for 24 h for cytokine secretion.

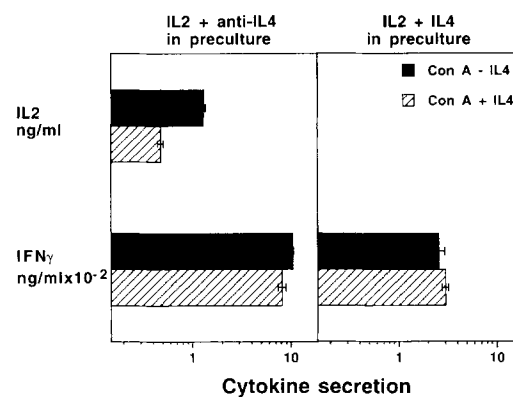


**Figure 5.** Inhibition of IL-2-synthesizing ability occurs when TC1 cells are incubated with IL-4 in the absence of APC. Allospecific TC1 cells were generated by stimulating C57BL/6 CD8<sup>+</sup> T cells with J774 cells in the presence of IL-2 plus IL-12 plus anti-IL-4. On day 7, TC1 cells were washed and cultured with either IL-2 plus anti-IL-4 or IL-2 plus IL-4 in the presence or absence of irradiated allogeneic M12.4.1 APC for another 6 d. On day 12, cells were washed and stimulated with Con A for cytokine estimation as mentioned in Fig. 1.

demonstrates that, even in the absence of APC, IL-4 impairs the subsequent IL-2-synthesizing capacity of TC1 cells. Thus, IL-4 appears to act directly on TC1 cells. To determine the kinetics of IL-4-induced loss of IL-2-synthesizing ability, we incubated differentiated TC1 cells on day 7 with IL-4 in the absence of APC. Cells were then harvested at the indicated intervals and stimulated with Con A for 24 h to determine cytokine secretion. Fig. 6 demonstrates that IL-2-synthesizing ability was substantially inhibited even after 3 h of incubation with IL-4 and was totally abrogated by 12 h. IL-4 was most effective at causing loss of IL-2 synthesis when added during the preculture of TC1 cells. When IL-4 was added only during



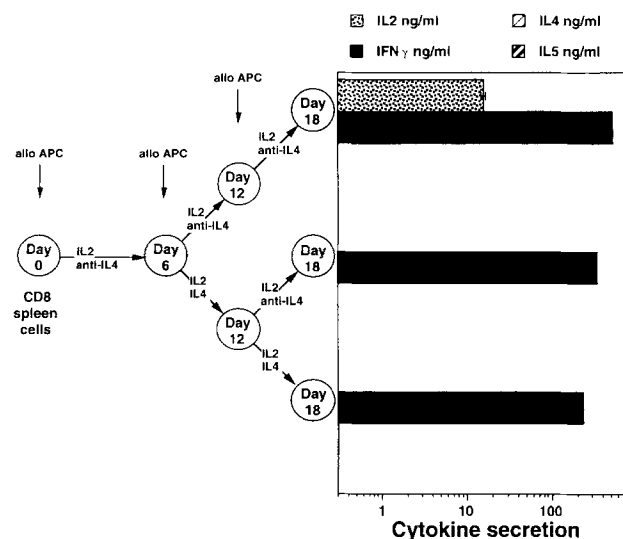
**Figure 6.** Abrogation of IL-2 synthesis occurs rapidly within 24 h of IL-4 treatment. Allospecific TC1 cells were generated by stimulating normal splenic CD8<sup>+</sup> T cells with allogeneic J774 cells in the presence of IL-2 plus IL-12 plus anti-IL-4. On day 7, cultures were washed and incubated with IL-2 in the presence or absence of IL-4 for various times. After each incubation, cells were washed thoroughly and stimulated ( $10^6$ /ml) with Con A ( $5 \mu\text{g}/\text{ml}$ ) for 24 h for cytokine synthesis.



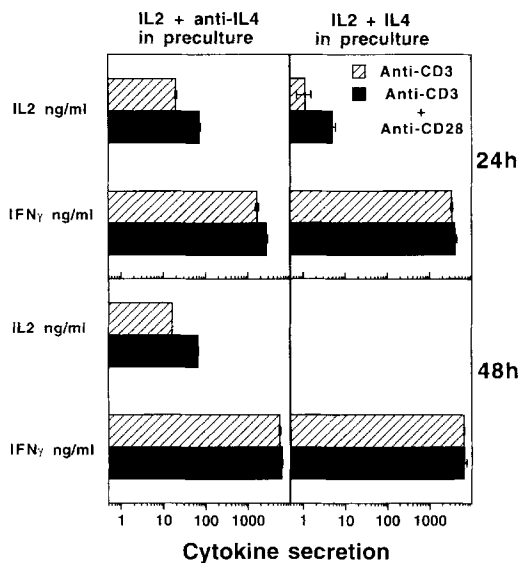
**Figure 7.** IL-4 does not abrogate IL-2 synthesis during short-term Con A activation of committed CD8<sup>+</sup> TC1 cells. Allospecific IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells were generated as mentioned in Fig. 1. On day 12, cells were thoroughly washed and stimulated with Con A for 24 h in the presence or absence of IL-4. Cytokines were measured in the 24-h supernatants.

the final Con A stimulation of IL-2<sup>+</sup> TC1 cells, IL-2 synthesis was partially inhibited but not abrogated (Fig. 7).

*IL-4-induced Loss of IL-2 Synthesis by TC1 Cells Is Irreversible.* To determine whether the IL-4-induced loss of the ability of TC1 cells to secrete IL-2 was a transient phenomenon, IL-2<sup>+</sup> or IL-2<sup>-</sup> TC1 cells were generated by stimulating effector TC1 cells on day 6 with allogeneic M12.4.1 cells in the presence or absence of IL-4 for another 6 d. On day 12, the resulting IL-2<sup>-</sup> TC1 cells were split into growth conditions containing either IL-2 plus anti-IL-4 or IL-2 plus IL-4 and were stimulated with



**Figure 8.** IL-4-induced inhibition of IL-2 synthesis by committed TC1 cells is irreversible. Normal C57BL/6 splenic CD8<sup>+</sup> T cells were stimulated with allogeneic M12.4.1 cells in the presence of IL-2 plus IL-12 plus anti-IL-4. On day 6, the resulting effector TC1 cells were split and stimulated with M12.4.1 cells in IL-4 or anti-IL-4. On day 12, aliquots of IL-2<sup>-</sup> TC1 cells (IL-2 plus IL-4 from days 6 to 12) were again stimulated in IL-2 plus IL-4 or washed and stimulated in IL-2 plus anti-IL-4. IL-2<sup>+</sup> TC1 cells (IL-2 plus anti-IL-4 from days 6 to 12) were restimulated in the same growth medium. On day 18, cells were harvested and stimulated ( $10^6$ /ml) with Con A ( $5 \mu\text{g}/\text{ml}$ ) for 24 h for cytokine secretion.

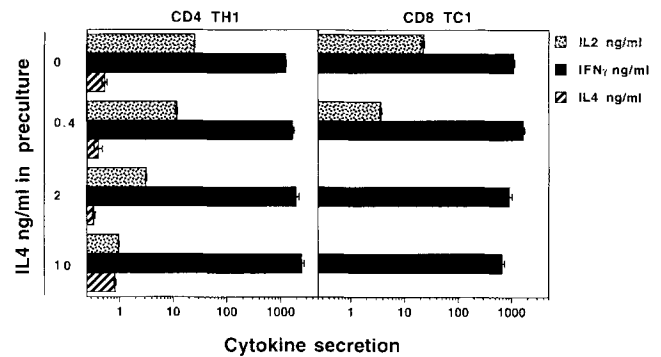


**Figure 9.** Stimulation of IL-2<sup>-</sup> TC1 cells in the presence of anti-CD28 does not reverse the IL-4-induced loss of IL-2 synthesis. Normal C57BL/6 splenic CD8<sup>+</sup> T cells were stimulated with irradiated allogeneic J774 cells in the presence of IL-2 plus IL-12 plus anti-IL-4. On day 6, the resulting effector TC1 cells were washed and cultured with either IL-2 plus anti-IL-4 or IL-2 plus IL-4. 3 d later (day 9), cells were washed and stimulated (10<sup>6</sup>/ml) with plate-bound anti-CD3 (10 μg/ml) in the presence or absence of plate-bound anti-CD28 (10 μg/ml). Supernatants were collected after 24 and 48 h for cytokine measurement.

M12.4.1 cells for another 6 d. On day 18, cells were harvested and stimulated with Con A for 24 h for cytokine secretion. TC1 cells that were continually grown in the presence of anti-IL-4 secreted substantial amounts of both IL-2 and IFN-γ (Fig. 8), whereas the cells grown in the presence of IL-4 from days 6 to 18 lost their capacity to secrete IL-2 while retaining their IFN-γ-producing capacity. IL-2 secretion was not restored if the IL-4-treated TC1 cells were stimulated with alloantigen in the presence of IL-2 plus anti-IL-4 from days 12 to 18.

After induction of the IL-2<sup>-</sup> phenotype, we also attempted to restore IL-2 synthesis by stimulation with plate-bound anti-CD3 and anti-CD28 antibodies. Fig. 9 shows that anti-IL-4-pretreated TC1 cells produced substantial amounts of IL-2, and, in this experiment, IL-4-pretreated cells produced a small amount of IL-2 at 24 but not 48 h. Anti-CD28 costimulation caused a fourfold enhancement of IL-2 secretion by both cell populations at 24 h. However, the IL-4-pretreated TC1 cells still produced 16-fold less IL-2 than controls. At 48 h, the control cultures maintained similar levels of IL-2, whereas no IL-2 was detectable in IL-4-pretreated cultures even with CD28 costimulation. Thus, in these experiments as well as others (results not shown), we have been unable to reverse the IL-4-induced defect in IL-2-producing capacity of TC1 cells.

**CD8<sup>+</sup> TC1 Cells Are More Susceptible than CD4<sup>+</sup> TH1 Cells to IL-4-induced Loss of IL-2 Synthesis.** As long-term CD4<sup>+</sup> clones can sometimes selectively lose the ability to synthesize IL-2 (Mosmann, T., unpublished observations), we tested whether IL-4 could also contribute to this effect.

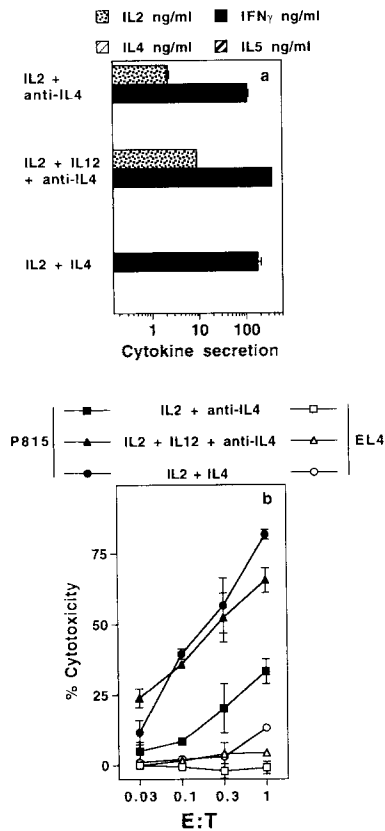


**Figure 10.** CD8<sup>+</sup> TC1 cells require less IL-4 than CD4<sup>+</sup> Th1 cells for inhibition of IL-2 synthesis. Allospecific CD4<sup>+</sup> Th1 and CD8<sup>+</sup> TC1 cells were generated by stimulating normal splenic C57BL/6 CD4<sup>+</sup> or CD8<sup>+</sup> T cells with allogeneic M12.4.1 cells in the presence of IL-2 plus IL-12 plus anti-IL-4 for 7 d. Effectors generated were then split and cultured with either IL-2 plus anti-IL-4 or IL-2 plus different doses of IL-4 as shown in the figure. On day 10, cells were washed and stimulated (2 × 10<sup>6</sup>/ml) with Con A (5 μg/ml) for 24 h for cytokine synthesis.

CD4<sup>+</sup> Th1 and CD8<sup>+</sup> TC1 cells were generated by stimulating normal splenic C57BL/6 CD4<sup>+</sup> or CD8<sup>+</sup> T cells with allogeneic M12.4.1 cells in the presence of IL-2 plus IL-12 plus anti-IL-4 for 7 d. Cultures were then split and incubated with either IL-2 plus anti-IL-4 or IL-2 plus IL-4. After 3 more days, cells were washed and stimulated with Con A for 24 h for cytokine synthesis. Fig. 10 demonstrates that IL-4 inhibited the IL-2-synthesizing ability of CD4<sup>+</sup> Th1 cells, but loss of IL-2 synthesis was only partial, even at 10 ng/ml IL-4. CD8<sup>+</sup> TC1 cells were more susceptible, showing complete loss of IL-2 synthesis in response to ≥2 ng/ml of IL-4.

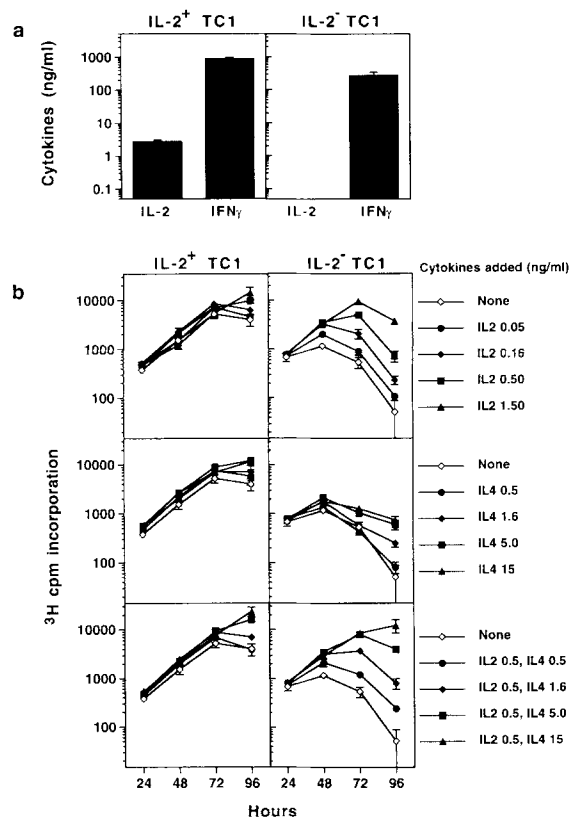
**Both IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 Cells Are Highly Cytolytic.** To determine whether the loss of IL-2-synthesizing ability of TC1 cells would alter their functions, we analyzed the cytolytic activity of IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells toward labeled allogeneic tumor cell lines. 6 d after the generation of TC1 cells in IL-2 plus anti-IL-4, the cultures were split and the cells were stimulated for another 6 d with M12.4.1 cells in the presence of (a) IL-2 plus anti-IL-4, (b) IL-2 plus IL-12 plus anti-IL-4, or (c) IL-2 plus IL-4. On day 12, cells were harvested to analyze their cytolytic ability and cytokine production. Fig. 11 a confirms that cells grown in the presence of IL-4 do not synthesize IL-2, whereas the cells grown in anti-IL-4 or IL-12 plus anti-IL-4 remained IL-2<sup>+</sup>IFN-γ<sup>+</sup>. TC1 cells grown in all three growth conditions were cytolytic toward allogeneic (P815) but not syngeneic (EL4) targets (Fig. 11 b). The cytotoxicity of the IL-2<sup>-</sup> cells was equal to that of the IL-2<sup>+</sup> cells grown in IL-12, suggesting that IL-2 synthesis does not affect cytotoxicity.

**Loss of IL-2 Synthesis Impairs Autonomous Proliferation of TC1 Cells.** We tested whether the lack of IL-2-synthesizing ability would render TC1 cells incapable of antigen-dependent proliferation. IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells (Fig. 12 a) were stimulated with irradiated allogeneic J774 cells in the presence or absence of different concentrations of IL-2 and IL-4. As a measure of proliferation, DNA synthe-



**Figure 11.** IL-4-derived CD8<sup>+</sup> TC1 cells that secrete IFN- $\gamma$  but not IL-2 are highly cytolytic. C57BL/6 spleen CD8<sup>+</sup> T cells were stimulated with M12.4.1 cells in the presence of IL-2 plus anti-IL-4 for 6 d. TC1 cells generated were then split and restimulated with allogeneic M12.4.1 cells in the presence of IL-2 plus anti-IL-4, IL-2 plus IL-12 plus anti-IL-4, and IL-2 plus IL-4. On day 12, aliquots of TC1 cells were analyzed for (a) cytokine secretion after Con A stimulation for 24 h, and (b) cytotoxicity against <sup>51</sup>Cr-labeled P815 (allogeneic) and EL4 (syngeneic) cells.

sis was then assessed by analyzing the incorporation of labeled thymidine every day for up to 4 d. Fig. 12 b demonstrates that in the absence of any exogenous cytokines, IL-2<sup>-</sup> and IL-2<sup>+</sup> TC1 cells proliferated to a similar degree for the first day after antigen stimulation, whereas at later times proliferation continued in the IL-2<sup>+</sup> population but declined rapidly in the IL-2<sup>-</sup> cells. Addition of exogenous growth factors slightly enhanced proliferation of IL-2<sup>+</sup> TC1 cells at day 4, presumably because the cells had used up the IL-2 synthesized during the initial stimulation. On the other hand, exogenous IL-2 strongly enhanced the proliferation of the IL-2<sup>-</sup> TC1 cells, and, at 1.5 ng/ml, almost completely restored proliferation to the levels of IL-2<sup>+</sup> cells. IL-4 also enhanced proliferation of IL-2<sup>-</sup> cells but did not fully restore proliferation even at 15 ng/ml. The combination of IL-2 (0.5 ng/ml) and IL-4 (15 ng/ml) fully restored proliferation of the IL-2<sup>-</sup> cells. Thus, antigen-stimulated IL-2<sup>-</sup> TC1 cells remained responsive to T cell growth factors but were dependent on the provision of these cytokines from other sources.



**Figure 12.** Exogenous cytokines are required to correct the proliferative defect in IL-2<sup>-</sup> TC1 cells. Allospecific (C57BL/6 CD8<sup>+</sup> anti-J774A.1) IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells were generated as described in Fig. 1. On day 12, TC1 cells ( $1.1 \times 10^4$ /ml) were restimulated with irradiated J774A.1 cells ( $5 \times 10^4$ /ml) in the presence or absence of various growth factors for up to 4 d. Radiolabeled thymidine was added (1  $\mu$ Ci/well) at the same time or after every 24 h. Cells were then harvested 24 h after the thymidine pulse, and radioactive incorporation was determined by liquid scintillation counting.

## Discussion

Antigen stimulation of naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the presence of IL-12 and/or IFN- $\gamma$  induces differentiation into the committed Th1 and TC1 phenotypes, both secreting IL-2 and IFN- $\gamma$ . However, long-term Th1 clones often lose the ability to synthesize IL-2 while retaining IFN- $\gamma$  synthesis (2). Among short-term CD8<sup>+</sup> clones, some do not synthesize IL-2 even when first tested (4, 44), suggesting that CD8<sup>+</sup> T cells may more rapidly lose the ability to synthesize IL-2. We now show that IL-4 rapidly induced committed TC1 cells to lose their subsequent ability to synthesize IL-2 but not IFN- $\gamma$ , GM-CSF, or TNF. Synthesis of IL-2 by Th1 clones was also reduced by IL-4, although this required higher IL-4 concentrations, and complete loss was not observed. These results raise the possibility that endogenous IL-4 production by APC might account for the eventual loss of IL-2 synthesis by long-term clones.

The mechanism of action of IL-4 on TC1 cells is not yet clear. IL-4-induced inhibition of IL-2-synthesizing ability occurs rapidly, within 24 h of IL-4 treatment, even in the

absence of APC. This proves that IL-4 acts directly on TC1 cells to impair their subsequent ability to synthesize IL-2. The IL-2<sup>+</sup> → IL-2<sup>-</sup> transition occurred when TC1 cells were treated with IL-4 during the resting phase, or during stimulation with either M12.4.1 (B cell-like) or J774 (macrophage-like) APC lines. This suggests that the IL-4 effect cannot be prevented by costimulatory signals supplied by these potent APC. Once established, the IL-2<sup>-</sup> phenotype cannot be reversed by stimulation with APC in the presence or absence of IL-2, or by activation with anti-CD3 and anti-CD28. Several mechanisms may account for the IL-4 effect: Direct modification of the IL-2 gene, for example, by methylation, may result in long-term inactivation. IL-4 may permanently alter expression of some of the factors that bind to the regulatory elements in the IL-2 gene promoter (45). A long-term, selective alteration in IL-2 messenger RNA processing or translation may be induced. Finally, IL-4 may prevent the expression of a component of the signaling pathways, particularly in the B7-CD28/CTLA4 pathway, which provides an essential costimulatory signal required for T cell stimulation, especially for IL-2 production (46). Expression of CD28 itself is similar on IL-2<sup>-</sup> and IL-2<sup>+</sup> TC1 cells, but CTLA4 expression or a subsequent step in the signaling pathway may be altered.

During stimulation of naive T cells, IL-4 inhibits the generation of cells secreting the Th1/TC1 pattern of cytokines and induces the appearance of cells that secrete the Th2/TC2 pattern (4, 6, 18, 19). IL-4 also inhibits the synthesis of IL-2 by naive CD4<sup>+</sup> T cells in short-term culture (47). These effects are distinct from our current observation, as IL-4 induces naive cells to switch to the TC2 phenotype, whereas TC1 cells only lose the ability to secrete IL-2 but do not show other changes. In both cases, however, IL-4 induces differentiation rather than short-term modulation, as neither the naive CD8<sup>+</sup> → TC2 nor the IL-2<sup>+</sup> TC1 → IL-2<sup>-</sup> TC1 transitions are reversible. The effects of IL-4 on naive and committed cells may be brought about by different mechanisms, as IL-4-induced differentiation of naive CD8<sup>+</sup> T cells into TC2 cells involves loss of IL-2 production accompanied by permanent alteration of the synthesis of several other cytokines, whereas the IL-2<sup>+</sup> TC1 → IL-2<sup>-</sup> TC1 transition appears to be very selective, involving only the IL-2 gene.

When CD4<sup>+</sup> Th1 cells are stimulated in the presence of fixed APC or anti-CD3 antibodies, they selectively lose the ability to synthesize IL-2 and proliferate, while retaining cytotoxicity, secretion of other cytokines, and responsiveness to IL-2 (27, 28). This state has been referred to as "anergy," although anergy previously meant a lack of effector function rather than a selective loss of autonomous proliferation. Most experiments on the induction of anergy have focused on CD4<sup>+</sup> cells, but a similar state can be induced by stimulation of CD8<sup>+</sup> cells with fixed APC (29). These conditions allow TCR engagement in the absence of the crucial second (costimulatory) signal mediated by APC. Although anergic CD4<sup>+</sup> cells are unable to synthesize IL-2 and proliferate autonomously, they remain fully antigen responsive, as assessed by the synthesis of other cytokines. In

contrast to the important role of costimulatory signals in previous reports on the induction or prevention of anergy, our results show that the IL-2<sup>-</sup> state of TC1 cells can be induced by IL-4 in the presence or absence of TCR or costimulatory signals. Although the mechanisms of induction are distinct, it is still possible that the IL-2<sup>-</sup> TC1 cells generated in the presence of IL-4 are equivalent to the "anergic" T cells derived by stimulation in the absence of costimulatory signals.

The state of anergy in CD4<sup>+</sup> cells can be reversed by stimulation with allogenic non-T cells and IL-2 in some systems (30, 31) but not others (33). Our results clearly indicate that IL-4-induced loss of IL-2-synthesizing ability in TC1 CD8<sup>+</sup> cells occurs even in the presence of excess IL-2 and is not reversed by stimulation in the presence of anti-CD28 antibodies, or by continued culture with IL-2, even with potent APC and anti-IL-4 antibodies.

The requirements for costimulation and the induction of anergy may be different for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. CD4<sup>+</sup> cells are stimulated by MHC class II<sup>+</sup> cells, which are often APC with strong costimulatory functions such as dendritic cells, B cells, and macrophages. In contrast, CD8<sup>+</sup> cells can potentially recognize antigen on almost any nucleated cell, and so will often be stimulated with antigen/MHC in the absence of costimulation. Also, CD8<sup>+</sup> effector T cells kill targets rapidly, so that there is little opportunity for the target cell to up-regulate expression of costimulators as occurs during CD4-APC interaction. Thus, CD8<sup>+</sup> cells may require less costimulation than CD4<sup>+</sup> cells, consistent with a report that fibroblasts can stimulate an efficient CD8<sup>+</sup> response (48), although IL-2 synthesis was not measured. However, some requirement for costimulation is indicated by the ability of fixed APC to induce anergy in CD8<sup>+</sup> cells (29). Another difference between CD4<sup>+</sup> and CD8<sup>+</sup> cells may relate to sensitivity to IL-4. Human CD4<sup>+</sup> cells can be anergized by APC lacking costimulators, and this is prevented by IL-4 (32). In contrast, our studies on mouse CD8<sup>+</sup> cells demonstrate that they are "anergized" by IL-4.

Both the IL-2<sup>+</sup> and IL-2<sup>-</sup> TC1 cells were highly cytotoxic when cultured in IL-12 and IL-4, respectively. Therefore, IL-2 secretion by a T cell during the effector-target interaction does not appear to affect cytotoxicity. Similarly, cytotoxicity was retained by both CD4<sup>+</sup> and CD8<sup>+</sup> anergic T cells lacking IL-2-synthesizing ability induced by fixed APC (29, 49). In contrast to their ability to kill target cells and synthesize other cytokines, TC1 cells that lacked IL-2-synthesizing ability were unable to proliferate when stimulated in the absence of exogenous growth factors. Proliferation was restored by the addition of IL-2, and to a lesser extent by IL-4. Thus, CD8<sup>+</sup> TC1 cells lacking IL-2-synthesizing ability appear to retain short-term effector function (cytotoxicity) while losing their ability to proliferate autonomously.

Thus, the loss of IL-2 production by TC1 cells may have more effect on clonal expansion than on effector functions. If TC1 responses coexist with Th2 or TC2 responses in vivo, TC1 cells might lose their ability to secrete IL-2 because of local IL-4 secretion. This may impair clonal ex-



pansion of the TC1 cells, as they would now be dependent on T cell growth factors produced by other cells, such as IL-2 and IL-4 produced by other T cells or IL-15 produced by nonlymphoid cells such as muscle cells, fibroblasts, or monocytes (50). Because of these other sources of T cell growth factors in vivo, anergic T cells may show damping of responses in vivo in contrast to the abrogation of responses seen in tissue culture systems that lack many of the cell types that contribute to in vivo responses.

The induction of the TC1 IL-2<sup>-</sup> phenotype by IL-4 is

consistent with other mechanisms whereby type 1 and type 2 responses cross-regulate each other. IL-10 and IL-4 produced by Th2 or TC2 cells inhibit IFN- $\gamma$  synthesis and differentiation of Th1 and TC1 cells (4, 18, 21, 22, 51). Conversely, IFN- $\gamma$  secreted by Th1 and TC1 cells inhibits the proliferation of Th2 cells (23, 24). Our results now suggest that Th2 and TC2 cells, via production of IL-4, may limit the extent of a TC1 response by preventing autonomous expansion in the absence of growth factors from other cells, but maintaining short-term effector functions.

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Address correspondence to Dr. Tim R. Mosmann, Department of Medical Microbiology and Immunology, 865 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

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