Control of CD4 Effector Fate: Transforming Growth Factor β1 and Interleukin 2 Synergize to Prevent Apoptosis and Promote Effector Expansion

By Xiaohong Zhang,* Lizzie Giangreco,* H. Elizabeth Broome,‡ Catherine M. Dargan,‡ and Susan L. Swain*

*From the *Department of Biology and the Cancer Center, and ‡Department of Pathology, University of California, San Diego, La Jolla, California 92093-0063*

Summary

The signals that determine the size and duration of the primary T cell immune response are not well defined. We studied CD4 T cells at an important checkpoint in their development: when they have become effectors and are ready to rapidly mediate effector functions, both via direct interaction with antigen (Ag)-presenting cells and via cytokine production. We determined the effects of specific Ag and the cytokines interleukin (IL) 2 and transforming growth factor (TGF) β1 on T helper cell type 2 (Th2) effector apoptosis versus expansion. Th2-polarized effector cells were generated in vitro from naive CD4 T of T cell receptor transgenic mice, and then restimulated with or without peptide Ag plus Ag-presenting cells and cytokines. In the absence of added cytokines, effector cells cultured without Ag died of apoptosis after 4–7 d. Paradoxically, Ag both induced proliferation and high levels of cytokine synthesis and accelerated effector cell death. IL-2 directly induced proliferation of effectors, supported and prolonged Ag-induced proliferation, and partially blocked apoptosis. TGF-β did not effect proliferation or influence cytokine secretion, but it also partially blocked apoptosis. Together, IL-2 and TGF-β synergized to almost completely block apoptosis, resulting in prolonged effector expansion and leading to the accumulation of a large pool of specific effectors. When Ag and both cytokines were present, the effector population increased 10^4–10^5-fold over 20 d of culture. The synergy of IL-2 and TGF-β suggests that they interfere with programmed cell death by distinct mechanisms. Since Th2 effectors are specialized to help B cells develop into antibody-secreting plasma cells, these results suggest that the availability of Ag and of the cytokines IL-2 and TGF-β is a key factor influencing the fate of Th2 effector cells and thus the size and duration of the primary antibody response.

The response of lymphocytes to external stimuli can include activation of cytokine production, proliferation, differentiation, and expansion or death by apoptosis (for review see references 1 and 2). Lymphocytes at different stages of development respond differentially to Ag stimulation. For example, as developing T cells express specific TCR in the thymus, they change from rapidly proliferating cells to small resting cells that express a default program for apoptosis unless they undergo positive selection (3). These same T cells are susceptible to activation-induced cell death (AICD)[1] if they recognize Ag with high avidity (negative selection) (4). In contrast, mature naive T cells persist for several weeks in situ without Ag stimulation (5, 6). Appropriate Ag stimulation results in IL-2 production, proliferation, and development of effector T cells (5, 7). The frequency of naive T cells able to recognize any particular protein Ag is ~1 in 10^4–10^6 (8). Thus, a protective response requires substantial expansion of a few specific cells to generate a large population of effector cells. After infection with a replicating organism, the effector population must be large enough to deal with a large pathogen load. On the other hand, once the Ag/organism has been cleared, the effectors are no longer needed and are taking up valuable space in the immune system, and, because of their potent effector functions and low activation requirements (9), they are potentially dangerous. Effector responses in vivo (5–7) are often transient, and effectors have a short life span in vitro (10). Indeed, responses to some viruses are accompanied by high rates of T cell apoptosis (11) and "exhaustion" of the immune response (12). In other studies, however, high numbers of effectors have been generated, and effector responses were found to be prolonged (13).

[1] *Abbreviations used in this paper:* AICD, activation-induced cell death; FL, fluoresceinated; mit C, mitomycin C; PCCF, pigeon cytochrome C fragment; PI, propidium iodide; Tg, transgenic; TdT, terminal deoxynucleotidyl transferase.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/09/0699/11 $2.00
Volume 182 September 1995 699–709

The AICD of activated normal T cells or T cell lines has been described in many recent reports (14–19). It has been suggested that IL-2 and high Ag dose play key roles in inducing apoptosis, and that this is responsible for the termination of effector responses and also perhaps for tolerance seen at high Ag dose (18). Most of these reports have used Th1-polarized populations, which secrete IL-2, IFN-γ, and TNF-α. The conditions favoring the expansion of effector populations are less well defined and have not been extensively studied. Experience in the generation of T cell lines and clones indicates that IL-2 and occasional Ag stimulation are required for the maintenance of both Th1 and Th2 lines (for review see references 20 and 21). IL-2 plays a central role in T cell response, acting as the major product of naive CD4 T, which can provide autocrine growth of responding cells. In many T cell responses, the degree of T cell expansion is proportional to the amount of IL-2 available, and IL-2 deprivation leads to apoptosis (19, 22).

TGF-β1 (and other TGF-β family members) have a large number of pleiotropic effects on T cells (for review see reference 23). Most studies have stressed its growth-inhibitory effects (23–25), but we found TGF-β to have long-term growth-promoting effects when it was added to naive CD4 (26), and addition of TGF-β to effector generation cultures resulted in effectors that secreted higher titers of IL-2 and expressed a distinct phenotype more like that of memory cells. TGF-β also increased cell proliferation and IFN-γ production in CD8 cells (27).

To investigate the factors that regulate expansion of Th2 effectors, we designed experiments to analyze the roles of Ag, IL-2, and TGF-β in effector cell recovery, proliferation, and apoptosis. Effector T cells are activated cells, ready to quickly mediate effector function upon restimulation without further differentiation (5, 8). They express high levels of adhesion and costimulatory receptors (5, 28), and they synthesize and secrete cytokines within a few hours of stimulation (29). This contrasts to resting naive and memory T lymphocytes, which require several days of proliferation and/or differentiation before they attain optimal functionality (8, 10). We generate Th2 effectors in short-term in vitro cultures from isolated, naive CD4 T cells of TCR transgenic mice (Tg) (28), allowing us to use a defined Ag (a pigeon cytochrome C peptide [PCCF]) to study Ag effects in a well-defined, recently ex vivo model. We focus here on Th2 effectors that produce high levels of IL-4 and IL-5 but little IL-2 (28), allowing uncomplicated analysis of IL-2 effects. Th2 effectors carry out unique functions in immune response, promoting B cell differentiation and IgG1, IgA, and IgE Ab production (9). Moreover, the fate of Th2 effectors has received little attention.

We report that, in the absence of added cytokines, effectors undergo apoptosis after several days of culture, but that Ag stimulation accelerates their death. However, IL-2 and TGF-β synergize to dramatically block apoptosis and sustain an impressive expansion of the population. These studies identify the effector stage as a key checkpoint at which the immune response can be either terminated or prolonged and expanded, and which might be amenable to therapeutic intervention.

Materials and Methods

Mice. H-$2^{b/k}$ or H-$2^{k/k}$ Vβ3/Vα11 and TCR Tg mice (28) were bred in the animal facilities at the University of California, San Diego, and used at 3-6 mo of age.

Reagents. Medium for all in vitro cultures was complete RPMI 1640 supplemented with penicillin (200 μg/liter), streptomycin (200 μg/liter), and glutamine (4 μM) plus 10% selected FCS from Hyclone Laboratories (Logan, UT). PCCF 88-104, used as Ag in all experiments, was synthesized by the peptide synthesis facility at the University of California, San Diego, and purified by HPLC.

Cytokines. Recombinant IL-2 (human, 10-20 U/ml from the Biological Response Modifiers Program, National Institutes of Health, Bethesda, MD) or murine IL-2 (derived from the X63-IL-2 line transfected with the murine IL-2 gene [30]) were used interchangeably and had equivalent effects. Porcine TGF-β1 (referred to as TGF-β1 throughout) was purchased from R&D Systems (Minneapolis, MN). Control cytokines used to standardize cytokine assays IL-2, IL-4, and IL-5 were purified from the X63.Ag8-653 lines transfected with the murine cytokine genes (30). Recombinant murine IFN-γ was obtained from Genzyme Corp. (Cambridge, MA).

Cell Preparations. Purified naive CD4$^+$ T cells from Tg mice were isolated as described previously (28). Spleen cells were passed over nylon columns to remove B, adherent, and activated cells, and then treated with anti-CD8 and anti-HSA (J11D) plus C'. Cells were >90% CD4$^+$ resting cells with naive phenotype (28, 31). APC for effector generation were prepared by T cell depletion of spleen cells with 2 anti-Thy 1.2 Ab (F7D5 and HO13.14), anti-CD4 (RL172.4), and anti-CD8 (HO2.2 and AD4) plus C' and treatment with 50 μg/ml of mitomycin C (mit C). For restimulation of effectors, mit C–treated cells of DCEK-ICAM, a fibroblast line transfected with I-E^k and ICAM-1 (32), which expresses high levels of B7.1 (Dubey, C., M. Croft, and S. L. Swain, unpublished observations), was used.

CD4 effectors were generated as previously reported (28). Purified T cells (3×10^5/ml) were cultured with APC and PCCF plus 20 U/ml IL-2. IL-4 (200 U/ml) plus 10 μg/ml anti–IFN-γ was added at the start of culture to generate Th2 effectors. Effectors were harvested, counted, and washed after 4-5 d, and then recultured at 5×10^5/ml viable CD4$^+$ cells with either 1.7×10^5/ml of APC plus 5 μM of PCCF (Ag/APC) or APC alone. Additions of IL-2 (20 U/ml), TGF-β (1 ng/ml), or IL-2 plus TGF-β were made to aliquots of the second cultures. Cells in flasks (T75; Falcon Labware, Oxnard, CA) were split if they became concentrated. According to the design of the experiment, recultured effector cells were harvested at different days, counted, and further analyzed.

DNA Fragmentation Assay. Effectors (2×10^6) were washed and resuspended in 15 μl of lysis buffer (15% Ficoll, 10 μg/ml boiled RNase A [Boehringer Mannheim Corp., Indianapolis, IN], 0.01% bromphenol blue in 1 × Tris acetate/EDTA electrophoresis buffer). Samples were stored at −70°C until separation by gel electrophoresis. DNA ladder gels were premade in two parts: an upper part of 0.8% agarose with 1.25 mg/ml of proteinase K and 2% SDS, and a lower part of 2% agarose. Samples were run in the upper part at 20 V for 1 h and then in the lower part at 90 V for 3 h. Gels were gently agitated overnight in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.8) containing 20 μg/ml RNase and stained with 5 μg/ml ethidium bromide for 30 min. Photographs were taken under UV light.

Terminal Deoxynucleotidyl Transferase (TdT) Staining for Detection of Nicked DNA. Using a method adapted from Gorczyca (33), effectors were harvested, stained with rat anti-murine CD4 conjugated with PE (PE anti-CD4), fixed in 1% formaldehyde for 15

min on ice, washed with PBS, and stored in 70% ethanol at 0-4°C. For analysis, cells were washed and incubated at 1×10^6 in 50 μl primary buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 2.5 mM cobalt chloride, 0.25 mg/ml BSA, 100 U/ml TdT, and 10 mM biotin-16-dUTP, both from Boehringer Mannheim Corp.) for 30 min at 37°C. Washed samples were resuspended in 100 μl of secondary buffer (2.5 μg/ml fluoresceinated [FL] streptavidin, 0.1% Triton X-100, and 5% nonfat dry milk in 4× saline sodium citrate buffer [Sigma Chemical Co., St. Louis, MO]). After 30 min at room temperature in the dark, gated $CD4^+$ cells were analyzed by FACScan® (Becton Dickinson and Co., Cockeysville, MD).

Cell Cycle Analysis. Cells were stained with FL-anti-CD4, fixed in 60% ethanol, washed, and incubated with 10 U/ml RNase A and 2 μg/ml propidium iodide (PI) as described by Nicoletti et al. (34). The programs MULTICYCLE and MCYCLE (Phoenix Flow Systems, San Diego, CA) were used for analysis.

Cytokine Analysis. Titers of IL-2, IL-4, IL-5, and IFN-γ were determined from supernatants collected 18-24 h after restimulation of effector cells as described previously (7).

Cell Recovery and Proliferation. Effectors were harvested either after the generation culture (day 4) or after further reculture. Cell recovery was determined by counting the density of live, trypan blue-excluding cells. Cells were stained with PE anti-CD4, and the percentage of $CD4^+$ cells was determined by FACScan® analysis. Fold expansion was calculated from the number of recovered cells divided by the CD4 in initial culture. For proliferation, [^{3}H]thymidine was added for the final 12-16 h of culture, and incorporation was determined on a betaplate counter. The mean counts per min per culture of triplicate cultures is reported.

Results

Experimental Design. All experiments had the same basic design. Th2 effectors were generated in vitro, harvested, and divided into groups for reculture. Aliquots were restimulated with APC, with or without PCCF, with either: no cytokine, IL-2 alone, TGF-β alone, or both. Recovered cells were analyzed at different times after restimulation as indicated. Tg CD4 cells are almost all naive (31), and after 4–5 d of stimulation they develop into a population of effectors (as judged by their functional activities and phenotype) (28). This first culture routinely results in a 10-fold increase in cells under standard conditions (IL-2 added at 20 U/ml) (see Fig. 1) and a much larger increase in the per-cell capacity for the secretion of IL-4 (28). The mit C–treated APC used to generate effectors disappear by the end of culture. The APC used to restimulate the effectors also disappear rapidly, constituting <5% of recovered cells at 24 h and <1–3% thereafter (not shown). We focus here on Th2 effectors by adding IL-4 (plus anti–IFN-γ) to the generation cultures, which results in effectors polarized for Th2 cytokine production (10, 28, and see Figs. 6 and 7). The effectors were routinely analyzed by staining and were invariably $CD4^+$, Tg TCR^+ cells with an activated phenotype including high expression of CD44, CD45RB, and IL-2R (28).

Effect of Ag Stimulation and Cytokines IL-2 and TGF-β on Effector Expansion. A representative analysis of live CD4 recovery at the end of the effector generation culture (day 4) and 4 d (day 8) and 7 d (day 11) after restimulation with APC is shown in Fig. 1. Th2 effectors were cultured with APC, with and without PCCF, and with and without recombinant cytokines IL-2 and/or TGF-β. IL-2 was added every 2 or 3 d, while TGF-β, PCCF, and APC were added only at the time of restimulation.

CD4 T expanded 10-fold in the Th2 effector generation culture. In the absence of reexposure to Ag (Fig. 1, *right*), effector recovery in cultures without cytokines was unchanged for >4 d of reculture (8 d total), but the population then declined, virtually disappearing over the next 3 d. In contrast, effectors restimulated with 5 μM PCCF (Fig. 1, *left*) remained roughly at prestimulation levels, even though Ag stimulation led to high levels of IL-4 secretion and proliferation (see Figs. 4, 6, and 7). Addition of TGF-β alone had no effect on cell recovery in "no Ag" cultures but led to an approximately twofold increase in recovered cells in cultures with Ag. Addition of IL-2 led to dramatic effector expansion in both PCCF and no Ag cultures. IL-2 directly supported expansion of effectors but became less effective in supporting proliferation after 4 d. Without Ag, the IL-2–driven populations died off within another week (not shown). Expansion in the absence of Ag, but with IL-2, was approximately five- to seven-fold. IL-2 and Ag together supported greater expansion. Addition of TGF-β to IL-2–supplemented cultures resulted in expansion that was just slightly greater than with IL-2 alone approximately two- to threefold in the series of experiments) for the first 4 d, but subsequent recovery showed

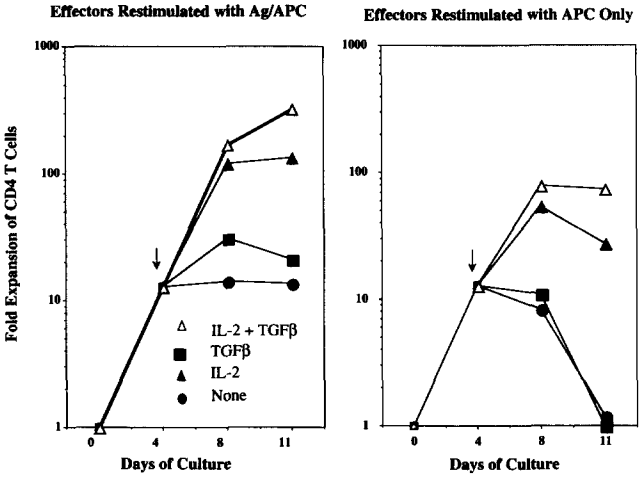

Figure 1. Effects of Ag and cytokines on effector expansion. Tg^+, Th2 effector cells were generated as described in Materials and Methods. 5×10^5/ml effectors were recultured with 1.7×10^5/ml APC (mit C–treated DCEK-ICAM) in the presence (*left*) or absence (*right*) of PCCF (5 μM). Replicate groups were harvested 4 and 7 d later. Expansion of CD4 T cells was calculated by determining the viable CD4 recovery and comparing it with the original number of naive CD4 cells cultured at day 0. Effectors were recultured with: no additional cytokine (●), 20 U/ml of IL-2 (▲), 1 ng/ml of TGF-β (■), or both TGF-β and IL-2 (△) added at the beginning of restimulation. IL-2 was given when fresh medium was added. Results are representative of three similar experiments.

more enhancement. By 11 d of culture (a week after restimulation), Ag-stimulated effectors that received periodic IL-2 and treatment with TGF-β at day 4 had expanded >30-fold (an expansion of 300-fold from the culture of naive cells). Similar levels of expansion have been observed in three experiments with Th2 effectors. The positive effects of Ag stimulation, IL-2, and TGF-β on effector expansion have been seen in six experiments.

Effect of Ag Stimulation, IL-2, and TGF-β on Effector Apoptosis. Net expansion of cells is a function of both increased number due to division and loss due to death. To assess the effects of Ag and cytokines on apoptosis, we compared the levels of DNA fragmentation in groups of naive CD4 T cells, fresh Th2 effector cells at day 4, and effector cells restimulated for 2, 4, or 7 d. We compared groups of effectors restimulated with Ag plus APC (Fig. 2, *top*) with those stimulated with APC alone (Fig. 2, *bottom*). Cells at each of these time points were >95% $CD4^+$ cells.

DNA from fresh, naive cells and from freshly isolated effectors was largely intact. DNA of recultured effectors without Ag or cytokines (Fig. 2, *bottom*) remained largely intact for 4 d, with ladders appearing by day 7. DNA from

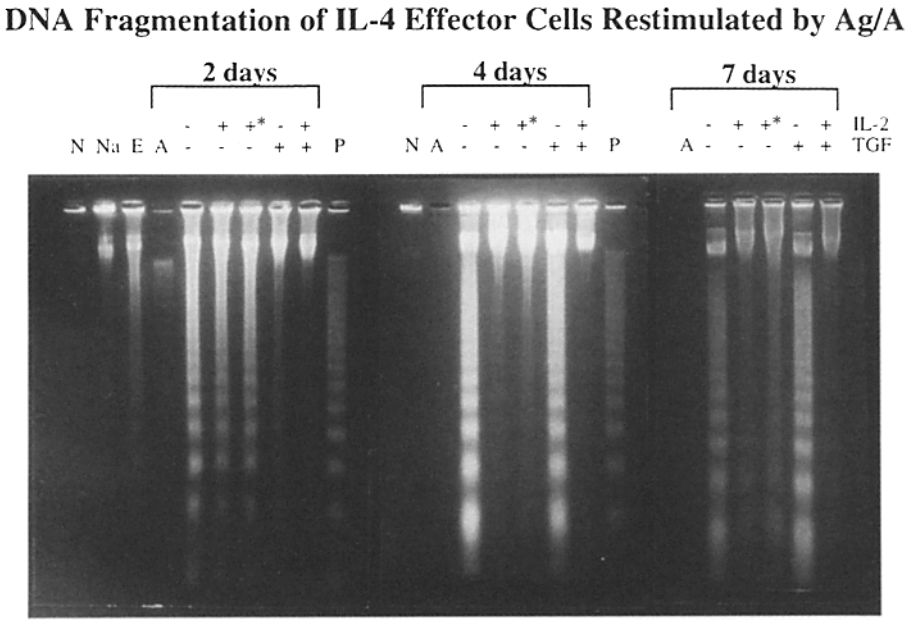

Figure 2. Effects of Ag and cytokines on DNA fragmentation. Th2 effectors were recultured with or without Ag and cytokines as described in Fig. 1. After 2, 4, and 7 d of reculture, the DNA was extracted from 2 × 10^6 cells from each group and run on gels for detection of DNA ladders. DNA from fresh Th2 effectors (*E*), from fresh naive CD4 T cells (*Na*), and from a sample of APC (*A*) were also analyzed. DNA from fresh thymocytes (negative; *N*) and from thymocytes treated with dexamethasone (positive; *P*) were included as controls. Effector cells were cultured with: no additional cytokines, IL-2 at 20 U/ml, IL-2 and anti-TGF-β (5 μg/ml) (indicated by asterisk), TGF-β at 1 ng/ml, or TGF-β and IL-2. Results are representative of three similar experiments.

PCCF-stimulated cells with no cytokines showed marked DNA ladders by 2 d of culture, which peaked at day 4 and continued to be prominent at day 7 (Fig. 2, *top*). Thus, Ag stimulation accelerated the rate of cell death. It should be noted that, in repeat experiments, maximum DNA fragmentation in Ag-stimulated cultures occurred slightly later, around day 4 (see Fig. 3), suggesting that Th2 effector cells undergo quite delayed apoptosis, even after stimulation with Ag. The level of Ag used here for restimulation (5 μM) is a level that stimulates optimum proliferation and cytokine secretion from both naive and effector CD4 T, but similar levels of apoptosis were seen at 10-fold lower and higher levels of PCCF (see Fig. 6).

In cultures with or without Ag, addition of IL-2 led to decreased DNA laddering at days 4 and 7. Addition of TGF-β decreased fragmentation early but on its own had little effect later. TGF-β is a ubiquitously produced cytokine, so we also tested whether Ab to it would block the effect of IL-2. A slight enhancement of laddering was seen only at 7 d in cultures both with and without Ag. Dramatically, addition of TGF-β with IL-2 was able to prevent all detectable fragmentation in Ag-stimulated as well as in no Ag effectors. In further studies we have concentrated on the effects of cytokines on PCCF-stimulated effectors, because the effects are more dramatic, but the effects of IL-2 and TGF-β in blocking apoptosis are also consistently seen when no Ag is present.

To better quantitate the DNA changes associated with apoptosis, we used the TdT UTP nick-end labeling method to visualize nicks in the DNA. Permeabilized cells were treated with TdT plus biotinylated dUTP followed by FL-avidin, allowing an assessment of the percentage of stained, apoptotic cells. Groups of restimulated Th2 effector populations were analyzed 2, 4, and 7 d after reculture with Ag. TdT staining of $CD4^+$-gated cells is shown in Fig. 3. In cultures without cytokines, nicked DNA was detectable in 18% of the $CD4^+$ population by 2 d and was apparent in >40% of the cells harvested at 4 d and >80% at 7 d. Addition of either IL-2 or TGF-β led to a reduction in cells with nicked DNA at 4 and 7 d after restimulation. The effects of the combination of IL-2 and TGF-β were dramatic. At 2, 4, and 7 d, only ~10% of cells in cultures receiving both cytokines showed evidence of damaged DNA. Thus, IL-2 plus TGF-β treatment of effectors led to prolonged resistance to AICD. Similar protection was achieved in non-Ag-stimulated Th2 effector cultures, although the TdT staining increased more slowly (not shown).

Kinetics of Cytokine Effects on Cell Cycle Progression and Proliferation. To analyze possible mechanisms involved in IL-2 and TGF-β effects, we analyzed the kinetics of DNA synthesis and cell cycle progression of Ag-restimulated Th2 effectors. Effectors were harvested after 0, 1, 2, and 4 d and stained with PI, and the proportion of cells in G_0/G_1, S, G_2, and M was determined. Fig. 4 (*left*) shows the total fraction of cycling effectors. The distribution of cells in each of the phases of cycle was proportional in each group (not shown). Before restimulation, approximately one-third of the cells were actively cycling (day 0), compared with <5% among naive CD4 T (not shown). Ag stimulation led to a large increase in the fraction of cells in active cycle 1 d later (day 1), regardless of cytokines added. No increase is seen in cultures not receiving Ag (not shown). Over the next 3 d, the proportion of cycling cells decreased in each group. Cultures without IL-2 had <20% cells in cycle by day 4, whereas significantly more were found in IL-2–treated cultures. The highest proportion of cycling cells at days 2–4 were found in the IL-2 plus TGF-β group. In some models, TGF-β has been shown to cause cell cycle arrest, especially in G_1, and suppression of cell growth (35–37). In this case, TGF-β did not cause cell cycle arrest in G_1, nor did it slow cell cycle progression.

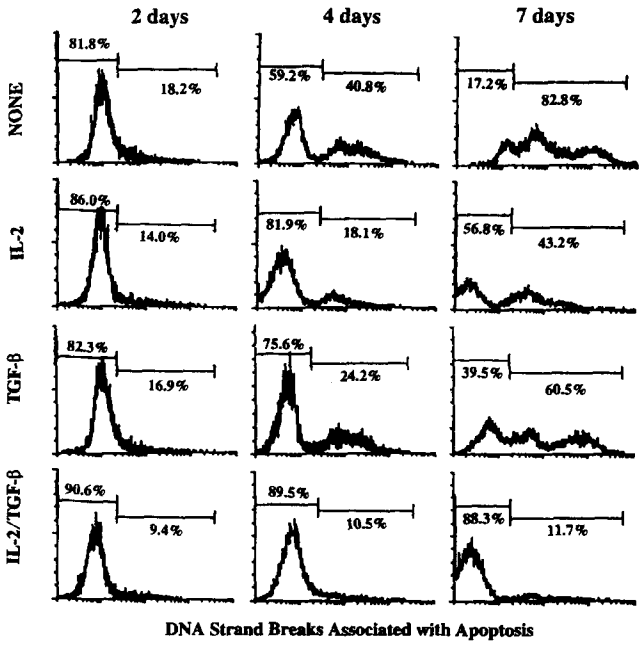

Figure 3. Effects of cytokines on DNA nick formation in Ag-stimulated Th2 effector cells. Th2 effector cells were restimulated with PCCF (5 μM) and APC with no additional cytokine (*NONE, top*), IL-2 (20 U/ml), TGF-β (1 ng/ml), or TGF-β plus IL-2. After 2, 4, and 7 d of reculture, cells were stained with the enzyme TdT, biotinylated dUTP, and FL-streptavidin. PE-conjugated anti-CD4 was used to counterstain. Histograms represent the log FL (TdT staining) of CD4 positive cells, analyzed by FACScan® Lysis II software (Becton Dickinson and Co.). The percentage of CD4 cells falling within the indicated negative (determined by omitting the TdT) and positive gates is indicated in each histogram. The results are representative of three similar experiments.

DNA synthesis, commonly used as an indicator of "proliferation," was analyzed in cultures of effectors restimulated with (Fig. 4, *middle*) or without (Fig. 4, *right*) PCCF. With Ag, effectors incorporated radiolabel with or without cytokines at days 1 and 2 (presumably responding to the IL-4 they secrete), but the IL-2–supplemented groups had prolonged synthesis of DNA. Effectors in cultures without PCCF (which produced no endogenous cytokine) synthesized little DNA (Fig. 4 *C*). IL-2, but not TGF-β, directly supported DNA synthesis of non-Ag-stimulated cells. Thus, TGF-β did not directly promote growth of the Th2 effectors, but it prolonged IL-2–supported proliferation.

IL-2 and TGF-β Synergize over a Broad Range of Doses. In

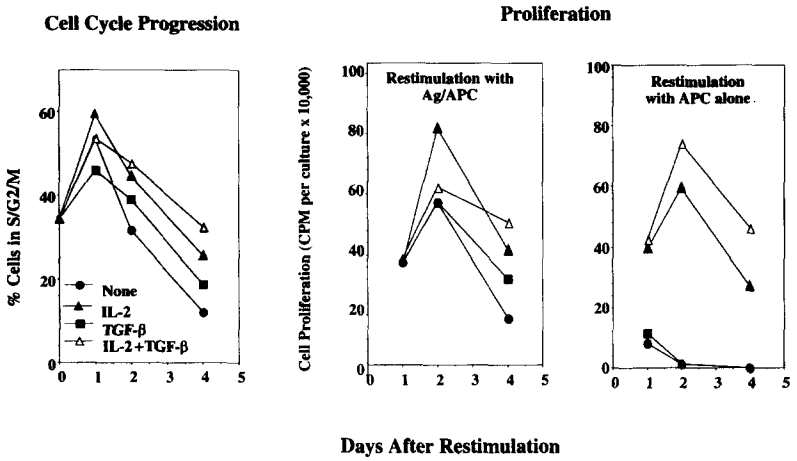

Figure 4. Kinetics of cytokine effects. Th2 effectors were restimulated and divided into groups for analysis of cell cycle progression at days 0, 1, 2, and 4 and proliferation at days 1, 2, and 4. For analysis of cell cycle (*left*), effectors were analyzed immediately (day 0) or after restimulation with PCCF (5 μM) plus APC with the indicated cytokine additions: none (●), IL-2 (▲), TGF-β (■), or both TGF-β and IL-2 (△), for 1, 2, or 4 d. Cells were stained with PI (and PE-CD4), and the fraction of gated CD4+ cells in G_0/G_1, S, G_2, and M was determined. The left panel shows the combined percentage in S, G_2, and M. The portion of cells in each of the phases of active cycling was very similar for each group (not shown). For proliferation (DNA synthesis), effectors were restimulated with PCCF plus APC (*middle*) or APC alone (*right*). Radiolabel was added during day 1, day 2, or day 4 of culture, and incorporation was determined after 18 h.

some cases, high doses of IL-2 have been found to promote rather than inhibit apoptosis (17, 18), so we investigated the effects of IL-2 dose (Fig. 5). Th2 effectors were restimulated for 4 d in the presence of different amounts of IL-2, with or without TGF-β. Representative results of two experiments are shown in Fig. 5. Doses of IL-2 from 0.6 to 600 U/ml led to enhanced effector cell recovery (not shown) and decreased apoptosis. Moreover, addition of TGF-β at each dose markedly enhanced the antiapoptotic effect of IL-2, indicating synergy between the two factors. We also compared different doses of TGF-β added to a constant dose of IL-2 (20 U/ml) for effects on Ag-stimulated cultures. Addition of TGF-β at 10, 1, and 0.1 ng reduced apoptosis as indicated by DNA fragmentation when IL-2 was present, and each of these doses also reduced fragmentation in the absence of IL-2 (not shown).

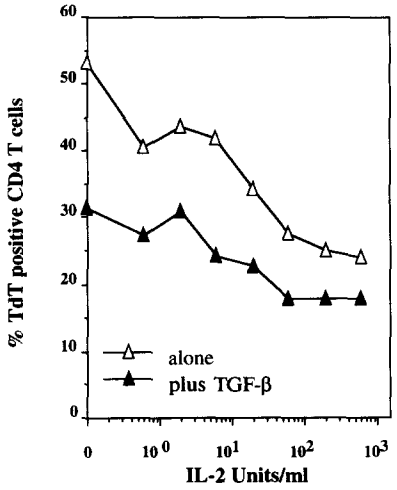

Figure 5. Effects of IL-2 concentration. Th2 effector cells were restimulated with PCCF plus APC plus IL-2 added in amounts ranging from 0.6 to 600 U/ml, with (▲) or without (△) TGF-β (1 ng/ml). After 4 d, cells from each group were stained with PE-CD4 and counted. Nicked DNA was determined as in Fig. 3, and the percentage of TdT-positive CD4+ T cells is shown.

IL-2 at 20 U/ml and TGF-β at 1–10 ng/ml were optimally effective and are the standard doses used in our experiments.

Effect of Ag Dose on Effector Fate. Since high Ag dose has been suggested to favor activated T cell apoptosis (18), we compared the effect of PCCF dose on apoptosis, IL-4 production, and DNA synthesis, each measured 2 d after restimulation. Results are shown in Fig. 6. TdT staining, IL-4 production, and DNA synthesis each increased with Ag dose, becoming maximum in the range of 1–10 μM PCCF. Thus, induction of each of these responses occurred at comparable Ag doses.

Effects of IL-2 and TGF-β on Cytokine Production. Effectors rapidly synthesize and secrete high titers of cytokines in an Ag-dependent fashion (29). Cytokines secreted by Ag-stimulated Th2 effectors could potentially act to drive sustained proliferation and protect effectors from apoptosis. IL-4 is a growth factor for effectors (Swain, S. L., unpublished observations) as well as Th2 cell lines. Thus, IL-2 and/or TGF-β might function by altering the pattern or amount of effector cytokines. To assess this possibility, Th2 effectors were restimulated with Ag and with or without IL-2 and/or TGF-β. After 24 h, effectors were harvested, washed to remove added cytokines, and recultured. Supernatants from each group were harvested after 18 h, and titers of IL-2, IFN-γ, IL-4, and IL-5 were determined. As shown in Fig. 7, effectors in each group made a Th2 pattern of cytokines: high titers of IL-4 and IL-5 and low to undetectable titers of IL-2 and IFN-γ. IL-2 and IL-2 plus TGF-β increased cytokine production slightly, but there was no change in the relative ratios of the different cytokines. Similar results were seen in supernatants collected in the first 24 h and from cells treated with cytokines for 2 or 4 d (not shown). Addition of exogenous IL-4 did not block apoptosis (not shown).

Prolonged Effects of TGF-β Treatment on Long-term Effector Expansion. Since IL-2 and TGF-β together effectively block apoptosis, and since IL-2 sustains proliferation of activated T cells, we reasoned that effector cells periodically restimulated with Ag, IL-2, and TGF-β might be able to undergo many rounds of proliferation. To test this prediction, Th2 effectors were generated, harvested, and counted after 6 d and

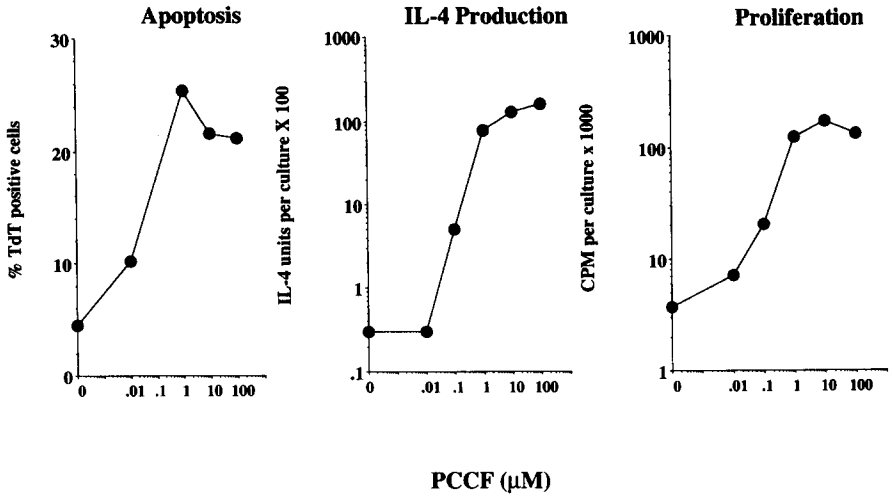

Figure 6. Effect of Ag dose on effector response. Th2 effectors were recultured with PCCF at doses ranging from 0.01 to 100 μM with APC but no cytokines. After 2 d groups were harvested, and groups were then stained with TdT as a measure of apoptosis (*left*); supernatant was harvested and assayed for IL-4 by ELISA (*middle*); or [^{3}H]thymidine was added and incorporation was determined for the final 18 h (*right*).

restimulated with Ag/APC plus either IL-2 alone or IL-2 plus TGF-β. Recovery was determined after 12 d, and, after 14 d, cultures were harvested and counted again, and an aliquot was restimulated with Ag/APC. Fresh medium with IL-2 was added whenever cultures became dense. Cultures were harvested after 20 d. At each harvest time CD4 recovery was determined. The long-term effector expansion shown in Fig. 8 was truly impressive. After 20 d, Th2 effectors, stimulated with TGF-β at day 6, had expanded >15,000-fold compared with the number of naive CD4 T cultured at day 0. Without TGF-β, total expansion was severalfold less. In other experiments, it was found that the addition of TGF-β at least one more time late in culture gave additional increases in CD4 effector recovery (not shown). At all time points the effector populations made impressive amounts of appropriate cytokines when restimulated with Ag/APC (not shown). It was particularly striking that the effects of TGF-β added only at day 6 were still detectable 2 wk later.

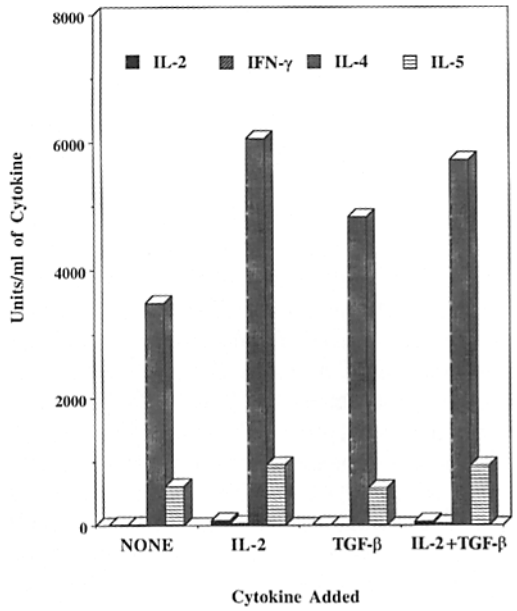

Fig. 7. Effects of cytokines on cytokine production. Th2 effectors were restimulated with PCCF plus APC for 1 d with no cytokine, with IL-2, with TGF-β, or with both IL-2 and TGF-β, as indicated. After that incubation, cells were washed and restimulated with PCCF and APC without cytokines. Culture supernatants were harvested 24 h later, and IL-2, IFN-γ, IL-4, and IL-5 titers were determined.

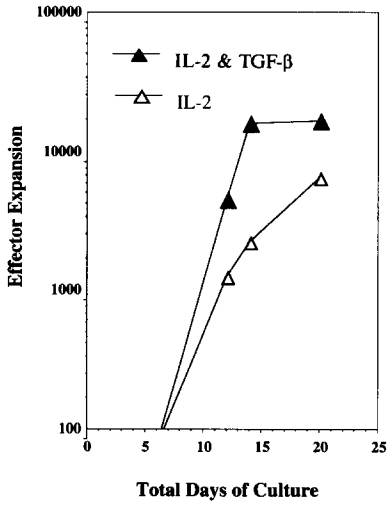

Figure 8. Effect of cytokines on long-term effector expansion. Th2 effectors were generated. After 6 d, 5 × 10^5/ml effector cells were recultured with PCCF plus APC in the presence of 20 U/ml of IL-2 (△) or IL-2 plus 1 ng/ml of TGF-β (▲). TGF-β was added only at day 6. IL-2 was added periodically when fresh medium was used. Cells were counted at 12 and 14 d. Aliquots of harvested cells were restimulated by PCCF plus APC at day 14, and cells were harvested again after a total culture of 20 d. Cell recovery, calculated by determining the number of CD4 projected if all effectors were recultured on day 14, was compared with the number of naive cells to calculate effector expansion over the entire culture period.

Discussion

The mechanisms underlying expansion and contraction of Ag-specific lymphocytes during immune response and the conditions that regulate the denouement or prolongation of the response have received far less attention than those factors that promote its initiation. Results from the experiments in this paper suggest that the effector stage is an important checkpoint for regulation of the size and duration of the primary response of CD4 T cells, and that the Th2 effector population can either be programmed or induced to die of apoptosis or can expand up to 1,000-fold over the next 2 wk. The experiments clearly identify several factors that regulate these fates of CD4 effector cells, including: (*a*) Ag stimulation, which promotes proliferation, cytokine production, and apoptosis, with the outcome of its effects depending on the presence of other factors; (*b*) IL-2, which supports expansion and also participates in blocking apoptotic death; and (*c*) TGF-β, which synergizes with IL-2 to block apoptosis and promote effector expansion, but which does not act as a growth factor. Thus, it appears that the array of signals that impinge on recently generated effector cells will determine whether the Th2 response is terminated or prolonged and increased, and that this stage of an immune response may be one at which therapeutic intervention is particularly relevant. While recent studies have focused on the conditions favoring exhaustion of effector responses by induction of apoptosis (14–19), the current study supports the concept that conditions can also be found that favor the opposite outcome of expanded response.

Ag-specific, Th2-polarized effectors generated in vitro from naive CD4 cells of TCR Tg mice were used to examine the consequences of restimulation with Ag, IL-2, and TGF-β. Effectors carry out their functions of cytokine production and helper function rapidly after restimulation (10, 29) and are easily detected after primary Ag injection in vivo as well as in vitro (7), attesting to their physiological relevance. Transfer of identical populations to adoptive hosts results in the development of long-term CD4 memory, indicating their potential for further development (28). Here we monitored the kinetics of effector expansion, cytokine production, DNA synthesis, cell cycle progression, and apoptotic death.

Ag stimulation induced each response, including production of high titers of specific cytokine by 24 h (Figs. 6 and 7; 28), entry into cell cycle and DNA synthesis (Fig. 4) within 1 d, and a delayed apoptosis (AICD), which commenced after the burst of cytokine production at 2–4 d (Figs. 1 and 2). Thus, in the absence of interfering cytokines, restimulated effectors synthesized and secreted cytokines and underwent cell division but did not expand in number (Fig. 1), because a majority of the population was dead or dying by 4 d. It is likely that TCR triggering induced expression of components of an apoptotic pathway, a result reported previously for bulk-activated T cells and Th1 lines (14–17). The AICD of Th2 effectors is considerably slower than that reported for cell lines or hybridomas (38). The dose of Ag optimal for each of these was comparable (Fig. 6), supporting a model in which common signal transduction events are shared by the different pathways (38). Critchfield et al., reported that apoptosis in Th1 clones and recently activated CD4 T cells was most evident at high Ag dose (18). These results suggest a paradigm in which effectors reencountering Ag but not receiving additional signals will carry out their effector functions and then die of apoptosis. This phenomenon is liable to be largely responsible for the apparent exhaustion of responses to viruses that remain present at high titers (12) and also to cases of high dose tolerance, as described by Lenardo and co-workers (18). It can also be postulated that this kind of mechanism of exhaustion of responses to persisting Ag may be an important component of protection against autoimmunity.

Interestingly, effectors not exposed to Ag survived several days and only then began to die of apoptosis, causing the loss of the effector population after 4–8 d of culture (Fig. 1). This in vitro data parallels the kinetics of an in vivo response to a soluble protein such as KLH, which shows a sharp peak of effector function at days 4–7, with a decline after that to baseline levels by day 14 (8). Thus, in the absence of Ag or other mitigating factors, the effector population is short lived and the response is transient. This kind of phenomenon would provide a natural termination of the effector response once the response had succeeded in clearing the organism or Ag that initially induced it.

The most novel finding is that TGF-β and IL-2 can synergize to prevent the apoptotic death (Figs. 2 and 3) of Th2 effectors, leading to prolonged expansion of the effector population (Figs. 1 and 8). Both IL-2 and TGF-β alone were able to partially block apoptosis. IL-2 is the major growth factor for T cells, directly supporting cell cycle progression in activated T cells expressing IL-2R (20) such as effectors. Activated lymphocytes are often addicted to growth factors, suffering apoptotic death when they are withdrawn (for review see references 19 and 39). This appears to be the case for activated T cells, which, in a number of models, die unless IL-2 is provided (22), and it is seen here for Th2 effectors. Prolonged, dramatic expansion of Th1 effectors was also seen under the same conditions (not shown).

Recent studies of Critchfield et al. described a facilitating effect of IL-2 in the apoptosis of in vitro activated CD4 T cells and Th1 clones (18). They found that the degree of apoptosis was correlated with the amount IL-2 during the first stage of culture and restimulation and that IL-2 was necessary for the apoptosis of a Th1 clone. Those results are in apparent contrast to the results reported here for Th2 effectors. We observed that IL-2, as expected, directly induced DNA synthesis of effectors, and enhanced cell cycle progression (Fig. 4), but here it led to a decrease in DNA laddering in both Ag-stimulated and unstimulated effectors (Fig. 2), as well as a decrease in the proportion of cells with damaged, nicked DNA (Fig. 3). At no concentration we tested, from 0.6 to 600 U/ml of IL-2, did the addition of IL-2 increase apoptosis (Fig. 5). Presumably as a consequence of these effects, IL-2, when present continuously, supported impressive expansion of effectors (Figs. 1 and 8). Several factors could explain the apparent differences between that report and the results here.

First, we have used Th2, not Th1-like cells, and there could be differences between the two subsets. Indeed, we have noted some differences between Th1 and Th2 effectors within the first few days after restimulation (Zhang, X., and S. L. Swain, unpublished observations). Second, we have analyzed expansion over a longer time frame. Further experiments will be needed to determine which of these factors accounts for the difference in results. We are currently exploring whether Th1 effectors behave more like the Th1 lines and undergo rapid, IL-2–dependent apoptosis.

In some models, apoptosis occurs preferentially during a particular phase of cell cycle such as G_1 (35, 36), G_2 (39), or S (40), although others report no such dependence (15, 19). IL-2 did not seem to protect effectors merely by driving them through cell cycle, since Ag stimulation, which was as efficient in driving cell cycle progression and DNA synthesis (Fig. 4), resulted in high levels of apoptosis. Moreover, the addition of IL-4, which also supports proliferation, did not block apoptosis (Zhang, X., and S. L. Swain, unpublished observations), and the stimulated Th2 effectors made abundant IL-4 (Fig. 6).

Particularly striking is the unexpected ability of TGF-β to block apoptosis. TGF-β is a highly pleiotropic cytokine with multiple positive and negative effects on growth and differentiation of a wide range of cell types. The TGF-β family and other related cytokines are noted for their effects on cell differentiation (41, 42). In the immune system it is best known for its negative effects on T and B cell proliferation (23–25) and its ability to drive B cells to switch to the IgA isotype of Ig (43). TGF-β has even been reported to induce apoptosis of some kinds of cells (44, 45). The effects of TGF-β thus seem to differ with the cell type and/or the stage of differentiation, and it has been suggested that it acts as a kind of switch to change whatever pattern of differentiation is currently in place (25). We found earlier that TGF-β had a series of interesting effects when it was added during effector generation. Addition of TGF-β enhanced the ability of effector populations generated to produce IL-2 and caused them to down-regulate CD45RB, an isoform of the intracytoplasmic CD45 phosphatase expressed on activated effectors but not on resting memory cells (26). Effectors generated in the presence of TGF-β also grew better in IL-2–containing medium than did those cells that were never exposed to TGF-β. In studies of CD8 cells, similar effects of TGF-β were noted (27). The effects of TGF-β did not appear to be mediated in this instance via a shift in production of the cytokines IL-2, IFN-γ, IL-4, and IL-5, commonly used to subdivide Th1 and Th2 effectors (Fig. 7). Of course, we cannot rule out an indirect effect via untested cytokines.

TGF-β alone partially blocked apoptosis of effectors and synergized with IL-2 to almost completely block apoptosis (Figs. 2 and 3). TGF-β alone modestly increased effector cell recovery, but, in contrast to IL-2, it did not act as a direct proliferation factor for unstimulated effectors (Figs. 1 and 4). This supports the hypothesis that TGF-β acts to block apoptosis via a different pathway from IL-2. Prolonged protection from apoptosis required IL-2. The relevance of the antiapoptotic effects is seen in the fact that, when both IL-2 and TGF-β were added, effectors were able to undergo multiple rounds of expansion, yielding populations 10,000–100,000-fold the size of the naive cell population (Fig. 8). Substantial growth also occurred in non–Ag-restimulated cultures, but total expansion was less and expansion was evident for a shorter time. These observations identify for the first time a set of factors–Ag, IL-2, and TGF-β–that can work together to promote extensive expansion of an effector population in vitro, without the period of massive death or "crisis" that usually accompanies the development of Ag-specific cell lines and clones.

The mechanism(s) of the TGF-β and IL-2 actions are not yet clear. Since effectors die in the absence of further stimulation, it seems likely that many of the components of the apoptotic pathway are already present and/or are constitutively produced, and that the action of TGF-β and perhaps IL-2 might include synthesis of products that block those components. Preliminary studies showed that Bcl-2, a protein implicated in blocking programmed death (1, 2), is found at reduced levels in Ag-stimulated effectors, and that treatment of effectors with either TGF-β or IL-2 led to increased maintenance of Bcl-2 levels (Zhang, X., L. Giangreco, H. E. Broome, and S. L. Swain, unpublished observations). We are currently studying in more detail the effects of Ag and cytokines on Bcl-2 and its family members to try to pinpoint how each cytokine may be functioning. Other aspects of the mechanism of apoptosis and its suppression, including the potential role of the Fas pathway, are also being investigated.

We suggest that the effector phase of a T cell response is an important checkpoint at which very different outcomes are possible. The potential impact of the different effector cell fates on in vivo immune response is great. Extrapolating from the in vitro studies, we would predict that the effector phase of a specific response can be either quite small and very short if Ag is rapidly cleared or if Ag is present but has failed to generate local cytokine levels of sufficient magnitude. On the other hand, if Ag levels continue to be high and a vigorous CD4 response including production of cytokines such as TGF-β and IL-2 occurs, effectors could continue to expand and function. It is likely that other factors that also regulate these decisions will be found, and it can be predicted that these might include not only other cytokines but also signals delivered by various costimulatory pathways during interaction of T cells with APC. Since many pathogens replicate rapidly, this would seem to be a useful mechanism to ensure that effector populations sufficiently large to effectively control the pathogen would be generated. Moreover, it might prove feasible to design forms of immune intervention at the time of effector response to a pathogen or after vaccination and to develop strategies either to exhaust unwanted responses or to enhance those that are desired.

We wish to thank Gail Huston, Deshon Hall-Bryant, and Edda Roberts for mechanical assistance and Terry Chamberlin for help in preparing the manuscript.

This work was supported by National Institutes of Health grant RO1Al26887.

Address correspondence to Dr. Susan L. Swain, Department of Biology and Cancer Center, University of California, San Diego, 9500 Gilman Drive, 0063, La Jolla, CA 92093-0063.

Received for publication 31 October 1994 and in revised form 15 February 1995.

References

1. Boise, L.H., M. Gonzalez-Garcia, C.E. Postema, L. Ding, T. Lindsten, L.A. Turka, X. Mao, G. Nunez, and C.B. Thompson. 1993. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell.* 74:597–608.
2. Oltavi, Z., C.L. Milliman, and S.J. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell.* 74:609–619.
3. Von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell.* 76:219–228.
4. Nossal, G.J.V. 1994. Negative selection of lymphocytes. *Cell.* 76:229–239.
5. Swain, S.L., L.M. Bradley, M. Croft, S. Tonkonogy, G. Atkins, A.D. Weinberg, D.D. Duncan, S.M. Hedrick, R.W. Dutton, and G. Huston. 1991. Helper T cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunol. Rev.* 123:115–144.
6. Sprent, J. 1994. T and B memory cells. *Cell.* 76:315–322.
7. Bradley, L.M., D.D. Duncan, S. Tonkonogy, and S.L. Swain. 1991. Characterization of antigen-specific CD4$^+$ effector T cells in vivo: immunization results in a transient population of MEL-14$^-$, CD45RB$^-$ helper cells that secretes interleukin 2 (IL-2), IL-3, IL-4, and interferon γ. *J. Exp. Med.* 174:547–559.
8. Bradley, L.M., D.D. Duncan, K. Yoshimoto, and L.S. Swain. 1993. Memory effectors: a potent, IL-4 secreting helper T cell population that develops after restimulation with antigen. *J. Immunol.* 150:3119–3130.
9. Croft, M., and S.L. Swain. 1992. Analysis of CD4$^+$ T cells that provide contact-dependent bystander help to B cells. *J. Immunol.* 149:3157–3165.
10. Swain, S.L., A.D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145:3796–3806.
11. Akbar, A.N., N. Borthwick, M. Salmon, W. Gombert, M. Bofill, N. Shamsadeen, D. Pilling, S. Pett, J.E. Grundy, and G. Janossy. 1993. The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. *J. Exp. Med.* 178:427–438.
12. Moskophidis, D., F. Lechner, H. Pircher, and R.M. Zinkernagel. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature (Lond.).* 362:758–761.
13. Lau, L., B.D. Jamieson, T. Somasudaram, and R. Ahmed. 1994. Cytotoxic T cell memory without antigen. *Nature (Lond.).* 369:648–652.
14. Russell, J.H., C.L. White, D.Y. Loh, and P. Meleed-key. 1991. Receptor-stimulated death pathway is opened by antigen in mature T cells. *Proc. Natl. Acad. Sci. USA.* 88:2151–2155.
15. Wesselborg, S., O. Janssen, and D. Kabelitz. 1993. Induction of activation-driven death (apoptosis) in activated but not resting peripheral blood T cells. *J. Immunol.* 150:4338–4345.
16. Radvanyi, L.G., G.B. Mills, and R.G. Miller. 1993. Religation of the T cell receptor after primary activation of mature T cell inhibits proliferation and induces apoptotic cell death. *J. Immunol.* 150:5704–5715.
17. Lenardo, M.J. 1991. Interleukin-2 programs mouse α/β T lymphocytes for apoptosis. *Nature (Lond.).* 353:858–861.
18. Critchfield, J.M., M.K., Racke, J.C. Zuniga-Pflucker, B. Cannella, C.S. Raine, J. Goverman, and M.J. Lenardo. 1994. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science (Wash. DC).* 263:1139–1143.
19. Kabelitz D., T. Pohl, and K. Pechhold. 1993. Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. *Immunol. Today.* 14:338–339.
20. Gillis, S., and J. Watson. 1981. Interleukin-2 dependent culture of cytolytic T cell lines. *Immunol. Rev.* 54:81–109.
21. Swain, S.L. 1991. Lymphokines and the immune response: the central role of interleukin-2. *Curr. Opin. Immunol.* 3:304–310.
22. Cohen, J.J. 1993. Apoptosis. *Immunol. Today.* 14:126–130.
23. Massague, J. 1990. The transforming growth factor-β family. *Annu. Rev. Cell Biol.* 6:597–641.
24. Kehrl, J.H., L.M. Wakefield, A.B. Roberts, S. Jakowlew, M. Alvarez-Mon , R. Derynck, M.B. Sporn, and A.S. Fauci. 1986. Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.* 163:1037–1050.
25. Moses, H.L., E.Y. Yang, and J.A. Pietenpol. 1992. TGFβ stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell.* 63:245–247.
26. Swain, S.L., G. Huston, S. Tonkonogy, and A.D. Weinberg. 1991. TGFb and IL-4 direct helper T cell precursors to develop into distinct CD4 helper cells which differ in lymphokine secretion pattern and cell surface phenotype. *J. Immunol.* 147:2991–3000.
27. Lee, H., and S. Rich. 1991. Co-stimulation of T cell proliferation by transforming growth factor-β1. *J. Immunol.* 147:1127–1133.
28. Swain, S.L. 1994. Generation and persistence of polarized CD4 cells. *Immunity.* 1:543–552.
29. Weinberg, A.D., M. English, and S.L. Swain. 1990. Distinct regulation of lymphokine production is found in fresh versus primed murine helper T cells. *J. Immunol.* 144:1800–1807.
30. Karasuyama, H., and F. Melchars. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4, 5 using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97–104.
31. Croft, M., D. Duncan, and S.L. Swain. 1992. Response of naive antigen-specific CD4$^+$ T cells: dendritic cells are a major

antigen-presenting cell. *J. Exp. Med.* 176:1431–1437.
32. Kuhlman, P., V.T. Moy, B.A. Lollo, and A.A. Brian. 1991. The accessory function of murine intracellular adhesion molecule-1 in T lymphocyte activation. *J. Immunol.* 146:1773–1782.
33. Gorczyca W., K. Bigman, A. Mittelman, T. Ahmed, J. Gong, M.R. Melamed, and Z. Darzynkiewicz. 1993. Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. *Leukemia (Basingstoke).* 7:659–670.
34. Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and cytometry. *J. Immunol. Methods.* 139: 271–279.
35. Koff, A., M. Ohsuki, K. Polyak, J.M. Roberts, and J. Massague. 1993. Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGFβ. *Science (Wash. DC).* 260:536–539.
36. Ewen, M.E., K. Sluss, L.L. Whitehouse, and D.M. Livingston. 1993. TGFβ inhibition of Cdk4 synthesis is linked to cell cycle arrest. *Cell.* 74:1009–1020.
37. Polyak, K., J.Y. Kato, M.J. Soloman, C.J. Sherr, J. Massague, J.M. Roberts, and A. Koff. 1994. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes & Dev.* 8:9–22.
38. Green, D.R., A. Mahboubi, W. Nishioka, S. Oja, F. Echieverri, Y. Shi, J. Glynn, Y. Yang, J. Ashwell, and R. Bissonette. 1994. Promotion and inhibition of activation-induced apoptosis in T-cell hybridomas by oncogenes and related signals. *Immunol. Rev.* 142:321–342.
39. Sorenson, C.M., M.A. Barry, and A. Eastman. 1990. Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. *J. Natl. Cancer Inst.* 82:749–755.
40. Boehme, S.A., and M.J. Lenardo. 1993. Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. *Eur. J. Immunol.* 23:1552–1560.
41. Testa, U., R. Masciulli, E. Tritarelli, R. Pustorino, G. Mariani, R. Martucci, T. Baberi, A. Camagna, M. Valtieri, and C. Peschle. 1993. Transforming growth factor-β potentiates vitamin D3-induced terminal monocyte differentiation of human leukemic cell lines. *J. Immunol.* 150:2418–2430.
42. Zentella, A., and J. Massague. 1992. Transforming growth factor-β induces myoblast differentiation in the presence of mitogens. *Proc. Natl. Acad. Sci. USA.* 89:5176–5180.
43. Coffman, R.L., D.A. Lebman, and B. Shrader. 1989. Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J. Exp. Med.* 170:1039–1044.
44. Alam, R., P. Forsythe, S. Stafford, and Y. Fukada. 1994. Transforming growth factor β abrogates the effects of hematopoietins on eosinophils and induces their apoptosis. *J. Exp. Med.* 179:1041–1045.
45. Bursch, W., F. Oberhammer, R.L. Jirtle, M. Askari, R. Sedivy, B. Grasl-Kraupp, A.F. Purchio, and R. Schulte-Hermann. 1993. Transforming growth factor-β1 as a signal for induction of cell death by apoptosis. *Br. J. Cancer.* 67:531–536.