

## **Antigen-independent Activation of Naive and Memory Resting T Cells by a Cytokine Combination**

By Derya Unutmaz, Piero Pileri, and Sergio Abrignani

*From Immunobiology Research Institute Siena, 53100 Siena, Italy*

### **Summary**

We investigated whether human resting T cells could be activated to proliferate and display effector function in the absence of T cell receptor occupancy. We report that combination of interleukin 2 (IL-2), tumor necrosis factor  $\alpha$ , and IL-6 activated highly purified naive (CD45RA<sup>+</sup>) and memory (CD45RO<sup>+</sup>) resting CD4<sup>+</sup> T cells to proliferate. Under this condition, memory resting T cells could also display effector function as measured by lymphokine synthesis and help for immunoglobulin production by B cells. This novel Ag-independent pathway of T cell activation may play an important role in vivo in recruiting effector T cells at the site of immune response and in maintaining the clonal size of memory T cells in the absence of antigenic stimulation. Moreover, cytokines can induce proliferation of naive T cells without switch to memory phenotype and this may help the maintenance of the peripheral pool of naive T cells.

It is well known that activation of resting T cells, in vivo, generally results from the occupancy of the TCR by immunogenic peptides bound to MHC molecules. However, there is circumstantial evidence that at infection sites in vivo many more T cells are activated, as measured by mRNA for lymphokines, than would be expected to be responding specifically to pathogens (1). Little is known about the activation mechanisms leading to nonspecific recruitment, i.e., the discrepancy in frequencies between activated and Ag-specific T cells (2).

The experiments presented here were aimed at assessing whether cytokine(s) secreted during Ag-specific activation could in turn activate bystander resting T cells that are not specific for the stimulating Ag. This study was prompted by the observation that highly purified resting CD4<sup>+</sup> T cells could be activated to proliferate in the presence of supernatants from Ag-stimulated T cell clones. Here we report that combination of IL-2, TNF- $\alpha$ , and IL-6 can activate highly purified naive (CD45RA<sup>+</sup>) and memory (CD45RO<sup>+</sup>) resting CD4<sup>+</sup> T cells to express activation markers, enter the cell cycle, and proliferate. Furthermore, memory resting T cells could also display effector function as measured by lymphokine synthesis and help for Ig production by B cells.

### **Materials and Methods**

**Purification of Resting T Cells.** After Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) separation of PBMC from buffy coats of healthy donors, most macrophages were removed by plastic adherence. To obtain a pure resting CD4<sup>+</sup> T cell population, cells were incubated with a cocktail of mAbs against HLA-DR (L-243; American Type Culture Collection [ATCC], Rockville, MD), CD19 (4G7), CD16 (B73.1), CD56 (MY31), CD57 (HNC-1, ATCC), CD8 (OKT8, ATCC), CD11b (OKM-1, ATCC), CD14 (M $\phi$ -P9),

TCR- $\gamma/\delta$  (B1, a gift of G. De Libero, ZLF Basel, Switzerland), CD25 (2A3), CD69 (L78), and CD71 (L01.1). After 30-min incubation on ice, cells were washed twice and incubated with magnetic beads (Dynabeads; Dynal, Oslo, Norway) conjugated with goat anti-mouse IgG and rat anti-mouse IgM, at a 1:4 target/bead ratio. After 30-min incubation, bead-bound cells were removed using rare earth magnet (Advanced Magnetics, Inc., Cambridge, MA). Remaining cells were further purified with four more incubations with beads at increasing target/bead ratios (1:10 to 1:100). Final population was used as a source of resting CD4<sup>+</sup> T cells when >99.3% of the population was TCR- $\alpha/\beta$ <sup>+</sup> (WT/31) and CD4<sup>+</sup> (Leu 3a), as determined by immunofluorescence analyses using a FACScan<sup>®</sup> flow cytometer (Becton Dickinson & Co., Mountain View, CA), and fulfilled the following criteria: (a) small size at the FACS<sup>®</sup> scatter; (b) absence of FACS<sup>®</sup>-detectable levels of the activation markers CD69, CD71, MHC-DR, and IL-2 receptor p55 chain (CD25); (c) absence of cells in the S and G<sub>2</sub>/M parts of the cell cycle; and (d) no significant incorporation of [<sup>3</sup>H]thymidine when exposed to IL-2. In some experiments, resting cells were further negatively sorted as CD45RO<sup>-</sup> (adding the mAb UCHL-1) or CD45RA<sup>-</sup> (adding the mAb L48). If not otherwise indicated, all the mAbs were from Becton Dickinson & Co.

**Preparation of Supernatants.** T cells ( $5 \times 10^5$ /ml) from a tetanus toxoid (TT)-specific clone were cultured with autologous macrophages ( $2.5 \times 10^5$ /ml) that had been prepulsed with or without TT (3  $\mu$ g/ml) (Biocine Sclavo, Siena, Italy). After 16 h, supernatants were collected and filtered with 0.2- $\mu$ m filters. Culture medium has been previously described (3) using 5% human serum or plasma. Effective supernatants were prepared using medium with either 5% human serum (from Florence blood bank) or serum-free media (HL-1; Ventrex, Portland, OR). Similar results were obtained with resting T cells derived from PBMC of six different healthy individuals and with supernatants from activated CD4<sup>+</sup> T cell clones, with different specificity (purified protein derivative [PPD] or pertussis toxin), from four different persons (see Fig. 2 and data not shown).

**Cell Cycle Analysis.** This was performed as described (4) using propidium iodide in combination with anti-CD4 mAb (FITC labeled) staining. Analyses were performed with the FACScan® Lysis II software and doublet discrimination program (Becton Dickinson & Co.).

**Purification of B Cells.** PBMC-derived B cells were stained with FITC-labeled anti-CD19 mAb and purified by positive sorting with FACStar® (Becton Dickinson & Co.). Purity was >98% as determined by staining with anti-CD20 and anti-Ig.

**Helper Assay.** Noncognate helper assays were performed as previously described (5). Briefly, purified autologous PBMC-derived B cells ( $2 \times 10^3$ /well) were cocultured for 12 d with CD4<sup>+</sup>CD45RO<sup>+</sup> resting T cells ( $3 \times 10^4$ /well) in the presence of cytokine combinations as described (see Fig. 3) or on anti-CD3-coated plates. To avoid an effect of cytokines on B cell differentiation, plates were washed after 4-d culture and cytokine combinations were replaced with IL-2 alone. Ig in the supernatants was measured by ELISA (5).

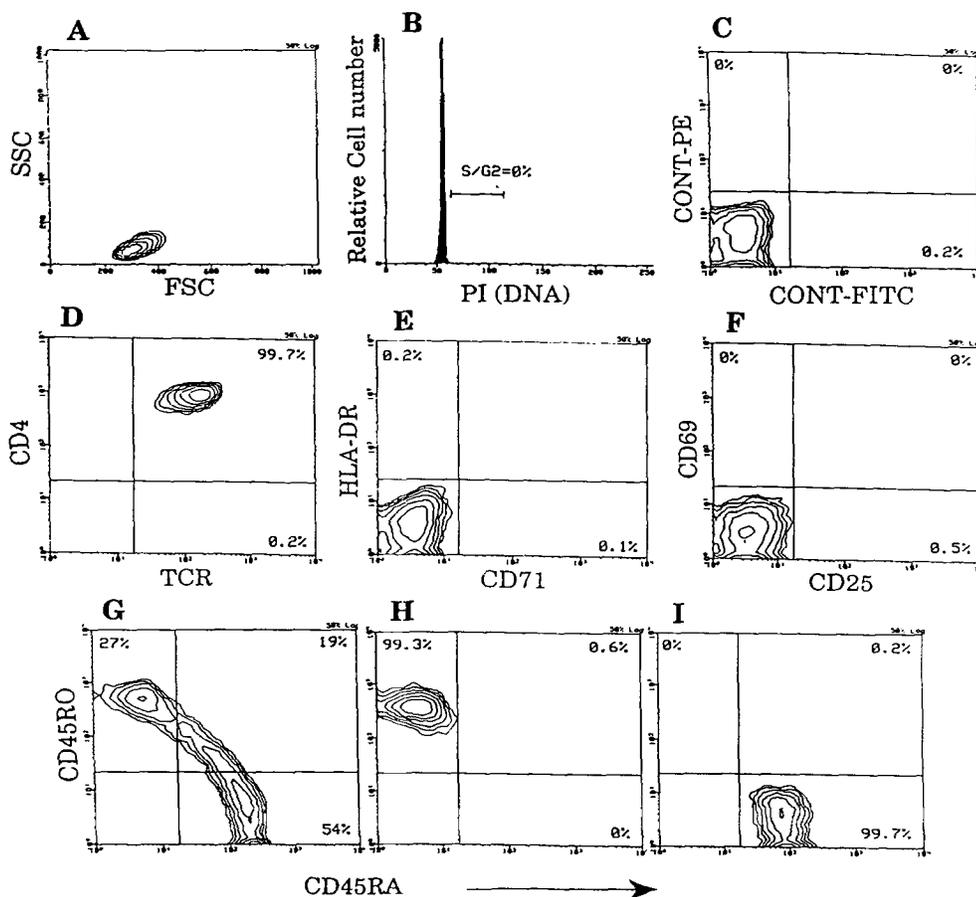
**Activation of Resting T Cells by Supernatants.** Resting T cells were cultured in 96-well flat-bottom plates ( $5 \times 10^4$ /well) with supernatant (50% vol/vol) from T cell clones cultured with autologous macrophages prepulsed with Ag, medium, or rIL-2 (Cetus Corp., Emeryville, CA) at a concentration corresponding to that found in the T cell supernatants (i.e., 200–300 U/ml). Activation was measured at various time points as expression of CD69 and CD25 or [<sup>3</sup>H]thymidine incorporation. In some experiments, [<sup>3</sup>H]thymidine incorporation of resting CD45RO<sup>+</sup> or CD45RA<sup>+</sup> T cells was measured in the presence of different concentrations of IL-2

plus either 1 µg/ml LPS (Difco, Detroit, MI) or supernatant (50% vol/vol) from LPS-activated macrophages. For the preparation of activated macrophage supernatant,  $5 \times 10^5$  macrophages were simulated with 1 µg/ml LPS (for 6–8 h). [<sup>3</sup>H]Thymidine incorporation experiments were performed as described (5). The results represent the mean of triplicate wells and SD was always 15%.

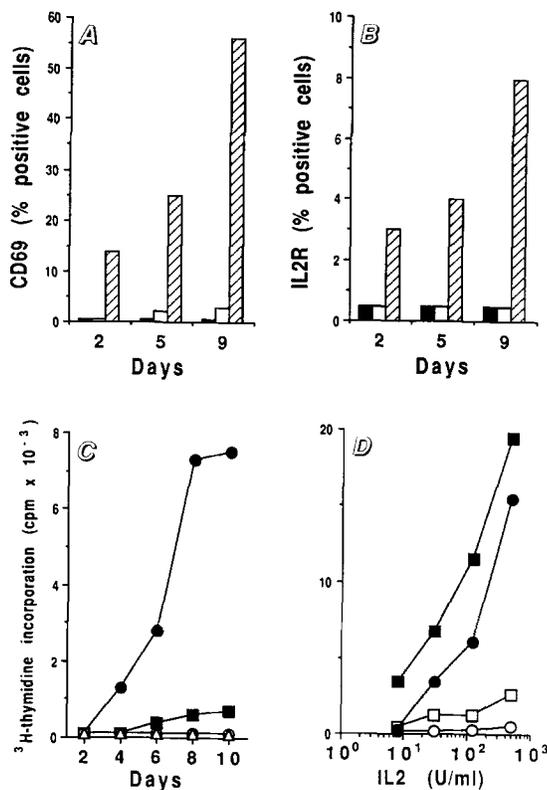
**Activation of Resting T Cells by Recombinant Cytokines.** Resting T cells ( $5 \times 10^4$ /well) in 96-well flat-bottom microplates were cultured for 8 d with various combinations of the following: rIL-2 (200–300 U/ml), rIL-6 (500 U/ml; Ciba-Geigy, Basel, Switzerland; IL-6 units were determined with the B9 assay), TNF-α (25 ng/ml; Genzyme Corp., Cambridge, MA), and supernatant (50% vol/vol) from LPS-stimulated macrophages. Thymidine incorporation and CD69 expression were measured as described in Fig. 2. IL-1b (up to 100 ng/ml, Biocine Sclavo Siena, Italy) in combination with IL-2 and TNF-α did not have any activities (data not shown). Recombinant cytokines from two different sources have been used with similar results. The optimal concentration of cytokines was established in preliminary dose-response experiments.

**PCR-assisted mRNA Amplification.** Purified resting CD4<sup>+</sup>CD45RO<sup>+</sup> T cells were cultured with TNF-α plus IL-2, or IL-2 alone. Total RNA was isolated after 60–100 h of culture from  $5 \times 10^5$  cells, by RNAzol™ B (Biotecx Laboratories, Houston, TX). cDNA was synthesized with murine reverse transcriptase as described (5). β-actin, IL-4, and IFN-γ specific primer pairs were purchased from Clontech (Palo Alto, CA). PCR was performed as described (5).

**Limiting Dilution Analyses.** CD45RO<sup>+</sup> resting T cells were



**Figure 1.** Phenotypic and cell cycle analysis of purified CD4<sup>+</sup> resting T cells. (A) Forward and side scatter profile. (B) Cell cycle analysis. (C) FITC- or PE-conjugated control antibodies. (D–F) Purity of CD4<sup>+</sup> cells and expression of activation markers. (G) Expression of CD45RA and CD45RO Ags on sorted CD4<sup>+</sup> cells. (H and I) CD4<sup>+</sup> cells purified as CD45RO<sup>+</sup> or CD45RA<sup>+</sup> subpopulations.

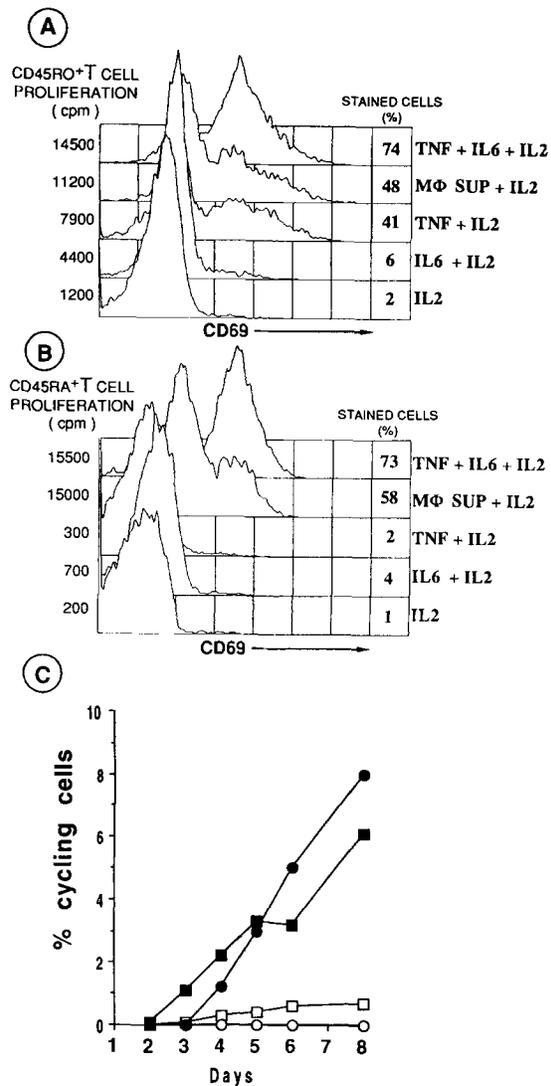


**Figure 2.** Activation of resting CD4<sup>+</sup> T cells by soluble factors. (A and B) Expression of activation markers on resting T cells cultured with supernatant from T cell clones cultured with autologous macrophages prepulsed with Ag (hatched bars) or medium (solid bars), or rIL-2 (open bars). Expression of CD69 or CD25 was analyzed in double staining with anti-CD4. (C) [<sup>3</sup>H]Thymidine incorporation of the same cells in A and B, cultured with medium alone (triangles), rIL-2 (squares), or supernatant from a T cell clone cultured with macrophages prepulsed with Ag (closed circle) or medium (open circle). (D) [<sup>3</sup>H]Thymidine incorporation of resting CD45RO<sup>+</sup> (squares) or CD45RA<sup>+</sup> (circles) T cells in the presence of different concentration of IL-2 plus 1 μg/ml LPS (open symbols), or IL-2 with supernatant from LPS-activated macrophages (closed symbols).

plated at different numbers in Terasaki plates (64 wells per condition) in 20 μl vol in the presence of purified autologous irradiated (2,500 rad) macrophages ( $3 \times 10^3$ /well), anti-DR mAb (L243, 20 μg/ml) with IL-2 alone (300 U/ml) or in combination with TNF-α (25 ng/ml) and IL-6 (500 U/ml). On day 14, cultures were visually inspected for growth. Randomly selected growing wells were positively stained with anti-CD4 and anti-TCR-α/β antibodies. Frequency analyses were done by the least squared method (6).

## Results and Discussion

A critical point of this study was to use a resting population devoid of activated T cells that would respond to IL-2 alone. We chose to work with resting CD4<sup>+</sup> T cells because, at variance with some CD8<sup>+</sup> or γ/δ T cells with resting phenotype, they do not express IL-2 receptor p75-chain in the absence of the p55-chain (7), which may be responsible for unwanted proliferative responses to IL-2 (8) and for which we did not have a good antibody to sort out. We therefore performed multistep exhaustive purifications to obtain



**Figure 3.** Combination of IL-2, TNF-α, and IL-6 activates resting T cells. CD45RO<sup>+</sup> (A) or CD45RA<sup>+</sup> (B) resting T cells were cultured for 8 d with various combinations of the following: rIL-2, rIL-6, TNF-α, and supernatant from LPS-stimulated macrophages. Thymidine incorporation and CD69 expression were measured as described in Fig. 1. (C) Cell cycle analyses of resting CD45RO<sup>+</sup> (squares) or CD45RA<sup>+</sup> (circles) T cells in the presence of IL-2 alone (open symbols) or in combination with TNF-α and IL-6 (closed symbols).

highly purified resting CD4<sup>+</sup> T cells from PBMC (Fig. 1). In preliminary experiments, resting CD4<sup>+</sup> T cells were cultured with supernatants from CD4<sup>+</sup> T cell clones that had been activated by Ag-pulsed macrophages. A representative experiment in Fig. 2 shows that a fraction of resting CD4<sup>+</sup> T cells is activated by the supernatant, but not by IL-2, to express CD69 (9) (Fig. 2 A) and IL-2 receptor p55-chain (Fig. 2 B), and to incorporate [<sup>3</sup>H]thymidine (Fig. 2 C).

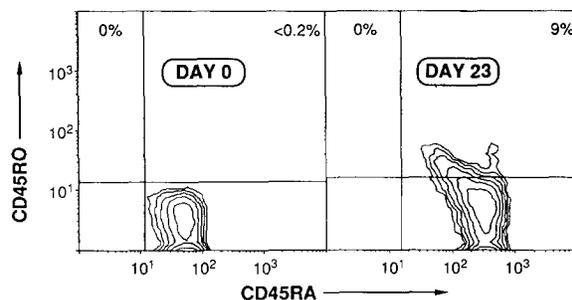
Since the activating supernatant is produced by the coculture of two cell types, we sought to determine the relative contribution of soluble factors produced by T cells and APCs. For this experiment, resting CD4<sup>+</sup> T cells were further

purified as CD45RO<sup>+</sup> (memory) and CD45RA<sup>+</sup> (naive) subpopulations (10), since they may have different activation requirements as already reported for TCR-mediated activation (11, 12). Fig. 2 D shows that supernatant from LPS-activated macrophages alone, as IL-2 alone, did not have any activity, whereas macrophage supernatant in combination with IL-2 induced thymidine incorporation in both CD45RA<sup>+</sup> and CD45RO<sup>+</sup> resting T cells. These results demonstrate that IL-2 and soluble factor(s) produced by APCs are required for the activation of resting T cells.

To identify the APC-derived factor(s), we tested the effect of recombinant cytokines known to be produced by macrophages and to have costimulatory activity on T cells, i.e., IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (13–15). In the absence of IL-2, all the possible combinations of these cytokines did not show any activity over a wide range of concentrations (data not shown). Fig. 3 A shows that TNF- $\alpha$  in combination with IL-2 induced resting CD45RO<sup>+</sup> T cells to express CD69 and to incorporate thymidine, whereas IL-6 in combination with IL-2 was much less effective. Remarkably, TNF- $\alpha$  and IL-6, in combination with IL-2, had a synergistic effect leading to a stronger activation. A similar effect of IL-2, IL-6, and TNF- $\alpha$  was also observed on CD45RA<sup>+</sup> resting T cells (Fig. 3 B), although, in this case, all three cytokines were required to induce activation. Furthermore, the cell cycle analyses in Fig. 3 C show that at day 8 of culture, ~8% of both CD45RO<sup>+</sup> and CD45RA<sup>+</sup> T cells are in the S or G<sub>2</sub>/M phases of the cell cycle. Activation by cytokines, measured as expression of activation markers, thymidine incorporation, or entry into cell cycle, was never inhibited by mAbs specific for DR, CD4, or CD3 (data not shown), thus confirming that TCR signaling is not involved in this type of activation.

It is interesting to note that we have observed that CD45RA<sup>+</sup> T cells activated by cytokines do not switch their phenotype to CD45RO, as was reported to occur within a few days after TCR engagement (16). CD45RA<sup>+</sup> T cells activated by combination of IL-2, TNF- $\alpha$ , and IL-6 were double stained with anti-CD45RA and anti-CD45RO antibodies at 3-d intervals up to day 23 of culture. We never found single positive CD45RO<sup>+</sup> cells at any time point, and only found a few percent of double positive CD45RA<sup>high</sup>/CD45RO<sup>dull</sup>. Indeed, Fig. 4 shows that naive T cells even 23 d after cytokine activation, when most cells are blastic and express CD69 (data not shown), are mainly CD45RA<sup>+</sup>. The same cells activated with anti-CD3 switched in few days to the CD45RO<sup>+</sup> CD45RA<sup>-</sup> phenotype (data not shown).

We next asked whether resting T lymphocytes can be activated by cytokines to display effector function. We performed PCR-assisted mRNA amplification for lymphokines. Fig. 5 shows that both IFN- $\gamma$  and IL-4 mRNA are expressed by CD45RO<sup>+</sup> T cells cultured with IL-2, TNF- $\alpha$ , and IL-6, but not with IL-2 alone. Moreover, CD45RO<sup>+</sup> T cells activated by cytokine combination are as effective as anti-CD3-stimulated T cells in helping B cells to produce Ig (Table 1). To exclude the possibility that T cell help to B cells could be due to activation of autoreactive cells, at the end of the helper assay, the B cells were removed by sorting, and the

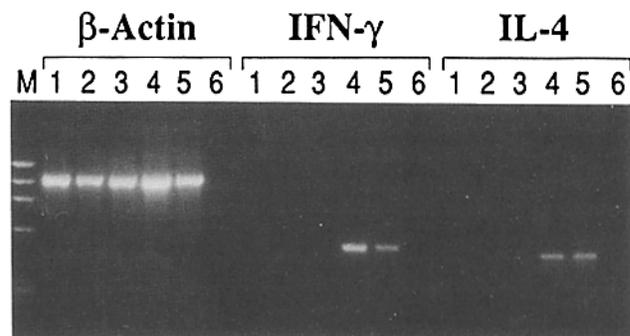


**Figure 4.** CD45RA<sup>+</sup> T cells activated by cytokines do not switch their phenotype to CD45RO. CD45RA<sup>+</sup> T cells were activated by combination of IL-2, TNF- $\alpha$ , and IL-6, and after 23 days were double stained with anti-CD45RA-FITC and anti-CD45RO-PE antibodies.

CD4<sup>+</sup> T cells were tested in proliferation against autologous purified B cells or macrophages. We never found any autoreactive proliferation (data not shown).

Neither cytokines nor anti-CD3 induced CD45RA<sup>+</sup> T cells to produce IFN- $\gamma$  (<1 IU/ml) and to help B cells (data not shown). Thus, we conclude that, similar to TCR-mediated activation (17), cytokines recruit CD45RA<sup>+</sup> T cells to proliferate but not to help Ig production, whereas they activate resting CD45RO<sup>+</sup> T cells to proliferate and display effector functions.

To evaluate the frequency of resting T cells with memory phenotype that could be stimulated by cytokines to grow, we performed limiting dilution experiments. CD45RO<sup>+</sup> CD4<sup>+</sup> resting T cells were cultured with IL-2 alone or in combination with TNF- $\alpha$  and IL-6, in the presence of autologous irradiated macrophages and anti-DR antibodies to prevent autoreactive responses. Fig. 6 shows that 1 of 33 resting CD45RO<sup>+</sup> CD4<sup>+</sup> T cells grew to a visible clone in response to IL-2, TNF- $\alpha$ , and IL-6. At present we do not know why only 3% of cells grew in response to cytokines. The cells that proliferated could have been a subset of resting T cells or could have been at a different stage of maturation/acti-



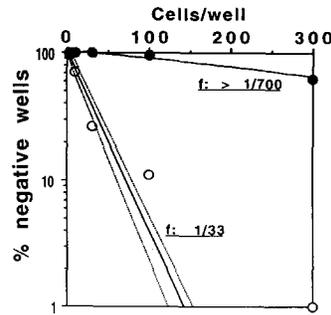
**Figure 5.** Expression of IFN- $\gamma$  and IL-4 mRNA by cytokine-activated T cells. Purified CD4<sup>+</sup>CD45RO<sup>+</sup> resting T cells are cultured with IL-2 alone for 60 (lane 1) and 100 h (lane 3) or with IL-2, TNF- $\alpha$ , and IL-6 for 60 (lane 2) and 100 h (lane 4) as described in Materials and Methods. (Lane 5) Positive template; (lane 6) negative control.

**Table 1.** Resting CD45RO<sup>+</sup> T Cells Activated by Cytokines Can Provide Help to B Cells

	IgM	IgG	IgA
	ng/ml		
B cells cocultured with:			
IL-2 plus TNF- $\alpha$ plus IL-6	<15	<5	<10
T cells plus medium	<15	<5	<10
T cells plus IL-2	<15	<5	<10
T cells plus IL-2 plus TNF- $\alpha$	32	23	<10
T cells plus IL-2 plus IL-6	<15	31	28
T cells plus IL-2 plus TNF- $\alpha$ plus IL-6	75	274	308
T cells plus anti-CD3 mAb plus IL-2	235	219	413

vation. It is possible that many cells ( $\approx 20\%$ ) respond to cytokines and express activation markers. Some of these cells will display effector functions and only a minority (3%) will be able to grow in vitro to a clone of visible size.

TNF- $\alpha$  and IL-6 both have been shown to upregulate IL-2R expression on T cells (15, 18). This could be a possible mechanism for the activation of resting T cells by this cytokine combination. However, resting T cells cultured for 1–3 d with TNF- $\alpha$  and IL-6, and washed and cultured for 4–5 d more with IL-2, did not show FACS<sup>®</sup>-detectable levels of IL-2R (p55) (data not shown), whereas IL-2R was expressed on  $\approx 20\%$  of the same cells cultured with TNF- $\alpha$ , IL-6, and IL-2 from the beginning of the culture. This experiment, however, does not rule out the possibility that low levels of IL-2R, below the FACS<sup>®</sup> sensitivity, are expressed and functionally relevant. Indeed, it has been reported that IL-2 is required for induction of IL-2R by TNF- $\alpha$  or IL-6 (19). Furthermore, IL-2 augments not only expression of its own



**Figure 6.** Frequency of resting T cells that grow in response to cytokine combination. CD45RO<sup>+</sup> resting T cells were plated in the presence of purified autologous macrophages, anti-DR mAb with IL-2 alone (closed circles) or in combination (open circles) with TNF- $\alpha$  and IL-6. (Dotted lines) 95% confidence limits.

receptor (20) but also upregulates TNF- $\alpha$  receptor (21). Elucidation of the mechanism of activation of resting T cells by cytokines will require additional biochemical and molecular analyses.

This novel Ag-independent pathway of T cell activation may play two important roles in vivo, by recruiting effector T cells at the site of immune response and by maintaining the peripheral pool of memory T cells. A scenario could be depicted where resting T cells at sites of Ag-specific response are activated by cytokines produced by specific T cells and macrophages to proliferate and to secrete other lymphokines that can further amplify the response. Indeed, the frequency of resting CD45RO<sup>+</sup> T cells that respond to cytokine combination is definitely higher than the usual frequency of T cells primed by any known Ags.

It has been postulated that memory can be carried by long-lived clones consisting of short-lived cells that require repeated, intermittent stimulation by persisting Ag, by recurrent infection, or by cross-reacting environmental Ags (22–24). In the light of our results, it is tempting to speculate that memory T cells may not require antigenic stimuli to maintain their clonal size, since resting T cells with memory phenotype (CD45RO<sup>+</sup>) can be expanded by cytokines secreted during responses to unrelated antigens. On the other hand, cytokines can induce proliferation of naive cells without switch to memory phenotype and may therefore help to maintain the naive (CD45RA<sup>+</sup>) T cell repertoire.

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Address correspondence to Dr. S. Abrignani, Immunobiology Research Institute Siena, IRIS, Via Fiorentina 1, 53100 Siena, Italy.

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