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Immunology

Interleukin 4 suppresses interleukin 2 and interferon γ production by naive T cells stimulated by accessory cell-dependent receptor engagement

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ABSTRACT **Interleukin 2 (IL-2) and interferon γ (IFN-γ) production by CD4+ T cells and IFN-γ production by CD8+ T cells from naive mice in response to soluble anti-CD3 and antigen-presenting cells (APCs) were strikingly inhibited by culture in the presence of IL-4. IL-4 decreased IL-2 and IFN-γ mRNA levels after 15–24 hr but gave relatively little decrease in these mRNAs at 6–12 hr after stimulation with soluble anti-CD3. A 16-hr preculture of T cells with anti-CD3, APCs, and IL-4 was sufficient to inhibit subsequent production of IL-2 and IFN-γ in response to restimulation in the absence of IL-4. Furthermore, IL-4 treatment of T cells purified 24 hr after stimulation inhibited their capacity to subsequently produce IL-2 in response to anti-CD3 and APCs, indicating that T cells were targets of IL-4-mediated inhibition. IL-4 blocked acute IL-2 production in response to a cytochrome *c* peptide of T cells derived from transgenic mice expressing T-cell receptors specific for cytochrome *c* but it did not block IL-2 production by such cells after they had been primed *in vitro*. Nor did IL-4 inhibit production of IFN-γ by cloned T cells in response to antigen and APCs or production of IL-2 and IFN-γ by naive T cells in response to phorbol ester and calcium ionophore. These results indicate that IL-4 strikingly inhibits IL-2 and IFN-γ production by naive T cells in response to accessory cell-dependent, receptor-mediated stimulation (i.e., soluble anti-CD3 and APCs or antigen and APCs) but does not inhibit accessory cell-independent stimulation of naive T cells or accessory cell-dependent receptor-mediated stimulation of recently primed T cells or cloned T-cell lines.**

Resting CD4+ T cells from naive donors can be stimulated to produce interleukin (IL-2) by polyclonal stimulants or antigens in the presence of antigen-presenting cells (APCs) but produce little or no IL-4 or interferon γ (IFN-γ) (1). *In vitro* priming gives rise to cells that produce the latter lymphokines, but whether IFN-γ or IL-4 is dominantly produced is determined by the conditions of priming. In particular, if IL-4 is present during the priming culture, the resultant cells produce IL-4 but little or no IL-2 or IFN-γ upon subsequent challenge (2–5). By contrast, if IL-4 is absent from the priming culture, the resultant cells produce IL-2 and IFN-γ but no IL-4. The dominant role of IL-4 in determining the outcome of priming leads one to ask what the acute effects of IL-4 are on lymphokine production by naive T cells. Prior studies have shown that addition of IL-4 limits production of IFN-γ (6) and of IL-2 (7) by human peripheral blood cells in response to mitogenic stimuli. By contrast, we have reported that mouse T cells stimulated with immobilized anti-CD3 show enhanced production of IL-2 or IFN-γ if IL-4 is present (8).

In the current study, we show that IL-4 powerfully inhibits acute production of IL-2 and IFN-γ by naive CD4+ and CD8+ T cells in response to accessory cell-dependent stimulation with soluble anti-CD3. Similarly, IL-4 inhibits IL-2 production in response to antigen and APCs of T cells from T-cell antigen receptor (TCR)-transgenic mice. By contrast, IL-4 fails to inhibit IL-2 or IFN-γ production by naive T cells stimulated with phorbol ester and calcium ionophore or by primed T cells or T-cell clones in response to antigen plus APCs.

MATERIALS AND METHODS

Culture Medium, Lymphokines, and Antibodies. RPMI 1640 (Biofluids, Rockville, MD) supplemented with 5% fetal bovine serum (Inovar Biologicals, Gaithersburg, MD), 2-mercaptoethanol (0.05 mM), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and sodium pyruvate (1 mM) was used as culture medium. Recombinant mouse IL-4 was obtained from a baculovirus expression system, utilizing a recombinant *Autographa californica* nuclear polyhedrosis virus (AcMNPV.IL-4) prepared by Cynthia Watson (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD). One unit of IL-4 is equal to ≈0.5 pg. Recombinant human IL-2 was a gift of Cetus. One unit of IL-2, defined as a "Cetus unit", is equal to 6 international units and is ≈0.3 ng. Purified rat anti-mouse IL-4 (11B11) (9) was prepared by Verax (Lebanon, NH). Purified mouse IFN-γ was purchased from Genzyme. Rat anti-mouse IFN-γ (XMG 1.2) (10) and fluorescein-conjugated rat anti-mouse B220 (RA3-6B2) (11), mouse anti-mouse I-A^d (AMS 32.1) (12), rat anti-mouse CD4 (RM 4-5) (13), rat anti-mouse CD8 (53-6.7) (14) and rat anti-mouse Thy 1.2 (30-H12) (13) antibodies were purchased from Pharmingen (San Diego). Anti-CD3 (2C11) (15) was purified by Carol Kinzer (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD) from tissue culture supernatants.

Preparation of T Cells and APCs. BALB/c female mice (8–12 weeks of age) were obtained from the Frederick Cancer Research Center. Lymph node cells were suspended at a concentration of 2×10^7 per ml in RPMI 1640 containing 5 mM EDTA (National Institutes of Health Media Unit, Bethesda, MD) and 5% fetal bovine serum. The cell suspension was incubated with fluorescein-conjugated anti-B220 and FITC anti-I-A^d antibodies for 30 min at 4°C on a turning wheel. The cells were then washed twice and resuspended with magnetic beads coated with sheep anti-fluorescein isothiocyanate antibodies (Advanced Magnetics, Cambridge, MA). Cells that had

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Abbreviations: IL, interleukin; IFN, interferon; APC, antigen-presenting cell; TCR, T-cell antigen receptor.

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bound antibody were depleted by two rounds of exposure to a magnetic field. The residual cells were collected, washed twice, resuspended in culture medium, and used as purified T cells. When $CD4^+$ or $CD8^+$ T cells were required, fluorescein-conjugated anti-CD8 or anti-CD4 antibody was included in the mixture of "depleting" antibodies. For selected experiments, T cells were stained with antibodies specific for CD4, CD8, Thy-1.2, or CD45RB (16A) (16), and the 10–15% brightest or dullest cells were purified by fluorescence-activated cell sorting on a FACStar^{Plus} flow cytometer (Becton Dickinson).

APCs were prepared by removing T cells from splenocytes by treatment with anti-Thy-1.2 (HO13.4; American Type Culture Collection, TIB 99), anti-CD4 (RL172) (17), and anti-CD8 (3.155) (18) antibodies plus Low-tox M rabbit complement (Cederlane Laboratories, Hornby, ON, Canada). The remaining cells were then layered onto a discontinuous Percoll (Pharmacia) gradient and centrifuged for 15 min at $1000 \times g$. The cells in the 50–60% fraction were collected and used as APCs.

For experiments involving lymphokine production in response to a cytochrome *c* peptide, T cells were purified from mice transgenic for genes, obtained from the helper T-cell line 5C.C7, specifying a TCR specific for pigeon cytochrome *c*-(88–104) peptide in conjunction with $I\text{-}E^k$ (B.F.d.S.G. and M. M. Davis, unpublished work). The conditions of priming and the preparation of dendritic cells used as APCs are described in ref. 4.

Lymphokine Assays. Purified T cells (10^6 per ml) and APCs (3×10^5 per ml) were cultured with soluble anti-CD3 (3 μg/ml) in a 24-well Costar plate. Supernatants were collected at 48 hr, and IL-2 concentration was measured with the IL-2-dependent indicator cell line CT.EV (19). IFN-γ concentration was measured with a two-site ELISA (20, 21).

Analysis of Expression of IL-2, IFN-γ, and IL-4 mRNA. Unseparated spleen cells (1.5×10^6 per ml) or purified T cells (10^6 per ml) plus APCs (3×10^5 per ml) were stimulated with soluble anti-CD3 (3 μg/ml) in the presence of anti-IL-4 or IL-4. RNA was isolated by the guanidinium method (22) and 10-μg samples were separated by electrophoresis in a 1% agarose/formaldehyde gel and blotted onto a nitrocellulose membrane (Nytran, Schleicher & Schuell). cDNA probes specific for mouse IL-2, IFN-γ, and IL-4 were ^{32}P-labeled by the random primer method to a specific activity of $0.5\text{–}2 \times 10^9$ cpm/μg. After baking, the filters were prehybridized at 42°C for 1 hr and then hybridized with labeled probe for 18 hr. The filters were washed twice with 300 mM NaCl/30 mM sodium citrate, pH 7/0.1% SDS at room temperature and twice at 60°C with 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% SDS.

RESULTS

IL-4 Suppresses IL-2 and IFN-γ Production by $CD4^+$ and $CD8^+$ T Cells. Lymph node T cells from naive BALB/c mice were stimulated *in vitro* with soluble anti-CD3 and APCs. Anti-IL-4 (10 μg/ml) or IL-4 (1000 units/ml, 38 pM) was added to the cultures, and the IL-2 and IFN-γ produced were measured at 48 hr (Fig. 1). In the presence of IL-4, virtually no IL-2 was detected in the culture fluid and IFN-γ concentration was diminished by a factor of $\approx$5. Half-maximal inhibition of IL-2 production required $\approx$0.5 pM IL-4; maximal inhibition was obtained with 42–127 pM. Purified $CD4^+$ lymph node T cells were excellent IL-2 producers and poor IFN-γ producers, whereas $CD8^+$ T cells were good IFN-γ producers. IL-4 strikingly inhibited production of IL-2 and IFN-γ by $CD4^+$ T cells and of IFN-γ by $CD8^+$ T cells. The $CD8^+$ T-cell population made very little IL-2. Although such IL-2 production was completely inhibited in the presence of IL-4, it is possible that a small contamination of the $CD8^+$ cells with $CD4^+$ cells could have accounted for the observed IL-2 production in this case.

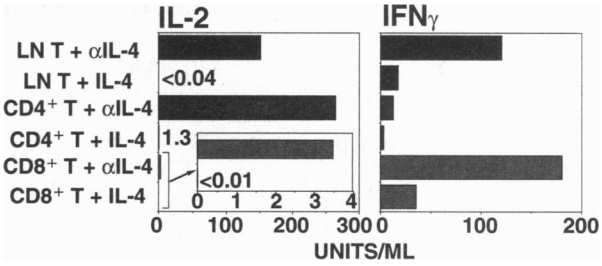

FIG. 1. IL-4 suppresses production of IL-2 and IFN-γ in response to anti-CD3 plus APCs. T cells purified from lymph nodes (LN) or separated into $CD4^+$ and $CD8^+$ populations were stimulated with anti-CD3 and APCs in the presence of IL-4 (1000 units/ml, 38 pM) or anti-IL-4 (αIL-4, 10 μg/ml). IL-2 (*Left*) and IFN-γ (*Right*) were measured at 48 hr.

We also examined IL-2 and of IFN-γ mRNA levels in spleen cell populations stimulated with anti-CD3 in the presence of IL-4 or anti-IL-4. Unstimulated spleen cells expressed no detectable IFN-γ mRNA (Fig. 2, Exp. A). Within 6–12 hr of stimulation, IFN-γ mRNA was detected but there was relatively little difference between cell populations stimulated in the presence of anti-IL-4 or IL-4. By 24 hr, IFN-γ mRNA levels had increased in the anti-IL-4 group; a very striking difference then existed between the "anti-IL-4" and the "IL-4" groups, which was even more prominent at 48 hr. In cells that had been stimulated for 48 hr, washed, and restimulated with soluble anti-CD3, there was little or no IFN-γ mRNA in the group treated with IL-4 but considerable IFN-γ mRNA in the group treated with anti-IL-4.

The analysis of IL-2 mRNA gave generally similar results (Fig. 2, Exp. B). Little or no difference in IL-2 mRNA between the anti-IL-4 and IL-4 groups was observed at 6 hr. At 15 hr, a difference was observed that was much more striking at 24 hr; no IL-2 mRNA was detected in either group at 48 hr. When purified T cells were stimulated with soluble anti-CD3 and APCs, IL-2 mRNA was detectable at 48 hr in the anti-IL-4 group but not in the IL-4 group (Fig. 2, Exp. C). By contrast, T cells stimulated in the presence of IL-4 had mRNA for IL-4 at 48 hr, whereas the cells stimulated in the presence of anti-IL-4 lacked detectable IL-4 mRNA.

Treatment of Purified Activated T Cells with IL-4 Diminishes Subsequent IL-2 Production. Purified lymph node T cells cultured overnight with APCs and anti-CD3 were washed and restimulated with additional anti-CD3; IL-2 levels were measured after 48 hr of the second culture. The inclusion of IL-4 in the initial culture resulted in a factor-of-5 diminution of IL-2 levels at the end of the second culture compared with T cells that had been preincubated with anti-CD3 plus anti-IL-4 (Fig. 3, Exp. 1). In both instances, anti-IL-4 was included in the second culture to preclude further action of IL-4. The addition of IL-4 in the second culture of cells that had been precultured in the presence of anti-IL-4 decreased IL-2 production by a factor of $\approx$3 compared with cells in which anti-IL-4 was present in the second culture. In keeping with these results, cells cultured with anti-IL-4 in both cultures produced 20-fold more IL-2 than cells cultured with IL-4 in both cultures.

The inhibition of IL-2 production obtained in T cells precultured with IL-4 was striking if anti-CD3 was present during the initial culture. Overnight culture of T cells with APCs in the presence of IL-4 or anti-IL-4 but without anti-CD3 led to only a modest diminution in IL-2 production in the second culture (Fig. 3, Exp. 2).

To test whether the activated T cell was a target of IL-4's inhibitory activity, lymph node T cells were stimulated

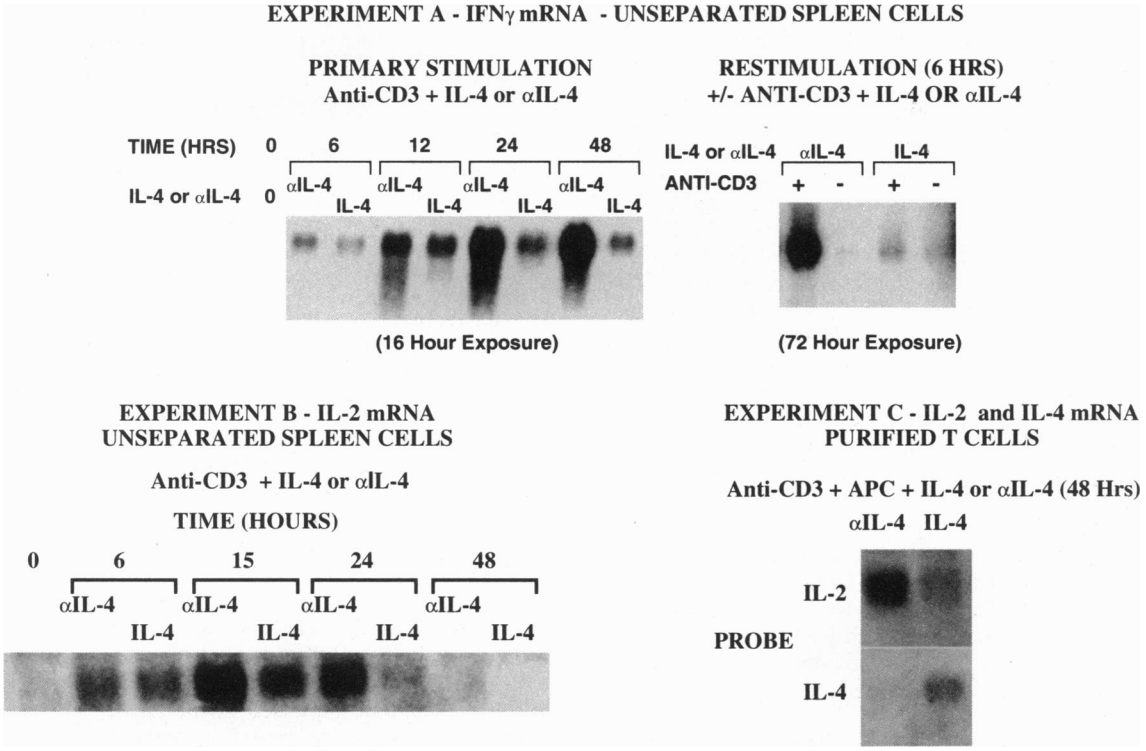

FIG. 2. IL-4 inhibits induction of IFN-γ and IL-2 mRNA in T cells stimulated with anti-CD3 and APCs. Spleen cells (Exps. A and B) or purified T cells plus APCs (Exp. C) were stimulated with anti-CD3 plus IL-4 or anti-IL-4 (αIL-4). RNA was prepared prior to stimulation (0 hr) (Exps. A and B) and at various times after stimulation and probed for mRNA for IFN-γ (Exp. A), IL-2 (Exps. B and C), and IL-4 (Exp. C). cDNA probes used were a 600-bp *Pst* I fragment for IL-2, a 643-bp *Pst* I fragment for IFN-γ, and a 373-bp *Eco*RI–*Hin*dIII fragment for IL-4.

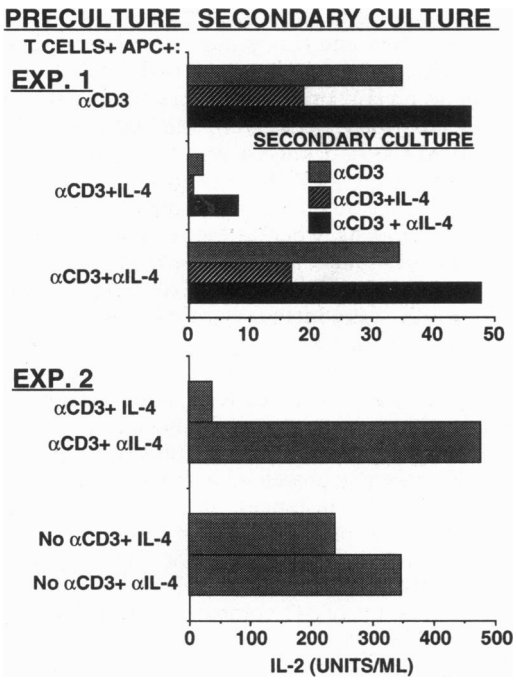

FIG. 3. Preculture with IL-4 and anti-CD3 inhibits subsequent production of IL-2. *Exp. 1.* Purified lymph node T cells were cultured with APCs and anti-CD3 (αCD3) with or without IL-4 or anti-IL-4 (αIL-4) for 16 hr, washed, and restimulated for 48 hr with fresh APC and anti-CD3 with or without IL-4 or anti-IL-4. IL-2 concentration was measured at the end of the second culture. *Exp. 2.* Purified lymph node T cells were cultured for 16 hr with APCs with or without anti-CD3, plus IL-4 or anti-IL-4. The cells were then washed and cultured with fresh APCs plus anti-CD3 for 48 hr. IL-2 was measured at the end of the second culture.

overnight with APCs and anti-CD3. The cells were then washed, stained with fluoresceinated anti-Thy-1.2 antibody, and purified by cell sorting. The purified T cells were cultured overnight, in the absence of APCs or anti-CD3, with IL-4 or anti-IL-4. The cells were washed again and stimulated for 48 hr with anti-CD3 and APCs. IL-2 levels were markedly lower in cultures of purified activated T cells that had been incubated with IL-4 (Fig. 4). It should be noted that the sorted cells produced considerably less IL-2 than did similar cells which had not been sorted but were subjected to an equivalent intermediate culture. In the unsorted cell population, as in the sorted cells, treatment with IL-4 during the intermediate culture inhibited IL-2 production in response to subsequent stimulation with anti-CD3 and APCs. These results indicate that IL-4 acts upon recently activated T cells to inhibit their subsequent production of IL-2.

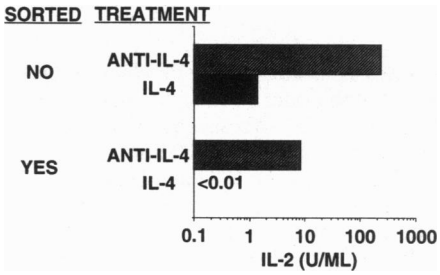

FIG. 4. IL-4 treatment of activated T cells inhibits their subsequent production of IL-2 in response to anti-CD3 plus APCs. Purified lymph node T cells were cultured for 16 hr with anti-CD3 plus APCs. A portion of the cells were stained with fluoresceinated anti-Thy-1.2 and the activated T cells were purified by fluorescence-activated cell sorting. Both the sorted T cells and the unsorted cells were cultured for 16 hr with IL-4 or anti-IL-4. The cells were then washed and restimulated with fresh APCs and anti-CD3 for 48 hr. IL-2 was measured at the end of the restimulation culture. U, units.

IL-4 Does Not Inhibit IL-2 or IFN-γ Production by Primed T Cells or by T Cell Clones, nor Does It Block Production of IL-2 by Naive T Cells in Response to Phorbol Ester and Ionomycin. In view of the striking inhibition by IL-4 of IL-2 and IFN-γ production by T cells from naive donors in response to soluble anti-CD3 and APCs and the prior failure to observe such inhibition in similar T cell populations stimulated with immobilized anti-CD3 (8), we examined the capacity of IL-4 to block IL-2 and IFN-γ production by lymph node T cells in response to phorbol 12-myristate 13-acetate and ionomycin. IL-4 did not diminish IFN-γ production in response to this stimulation although, in the same experiment, it blocked production in response to soluble anti-CD3 and APCs (Fig. 5). Similarly, IL-4 failed to inhibit IFN-γ production by two cloned T-cell lines, CDC35 (23) and D1.6 (24), in response to their cognate antigen, rabbit IgG, in the presence of I-A^d-positive APCs (Fig. 5).

To more directly test the differential susceptibility of naive and primed T cells to the inhibitory effects of IL-4, we utilized T cells from mice transgenic for the genes for the α and β chains of a TCR specific for pigeon cytochrome *c*-(88–104) peptide in association with I-E^k. These cells proliferate when initially challenged with peptide and APCs and produce substantial amounts of IL-2 but little IL-4 or IFN-γ (4). IL-2 production by T cells freshly prepared from these mice was strikingly inhibited by IL-4 (Fig. 6). Culture of the cells with peptide and APCs for 4 days in the absence of IL-4 "primed" the cells, allowing them to make IFN-γ and to retain the capacity to produce IL-2 upon restimulation with peptide and APCs. In contrast to the inhibitory effect of IL-4 upon IL-2 production by naive transgenic T cells, the production of IL-2 in response to antigen and APCs by primed T cells was not diminished by IL-4.

To further examine the cellular target of action of IL-4, T cells from normal mice were sorted into $CD45RB^-$ and $CD45RB^+$ cells. $CD45RB^+$ cells produced substantial amounts of IL-2 and IFN-γ. IL-4 strikingly inhibited IL-2 production by these cells and partially inhibited their IFN-γ production (Table 1). $CD45RB^-$ cells were poor producers of IL-2 and IFN-γ but were only marginally inhibited by IL-4.

DISCUSSION

The results demonstrate that IL-4 is a potent inhibitor of IL-2 and IFN-γ production by naive $CD45RB^+$ T cells stimulated with soluble anti-CD3 plus APCs or, when T cells from TCR-transgenic mice are employed, of IL-2 production in response to antigen and APCs. Our studies show that IL-4 inhibits IL-2 production by $CD4^+$ T cells and IFN-γ production by both $CD4^+$ and $CD8^+$ T cells.

One very striking observation is that IL-4 has no inhibitory effect on IL-2 production by naive T cells in response to immobilized anti-CD3 (8) or to phorbol ester and ionomycin. IL-4 also fails to inhibit IFN-γ production by cloned T-cell lines in response to their cognate antigens and APCs. Similarly, the production of IL-2 and of IFN-γ by *in vitro* primed T cells from TCR-transgenic mice stimulated with cytochrome *c* peptide and APCs is resistant to IL-4-mediated inhibition. These results imply that the effects of IL-4 in the regulation of IL-2 and IFN-γ production occur at a very precise stage of activation and differentiation, presumably while the cells are still in a naive state, and in response to stimuli that depend upon receptor engagement and the participation of APCs. It is interesting that mRNA levels for IL-2 and IFN-γ are not substantially diminished until 15–24 hr after initiation of culture. This could imply that the action of IL-4 is to block a late amplification of lymphokine mRNA in all stimulated cells from naive donors. Alternatively, there may be two populations of IL-2- and IFN-γ-producing cells: a small population of cells that makes IL-2 and IFN-γ promptly and is resistant to inhibition by IL-4 and a large population that produces IL-2 and IFN-γ at a slower pace and is completely susceptible to inhibition by IL-4. Although our data do not allow a definitive choice between these possibil-

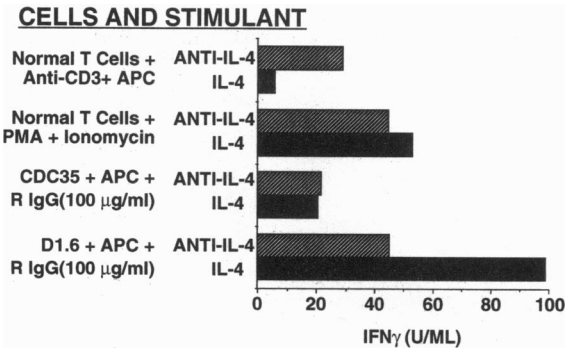

FIG. 5. IL-4 does not block IFN-γ production by cloned T-cell lines or by normal T cells in response to phorbol ester and ionomycin. Normal T cells freshly prepared from BALB/c lymph nodes and T cells from the cloned lines CDC35 and D1.6 were tested for IFN-γ production. The normal cells were stimulated with anti-CD3 and APCs or with phorbol 12-myristate 13-acetate (PMA, 1 ng/ml) and ionomycin (1 μM) in the presence of anti-IL-4 or IL-4. The cloned cells were stimulated with their cognate antigen, rabbit IgG (RIgG, 100 μg/ml), and APCs derived from BALB/c mice in the presence of anti-IL-4 or IL-4. IFN-γ was measured in supernatants after 48 hr of stimulation. U, units.

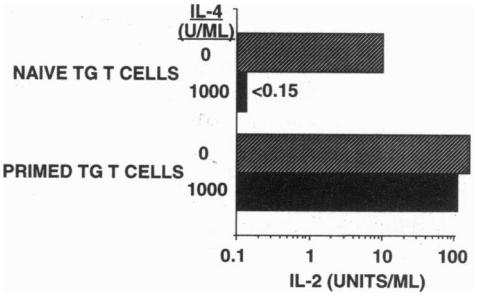

FIG. 6. IL-4 inhibits IL-2 production in response to antigen by naive but not primed T cells from TCR-transgenic (TG) donors. T cells were prepared from lymph nodes and spleen of mice transgenic for genes encoding a TCR specific for cytochrome *c*-(88–104) peptide in association with I-E^k. A portion of the cells were acutely stimulated with peptide and dendritic cells in the presence or absence of IL-4, and IL-2 content of supernatants was measured 48 hr later. A second portion of the cells were cultured with peptide and dendritic cells for 4 days, washed, and restimulated with peptide and dendritic cells in the presence or absence of IL-4 (1000 units/ml). IL-2 content of supernatants from these cells was measured after 48 hr of stimulation.

Table 1. IL-4 inhibits IL-2 and IFN-γ production by $CD45RB^+$ cells

Cells	Treatment	IL-2, units/ml	IFN-γ, units/ml
Unsorted	Anti-IL-4	89.3	10.8
	IL-4	12.1	1.3
$CD45RB^+$	Anti-IL-4	60.1	33.3
	IL-4	8.9	16.9
$CD45RB^-$	Anti-IL-4	0.5	2.7
	IL-4	0.7	1.8

Purified T cells were either unstained or stained with fluoresceinated anti-CD45RB antibody and sorted for $CD45RB^+$ or $CD45RB^-$ cells. These cells were then stimulated with APCs, anti-CD3, and anti-IL-4 or IL-4. Culture supernatant was collected after 48 hr of stimulation, and IL-2 and IFN-γ were measured.

ities, the finding that CD45RB$^+$ cells produce substantial amounts of both IL-2 and IFN-γ and are strikingly inhibited in such production by IL-4 whereas CD45RB$^-$ cells are poor producers of these lymphokines but are relatively insensitive to the effects of IL-4 is consistent with the second possibility.

The inhibition of IL-2 and IFN-γ production by IL-4 has features in common with the capacity of IL-4 present at the outset of *in vitro* or *in vivo* responses to determine the subsequent lymphokine-producing phenotype of CD4$^+$ T cells. In *in vitro* experiments, one observes that if IL-4 is present at the time of priming, T cells are obtained that produce IL-4 upon subsequent challenge and that fail to produce either IL-2 or IFN-γ (4, 5). By contrast, the absence of IL-4 strikingly diminishes the capacity to produce IL-4 and allows IFN-γ and IL-2 to be made. The *in vivo* counterpart of these experiments involves the inhibition of development of IL-4-producing T cells in mice treated with anti-IL-4 antibodies at the time of infection with *Leishmania major* (25) or *Candida albicans* (26) or upon immunization with hemocyanin (27). Further, the injection of IL-4 at the time of infection with *L. major* (28) or immunization with hemocyanin (S. Z. Ben-Sasson, and W.E.P., unpublished work) strikingly enhances the IL-4-producing capacity of the primed cells. It seems likely that the factors that cause the suppression of acute production of IL-2 by CD4$^+$ T cells may also be involved in inhibiting the differentiation of naive CD4$^+$ T cells into IFN-γ producers.

Finally, although most work on the role of IL-4 in determining lymphokine-producing phenotype during priming has focused on CD4$^+$ T cells, we have shown that IL-4 causes CD8$^+$ T cells to develop into IL-4-producing cells if they are simultaneously primed with immobilized anti-CD3 and IL-2 (29). These results, together with the current demonstration that IL-4 inhibits IFN-γ production by CD8$^+$ T cells, suggest that the establishment of lymphokine-producing phenotype in CD4$^+$ and CD8$^+$ T cells may have certain common features.

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