

Interleukin 12 Induces Stable Priming for Interferon γ (IFN- γ) Production During Differentiation of Human T Helper (Th) Cells and Transient IFN- γ Production in Established Th2 Cell Clones

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Summary

Interleukin 12 (IL-12) facilitates the generation of a T helper type 1 (Th1) response, with high interferon γ (IFN- γ) production, while inhibiting the generation of IL-4-producing Th2 cells in polyclonal cultures of both human and murine T cells and in vivo in the mouse. In this study, we analyzed the effect of IL-12, present during cloning of human T cells, on the cytokine profile of the clones. The culture system used allows growth of clones from virtually every T cell, and thus excludes the possibility that selection of precommitted Th cell precursors plays a role in determining characteristics of the clones. IL-12 present during the cloning procedures endowed both CD4⁺ and CD8⁺ clones with the ability to produce IFN- γ at levels severalfold higher than those observed in clones generated in the absence of IL-12. This priming was stable because the high levels of IFN- γ production were maintained when the clones were cultured in the absence of IL-12 for 11 d. The CD4⁺ and some of the CD8⁺ clones produced variable amounts of IL-4. Unlike IFN- γ , IL-4 production was not significantly different in clones generated in the presence or absence of IL-12. These data suggest that IL-12 primes the clone progenitors, inducing their differentiation to high IFN- γ -producing clones. The suppression of IL-4-producing cells observed in polyclonally generated T cells in vivo and in vitro in the presence of IL-12 is not observed in this clonal model, suggesting that the suppression depends more on positive selection of non-IL-4-producing cells than on differentiation of individual clones. However, antigen-specific established Th2 clones that were unable to produce IFN- γ with any other inducer did produce IFN- γ at low but significant levels when stimulated with IL-12 in combination with specific antigen or insoluble anti-CD3 antibodies. This induction of IFN- γ gene expression was transient, because culture of the established clones with IL-12 for up to 1 wk did not convert them into IFN- γ producers when stimulated in the absence of IL-12. These results suggest that Th clones respond to IL-12 treatment either with a stable priming for IFN- γ production or with only a transient low level expression of the IFN- γ gene, depending on their stage of differentiation.

Recent work from our and other laboratories (1–5) has shown that human CD4⁺ Th cell clones, in analogy to the murine system (6, 7), can be subdivided into at least three distinct functional subsets based on their cytokine secretion profile. One type of CD4⁺ clone (Th1) produces IL-2, IFN- γ , and TNF- β , but not IL-4 or IL-5; a second type (Th2) produces IL-4 and IL-5, but not IL-2, IFN- γ , or TNF- β ;

and a third type (Th0) produces both Th1- and Th2-type cytokines (1, 3, 5, 6). Although most CD8⁺ human T cell clones exhibit a Th1-like cytokine profile (3, 5), some CD8⁺ clones showing a Th2-like phenotype have also been described (8, 9).

Several factors can affect the development of Th1 or Th2 responses both in vivo and in vitro. Attention has been focused on cytokines produced by either APCs or lymphocytes. Thus important effects have been demonstrated for IFN- γ , IFN- α , IL-12, and TGF- β on CD4⁺ subset maturation by inducing Th1 expansion in various systems (10–19). Most

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striking is the requirement for IL-4 in maturation of Th2 cells (20–22).

The *in vitro* studies with human cells have investigated the generation of Th clones in response to recall antigens, analyzing prevalently the clonal expansion of *in vivo* primed memory T cells (1, 12). Mice transgenic for the genes encoding an antigen-specific TCR have been used to analyze the ability of naive T cells to develop into a Th1 or Th2 pattern of response or to give rise to Th1 or Th2 clones in response to antigen in different culture conditions (20–22). *In vitro* studies with transgenic mice have demonstrated that, at the polyclonal level, IL-12 induces the development of Th1 CD4⁺ cells by priming Th cells for efficient IFN- γ production and preventing development of IL-4-producing cells (17, 18, 23). However, IL-12 did not prevent the development of IL-4-producing cells induced by optimal concentrations of IL-4, and IL-4 significantly, although not completely, prevented the priming for IFN- γ production induced by IL-12 (18, 23).

In vivo, IL-12 protects susceptible BALB/c mice from *Leishmania major* infection (24, 25) and acts as an efficient adjuvant in vaccination with soluble *L. major* antigens (26). These effects of IL-12 are dependent on its ability to partially inhibit IL-4 production and to significantly increase IFN- γ production in the infected animals, i.e., shifting the response from a prevalent Th2-type to the Th1-type, which is characteristic in the resistant mice strains. The probable physiologic role of IL-12 in the development of an efficient Th1 response is suggested by the ability of anti-IL-12 antibodies *in vivo* to suppress the natural resistance of C3H mice to *L. major* infection (25). *In vitro*, however, IL-12 is not an absolute requirement for antigen-induced priming for a Th1 response, although its presence during priming enhances the ability of antigen-primed Th cells to produce IFN- γ (17, 18).

The experimental systems used to date have not permitted determination of whether the different cytokines affecting Th cell development, IL-12 in particular, induce differentiation of bipotential Th precursors or rather a selective priming and/or expansion of already committed Th1 and Th2 precursor cells (10, 19, 27, 28). This question is particularly relevant in the case of human studies which have analyzed clonal expansion of memory Th cells (1, 15). However, once a Th1 or Th2 response has been established, it appears to be relatively stable, and no factors capable of inducing qualitative changes in the cytokine profile of established murine or human T cell clones have been reported.

In this study, we analyzed the effect of IL-12 on the differentiation of Th1-, Th0-, and Th2-like human T cells from clones of resting peripheral blood T cells and on the production of IL-4 and/or IFN- γ by established antigen-specific human T cell clones. We show that IL-12, when present during the cloning of human T cells in a culture system that ensures clonal expansion of almost every CD4⁺ and CD8⁺ cell, induced the differentiation of clones with greatly enhanced ability to produce IFN- γ but with the same pattern of IL-4 production observed in clones generated in cultures without IL-12. Once established, clones show only minimal change in their ability to produce IFN- γ or IL-4 throughout continuous cul-

ture in the presence or absence of IL-12. IL-12 had no effect on IL-4 production by established antigen-specific Th0- and Th2-like T cell clones in response to antigens or anti-CD3 antibodies, but it strongly potentiated IFN- γ production by Th1- and Th0-like clones. IL-12 also induced transient expression of IFN- γ mRNA and detectable amounts of IFN- γ in Th2-like clones. Comparable effects were observed with CD8⁺ human T cell clones.

Materials and Methods

Reagents. PHA was purchased from Grand Island Biological Co. (Grand Island, NY). Purified protein derivative (PPD)¹ of *Mycobacterium tuberculosis* and purified tetanus toxoid (TT) were obtained from Istituto Sieroterapico e Vaccinogeno Sclavo (Siena, Italy). *Dermatophagoides pteromyssinus* group I (Der p I) was obtained from Lofarma Allergeni (Milan, Italy), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was from Sigma Chemical Co. (St. Louis, MO). Anti-CD3, anti-CD4, and anti-CD8 mAbs were purchased from Ortho Pharmaceutical Corp. (Raritan, NJ) and Becton Dickinson & Co. (Mountain View, CA), or produced from cells obtained from American Type Culture Collection (ATCC; Rockville, MD). Human recombinant IL-2 (rIL-2) was a generous gift of Eurocetus (Milan, Italy) and of Dr. T. Taguchi (Osaka University, Osaka, Japan). Human rIL-12 (Chinese hamster ovary-cell derived) was provided by Dr. Stan Wolf (Genetics Institute, Cambridge, MA).

Subjects. PBMC were obtained by Ficoll-Hypaque gradient separation from peripheral blood of healthy volunteer donors; donors showing delayed-type hypersensitivity skin reaction to PPD; atopic house dust mite-sensitive subjects suffering from atopic dermatitis and/or extrinsic asthma and showing immediate-type skin reactivity to Der p I; and HIV-1-infected individuals.

Generation of CD4⁺ and CD8⁺ T Cell Clones in the Presence or Absence of rIL-12. Clonal growth of almost every T cell from peripheral blood was obtained using a modification of the method described by Moretta et al. (29). Briefly, PBMC from healthy donors were cloned by limiting dilution in 96-well round-bottom tissue culture plates in 200 μ l RPMI 1640 medium with 10% FCS in the presence of 5 μ g/ml PHA, and γ -irradiated (50 Gy) autologous PBMC (25 \times 10³ cells/well) and RPMI 8866 B lymphoblastoid cells (12 \times 10³ cells/well). rIL-12 (2.5 ng/ml) was added to half of the wells. After 3 d, rIL-2 (50 U/ml final concentration) was added to all cultures by replenishing half of the medium (100 μ l). After 1 wk, irradiated feeder PBMC and RPMI 8866 (25 and 12 \times 10³, respectively) cells were again added. Cultures were then maintained in rIL-2 (50 U/ml) or a combination of rIL-2 and rIL-12 (1 ng/ml) and fed or split as required. In the first experiment (detailed in Results), 1 PBMC/well, corresponding to \sim 0.7 T cell/well, was plated and growth was observed in 38 of 88 seeded wells with IL-2 only, whereas in wells maintained in IL-2 and IL-12, growth was observed in 48 out of 96. These data, analyzed according to Poisson distribution, indicate clonal efficiency for T cells of >80% in IL-2 and of >90% in IL-2 + IL-12. All the clones derived in IL-2 were either CD4⁺ or CD8⁺, whereas four of the IL-2 + IL-12-derived clones were CD4⁻ CD8⁻ and not further analyzed. On the basis

¹ Abbreviations used in this paper: Der p I, *Dermatophagoides pteromyssinus*; PPD, purified protein derivative; RIA, radioimmunoassay; RT-PCR, reverse transcriptase-polymerase chain reaction; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; TT, tetanus toxoid.

of the calculated clonal efficiency and the observed number of wells containing both CD4⁺ and CD8⁺ cells, ~20% of the wells are expected to contain more than one clone; because CD4⁺ CD8⁺ wells were excluded from the study, ~15% of the wells analyzed probably contained two clones, both either CD4⁺ or CD8⁺. Most of the clones (34/38 IL-2-generated and 42/48 IL-2 + IL-12-generated) grew sufficiently to allow assessment of phenotype and cytokine production. The clones generated and maintained in the presence of IL-12 + IL-2 were washed after 23 d of culture, and divided into two parallel sets of cultures and maintained for a further 11 d in IL-12 + IL-2 or in IL-2 alone. Clones generated in the presence of IL-2 alone were washed after 27 d of culture, and divided into two parallel sets (IL-2 or IL-2 + IL-12) and cultured for a further 7 d. Fig. 1 A summarizes the cloning protocol. In a second experiment, PBMC were plated at 0.6 and 0.3 T cell/well. Cloning efficiency was ~80% for culture in the presence of IL-12 and ~70% for culture in the absence of IL-12. In this second experiment, in the cultures without IL-12, anti-IL-12 mAb C8.6 (ascites, 1:500) was added to neutralize any endogenously produced IL-12.

Generation of Antigen-specific CD4⁺ T Cell Clones. PPD-, Der p I-, and TT-specific T cell lines were generated as described (1, 30). Briefly, 10⁶ PBMC in 2 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 2 × 10⁻⁵ M 2-ME and 5% human serum (complete medium) were stimulated in 24-well flat-bottomed plates for 5 d with PPD, TT, or Der p I (10 μg/ml). Human rIL-2 (20 U/ml) was then added and cultures continued for an additional 9 d. Visible T blasts were resuspended in complete medium and tested for antigen specificity before cloning. To assess the antigen specificity of T cell lines, 2 × 10⁴ T blasts were seeded in microplates and cocultured for 48 h with irradiated (60 Gy) autologous PBMC (10⁵) in the presence of medium alone, PPD, TT, or Der p I (1 μg/ml). After a 16-h pulse with 0.5 μCi [³H]thymidine (Amersham International, Amersham, UK), cultures were harvested and radioactivity measured by liquid scintillation. To generate PPD-, TT-, or Der p I-specific T cell clones, T blasts obtained from antigen-specific T cell lines were seeded under limiting dilution conditions (0.3 cell/well) in six round-bottomed microwell plates containing 10⁵ irradiated (60 Gy) allogeneic PBMC (as feeder cells) and PHA (1% vol/vol) in a final volume of 0.2 ml complete medium supplemented with IL-2 (20 U/ml) and 10% FCS (Hyclone Laboratories Inc., Logan, UT). Growing microcultures were then supplemented with IL-2 (20 U/ml) and 10⁵ irradiated feeder cells at weekly intervals. The antigen specificity of T cell clones was assessed by measuring [³H]thymidine uptake after 60-h stimulation with the appropriate antigen under MHC-restricted conditions (1, 30). When the stimulation index (mean cpm in cultures stimulated with APC plus antigen/mean cpm in cultures with APC alone) was >10, responses were considered positive.

Generation of CD8⁺ T Cell Clones from HIV-infected Individuals. CD8⁺ T cell clones were generated from the peripheral blood of HIV-infected individuals showing low proportions of CD4⁺ T cells, according to the technique of Moretta et al. (29). Briefly, purified T cells (mostly CD8⁺) were seeded in limiting numbers in round-bottom microwells (0.3 cells/well) containing 10⁵ irradiated (50 Gy) feeder cells and 1% PHA (vol/vol) in a final volume of 0.2 ml complete medium. After 48 h, microcultures were supplemented with IL-2 (20 U/ml) and, at weekly intervals, with 10⁵ irradiated feeder cells and IL-2. Growing microcultures were expanded by repeated addition of feeder cells and IL-2 until a number of cells suitable for phenotypic and functional studies was obtained.

Surface Phenotype of T Cell Clones. The phenotype of T blasts

was examined by immunofluorescence (flow cytometry) using FITC-conjugated anti-CD3, anti-CD4, and anti-CD8 mAbs.

Induction of Cytokine Production by T Cell Clones. To induce cytokine production, T cell clones were resuspended in complete medium to a concentration of ~10⁶/ml and stimulated for 3–24 h with insolubilized anti-CD3 mAb (microtiter well-bound, 10 μg/ml; no accessory cells added) or antigen and irradiated autologous PBMC as APC (as described above for antigen-specific proliferation) in the presence or absence of different concentrations of IL-12. Soluble anti-CD3 mAb (OKT3, ascites 1:10,000) and TPA (10 ng/ml) were also used as a maximal stimulus. Cell-free culture supernatants were collected and stored in aliquots at -70°C until used.

IL-4 and IFN-γ mRNA Expression in T Cell Clones. IL-4 and IFN-γ mRNA expression was evaluated in T cell clones by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was prepared from T blasts by a single step guanidium isothiocyanate-phenol-chloroform extraction method. First-strand cDNA was synthesized using 5 μg total RNA, reverse transcriptase and oligo-dT primer in a final volume of 50 μl at 42°C for 1 h. The products obtained were denatured and 5 μl was used for amplification with specific primers for IL-4 and IFN-γ. PCR amplification was carried out in buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.1% (wt/vol) gelatin, 200 mM dNTP, 2.5 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 500 pmol of each primer. The reaction consisted of 30 cycles of amplification followed by a 15-min final extension at 72°C. Each cycle included denaturation at 95°C for 30 s, annealing at 65°C for 60 s and extension at 72°C for 2 min. Amplified products were electrophoresed on a 1.5% agarose gel.

Quantitation of IFN-γ and IL-4 in T Cell Clone Supernatants. IFN-γ was quantitated by radioimmunoassay (RIA) using our mAbs (31) or a commercial kit (Centacor Corp., Malvern, PA); IL-4 was analyzed by ELISA (Quantikine; R & D Systems Inc., Minneapolis, MN). Levels of cytokine 3 SD over those of control supernatants obtained by stimulation of irradiated feeder cells alone were considered positive.

Results

Effect of IL-12 Present During the Cloning Procedure on IFN-γ and IL-4 Production by the Clones. CD4⁺ and CD8⁺ clones generated in the presence or absence of IL-12 were analyzed for the ability to produce IFN-γ and IL-4 in response to anti-CD3 mAb and TPA. As shown in Fig. 1 B for eight representative clones, IFN-γ levels were highly variable among clones, but the high or low IFN-γ producer phenotype of the clones was stable throughout repeated testing between days 18 and 34 of culture. At day 34 of culture, all clones were tested for production of both IFN-γ and IL-4 (Fig. 2 and Tables 1 and 2). The CD4⁺ and CD8⁺ clones generated in the presence of IL-12 and IL-2 produced significantly more (eight- and threefold, respectively) IFN-γ than those generated in the presence of IL-2 only. The effect was less dramatic among CD8⁺ clones due to a higher proportion of high IFN-γ producers in CD8⁺ than in CD4⁺ clones generated in the absence of added rIL-12. These results were fully confirmed in a second cloning experiment (Table 3). Unlike IFN-γ, IL-4 production by CD4⁺ clones was not affected by the presence or absence of IL-12 during cloning (Fig. 2 and Tables 1, 2, and 3). The very low proportion of

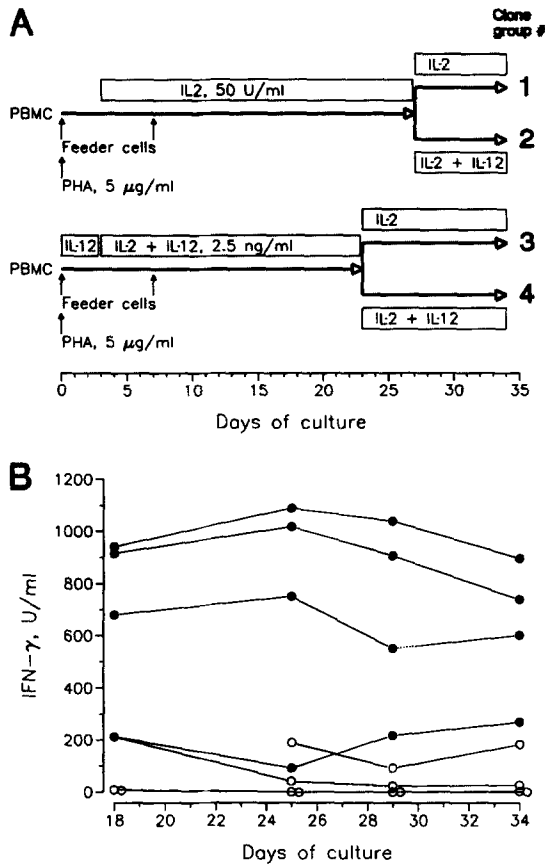


Figure 1. Cloning protocol (A) and production of IFN- γ by representative clones at different times of culture (B). (A) PBMC from one donor were plated at 1 cell/well (~ 0.7 T cell/well) and cultured as described in Materials and Methods. Clone groups 1–4 correspond to those analyzed in Table 1. (B) At the indicated time, cells from eight representative CD4⁺ clones [O, clones generated in IL-2 only (group 1); ●, clones generated in IL-12 + IL-2 (group 4)] were washed and tested for IFN- γ production in response to anti-CD3 mAb and TPA.

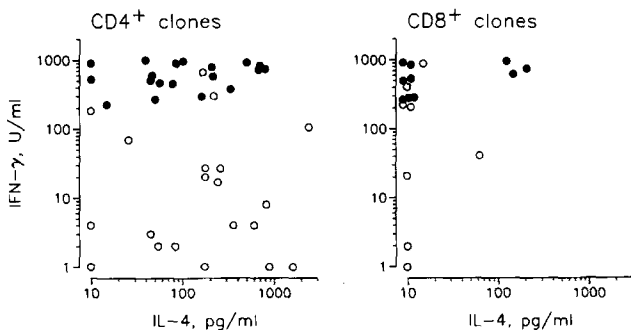


Figure 2. Production of IFN- γ and IL-4 by individual CD4⁺ and CD8⁺ clones in response to anti-CD3 mAb and TPA. All clones were harvested on day 34 of culture, washed, and stimulated in the absence of APC or feeder cells. Cell-free supernatant fluids were collected after 24 h and assayed IFN- γ and IL-4 by RIA and ELISA, respectively. O, clones generated in the presence of IL-2 only (group 1); ●, clones generated in the presence of IL-12 + IL-2 (group 4).

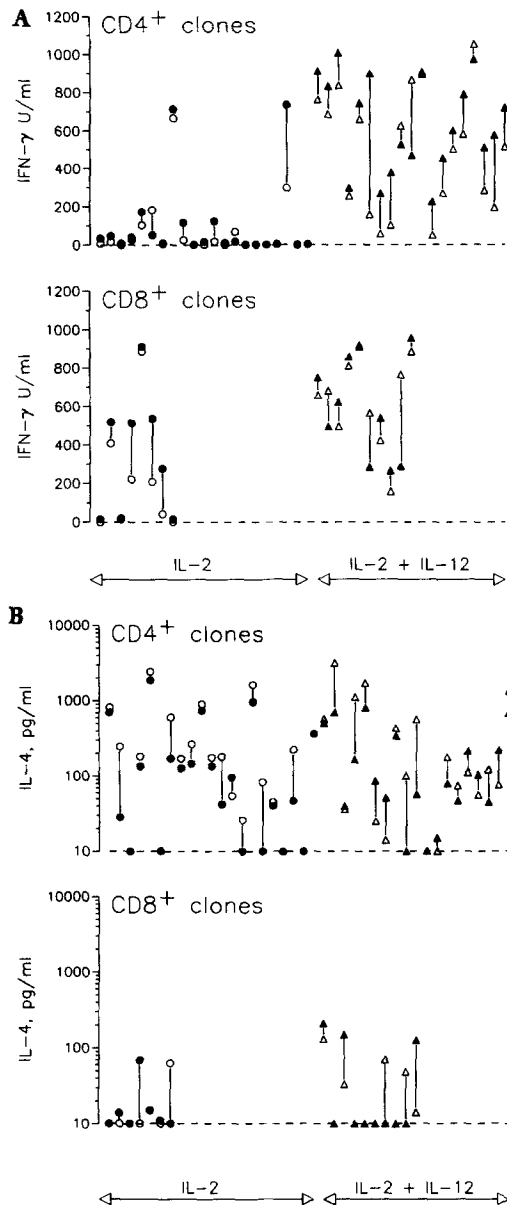


Figure 3. Effect of IL-12 withdrawal or addition during culture of established clones on IFN- γ (A) and IL-4 (B) production. CD4⁺ and CD8⁺ clones were all tested at day 34 after cloning. Clones generated in the presence of IL-2 alone were cultured continuously with IL-2 alone (O, group 1), or IL-12 was added in the last 7 d of culture (●, group 2). Clones generated in IL-12 + IL-2 were cultured continuously in the presence of the two cytokines (\blacktriangle , group 4) or IL-12 was omitted during the last 11 d of culture (\triangle , group 3). Clones were washed free of cytokines before stimulation with anti-CD3 mAb and TPA for 24 h; IFN- γ and IL-4 were measured in the cell-free supernatant fluid. The joined closed and open symbols represent the results with each single clone, cultured in the presence or absence of IL-12, respectively, in the last 7 (groups 1 and 2) or 11 (groups 3 and 4) d of culture. Corresponding positions in A and B refer to the same clones.

CD8⁺ clones producing IL-4 precluded analysis of any effect of IL-12 on their ability to produce IL-4.

Because the cloning protocol used allowed the clonal growth and analysis of the large majority of peripheral blood T cells,

Table 1. *IFN- γ and IL-4 Production in CD4⁺ Human T Cell Clones*

Group	Clones*	Initial condition	Final condition	No. of clones	mean \pm SE			
					IFN- γ U/ml	IL-4 pg/ml		
1	CD4 ⁺	IL-2	IL-2	21	69 \pm 34	399 \pm 133		
2	CD4 ⁺	IL-2	IL-2 + IL-12	21	101 \pm 46	268 \pm 99		
3	CD4 ⁺	IL-2 + IL-12	IL-2	19	493 \pm 72	511 \pm 187		
4	CD4 ⁺	IL-2 + IL-12	IL-2 + IL-12	19	635 \pm 57	220 \pm 60		
Student's <i>t</i> test					IFN- γ		IL-4	
Comparison	Type	t	P	t	P			
Group 1 vs. 2	paired	1.42	NS [†]	3.14	0.005			
Group 1 vs. 3	indep.	5.47	<0.001	0.49	NS			
Group 1 vs. 4	indep.	8.70	<0.001	1.19	NS			
Group 2 vs. 3	indep.	4.65	<0.001	1.17	NS			
Group 2 vs. 4	indep.	7.31	<0.001	0.59	NS			
Group 3 vs. 4	paired	2.79	0.012	1.48	NS			

* CD4⁺ and CD8⁺ human T cell clones of groups 1–4 from the first cloning experiment (as shown in Fig. 1, A and B, groups 1 and 2 and groups 3 and 4 are the same clones maintained in the presence of different cytokines in the final 7–11 d of culture) were stimulated with anti-CD3 mAb and TPA, and IFN- γ and IL-4 production was determined.

[†] *p* > 0.05.

Table 2. *IFN- γ and IL-4 Production in CD8⁺ Human T Cell Clones*

Group	Clones*	Initial condition	Final condition	No. of clones	mean \pm SE	
					IFN- γ U/ml	IL-4 pg/ml
1	CD8 ⁺	IL-2	IL-2	8	224 \pm 108	17 \pm 7
2	CD8 ⁺	IL-2	IL-2 + IL-12	8	350 \pm 116	25 \pm 9
3	CD8 ⁺	IL-2 + IL-12	IL-2	10	636 \pm 74	34 \pm 12
4	CD8 ⁺	IL-2 + IL-12	IL-2 + IL-12	10	597 \pm 84	55 \pm 24
Student's <i>t</i> test					IFN- γ	
Comparison	Type	t	P			
Group 1 vs. 2	paired	2.61	0.035			
Group 1 vs. 3	indep.	3.26	<0.001			
Group 1 vs. 4	indep.	2.78	0.012			
Group 2 vs. 3	indep.	2.17	0.044			
Group 2 vs. 4	indep.	1.77	NS [†]			
Group 3 vs. 4	paired	0.60	NS			

* CD8⁺ human T cell clones of groups 1–4 from the first cloning experiment. See legend to Table 1.

[†] *p* > 0.05.

the results above indicate that the presence of IL-12 during the clonal expansion of CD4⁺ and, in part, CD8⁺ T cells induces a differentiation of T cells to high IFN- γ -producer clones and does not select or induce preferential growth of Th1 or Th0 precursor cells already committed to the high IFN- γ -producer status. We therefore tested the effect of IL-12 on the IFN- γ -producer phenotype of already established clones. CD4⁺ clones generated in the presence of IL-2 alone did not produce significantly more IFN- γ after culture for 1 wk in the presence of IL-12, whereas clones generated in the presence of IL-12 and IL-2 produced 25% less IFN- γ when cultured in the absence of IL-12 for the last 11 d (Fig. 3 A and Tables 1 and 2). IL-2-generated clones cultured in IL-2 and IL-12 for 1 wk also produced slightly lower levels of IL-4 ($p = 0.005$), whereas the removal of IL-12 from the clones established in the presence of IL-2 and IL-12 did not result in a significant increase in IL-4 production (Fig. 3 B and Tables 1 and 2). Similar results were obtained with CD8⁺ cells; however, the intermediate levels of IFN- γ production observed in half of the CD8⁺ clones generated in the presence of IL-2 alone rose after 1 wk in culture with IL-12.

Effect of IL-12 with anti-CD3 Antibodies or with Specific Antigens and APC on IFN- γ Production in Th1, Th0, and Th2 Human T Cell Clones. T blasts of ten established human CD4⁺ T cell clones with defined cytokine secretion profile (four Th1, three Th0, and three Th2) (Fig. 4) were stimulated for 24 h with insolubilized anti-CD3 mAb and T blasts from six other CD4⁺ clones with defined antigen specificity (two Th1, one Th0, and three Th2) (Fig. 5) were stimulated for 24 h with specific antigen and APC. Cell cultures were performed in the absence or presence of increasing concentrations of IL-12 and assessed for production of IL-4, and IFN- γ . As shown in Figs. 4 and 5, the presence of IL-12 had no effect on IL-4 production by all types of clones, but strongly increased the IFN- γ production by both Th1 and Th0 clones, and induced small but detectable IFN- γ production by Th2 clones. To exclude the possibility that Th2 clones were already capable of producing small but undetectable amounts of IFN- γ and that the IL-12-mediated effect simply

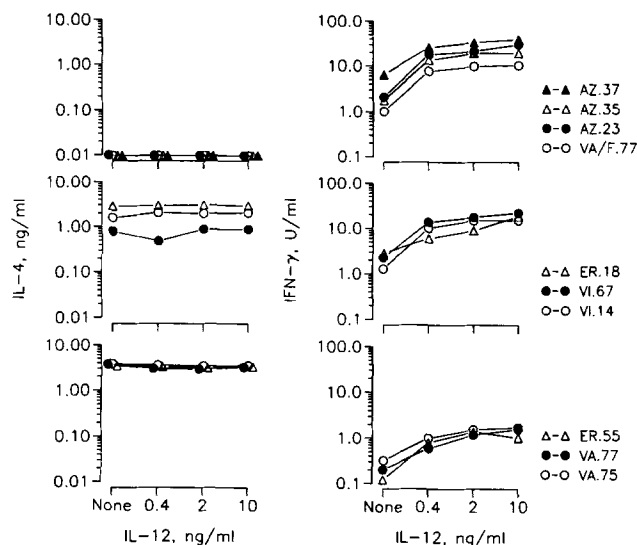


Figure 4. Effect of IL-12 on IL-4 and IFN- γ production by established Th1 (top), Th0 (middle), and Th2 CD4⁺ human antigen-specific T cell clones (bottom). Cells from the indicated clones were stimulated with insolubilized anti-CD3 mAb and the indicated concentrations of IL-12 in the absence of APC or feeder cells. IFN- γ and IL-4 were measured in the cell-free supernatant fluids after 24-h incubation by RIA and ELISA, respectively.

reflects increased IFN- γ production over the detection limits of the assay, IFN- γ mRNA expression was evaluated by RT-PCR in two representative Th2 clones and one Th0 clone stimulated with anti-CD3 mAb in the absence or presence of IL-12. IFN- γ and IL-4 mRNA was easily detected in the Th0 clone. Neither of the two Th2 clones expressed mRNA for IFN- γ when stimulated with insoluble anti-CD3 mAb in the presence of medium alone, but both clones expressed it in the presence of IL-12 at 5 or more ng/ml (Fig. 6). IL-4 mRNA was always detectable in all experimental conditions (Fig. 6). IFN- γ mRNA expression was already detectable at 3 h after incubation with IL-12 and disappeared by 24 h, when the protein product became clearly detectable in the

Table 3. IFN- γ and IL-4 Production in CD4⁺ and CD8⁺ Human T Cell Clones (Second Cloning Experiment)

Clones*	Conditions	No. of Clones	mean \pm SE	
			IFN- γ U/ml	IL-4 pg/ml
CD4 ⁺	IL-2 + anti-IL-12	23	46 \pm 11	159 \pm 69
CD4 ⁺	IL-2 + IL-12	23	447 \pm 42 ($p < 0.001$) [†]	41 \pm 10 (NS) [§]
CD8 ⁺	IL-2 + anti-IL-12	20	59 \pm 17	10 \pm 0.3
CD8 ⁺	IL-2 + IL-12	15	491 \pm 33 ($p < 0.001$)	13 \pm 3 (NS)

* CD4⁺ and CD8⁺ human T cell clones, generated and maintained for 20 d in the indicated conditions, were stimulated with anti-CD3 and mAb and TPA, and IFN- γ and IL-4 production was determined.

[†] p for significance of the difference between clones cultured in IL-2 + anti-IL-12 and those cultured in IL-2 + IL-12 is reported (Student's t test, independent samples).

[§] $p > 0.05$.

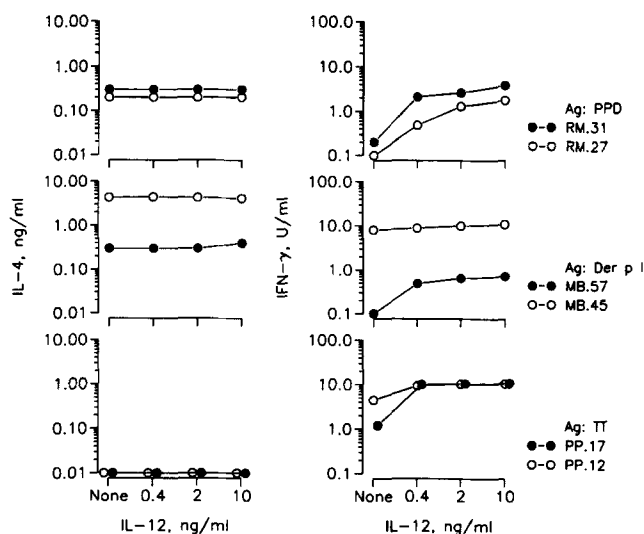


Figure 5. Cooperative effect of specific antigens and IL-12 in inducing IFN- γ production from human CD4⁺ clones. Cells from two PPD (*top*), Der p I (*middle*), and TT-specific (*bottom*) human CD4⁺ clones were stimulated for 24 h with the corresponding antigen (1 μ g/ml) and the indicated concentrations of IL-12. Irradiated autologous PBMC were used as APC. IL-4 and IFN- γ were measured in the cell-free supernatant fluids by ELISA and RIA, respectively.

T cell clone supernatants (Fig. 7). The inability of the Th2 clones to produce IFN- γ and to accumulate IFN- γ mRNA in the absence of IL-12 was confirmed at both the protein and the mRNA level upon stimulation of the cells with anti-CD3 (100 ng/ml) and TPA (10 ng/ml) or with TPA and Ca²⁺ ionophore, two protocols that induce maximal stimulation of IFN- γ production in Th1 and Th0 clones (not shown).

We then analyzed whether the IL-12-induced production of IFN- γ was due to a transient or a permanent change in the cytokine production profile of the Th2 clones. Two Th2 clones that had been induced to produce IFN- γ by IL-12 and anti-CD3 mAb were extensively washed, cultured with IL-2 alone for 7 d, and stimulated again with anti-CD3 mAb in the presence or absence of IL-12. Th2 cells stimulated with anti-CD3 mAb alone no longer produced IFN- γ , whereas IFN- γ production was restored in the presence of freshly added IL-12 (Fig. 8).

The effect of IL-12 on the cytokine production profile of CD8⁺ T cell clones established from HIV⁺ patients was also investigated. Three CD8⁺ with a Th1-like cytokine profile and three Th2-like CD8⁺ clones were stimulated with anti-CD3 mAb in the presence or absence of IL-12. As shown in Fig. 9, IL-12 had no effect on IL-4 production, but it strongly potentiated IFN- γ production by Th1-like CD8⁺ clones and induced production of detectable amounts of IFN- γ by Th2-like CD8⁺ clones.

Discussion

IL-12, a novel heterodimeric cytokine, was shown to be a potent inducer of IFN- γ , acting synergistically with IL-2

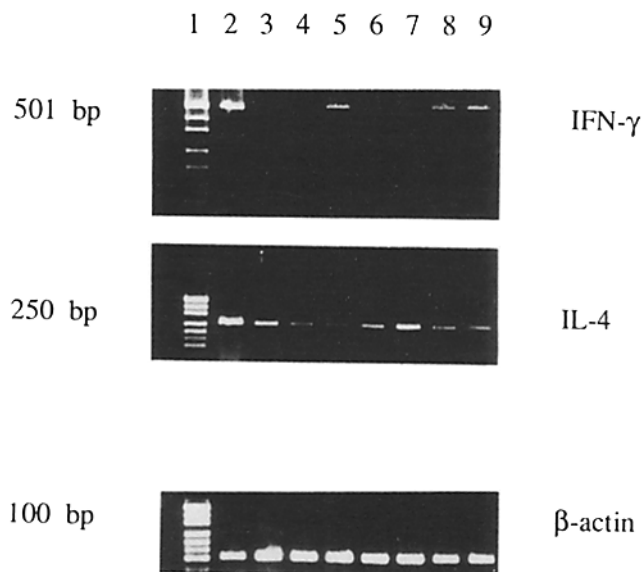


Figure 6. Accumulation of mRNA for IFN- γ and IL-4, as analyzed by RT-PCR and agarose electrophoresis, in one human CD4⁺ Th0 clone (ER14) and two Th2 clone (ER55 and ER77) induced for 6 h with insolubilized anti-CD3 mAb in the presence of the indicated concentrations of IL-12. First-strand cDNA was synthesized and amplified by PCR with IFN- γ and IL-4 specific primers. After amplification, 10 μ l of the reaction mixture was removed, analyzed by agarose gel electrophoresis, and visualized by ethidium bromide staining. The size of the expected PCR products is indicated on the left; β -actin mRNA amplification was used as a control for a constitutively expressed gene. Lane 1, Φ X 174 DNA/Hinf 1 size markers. Lane 2, Th0 (ER 14) clone without IL-12. Lane 3, Th2 (ER55) clone without IL-12. Lane 4, Th2 (ER55) clone with 1 ng/ml of IL-12. Lane 5, Th2 (ER55) clone with 50 ng/ml of IL-12. Lane 6, Th2 (ER77) clone without IL-12. Lane 7, Th2 (ER77) clone with 1 ng/ml of IL-12. Lane 8, Th2 (ER77) clone with 5 ng/ml of IL-12. Lane 9, Th2 (ER77) clone with 50 ng/ml of IL-12.

and several inducing stimuli (25, 32–34). More recently, IL-12 was found to favor the *in vitro* development of Th1-like CD4⁺ human T cell clones (15). The effect of IL-12 on the development of Th1 cells has been confirmed in the murine system *in vitro* (23) and *in vivo* (24, 25). Both in the human and murine systems, IL-12 enhances IFN- γ production in polyclonally or clonally expanded Th cell populations and inhibits the priming for IL-4 production (17, 18, 23–25, 35). However, it has not been clear whether these effects reflect the action of IL-12 on Th cell precursors, directly or through secretion of secondary cytokines such as IFN- γ or TNF, by inducing a differentiation effect on single clones to produce the various cytokines, or rather by selectively stimulating the growth of IFN- γ -producing Th1 cells. There is clearcut evidence that IL-12 can directly activate expression of IFN- γ genes in T cells, both CD4⁺ and CD8⁺, and in NK cells (34, 36). However, there was no evidence indicating whether IL-12 induces a stable phenotype change in Th cells leading to high IFN- γ production, or instead, selects for Th1 cell expansion when present during antigenic or mitogenic stimulation. IL-12 has a direct mitogenic effect on murine Th1 clones ([37] and Gerhard, W., and G. Trinchieri, unpublished

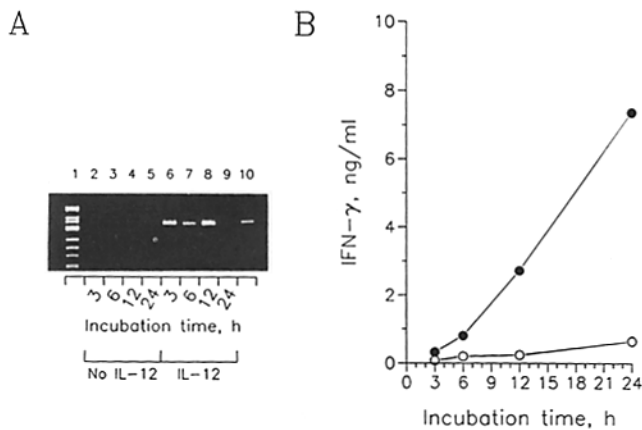


Figure 7. Kinetics of IL-12-induced IFN- γ mRNA and protein. Cells of the Th2 CD4⁺ clone B14 were stimulated with insolubilized anti-CD3 mAb in the absence or presence of IL-12 (10 ng/ml) and at the indicated times of culture, IFN- γ mRNA accumulation was evaluated by RT-PCR (A) and IFN- γ production by RIA (B). (A) Cells were stimulated in the absence (lanes 2–5) or presence (lanes 6–9) of IL-12 for 3 h (lanes 2 and 6), 6 h (lanes 3 and 7), 12 h (lanes 5 and 8), and 24 h (lanes 6 and 9); lane 1 shows Φ X 174 DNA/Hinf 1 size markers and lane 10, a positive control for PCR, carried out under conditions as in Fig. 6. (B) IFN- γ production was tested by RIA at the indicated times in the cell-free supernatant fluids from B14 cells cultured in the absence (O) or presence (●) of IL-12.

results) but not on Th2 clones and is a potent inducer of production of IFN- γ , which suppresses Th2 cell proliferation (28). Another open question is whether cytokines such as IL-12, which have major effects during primary and secondary antigen priming and clone generation, can still modify the phenotype of established Th clones with a defined pattern of cytokine production.

To address some of these questions, we cloned human peripheral blood lymphocytes in the presence of PHA, IL-2,

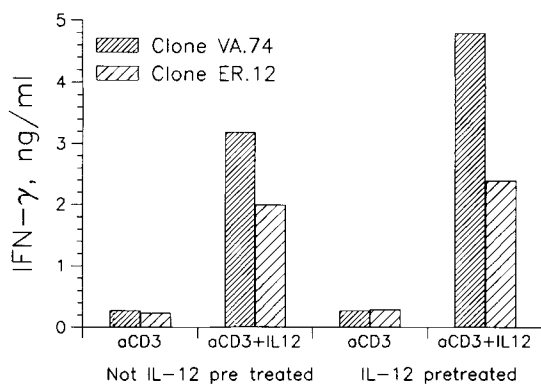


Figure 8. Transient IFN- γ production induced by IL-12 in Th2-like CD4⁺ clones. Two Th2-like clones, VA74 and ER12, were stimulated for 24 h with insolubilized anti-CD3, alone, or with IL-12 (10 ng/ml), and IFN- γ production was measured. Cells treated with anti-CD3 and IL-12 (IL-12 pretreated) were then washed, maintained in culture for 7 d in complete medium, and again stimulated with insolubilized anti-CD3 in the presence or absence of IL-12; IFN- γ production was measured.

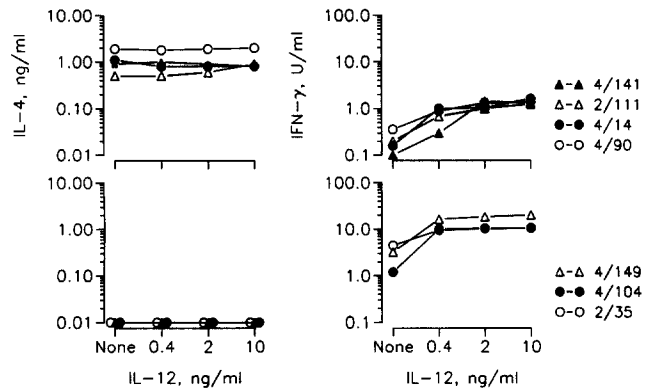


Figure 9. Effect of IL-12 on cytokine production by human CD8⁺ clones from HIV⁺ patients. Cells from four Th1-like (top) and from 3 Th-2-like human CD8⁺ clones (bottom) were stimulated with insoluble anti-CD3 mAb and the indicated concentrations of IL-12 for 24 h. Cell-free supernatant fluids were measured for IL-4 and IFN- γ by ELISA and RIA, respectively.

and feeder cells (29), conditions that allowed us to clone virtually every T cell present in the initial population, and analyzed the effect of IL-12 present during the cloning on the phenotype of the clones. Because irradiated feeder cells (PBMC and RPMI 8866) were present in the first week of culture of the clone, we cannot completely rule out an indirect effect of IL-12 on the clone precursor cells, especially since NK cells, contained in the feeder PBMC, play a role in the effect of IL-12 on Th1 cell development both in humans (15) and in the mouse (26). IL-12 might also induce production of IFN- γ or other cytokines from the feeder PBMC. However, a direct effect of IL-12 on CD4⁺ cells has been demonstrated in TCR transgenic mice (18), suggesting that at least part of the effect of IL-12 is directly on the T cell clones and their precursor cells in our system. It is also possible that both PBMC and RPMI 8866 cells, despite irradiation and brief survival in our culture conditions, produce low levels of IL-12 (38). However, addition of optimal concentrations of rIL-12 dramatically enhanced IFN- γ production by all clones in response to a maximal nonspecific stimulation (anti-CD3 mAb and TPA), but did not significantly change IL-4 production. In the second cloning experiment, endogenous IL-12 was neutralized using anti-IL-12 mAb: in these conditions the differential IFN- γ production between clones generated in the presence or absence of IL-12 was even more marked. Because all of the CD4⁺ clones generated in the presence of IL-12 were high IFN- γ producers whereas most of the clones generated in the absence of IL-12 were nonproducers or low producers, and because the cloning efficiency of T cells was more than 80%, these results clearly show that the generation of high IFN- γ producer clones in the presence of IL-12 reflects induced differentiation of individual clones and not selection. Although ~15% of the cultures analyzed in the first experiment were expected to contain more than 1 clone, the presence of few oligoclonal wells would lead to an overestimation of IFN- γ producer clones in the culture without IL-12, because the mixture of a low and a high producer clone would

be interpreted as a high producer, and therefore does not weaken our conclusion. The question of whether IL-12 acts on Th cells through the induction of IFN- γ is controversial; some studies (15, 18) report on the inability of anti-IFN- γ antibodies to prevent the IL-12-mediated effect on Th1 differentiation, whereas others indicate that IFN- γ may be required at least for the priming of IFN- γ production but not for inhibition of development of IL-4-producing cells (17, 24). Although IFN- γ can upregulate its own gene expression (39), and the induced IFN- γ may play a role in some of the effects of IL-12, IFN- γ cannot replace IL-12 in inducing Th1 cell development (18, 40). Because generation of T cell clones in the presence of IL-12 had no significant effect on IL-4 production, it is possible that while the priming effect for IFN- γ is a true differentiation effect of IL-12, the inhibition of IL-4-producing cells in polyclonal cultures or in vivo might be more of a selection process. It is interesting to note that although the IL-12 inhibition of IL-4-producing cells was first demonstrated in vitro in the human system (15), this effect is particularly evident in vivo in the *L. major* infection model (24–26), whereas in vitro IL-12 has little or no ability to prevent the priming for IL-4 production induced in the presence of IL-4 (18, 35). It is, however, likely that the negative effect of IL-12 on IL-4 producing cells, observed in bulk cultures or in vivo, is based on a selection process more effective in a physiologic in vivo environment than in tissue culture conditions. It should be pointed out that in our cloning conditions containing accessory cells, low levels of IL-4 might have been present and had an effect on the ability of the clones to produce IL-4; studies testing the effect of adding exogenous IL-4 or neutralizing endogenous IL-4 in our cloning conditions are in progress.

The effect of IL-12 in inducing differentiation of the clones to a high IFN- γ producer phenotype was relatively stable: removal of IL-12 from the culture medium of the clones for a period of 11 d resulted in only 25% reduction of the overall ability of the clones to produce IFN- γ . On the other hand, most of the clones generated in the absence of IL-12, which were not high IFN- γ producers, were not primed for IFN- γ even after culture for 1 wk in the presence of optimal concentrations of IL-12. In the second cloning experiment, addition to or removal from established clones of IL-12 for 1 wk also did not affect the ability of the clones to produce IFN- γ (results not shown). These results suggest that there is a stage during the expansion of the Th clones in which cells are susceptible for priming for IFN- γ production, perhaps through the induction of a stable alteration in IFN- γ gene conformation that poises it for transcription or that renders it responsive to positive regulatory mechanisms. In established clones that display a non-IFN- γ -producing phenotype (often Th2-like with IL-4 production), the IFN- γ gene might no longer be susceptible to the priming effect of IL-12. Since no attempt was made to isolate T cells at distinct functional stages prior to limiting dilution analysis, the clones analyzed were likely derived from both memory and naive T cells. The priming/differentiative effect of IL-12 in reducing high IFN- γ -producing clones was observed with all clones and did not appear to be affected by the nature of the clonal

T progenitor cells, although other effects of IL-12 on Th cell generation, e.g., selection, would likely be influenced by the immune status of the Th cells (10, 27).

Because murine Th1 but not Th2 clones were reported to respond to IL-12 with proliferation (37), it was of interest to investigate the direct effect of IL-12 on CD4⁺ T cell clones with already established cytokine profiles. IL-12 strongly potentiated IFN- γ production induced by anti-CD3 mAb or specific antigen in both Th1- and Th0-type T cell clones, without affecting the ability of Th0- and Th2-type clones to produce IL-4. More importantly, IL-12 promoted mRNA expression and production of detectable amounts of IFN- γ by Th2 clones, demonstrating that the cytokine profile of T cells exhibiting a well-established functional phenotype can be qualitatively altered. Previous attempts to change the cytokine profile of established CD4⁺ human T cell clones by using different cytokines, hormones, or drugs have been consistently unsuccessful. For example, IL-4, which is the dominant factor in determining the likelihood for Th2 polarization in cultured cells, had no effect on the cytokine secretion profile of Th0- or Th1-type clones (our unpublished results). However, IFN- γ production induced by IL-12 on Th2 clones was a transient phenomenon, and after IL-12 removal, the same antigen-specific clones no longer produced IFN- γ in response to stimulation with anti-CD3 mAb, similar to the results obtained with the PHA/IL-2-induced clones. Nevertheless, this finding suggests that even highly polarized T cells can produce all types of cytokines, given the appropriate microenvironmental conditions. Indeed, we recently found that growth transformation with herpesvirus saimiri induces stable and even constitutive IFN- γ production by established Th2-type CD4⁺ human T cell clones (De Carli, M., E. Maggi, and S. Romagnani, manuscript in preparation). Explanation of the paradoxical observation that IL-12 can induce IFN- γ gene expression in Th2 clones, although at low levels, but fails to induce priming for subsequent IFN- γ on the same clones awaits information on the molecular mechanisms of the priming effect. Because both transcriptional and posttranscriptional mechanisms are involved in induction of IFN- γ expression by IL-12 (36), it is possible that different levels of regulation are involved in direct gene expression and in priming.

Our results also suggest that human CD8⁺ and CD4⁺ T cell clones are similarly regulated. Although a higher proportion of CD8⁺ clones than of CD4⁺ generated in the absence of IL-12 were high IFN- γ producers, a significant effect of IL-12 on the generation of high IFN- γ producer CD8⁺ clones was observed and every CD8⁺ clone generated in the presence of IL-12 was a high IFN- γ producer. Interestingly, Th2-like CD8⁺ T cell clones were derived from both peripheral blood and skin biopsy specimens of HIV-seropositive patients, and the proportion of such clones was even higher when IL-4 was added in bulk culture before cloning (Maggi, E., and S. Romagnani, manuscript in preparation). However, even these Th2-type CD8⁺ clones were induced to IFN- γ production when stimulated with anti-CD3 mAb in the presence of IL-12.

The present study confirms that IL-12 is a key component

in the cytokine network with complex regulatory mechanisms that can not only determine the type of specific immune response at early stages of antigenic challenge, but also

alter, at least transiently, the profile of highly polarized CD4⁺ and CD8⁺ T cell responses.

We thank Drs. Phillip Scott, Walter Gerhard, and Anne O'Garra for discussion and suggestions.

This work was supported in part by grants from Associazione Italiana Ricerca Cancro and Consiglio Nazionale Ricerche (target projects "Biotechnologies and Bioinstrumentations" and "Clinical Application of Research in Oncology," 92.02256.PF39) to S. Romagnani, and by United States Public Health Service grants CA-10815, CA-20833, CA-32898, and CA-40256 to G. Trinchieri. F. Gerosa was supported in part by a fellowship from Regione Veneto and by Consiglio Nazionale Ricerche (target project "Clinical Application of Research in Oncology," 93.02256.PF39).

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Received for publication 15 October 1993 and in revised form 28 December 1993.

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