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Immunology

Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming

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ABSTRACT **Naive CD4+ T cells produce interleukin 2 (IL-2) but little IL-4 or interferon γ (IFN-γ). *In vitro*, they develop into IL-4 or IFN-γ producers depending on the conditions of the priming culture. Using T-cell receptor transgenic CD4+ T cells, the role of IL-12 and IL-4 in antigen-specific priming was examined. IL-12 substantially enhanced the ability of naive CD4+ T cells to develop into cells that produced IFN-γ upon restimulation. However, it was not essential since anti-IL-12 antibodies failed to block the priming for IFN-γ observed in the absence of exogenous IL-12. When both IL-12 and IL-4 were present in the priming culture, IL-12 did not inhibit priming for IL-4 production. In contrast, IL-4 diminished but did not abolish priming for IFN-γ production. In an accessory cell-independent priming system, IL-12 strikingly augmented priming for IFN-γ production, indicating that it acts directly on T cells. IFN-γ itself did not enhance priming for IFN-γ production in either accessory cell-dependent or independent systems. In an accessory cell-dependent system, the IL-12-mediated enhancement was not blocked by adding neutralizing anti-IFN-γ monoclonal antibody. However, in an accessory cell-independent system, anti-IFN-γ antibody did inhibit priming for IFN-γ production leaving open a role for IFN-γ in the priming process. These data indicate that IL-12 has a major effect on the inductive phase of T-cell priming by enhancing commitment to IFN-γ production and thus can profoundly influence the state of immunity that develops.**

Naive CD4+ T cells produce interleukin 2 (IL-2) in response to challenge with antigen and antigen-presenting cells (APCs) or to polyclonal stimulants such as anti-CD3 antibodies (1, 2). However, these cells produce little or no IL-4 or interferon γ (IFN-γ). Such cells can be primed to become IL-4 or IFN-γ producers. T cells from normal mice stimulated with immobilized anti-CD3 antibody develop into IL-4 producers only if IL-4 is added to the priming culture (3). Using T cells from mice transgenic for genes specifying the α and β chains of a T-cell receptor specific for a cytochrome *c* peptide or for ovalbumin, it was demonstrated that the addition of IL-4 to priming cultures resulted in high levels of IL-4 being produced when the cells were subsequently challenged with antigen and APCs (4, 5). This priming procedure did not lead to the appearance of IFN-γ-producing cells. By contrast, if the initial culture was carried out in the absence of IL-4, as ensured by the presence of a monoclonal anti-IL-4 antibody, there was virtually no priming for IL-4 production. Rather, the cells produced IFN-γ upon restimulation. These results were in keeping with *in vivo* observations in which treatment with anti-IL-4 antibody at the time of infection with *Leishmania major* or *Candida albicans* or immunization with hemocyanin markedly diminished the appearance of antigen-specific IL-4-producing cells (6–8).

Although these results provide strong evidence that IL-4 plays an important role in the process through which naive CD4+ T cells develop into cells capable of producing IL-4 but not IFN-γ, they leave open the question of the control of priming for IFN-γ production. It is known that peripheral blood T cells and natural killer (NK) cells from humans will produce increased amounts of IFN-γ if they are cultured in the presence of IL-12, a recently characterized cytokine (9, 10). T cells from allergic patients grown in the presence of IL-12 will develop clones producing less IL-4 and more IFN-γ than such cells grown in IL-12-free conditions (11). Furthermore, T cells from patients positive for purified protein derivative of tuberculin (PPD), if grown *in vitro* with PPD and anti-IL-12 antibody, develop into cells with an increased ability to make IL-4 compared to cells cultured in the absence of anti-IL-12 antibody. These results suggest that IL-12 may be a critical mediator in controlling the expansion from primed donors of T cells that can produce or develop into producers of IL-4 or IFN-γ. Since these studies examined the capacity of IL-12 to alter the lymphokine-producing phenotype of T lymphocytes from donors that have been primed *in vivo*, they leave open the question of the extent to which the regulation of lymphokine-producing phenotype in such cells resembles the normal process of subset differentiation occurring in naive CD4+ T cells.

Hsieh *et al.* (12) have recently shown that IL-12 enhances priming for IFN-γ production by T cells from mice transgenic for a T-cell receptor (TCR) specific for ovalbumin. We wished to determine whether this effect of IL-12 was mediated directly on CD4+ T cells or through an intermediate stimulation by IFN-γ and whether IL-12 was required for priming for IFN-γ production or acted to enhance such priming. Here we present *in vitro* data on the influence of IL-12 on naive CD4+ T cells, which show that IL-12 is not required for, but strikingly enhances, IFN-γ production and that this effect is directly mediated on T cells. Moreover, we demonstrate that when both IL-4 and IL-12 are present in the same culture, IL-12 has little effect on subsequent priming for IL-4 production compared to IL-4 alone but allows significant priming for IFN-γ production to occur even in the presence of IL-4.

MATERIALS AND METHODS

Animals. TCR transgenic mice were produced as described (4). All mice used in experiments were heterozygous for the integration of TCR variable region $V_\alpha 11$ and $V_\beta 3$ chains. Virus-free B10.A and BALB/c female mice (8–12 wk old)

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Abbreviations: IL, interleukin; APC, antigen-presenting cell; IFN-γ, interferon γ; TCR, T-cell receptor; r, recombinant; V, variable region; TDS, T-cell-depleted spleen cell.

were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Cytokines and Antibodies. Human IL-2 was a gift of Cetus. Mouse recombinant IL-4 (rIL-4) was produced in a baculovirus expression system, utilizing a vector into which the IL-4 gene had been inserted by C. Watson (Laboratory of Immunology, NIAID). Mouse rIFN-γ was purchased from Genzyme (Cambridge, MA). Mouse rIL-12 was a generous gift of Genetics Institute and Hoffman–LaRoche (13). Purified monoclonal rat anti-mouse IL-4 antibody (11B11) (14) was prepared by Verax (Lebanon, NH). Rat anti-mouse IFN-γ antibody (XMG 1.2) (15) was purchased from PharMingen. Rabbit anti-IL-12 antibody was a generous gift from Stan Wolf, and the Immunology Department of Genetics Institute. Purified monoclonal hamster anti-mouse CD3 antibody (2C11) (16) was prepared by C. Kinzer (Laboratory of Immunology, NIAID).

Measurement of Lymphokine Production. IFN-γ, IL-4, and IL-2 were assayed by specific two-site ELISAs (17, 18). The IL-4-dependent cell line CT.4S (19) was also used to measure IL-4 production by using serial dilutions of supernatant and comparing responses to those elicited by known amounts of murine rIL-4. Lymphokine production is reported in units/ml.

Preparation of T Cells and Accessory Cells. Pooled lymph node cells were removed from TCR transgenic mice or BALB/c mice (4–8 wk old) and passed through a nylon wool column. Cells were then collected and stained with either phycoerythrin (PE)-labeled anti-CD4 and fluorescein isothiocyanate (FITC) anti-V_α11 (RR8-1) or PE-labeled anti-CD4 and FITC anti-LECAM-1 (Mel 14) antibodies (20). These cells were subjected to fluorescence-activated cell sorting with a FACStar Plus (Becton Dickinson). Post-sort analysis revealed >99.6% CD4+/V_α11+ or CD4+/LECAM-1+ for TCR transgenic T cells and BALB/c T cells, respectively. T-cell-depleted spleen cells (TDS) were prepared from B10.A mice by removing T cells from splenocytes by treatment with a mixture of anti-Thy1.2 (HO134; ATCC TIB 99) (21), anti-CD4 (RL172) (22), and anti-CD8 (3.155) (23) antibodies plus Low-tox M rabbit complement (Cedarlane Laboratories). Fibroblasts expressing I-E^k as a result of transfection (DCEK) were a generous gift of Ronald Germain (Laboratory of Immunology, NIAID).

Primary and Secondary Stimulation of T Cells. Primary stimulation of transgenic T cells was carried out by adding 3–5 $\times$ 10^5 sorted CD4+/V_α11 T cells to individual wells of 24-well plates in a total vol of 1 ml with accessory cells, peptide, and lymphokines. After 36–48 h, cells were transferred to 5-ml dishes supplemented with fresh medium for an additional 48 h to allow further expansion. Cultures were washed three times and 5 $\times$ 10^4 T cells were restimulated with peptide and APCs in a total vol of 200 μl for 36 h. Supernatants were collected and assayed for lymphokine. Primary stimulation of sorted CD4+/LECAM-1+ from BALB/c mice was carried out by adding 3 $\times$ 10^5 T cells to 5-ml dishes coated with anti-CD3 antibody (10 μg/ml). Various combinations of lymphokines were added. After 4 days, cells were collected, washed extensively, and restimulated by adding 3 $\times$ 10^4 T cells in 200 μl to 96-well plates coated with anti-CD3 antibody in the presence of IL-2 or IL-12. Thirty-six hours later, supernatants were collected and assayed for lymphokine. T cells reexamined by fluorescence-activated cell sorting analysis after the 4-day culture, prior to restimulation, were found to be >99% CD4+.

RESULTS

IL-12 Enhances the Priming of T Cells to Produce IFN-γ Upon Restimulation. Sorted CD4+ T cells from TCR transgenic mice were cultured with DCEK cells, peptide, and IL-2. We chose to use DCEK as APCs since they express no detectable mRNA for the p35 or p40 chains of IL-12 (data not shown). After a 4-day priming culture, the cells were restimulated with fresh DCEK and peptide. In the secondary culture, 5 $\times$ 10^4 cells produced 115 units of IFN-γ per ml (Fig. 1). Addition of IL-12 to the priming culture resulted in cells that produced 800 units of IFN-γ per ml on secondary stimulation. In a series of experiments with DCEK or TDS as APCs, IL-12 was observed to increase priming for IFN-γ production by 1.8- to 25.1-fold with a mean of 9.0 $\pm$ 3.2 (Table 1). Addition of an anti-IL-12 antiserum neutralized the increase in priming for IFN-γ production caused by exogenous IL-12 (Fig. 1). A control serum failed to alter the IL-12 effects.

Priming for IFN-γ Can Occur in the Absence of IL-12 if IL-2 Is Present. To determine whether IL-12 was essential for priming naive CD4+ T cells for IFN-γ production, we tested the capacity of anti-IL-12 to inhibit the priming for IFN-γ production that is observed in the absence of added IL-12. In five experiments (Table 1), priming for IFN-γ production was observed in the presence of peptide and IL-2. In each case, as already noted, IL-12 enhanced such priming. Although anti-IL-12 antibody caused a modest inhibition of priming for IFN-γ production in the absence of exogenous IL-12 in some experiments, the mean degree of inhibition was by a factor of 0.0 $\pm$ 0.21. Thus, T cells could be primed for IFN-γ production in the absence of IL-12, if IL-2 was present.

IL-12 Can Prime for IFN-γ Production in the Presence of IL-4. IL-4 has been shown to strikingly inhibit priming for IFN-γ production in the absence of exogenous IL-12 (4, 5). The addition of IL-12 to these cultures allows priming for IFN-γ. As little as 0.1 ng of IL-12 per ml is sufficient for detectable priming, while optimal priming required 1–10 ng/ml (Fig. 2). Nonetheless, IL-4 still exerts an inhibitory effect on IL-12-enhanced priming for IFN-γ production. Thus, in four experiments, using 10 ng of IL-12 per ml, addition of IL-4 to the priming culture reduced subsequent production of IFN-γ by a factor of 0.52 $\pm$ 0.04. This should be contrasted with the almost complete inhibition of priming for IFN-γ production exerted by IL-4 in the absence of IL-12. In those experiments, the mean inhibition was by a factor of 0.95 $\pm$ 0.03 (Table 2).

IL-12 Does Not Inhibit Priming for IL-4 Production. The effect of IL-12 on priming for IL-4 production was studied by culturing CD4+ T cells with peptide, TDS, IL-2, and IL-4 with or without IL-12. In four experiments, IL-12 did not have a major effect of priming for IL-4 production (Table 2).

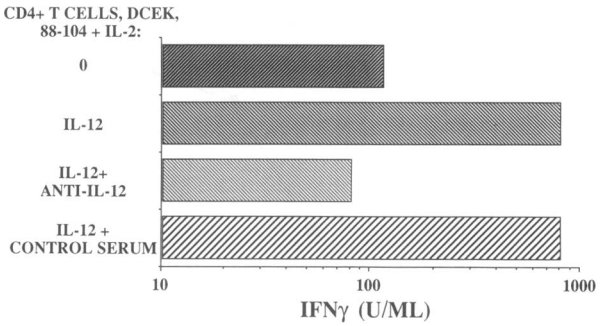

FIG. 1. IL-12 enhances priming of transgenic CD4+ T cells to become IFN-γ producers. Sorted CD4+ transgenic T cells (5 $\times$ 10^5) were primed by stimulation of cells cultured for 4 days with DCEK cells (1.5 $\times$ 10^5), peptide (1 μM), and IL-2 (10 units/ml), with IL-12, IL-2 and anti-IL-2 antibody (1:2000 dilution), or IL-12 and control serum. Cells were washed and recultured at 5 $\times$ 10^4 cells per 200 μl with DCEK cells (2 $\times$ 10^4) and peptide (1 μM) for 36 h. Supernatants were harvested and IFN-γ content was measured. Results are reported as IFN-γ units/ml.

Table 1. IL-12 enhances but is not essential for priming for IFN-γ production

Exp.	APC	IFN-γ, units/ml IL-2	IL-2 + anti-IL-12 antibody	Relative inhibition	IL-2 + IL-12	Relative increase
1	TDS	400	300	0.25	2200	7.3
	DCEK	200	370	−0.85	660	1.8
2	DCEK	680	400	0.42	1700	4.3
3	DCEK*	440	325	0.26	1200	3.7
4	DCEK	115	210	−0.82	800	3.8
	TDS	60	35	0.42	880	25.1
5	TDS	90	60	0.33	1000	16.7
		Mean ± SE†		0.00 ± 0.21		9.0 ± 3.2

Sorted CD4+ transgenic T cells (5×10^5) were primed by stimulation for 4 days with TDSs (1.5×10^5) or DCEK cells, peptide (1 μM), IL-2 (10 units/ml), with IL-12 (10 ng/ml), anti-IL-12 antibody (1:2000 dilution), or nothing. Supernatants were assessed for IFN-γ production after restimulation, and results are reported as described in Fig. 1. Relative inhibition is IFN-γ production in the presence of IL-2 plus anti-IL-12 antibody/IFN-γ production in the presence of IL-2. Relative increase is IFN-γ production in response to IL-2 plus IL-12/IFN-γ production in response to IL-2 plus anti-IL-12.

*T cells used in this experiment were sorted on the basis of CD4+/LECAM-1^{hi}.

†Mean ± SE of five experiments.

The mean inhibition was by a factor of 0.13 ± 0.45. These results indicate that in the presence of optimal concentrations of IL-4 and IL-12, priming for production of both IL-4 and IFN-γ will occur.

IL-12 Acts Directly on CD4+ T Cells to Increase Priming for IFN-γ Production. To determine whether IL-12 acts directly on CD4+ T cells to enhance priming for IFN-γ production, we used a culture system devoid of cells other than CD4+ T cells. CD4+/LECAM-1^{hi} T cells from BALB/c mice were purified by cell sorting and cultured on dishes coated with anti-CD3 antibody (Fig. 3). In experiment 1, cells were primed with immobilized anti-CD3 antibody in the presence of IL-2 and anti-IL4 antibody with or without IL-12. Cells primed in the presence of IL-12 produced >300 units of IFN-γ per ml after restimulation; cells primed in the absence of IL-12 produced amounts of IFN-γ that were below the limits of detection (<5 units/ml). Experiment 2 showed a similar result. Furthermore, IFN-γ had no effect on enhancing priming for IFN-γ production in the absence of IL-12.

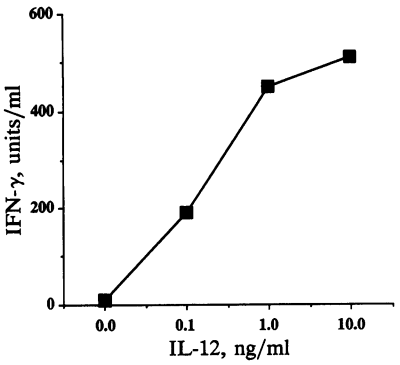

FIG. 2. IL-12 induces priming for IFN-γ production in the presence of IL-4. Sorted CD4+ transgenic T cells (5×10^5) were primed by stimulation for 4 days with TDSs (1.5×10^5), peptide (1 μM), IL-2 (10 units/ml), and IL-4 (1000 units/ml) in the presence of 0–10 ng of IL-12 per ml. Supernatants were assessed for IFN-γ content after stimulation, and results are reported as described in Fig. 1.

Does IFN-γ Play a Role in IL-12-Mediated Enhancement of Priming for IFN-γ Production? IFN-γ has been proposed as an inducer of priming for IFN-γ production (24–27). We wished to determine whether neutralizing IFN-γ during the priming culture would affect the capacity of IL-12 to enhance priming for subsequent IFN-γ production. CD4+ TCR transgenic T cells were primed with DCEK or TDS, peptide 88–104, IL-2, and exogenous IFN-γ or IL-12. In two separate experiments with DCEK as APCs exogenous IFN-γ did not increase the priming for IFN-γ production, while IL-12 enhanced priming for IFN-γ production 3-fold in experiment 1 and 8-fold in experiment 2 (Fig. 4). To further demonstrate that it was IL-12 and not IFN-γ that was mediating the increase in priming for IFN-γ production, we added IL-12 in the presence of a neutralizing monoclonal anti-IFN-γ antibody. Anti-IFN-γ failed to diminish the effect of IL-12 in enhancing priming for IFN-γ production. Moreover, several experiments using TDSs as APCs gave similar results (data not shown). By contrast, in an accessory cell-independent system, the ability of IL-12 to enhance production of IFN-γ was substantially diminished in the presence of anti-IFN-γ antibody (Fig. 4). Thus, this leaves open the possibility that IFN-γ has a role in enhancing IFN-γ production from naive T cells.

DISCUSSION

Several recent studies have demonstrated that IL-12 has a striking effect on increasing production of IFN-γ from T lymphocytes and NK cells (9, 10). We show here, as Hsieh *et al.* (12) have recently reported, that IL-12 enhances priming for IFN-γ production. In our experiments, IL-12 caused a 9-fold increase in IFN-γ production by purified CD4+ T cells from TCR transgenic mice in response to antigen and APCs. On the other hand, priming for IFN-γ production can be achieved in the absence of added IL-12, even when no endogenous source of IL-12 is present. Thus, using DCEK cells, which fail to express mRNA for either the p35 or p40 chains of IL-12, as APCs and purified CD4+ T cells, priming for IFN-γ does occur. Furthermore, in a series of five experiments, using either DCEK or TDS as APCs, anti-IL-12 antibody did not significantly inhibit priming for IFN-γ production in the absence of added IL-12. In one of these experiments, the transgenic T cells had been sorted for expression of high levels of LECAM-1, further indicating that naive CD4+ T cells can be primed to produce IFN-γ in the absence of IL-12. In these experiments, as in priming experiments in which IL-12 was added, IL-2 was essential for priming for IFN-γ production (R.A.S. and W.E.P., unpublished data). Thus, we conclude that IL-12 enhances but is not required for priming for IFN-γ production.

IL-12 can enhance priming for IFN-γ production by direct action on T cells. Its action on T cells was shown in experiments in which sorted CD4+, LECAM-1+ T cells from BALB/c mice were cultured with immobilized anti-CD3 antibody and IL-2. In this system, IL-12 strikingly increased priming for IFN-γ production. Since the only cells in this system are CD4+ T cells and those cells used have been selected for expression of a marker (LECAM-1) associated with a naive phenotype (28), these experiments provide a clear demonstration that the IL-12 effect in priming can be mediated directly on resting T cells.

Others have suggested that IFN-γ itself may act to induce or enhance priming for IFN-γ production *in vitro* from naive T cells (24–27). Although we could find no evidence of this in our previous studies carried out by priming with peptide, APCs, and IL-2 (4), we directly tested whether IFN-γ might be required for the enhanced priming mediated by IL-12. Similar to a recent report by Macatonia *et al.* (27), the presence of exogenous IFN-γ did not enhance subsequent

Table 2. Cross-regulatory effects of IL-4 and IL-12

	IFN-γ, units/ml						IL-4 units/ml		
	No IL-12			IL-12 (10 ng/ml)					
Exp.	Anti-IL-4 antibody	IL-4	Inhibition	Anti-IL-4 antibody	IL-4	Inhibition	No IL-12	IL-12	Inhibition
1	1300	0	1.00	7000	2800	0.60	5300	13,000	−1.45
2	400	36	0.91	2200	1270	0.42	9000	4,500	0.50
3	60	0	1.00	880	380	0.57	2100	1,700	0.19
4	90	10	0.90	1000	510	0.50	2700	2,000	0.26
		Mean ± SE*	0.95 ± 0.03			0.52 ± 0.04			0.13 ± 0.45

For IFN-γ production, sorted CD4+ transgenic T cells (5×10^5) were primed by stimulation for 4 days with TDS (1.5×10^5), peptide (1 μM), IL-2 (10 units/ml), IL-4, or anti-IL-4 antibody in the presence or absence of exogenous IL-12. Inhibition of IFN-γ production in the presence or absence of IL-12 was calculated by dividing the amount of IFN-γ produced when IL-4 was present in the primary culture by that produced in the presence of anti-IL-4 antibody. To evaluate priming for IL-4 production, sorted CD4+ transgenic T cells (5×10^5) were primed by stimulation for 4 days with TDS (1.5×10^5), peptide (1 μM), IL-2 (10 units/ml), and IL-4 (1000 units/ml) in the presence or absence of exogenous IL-12 (10 ng/ml). Inhibition of IL-4 production due to the presence of IL-12 was calculated by dividing the amount of IL-4 produced when IL-12 plus IL-4 was present in the primary culture by that produced when IL-4 alone was present.
*Mean ± SE of four experiments.

IFN-γ production. Moreover, we observed that anti-IFN-γ antibody did not substantially diminish the enhanced priming for IFN-γ production caused by addition of IL-12 in accessory cell-dependent priming. However, in accessory cell-independent priming, the addition of anti-IFN-γ antibody to cultures substantially diminished the IL-12-mediated increase in production of IFN-γ. The fact that IFN-γ does not replace the action of IL-12 rules out the possibility that the sole function of IL-12 is to induce IFN-γ, with the latter being the actual priming stimulant for IFN-γ-producing cells. Our failure to block priming for IFN-γ production in the accessory cell-dependent system but not in the accessory cell-independent system suggests that the requirements for priming in the two systems may be different. Indeed, IFN-γ may act as cofactor with IL-12 providing a stimulant that can also be mediated by APCs in the accessory cell-dependent priming system. However, the possibility that we failed to completely neutralize all the IFN-γ in the accessory cell-dependent system cannot be unequivocally excluded. This would be a tenable explanation only if very small amounts of IFN-γ were required to mediate its inducing effect since little or no IFN-γ is detected early in the priming culture. Furthermore, the amounts of anti-IFN-γ antibody added to the priming cultures should be sufficient to neutralize virtually all such IFN-γ. Thus, if IFN-γ is essential, a small nonneutralized residuum would have to mediate this effect. IFN-γ does appear to have a substantial effect in priming for IFN-γ production *in vivo*. In infection with *Leishmania*, Scott (25) and Chatelain *et al.* (26) have demonstrated that anti-IFN-γ antibody treatment diminished subsequent production of IFN-γ as measured *in vitro* from whole lymph nodes. The mechanism through which anti-IFN-γ antibody treatment suppresses priming for IFN-γ production may be indirect (i.e., by enhancing IL-4 production) or it may be due to a direct action of IFN-γ on T cells that is not reflected in the *in vitro* priming system.

In physiologic situations, it seems likely that priming will often occur in the presence of mixtures of IL-4 and IL-12. To gain insight into the effects of these two agents in one anothers' presence, we carried out priming experiments

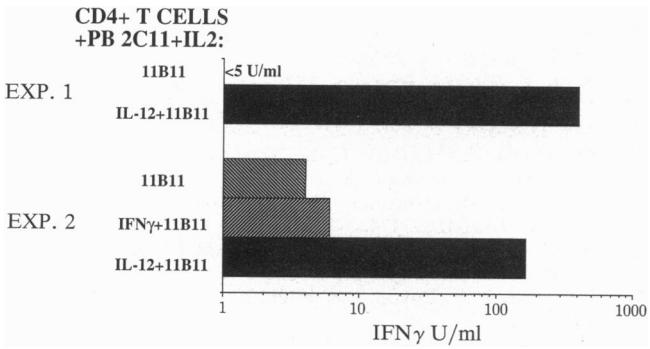

FIG. 3. IL-12 has a direct effect on T cells to increase priming for IFN-γ production. Sorted CD4+/LECAM-1+ T cells (3×10^5) from naive BALB/c mice were stimulated for 4 days with immobilized anti-CD3, IL-2, and anti-IL-4 antibodies (10 μg/ml) in the presence of IFN-γ or IL-12. Cells were washed three times, and 3×10^4 cells per 200 μl were restimulated for 36 h in 96-well plates coated with anti-CD3 antibody in the presence of IL-2. Supernatants were assessed for IFN-γ content, and results are reported as described in Fig. 1.

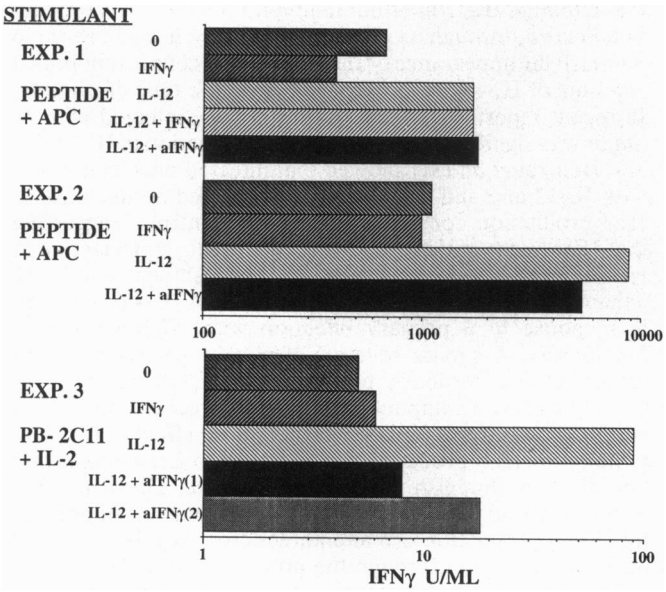

FIG. 4. Role of IFN-γ in the IL-12-mediated enhancement of priming for IFN-γ production. Experiments 1 and 2: sorted CD4+ transgenic T cells (5×10^5) were primed by stimulation for 4 days with DCEK cells (1.5×10^5), peptide (1 μM), and IL-2 (10 units/ml) in the presence of IFN-γ (500 units/ml), IL-12 (10 ng/ml), IL-12 and IFN-γ, or IL-12 and anti-IFN-γ antibody (10 μg/ml). Experiment 3: sorted CD4+/LECAM-1+ T cells (3×10^5) from naive BALB/c mice were stimulated for 4 days with immobilized anti-CD3 antibody, IL-2, and either IFN-γ (2000 units/ml), or IL-12 (10 μg/ml) in the presence or absence of anti-IFN-γ antibody (10 μg/ml). Two separate preparations of anti-IFN-γ antibody were used. Cells were washed three times, and 3×10^4 cells per 200 μl were restimulated for 36 h in 96-well plates coated with anti-CD3 antibody in the presence of IL-2. Supernatants were assayed for IFN-γ content after restimulation, and results are reported as described in Fig. 1.

using both IL-4 and IL-12. We and others have previously demonstrated that IL-4 is essential for the *in vitro* development of naive CD4+ T cells into IL-4 producers (3–5, 29) and, when present at concentrations of 1000 units/ml ($\approx 0.4 \times 10^{-10}$ M), is strikingly inhibitory for priming for IFN-γ production. When suboptimal amounts of IL-4 were used (100 units/ml) in the priming culture, inhibition for priming for IFN-γ production was only partial. Here we show that even in the presence of optimal concentrations of IL-4, IL-12 allows priming for IFN-γ production. Indeed, as little as 0.1 ng of IL-12 per ml is sufficient to obtain measurable priming for IFN-γ production. Nonetheless, IL-4 does have cross-regulatory activity even in the presence of optimal amounts of IL-12; it caused inhibition of such priming by a factor of 0.52 ± 0.04. However, this should be contrasted with its virtual complete inhibition of priming for IFN-γ production in the absence of IL-12.

By contrast, IL-12 has little effect on priming for IL-4 production. Thus, if sufficient IL-4 is present to prime for IL-4 production, even in the presence of IL-12 such priming will occur. We have previously shown that when suboptimal concentrations of IL-4 are used (100 units/ml; 0.4×10^{-11} M), IFN-γ diminished the amount of IL-4 produced upon restimulation, suggesting a possible cross-regulatory role for IFN-γ and, thus, an indirect cross-regulatory role for IL-12. This is consistent with reports showing that IFN-γ diminishes the appearance of IL-4-producing cells in mice infected with *L. major*. Moreover, during the derivation of T-cell clones, IFN-γ was inhibitory for the appearance of IL-4-producing T-cell clones (30).

Our *in vitro* studies and those of others suggest that *in vivo* establishment of a lymphokine-producing phenotype may be quite complex. Thus, we and Hsieh *et al.* (12) have shown that although IL-12 does not inhibit priming for IL-4 production *in vitro*, through its induction of IFN-γ, it might serve to diminish the appearance of IL-4-producing cells if suboptimal amounts of IL-4 were present *in vivo* at the time of priming. In recent reports, IL-4 production in mice infected with *L. major* was significantly decreased after injection of IL-12 (31, 32). Heinzel *et al.* (31) showed that infected mice coinjected with IL-12 and anti-IFN-γ antibody showed no decrease in IL-4 production compared to infected controls, suggesting that IFN-γ was critical in downregulating IL-4 production *in vivo*. Similarly, Morris *et al.* (33) have shown that IL-12 enhances IFN-γ production and diminishes IL-4 production in response to a primary infection with *Nippostrongylus brasiliensis*. A similar reversal of phenotype has been observed in the response to injected schistosome eggs (I. Oswald and A. S., unpublished data). Moreover, anti-IFN-γ antibody completely blocked the inhibitory effect of IL-12 on priming for IL-4 production in response to *Leishmania* (31) but did not diminish priming for IFN-γ. These *in vivo* experiments all point to IL-12 having a direct role in priming for IFN-γ production and an indirect cross-regulatory effect on IL-4 production through the production of IFN-γ.

These results indicate that the cytokines present at the outset of a response powerfully determine the character of the response that ensues and suggest that optimal vaccine strategies for protection against intracellular organisms might include both administration of IL-12 and neutralization of IL-4. A major question that remains open is whether established Th1 or Th2 phenotypes can be altered by manipulations of the cytokine environment.

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1. Powers, G. D., Abbas, A. K. & Miller, R. A. (1988) *J. Immunol.* **140,** 3352–3357.
2. Weinberg, A. D., English, M. & Swain, S. L. (1990) *J. Immunol.* **144,** 1800–1807.
3. LeGros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D. & Paul, W. E. (1990) *J. Exp. Med.* **172,** 921–929.
4. Seder, R. A., Paul, W. E., Davis, M. M. & de St. Groth, B. F. (1992) *J. Exp. Med.* **176,** 1091–1098.
5. Hsieh, Y.-S., Heimberger, A. B., Gold, J. S., O'Garra, A. & Murphy, K. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6065–6069.
6. Heinzel, F. P., Sadick, M. D., Holaday, B. J., Coffman, R. L. & Locksley, R. M. (1989) *J. Exp. Med.* **169,** 59–72.
7. Romani, L., Mencacci, A., Mocci, S., Mosci, P., Puccetti, P. & Bistoni, F. (1992) *J. Exp. Med.* **176,** 19–25.
8. Gross, A., Ben-Sasson, S. Z. & Paul, W. E. (1993) *J. Immunol.* **150,** 2112–2120.
9. Wolf, S. F., Temple, P. A., Kobayashi, M., Young, D., Dicig, M., Lowe, L., Dzialo, R., Fitz, L., Ferenz, C., Hewick, R. M., Kelleher, K., Herrmann, S. H., Clark, S. C., Azzoni, L., Chan, S. H., Trinchieri, G. & Perussia, B. (1991) *J. Immunol.* **146,** 3074–3081.
10. Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospisil, M., Young, H. A., Wolf, S. F., Young, D., Clark, S. C. & Trinchieri, G. (1991) *J. Exp. Med.* **173,** 869–879.
11. Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M.-P., Maggi, E., Trincheri, G. & Romagnani, S. (1993) *J. Exp. Med.* **177,** 1199–1204.
12. Hsieh, C.-S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A. & Murphy, K. M. (1993) *Science* **260,** 547–549.
13. Schoenhaut, D. S., Chua, A. O., Wolitzky, A. G., Quinn, P. M., Dwyer, C. M., McComas, W., Familletti, P. C., Gately, M. K. & Gubler, U. (1992) *J. Immunol.* **148,** 3433–3440.
14. Ohara, J. & Paul, W. E. (1985) *Nature (London)* **315,** 333–336.
15. Cherwinski, H., Schumacher, J., Brown, K. & Mosmann, T. (1987) *J. Exp. Med.* **166,** 1229–1244.
16. Leo, O., Foo, M., Sachs, D., Samelson, L. E. & Bluestone, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378.
17. Curry, R. C., Keiner, P. A. & Spitalny, G. L. (1987) *J. Immunol. Methods* **104,** 137–142.
18. Mosmann, T. R. & Fong, T. A. T. (1989) *J. Immunol. Methods* **116,** 151–158.
19. Hu-Li, J., Ohara, J., Watson, C., Tsang, W. & Paul, W. E. (1989) *J. Immunol.* **142,** 800–807.
20. Fink, P., Gallatin, W., Reichert, R., Butcher, E. & Weissman, I. (1985) *Nature (London)* **313,** 233–235.
21. Marshak-Rothstein, A., Fink, P., Gridley, T., Raulet, D. H., Bevan, M. J. & Gefter, M. L. (1979) *J. Immunol.* **122,** 2491–2497.
22. Dialynas, D. P., Quan, Z. S., Wall, K. A., Pierres, A., Quintan, J., Loken, M. R., Pierres, M. & Fitch, F. (1983) *J. Immunol.* **131,** 2445–2451.
23. Sarminento, M., Glasebrook, A. L. & Fitch, F. W. (1980) *J. Immunol.* **125,** 2665–2672.
24. Swain, S. L., Weinberg, A. D., English, M. & Huston, G. (1990) *FASEB J.* **4,** 2020 (abstr.).
25. Scott, P. (1991) *J. Immunol.* **147,** 3149–3155.
26. Chatelain, R., Varkila, K. & Coffman, R. L. (1992) *J. Immunol.* **148,** 1182–1187.
27. Macatonia, S. E., Hsieh, C.-S., Murphy, K. M. & O'Garrar, A. (1993) *Int. Immunol.*, **5,** in press.
28. Bradley, L. M., Duncan, D. D., Tonkonogy, S. & Swain, S. L. (1991) *J. Exp. Med.* **174,** 547–559.
29. Swain, S. L., Weinberg, A. D., English, M. & Huston, G. (1990) *J. Immunol.* **145,** 3796–3806.
30. Gajewski, T. F., Joyce, J. & Fitch, F. W. (1989) *J. Immunol.* **143,** 15–22.
31. Heinzel, F. P., Schoenhaut, D. S., Rerko, R. M., Rosser, L. E. & Gately, M. K. (1993) *J. Exp. Med.* **177,** 1505–1509.
32. Sypek, J. P., Chung, C. L., Mayor, S. E. H., Subramanyam, J. M., Goldman, S. J., Sieburth, D. S., Wolf, S. F. & Schaub, R. G. (1993) *J. Exp. Med.* **177,** 1797–1802.
33. Morris, S. C., Hubbard, B., Gately, M., Gause, W. C. & Finkelman, F. D. (1993) *J. Immunol.* **150,** 45A (abstr.).