

Differentiation and Stability of T Helper 1 and 2 Cells Derived from Naive Human Neonatal CD4⁺ T Cells, Analyzed at the Single-cell Level

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Summary

The development of CD4⁺ T helper (Th) type 1 and 2 cells is essential for the eradication of pathogens, but can also be responsible for various pathological disorders. Therefore, modulation of Th cell differentiation may have clinical utility in the treatment of human diseases. Here, we show that interleukin (IL) 12 and IL-4 directly induce human neonatal CD4⁺ T cells, activated via CD3 and CD28, to differentiate into Th1 and Th2 subsets. In contrast, IL-13, which shares many biological activities with IL-4, failed to induce T cell differentiation, consistent with the observation that human T cells do not express IL-13 receptors. Both the IL-12-induced Th1 subset and the IL-4-induced Th2 subset produce large quantities of IL-10, confirming that human IL-10 is not a typical human Th2 cytokine. Interestingly, IL-4-driven Th2 cell differentiation was completely prevented by an IL-4 mutant protein (IL-4.Y124D), indicating that this molecule acts as a strong IL-4 receptor antagonist. Analysis of single T cells producing interferon γ or IL-4 revealed that induction of Th1 cell differentiation occurred rapidly and required only 4 d of priming of the neonatal CD4⁺ T cells in the presence of IL-12. The IL-12-induced Th1 cell phenotype was stable and was not significantly affected when repeatedly stimulated in the presence of recombinant IL-4. In contrast, the differentiation of Th2 cells occurred slowly and required not only 6 d of priming, but also additional restimulation of the primed CD4⁺ T cells in the presence of IL-4. Moreover, IL-4-induced Th2 cell phenotypes were not stable and could rapidly be reverted into a population predominantly containing Th0 and Th1 cells, after a single restimulation in the presence of IL-12. The observed differences in stability of IL-12- and IL-4-induced human Th1 and Th2 subsets, respectively, may have implications for cytokine-based therapies of chronic disease.

The outcome of a protective immune response against a pathogenic threat depends to a great extent on the differentiation of specialized Th subsets secreting specific cytokines. Two subsets of CD4⁺ Th cells, referred to as Th1 and Th2, have been identified, initially in the mouse and later in the human (1–5). Murine Th1 cells produce IL-2 and IFN- γ , but no IL-4 and IL-5, and have been shown to play an important role in cell-mediated immune responses against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-10, but no IFN- γ , and are involved in humoral immunity and allergic responses. A subset of Th cells, designated Th0 cells, simultaneously secretes Th1 as well as Th2 cytokines (6–9). Th0 cells may represent a stable and distinct population of differentiated Th cells or an intermediate stage of Th cells in the process of differentiating into Th1 or Th2 cells (10).

Although cytokine production profiles of human CD4⁺ Th1 and Th2 T cell clones are reminiscent of those of mu-

rine Th1 or Th2 clones, the range of cytokines secreted is generally less polarized. Human Th1 and Th2 clones can still produce IL-4 or IFN- γ , respectively, depending on their mode of activation (3, 11). In addition, human Th2 clones are able to produce significant amounts of IFN- γ when activated in the presence of IL-12 (12, 13) or when stimulated with phorbol ester and calcium ionophore (11), demonstrating that the IFN- γ gene is not permanently silent in these cells.

It is clear from studies in the mouse, as well as in the human, that the presence of cytokines at the onset of an immune response determines the development of a particular Th phenotype. Priming of naive cells *in vitro* in the presence of IL-4 results in the generation of IL-4-producing Th2 effector cells (14–18). Furthermore, IL-4 has also been shown to induce Th2 responses *in vivo* (19, 20). Conversely, IL-12, produced by monocytes and dendritic cells, drives the differentiation of naive T cells toward IFN- γ -pro-

ducing cells (21–23) and also enhances the production of IFN- γ by allergen-specific Th2 clones (12, 13).

In view of the important role that Th subsets seem to play in the regulation of immune responses, it is important to obtain information about the stability of the Th1 and Th2 phenotypes. It has been shown that polarized IL-12-induced mouse Th1 populations still responded to the Th2 cell differentiation-inducing effects of rIL-4 *in vitro* (24, 25). In contrast, the phenotype of Th2 populations derived from transgenic mice was not reversible by stimulation with rIL-12 (24), whereas stimulation of *Leishmania major*-specific Th2 cells in the presence of rIFN- γ and rIL-12 enhanced the production of IFN- γ , but was not able to induce a total conversion of these cells into Th1 cells (25). The resistance of CD4⁺ murine Th2 cells to the effects of rIL-12 may be due to an IL-12-signaling defect reflecting a downregulation of IL-12 receptors on these cells (26).

In the human, it has been reported that purified protein derivative-specific T cell clones that normally show a Th1-like cytokine production profile secreted high levels of IL-4, as well as IFN- γ , when they were generated in the presence of IL-4 *in vitro* (17). Conversely, allergen-specific T cell clones, generated in the presence of IFN- α , IFN- γ (17), or IL-12 (23), produced little IL-4 and high levels of IFN- γ . Although the latter results show that antigen-specific T cells can be induced to produce certain cytokines in response to stimulation with recall antigens, little is known about the conditions responsible for the development and reversibility of committed Th1 and Th2 populations.

In the present study, we have studied the effect of the differentiation-inducing cytokines IL-4 and IFN- γ on the *in vitro* development of Th1 and Th2 subsets from naive human neonatal CD4⁺ T cells. In addition, using flow cytometry we have analyzed the production of IL-4 and IFN- γ at the single-cell level to determine the plasticity of polarized Th1 and Th2 subsets.

Materials and Methods

Human Neonatal CD4⁺ T Cells. Human neonatal leukocytes were isolated from freshly collected, heparinized, neonatal blood by density gradient centrifugation using Histopaque 1077 (Sigma Chemical Co., St. Louis, MO). Adherent cells were removed by incubation of the cells on a plastic petri dish (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) for 1 h at 37°C in the presence of 6 μ g/ml DNase (Sigma Chemical Co.). Neonatal CD4⁺ T cells were purified from nonadherent lymphocytes using magnetic microspheres. Briefly, nonadherent cells were incubated with anti-CD4 mAb-coated Dynabeads (Dyna, Great Neck, NY) at a 10:1 bead to cell ratio for 1 h at 4°C with gently rotating. CD4⁺ T cells were isolated from the bead/cell mixture by exposure to a magnetic field using a magnetic particle concentrator (Dyna), according to the manufacturer's instructions. Cells were washed five times with PBS, supplemented with 2% FCS, and the bead/cell complexes were incubated with Dynal Detach-ahead reagent for 1 h at room temperature with vigorous shaking. The detached cells were collected by applying a magnetic field, followed by washing twice with PBS/2% FCS. The purity of

positively selected neonatal CD4⁺ T cells, using this procedure, was typically >97%, as determined by flow cytometry.

Mouse L Cell Transfectants. Mouse L cells, transfected with CD32 (Fc γ RII) (27), human CD58 (LFA-3), and human CD80 (B7.1), were generated as described previously (28, 29) and were kind gifts from Drs. R. de Waal Malefyt and L.L. Lanier (DNAX Research Institute). The expression of CD58 and CD80 on the L cell transfectants was monitored periodically by flow cytometry.

mAbs and Reagents. mAbs used for activation of T cells were the anti-CD3 mAb SPV-T3b (30) and the anti-CD28 mAb L293.1 (a kind gift of Dr. L. Lanier). mAbs used for intracellular cytokine staining were the anti-hIL-4 mAb 25D2 (31) and the anti-hIFN- γ mAb B27 (32). Cytokines used in this study were rIL-2, rIL-13 (generously provided by Drs. G. Zurawski and S. Menon, respectively, from the DNAX Research Institute), rIL-4 (Schering-Plough Research Institute, Kenilworth, NJ), and rIL-12 (purchased from R & D Systems, Minneapolis, MN). The IL-4R antagonist IL-4.Y124D (33) was a generous gift of Dr. G. Zurawski. Cells were cultured in DMEM/High, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin (JRH Biosciences, Lenexa, KS), and 10% FCS (Sigma Chemical Co.).

Culture Conditions. L cell transfectants, used in coculture experiments were detached by 20-min incubation at 37°C in EDTA (Versene; Flow Laboratories, McLean, VA) and were washed twice in PBS (supplemented with 2% FCS), irradiated at 7,000 rad, and washed once in DMEM/10% FCS. 2×10^5 neonatal CD4⁺ T cells were incubated with 2×10^5 irradiated L cell transfectants, 100 ng/ml of anti-CD3 mAb, and 100 U/ml of rIL-2 in the presence or absence of combinations of 10 ng/ml rIL-4, 0.5 ng/ml rIL-12, 200 U/ml rIL-13, or various concentrations of the IL-4R antagonist IL-4.Y124D, in 24-well tissue culture plates (Linbro, Flow Laboratories) in a final volume of 1 ml. Neonatal CD4⁺ T cells were cultured for 6 d, washed with fresh culture medium, and restimulated under the same conditions for another 6 d after which the production of cytokines was measured, as described below.

Restimulation of Cells for Analysis of Cytokine Production by Cytokine-specific Immunoassay. Anti-CD3 mAb was coated overnight on tissue culture plates at a concentration of 10 μ g/ml in PBS and plates were washed twice with medium before the addition of the cells. 10^6 cultured neonatal T cells/ml were stimulated with plastic-coated anti-CD3 mAb and 1 μ g/ml of soluble anti-CD28 mAb, in the presence of 100 U/ml rIL-2. After 48 h of activation, supernatants were harvested, spun at 250 g to remove remaining cells, aliquoted, and stored at -80°C before testing. Cytokine levels in the supernatants of activated neonatal CD4⁺ T cells were determined by cytokine-specific immunoassays, using antibodies specific for IL-4, IL-5, IL-10, IL-13, and IFN- γ , as described previously (34). Sensitivity of the assay for IL-13 production was 100 pg/ml, whereas the sensitivity of all other assays was 50 pg/ml.

Restimulation of Cells for Analysis of Intracellular Cytokine Production by Flow Cytometry. Intracellular cytokines were detected by flow cytometry using the method of Andersson et al. (9), with modifications (35–38). Briefly, 10^6 neonatal CD4⁺ T cells/ml were stimulated with plate-bound anti-CD3 mAb and 1 μ g/ml of soluble anti-CD28 mAb in the presence of 100 U/ml rIL-2. After 3 h of activation, 10 μ g/ml of Brefeldin A (Sigma Chemical Co.) was added to the cultures and the cells were cultured for an additional 2 h. After a total of 5 h of activation, neonatal CD4⁺ T cells were washed in PBS, fixed by incubation for 20 min with 2% formaldehyde (Sigma Chemical Co.), washed twice with

PBS, and permeabilized by incubation for 10 min in PBS (supplemented with 2% FCS, 2 mM NaN₃, and 0.5% saponin; Sigma Chemical Co.). All incubations were carried out at room temperature. Permeabilized T cells were incubated with PE-labeled anti-IL-4 mAb and FITC-labeled anti-IFN- γ mAb, diluted in PBS/FCS/NaN₃/saponin. After 30 min of incubation at room temperature, cells were washed twice with the same buffer, resuspended in PBS (supplemented with 2% FCS and 2 mM NaN₃), and analyzed using a FACScan[®] flow cytometer (Becton Dickinson & Co., San Jose, CA). Data are displayed as dot plots of PE (x-axis) and FITC (y-axis) fluorescence (four decade log scales). Quadrant markers were positioned to include >98% of control Ig stained cells in the lower left quadrant. Methods of flow cytometry and data analysis were carried out as described by Lanier and Recktenwald (39).

Results

rIL-12 Drives Th1 Development, and rIL-4 but Not rIL-13 Drives Th2 Development In Vitro. To determine the effects of IL-4, IL-12, and IL-13 on the differentiation of naive T cells, highly purified neonatal CD4⁺, CD45RA⁺ T cells were repeatedly activated with anti-CD3 mAb, cross-linked on CD32⁺ mouse L cells, expressing human CD58 and CD80, in the presence of these cytokines. All cultures contained 100 U/ml rIL-2 to ensure cell viability and proliferation. At the end of each of the two consecutive 6-d culture periods, referred to as first and second stimulation, respectively, the cells were activated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the presence of rIL-2, and the cytokine production profile was analyzed by immunoassay. As is shown in Table 1, neonatal CD4⁺ T cells stimulated twice in the presence of rIL-4 differentiated into Th2 populations that produced high amounts of IL-4, IL-5, and IL-13, upon subsequent activa-

tion with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the presence of rIL-2. The production of IFN- γ in these cultures was generally low (but not absent) and varied between donors. In contrast, in the presence of rIL-12, neonatal CD4⁺ T cells from seven out of seven donors differentiated into Th1 cells, secreting high amounts of IFN- γ . However, in three out of seven donors, neonatal CD4⁺ T cells cultured in the presence of rIL-12 also produced significant levels of IL-4 and IL-13 after activation, whereas in the other four donors, IL-4 and IL-13 production was below the level of detection. The production of IL-5, however, was never detectable, suggesting that IL-12 induced the differentiation of an IL-4⁻ and IL-13⁻, but not IL-5⁻ producing T cell subset under these conditions. The Th1 and Th2 subsets generated in the presence of rIL-4 or rIL-12, respectively, both produced high levels of IL-10, confirming previous results indicating that IL-10 is produced by both human Th1 and Th2 cells (40). Furthermore, these results are consistent with data showing that IL-12 synergizes with IL-2 to induce IL-10 production (41), although in all experiments, rIL-4-driven cultures tended to produce higher levels of IL-10. Activation of neonatal CD4⁺ T cells in the presence of a combination of rIL-4 and rIL-12 resulted in the generation of a population of T cells that produced relatively high amounts of both Th1 and Th2 cytokines, including IL-4, IL-5, IL-10, IL-13, and IFN- γ (Table 1).

T cells stimulated in the presence of both rIL-4 and rIL-12 produced higher levels of IL-4, IL-5, and IL-13 after activation, as compared with T cells that developed in the presence of rIL-12 alone. Levels of IFN- γ production in these cultures, however, were comparable. Conversely, the levels of IL-4 production were reduced by ~30–50%, but a strong increase in IFN- γ production was observed, as com-

Table 1. Cytokine Production Profile of Th1 and Th2 Populations, Generated from Human Neonatal CD4⁺ T Cells

Cytokines added	Cytokine production (ng/10 ⁶ cells)									
	IL-4		IL-5		IL-10		IL-13		IFN- γ	
Control	0.1	(<0.1–0.7) (n = 7)	<0.1	(<0.1–0.12) (n = 7)	4.4	(1.6–6.4) (n = 6)	0.6	(<0.2–1.9) (n = 3)	0.2	(<0.1–1.3) (n = 7)
IL-4	2.5	(0.67–7.0) (n = 7)	1.7	(0.4–2.9) (n = 7)	68.8	(42.9–>100) (n = 6)	9.3	(6.6–11.4) (n = 3)	0.2	(0.1–0.6) (n = 7)
IL-12	0.8	(<0.1–3.6) (n = 7)	0.1	(<0.1–0.2) (n = 7)	33.0	(19.3–53.0) (n = 6)	2.3	(<0.2–5.6) (n = 3)	3.9	(1.3–>10.0) (n = 7)
IL-4 + IL-12	1.8	(0.3–4.3) (n = 7)	0.9	(<0.1–1.7) (n = 7)	27.4	(4.0–59.0) (n = 6)	6.2	(1.2–10.8) (n = 3)	3.8	(1.22–>10.0) (n = 7)
IL-13	0.1	(<0.1–0.1) (n = 4)	<0.1	(<0.1–0.1) (n = 4)	4.6	(1.8–7.5) (n = 4)	0.5	(<0.2–1.4) (n = 3)	0.3	(<0.1–1.4) (n = 4)

Neonatal CD4⁺ T cells were stimulated twice, at 6-day intervals, with anti-CD3 mAb and L cell transfectants in the presence of rIL-2 (Control), or in the presence of rIL-2 and combinations of rIL-4, rIL-12 or rIL-13, respectively. Cells were harvested and cytokine production levels were analyzed by cytokine-specific immunoassay. Data are presented as average of “n” independent donors. Maximum and minimum cytokine titers for each group are included. Refer to Materials and Methods for conditions of culture and analysis for cytokine production.

pared with IL-4 and IFN- γ production by T cells stimulated in the presence of rIL-4 alone. Finally, in this culture system, rIL-13 added at concentrations as high as 200 U/ml was unable to induce the differentiation of neonatal CD4⁺ T cells into mature effector T cells, consistent with the observation that T cells do not express IL-13R.

The IL-4R Mutant Protein IL-4.Y124D, Specifically Inhibits IL-4-mediated Th2 Differentiation. Recently, we described an IL-4 mutant protein in which the Tyr residue at position 124 is replaced by an Asp (IL-4.Y124D) (33). IL-4.Y124D binds with high affinity to the IL-4R α chain without receptor activation. Since the IL-4R and the IL-13R chain share the IL-4R α chain that is required for signal transduction, IL-4.Y124D acts as an antagonist for both the IL-4R and IL-13R in vitro and in vivo (33, 42, 43). Here, we investigated whether the mutant IL-4 protein could inhibit IL-4-driven Th2 differentiation. As is shown in Fig. 1, IL-4.Y124D, added at a 20-fold excess over the concentration of rIL-4, completely prevented IL-4-induced differentiation of IL-5- and IL-13-producing cells. The addition of IL-4.Y124D also inhibited the generation of IL-4-producing cells in a dose-dependent way. Prevention of IL-4-induced differentiation of neonatal cells into IL-4-producing Th2 cells by IL-4.Y124D was confirmed by Northern analysis. No IL-4 mRNA could be detected in neonatal CD4⁺ T cells cultured in the presence of rIL-4 and a 20-fold excess of IL-4.Y124D (results not shown).

IL-4 and IL-12 Induce Neonatal CD4⁺ T Cells to Differentiate into Polarized Subsets of Th Cells. It is not possible to determine, using cytokine immunoassays, whether Th1 and Th2 cytokines are produced by the same cell or different cells within a polyclonal population. Therefore, we analyzed IL-4- and IL-12-induced differentiation of neonatal CD4⁺ T cells at the single-cell level, by measuring their intracellular production of IL-4 and IFN- γ by flow cytometry. Neonatal CD4⁺ T cells stimulated twice with cross-linked anti-CD3 mAb in the presence of exogenous rIL-4 gave rise to a population of Th2 cells (12–20%) that produced only IL-4 and no IFN- γ (Figs. 2 and 3). In addition, a small proportion (4–6%) of IFN- γ -producing cells was reproducibly detected in this population, indicating that

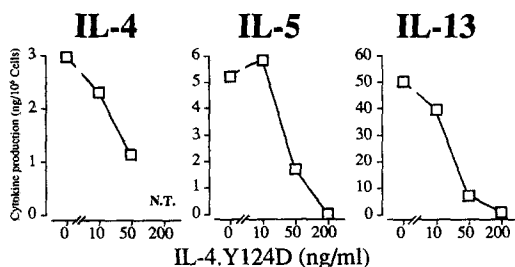


Figure 1. Effect of the IL-4 mutant protein IL-4.Y124D on IL-4-induced production of IL-4, IL-5, and IL-13 by differentiated neonatal CD4⁺ T cells. Neonatal CD4⁺ T cells were stimulated twice, at 6-d intervals, with anti-CD3 mAb and L cell transfectants, in the presence of rIL-2, rIL-4, and increasing concentrations of IL-4.Y124D. Cells were harvested and cytokine production levels were analyzed by cytokine-specific immunoassay. (N.T.) Not tested.

IL-4 induces the generation of a subset of IFN- γ -producing Th1 cells that appears relatively late after activation (Fig. 3). The majority (70–80%) of the cells did not appear to be committed to the production of IFN- γ or IL-4. However, it is not possible to conclude whether these cells are truly uncommitted or whether their cytokine production levels are below the sensitivity of the assay. These results, analyzed at the single-cell level, are consistent with the cytokine production levels measured by immunoassay (Table 1) and strongly suggest that the low levels of IFN- γ detected in rIL-4-primed cultures are produced by a subset of IFN- γ -producing Th1 cells.

In contrast, >30% of neonatal CD4⁺ T cells stimulated repeatedly in the presence of rIL-12 differentiated into Th1 cells, producing high levels of IFN- γ but no IL-4. However, a small fraction of neonatal CD4⁺ T cells stimulated in the presence of rIL-12 produced IL-4 and IFN- γ simultaneously. This is consistent with the observation that rIL-12 induced low levels of IL-4 protein at the population level (Table 1), and indicates that this IL-4 is derived from Th0 cells. Stimulation of neonatal CD4⁺ T cells in the presence of both rIL-4 and rIL-12 resulted in a two- to three-fold reduction in IL-4-producing Th2 cells and a strong increase (6–10-fold) in IFN- γ -producing Th1 cells, as compared with neonatal CD4⁺ T cells, stimulated in the presence of rIL-4 alone (Figs. 2 and 3). On the other hand, the

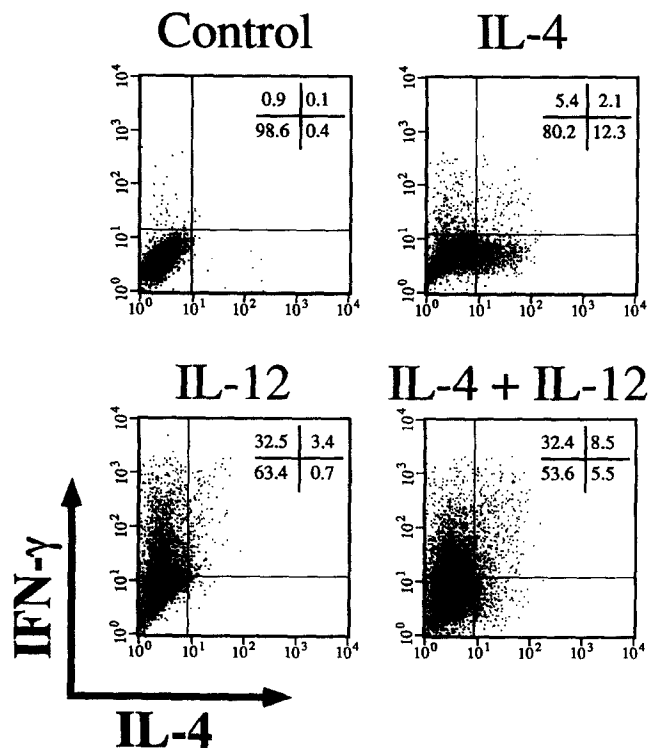


Figure 2. Intracellular cytokine production of Th1 and Th2 populations. Neonatal CD4⁺ T cells were stimulated twice, at 6-d intervals, with anti-CD3 mAb and L cell transfectants in the presence of rIL-2 (Control), or in the presence of rIL-2 and combinations of rIL-4 and rIL-12, respectively. After this culture period, cells were harvested and the intracellular production of IL-4 and IFN- γ was analyzed by flow cytometry.

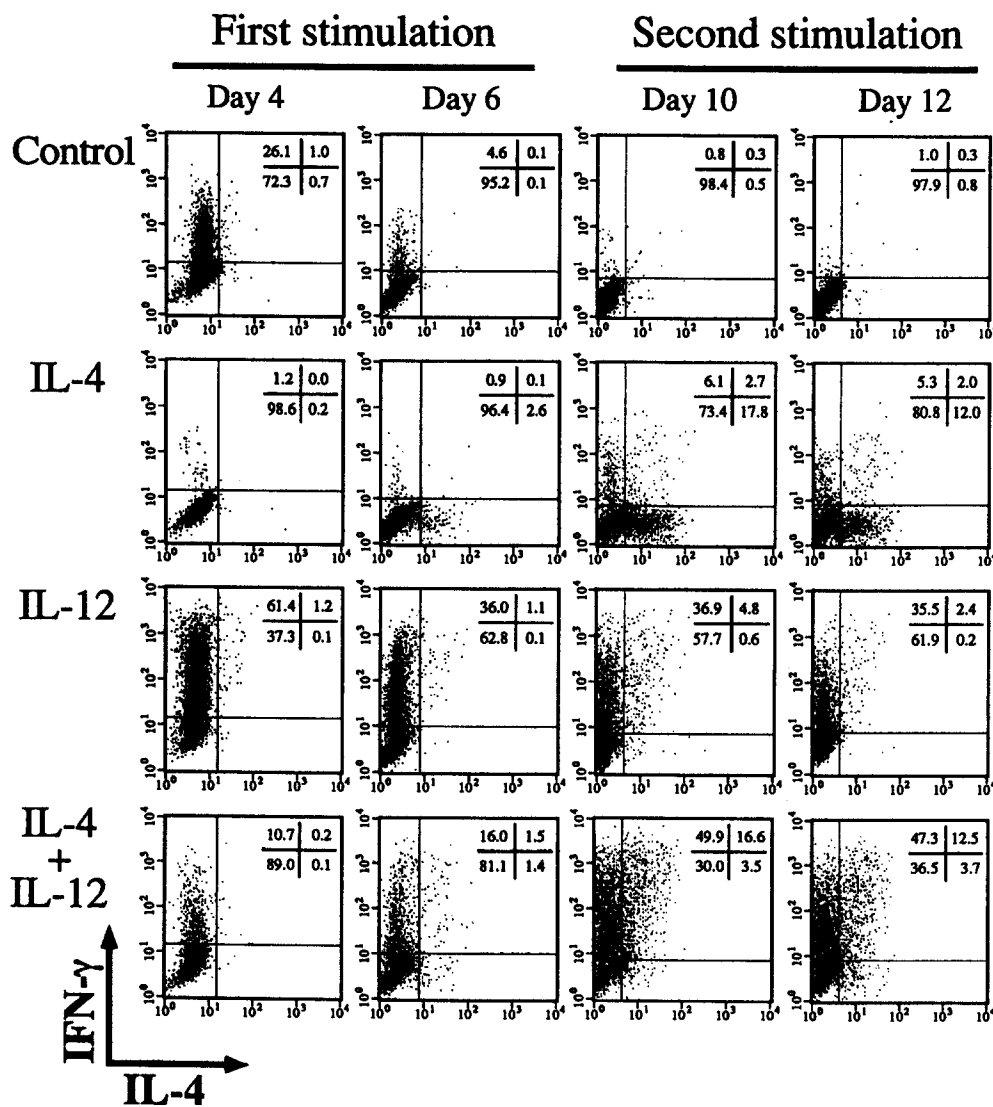


Figure 3. Kinetics of differentiation of Th1 and Th2 subpopulations. Neonatal CD4⁺ T cells were stimulated with anti-CD3 mAb and L cell transfectants in the presence of rIL-2 (*Control*), or in the presence of rIL-2 and combinations of rIL-4 and rIL-12, respectively. At 4 and 6 d after the first and the second stimulation, the cells were harvested and the intracellular production of IL-4 and IFN- γ was analyzed by flow cytometry.

presence of rIL-4 failed to reduce the frequency of IL-12-induced IFN- γ -producing cells, but induced a small population of IL-4-producing Th2 cells (Figs. 2 and 3). In addition, considerable numbers (8–15%) of individual Th0 cells that simultaneously secreted IL-4 and IFN- γ were generated in these cultures. Control cultures, containing neonatal CD4⁺ T cells, stimulated twice in the presence of rIL-2 alone, failed to differentiate into IL-4- or IFN- γ -producing Th0, Th1, or Th2 cells. No IL-4- or IFN- γ -producing cells could be detected in control T cells that had not been activated by plate-bound anti-CD3 and soluble anti-CD28 mAbs (data not shown), indicating that IL-4 and IFN- γ production detected in these populations reflected neosynthesis of these cytokines. Taken together, the results indicate that IL-12 seems to be more potent in redirecting IL-4-driven Th2 differentiation of neonatal CD4⁺ T cells, than IL-4 in redirecting IL-12-mediated Th1 cell differentiation.

Kinetics of Differentiation of Neonatal CD4⁺ T Cells, Induced by rIL-4 and rIL-12. We analyzed the kinetics of in-

duction of differentiation of neonatal CD4⁺ T cells into Th0, Th1, and Th2 cells, after stimulation in the presence of rIL-4 and/or rIL-12. After stimulation in the presence of rIL-2 alone, a subset of IFN- γ -producing Th1 cells appeared rapidly during the first 4 d of culture, but could no longer be detected at day 6 of stimulation (Fig. 3). In the presence of rIL-4, no IFN- γ -producing cells were generated, indicating that rIL-4 inhibited the development of these early-appearing IFN- γ -producing Th1 cells. Interestingly, only very low numbers (2%) of IL-4-producing Th2 cells could be detected after the cells had been restimulated in the presence of rIL-4 for 6 d. The frequency of IL-4-producing cells, however, increased dramatically to ~20% after restimulation, suggesting that neonatal CD4⁺ T cells require at least two consecutive stimulations in the presence of IL-4 in order to optimally differentiate into Th2 cells. rIL-4 also induced the development of a subset of IFN- γ -producing Th1 cells, which appeared late, concomitantly with IL-4-producing cells during the second stimulation. This

subset of Th1 cells differs from the one appearing early in the presence of rIL-2, since the generation of the latter population is suppressed by IL-4 (Fig. 3).

In contrast to the slow development of IL-4-producing Th2 cells, high frequencies (60–70%) of IFN- γ -producing Th1 cells were detected as early as 4 d after stimulation in the presence of rIL-12 (Fig. 3). However, after 6 d of culture, the proportion of IFN- γ -producing Th1 cells stabilized at lower frequencies (30–40%). The decrease in frequency of these cells paralleled the progressive loss of IFN- γ -producing cells, observed in the control cultures, which contained only rIL-2, suggesting that the high percentages of IFN- γ -producing Th1 cells detected early in these cultures can be attributed to the additive effects of rIL-2 and rIL-12. The presence of rIL-4 in rIL-12-containing cultures partially prevented this early burst of IFN- γ -producing T cells. However, during the second stimulation of neonatal CD4⁺ T cells in the presence of rIL-4 and rIL-12, additive effects were observed on the frequencies of IFN- γ -producing Th1 cells, confirming the notion that rIL-4 enhanced the late differentiation of a subset of IFN- γ -producing cells, consistent with the protein data shown in Table 1. IL-4 and IL-12 clearly synergized in inducing Th0 cell differentiation, whereas a strong reduction in the generation of Th2 single cells was observed, as compared with cultures carried out in the presence of rIL-4 alone. Kinetics of differentiation of IL-4-, as well as IFN- γ -producing Th cells, induced by the combination of rIL-4 and rIL-12 (Fig. 3), paralleled differentiation kinetics of these cells induced by each of the cytokines alone. The majority of IL-4-producing Th cells detected among this population also produced IFN- γ and thus belonged to the Th0 subset.

It is important to note that differences in differentiation kinetics of Th1 and Th2 cells were unlikely to be due to a difference in growth of neonatal CD4⁺ T cells in response to rIL-4 and rIL-12, since similar cell numbers were recov-

Table 2. Growth Characteristics of Cultured Human Neonatal CD4⁺ T Cells

Cytokine added	Cell recovery (fold of initial population)			
	First stimulation		Second stimulation	
	Day 0→4	Day 0→6	Day 6→10	Day 6→12
Control	6.9	3.8	0.8	1.9
IL-4	6.4	17.8	3.0	3.2
IL-12	6.5	13.3	1.7	3.0
IL-4 + IL-12	5.4	33.2	1.7	4.0

Neonatal CD4⁺ T cells were stimulated twice, at 6-d intervals, with anti-CD3 mAb and L cell transfectants, in the presence of rIL-2 (Control), or in the presence of rIL-2 and combinations of rIL-4 and rIL-12, respectively. At the indicated culture periods, cell recovery was determined by trypan blue exclusion.

ered during stimulation in the presence of these cytokines (Table 2).

Stability of Th1 and Th2 Populations Derived from Neonatal CD4⁺ T Cells. The use of cytokines to manipulate Th differentiation may have clinical utility in the treatment of several human diseases. However, it is still unclear whether it is possible to modulate the phenotype of polarized human Th populations. To address this question, we analyzed the distribution of IL-4- and IFN- γ -producing cells among differentiated Th1 and Th2 populations that were restimulated in the presence of rIL-4 or rIL-12, respectively. Restimulation in the presence of rIL-12 of a Th2 population that was generated by two consecutive stimulations in the presence of rIL-4 (Fig. 4 A) resulted in the emergence of IFN- γ -producing Th1 and IFN- γ - and IL-4-producing Th0 cells and, concomitantly, in a strong

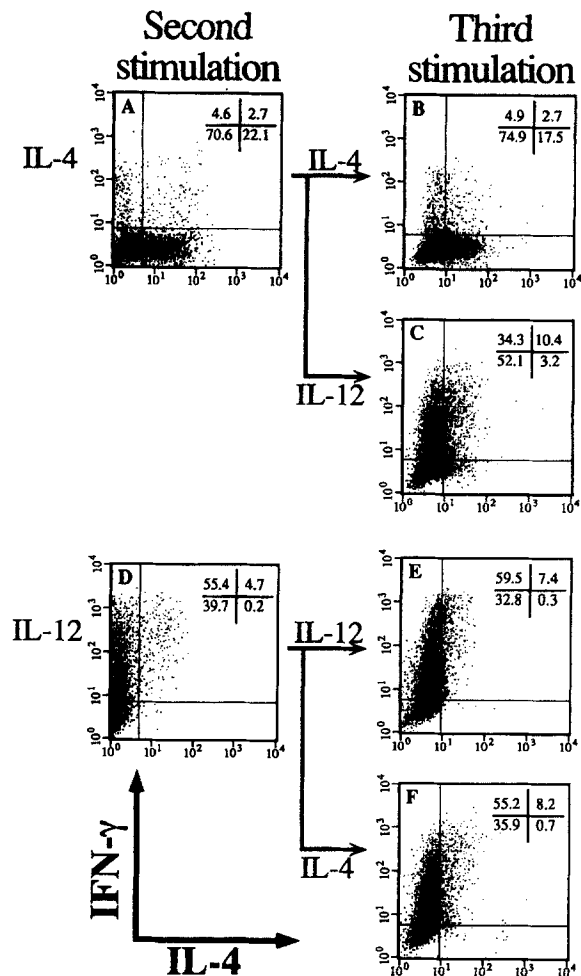


Figure 4. Phenotype of Th2, but not of Th1 populations is reversible after stimulation with oppositely polarizing stimuli. Neonatal CD4⁺ T cells were stimulated twice, at 6-d intervals, with anti-CD3 mAb and L cell transfectants and rIL-2 in the presence of rIL-4 (A) or rIL-12 (D), respectively. After this culture period, the cells were harvested, washed, and restimulated under identical conditions with rIL-4 (B and E) or with rIL-12 (C and F). 6 d later, cells were harvested and the intracellular production of IL-4 and IFN- γ was analyzed by flow cytometry.

reduction in the frequencies of Th2 cells (Fig. 4 C). However, the same Th2 cells, restimulated in the presence of rIL-4, retained their phenotype (Fig. 4 B). In contrast, Th1 populations derived from neonatal CD4⁺ T cells in the presence of rIL-12 (Fig. 4 D) and restimulated in the presence of rIL-4 (Fig. 4 F) retained their Th1 phenotype and did not differ significantly from their counterparts that had been restimulated in the presence of rIL-12 alone (Fig. 4 E). Furthermore, Th1 populations derived after only one stimulation of neonatal T cells in the presence of rIL-12 (Fig. 5 B) retained their predominant Th1 phenotype even after two restimulations in the presence of rIL-4 (Fig. 5, E and I), suggesting that even early IL-12-induced Th1 populations cannot be readily skewed toward a Th0/Th2 phenotype in the presence of rIL-4. Conversely, neonatal T cells initially stimulated in the presence of rIL-4 (Fig. 5 A) and then restimulated in the presence of rIL-12 differentiated into a T cell population in which Th1 and Th0 cells frequencies were enhanced five- to seven- and three- to five-fold, respectively (Fig. 5 D). However, in spite of their apparent Th1 phenotype after the second stimulation, a third stimulation of this Th1/Th0 population in the presence of rIL-4 gave rise to a Th2/Th0 phenotype (Fig. 5 H), with frequencies of IL-4-producing cells comparable with those in populations of neonatal CD4⁺ T cells, stimulated three times in the presence of rIL-4 (Fig. 5 G). This result suggests that priming in the presence of rIL-4 was pivotal for the subsequent generation of IL-4-producing Th2 cells from this population. In contrast, the population that had been initially stimulated in the presence of rIL-12 retained a Th1 phenotype even after two consecutive stimulations in the presence of rIL-4 (Fig. 5 I), indicating that the presence of rIL-12 during the initial stimulation directly induced a stable differentiation of Th1 cells. However, both the proportion of IFN- γ -producing cells and the intensity of production of this cytokine among this population were significantly lower than the proportion of IFN- γ -producing Th1 cells and the intensity of production of IFN- γ detected among the control population that had been repeatedly stimulated in the presence of rIL-12 only (Fig. 5, F and J). The latter result indicates that despite the inability of IL-4 to reverse the phenotype of Th1 populations, it may reduce the frequencies of Th1 cells and the levels of IFN- γ production per cell.

Discussion

We report here that repeated stimulation of neonatal CD4⁺ T cells develops into Th1 and Th2 subsets when the differentiation-inducing cytokines, IL-12 or IL-4, respectively, are present during the priming of the cells, but that differentiation requirements and stability with respect to cytokine production profiles differ between subsets. Repeated stimulation of neonatal CD4⁺ T cells with anti-CD3 mAb, cross-linked on CD32⁺ mouse L cells expressing CD80 and CD58 in the presence of rIL-4, resulted in the generation of a Th2 population, producing high levels

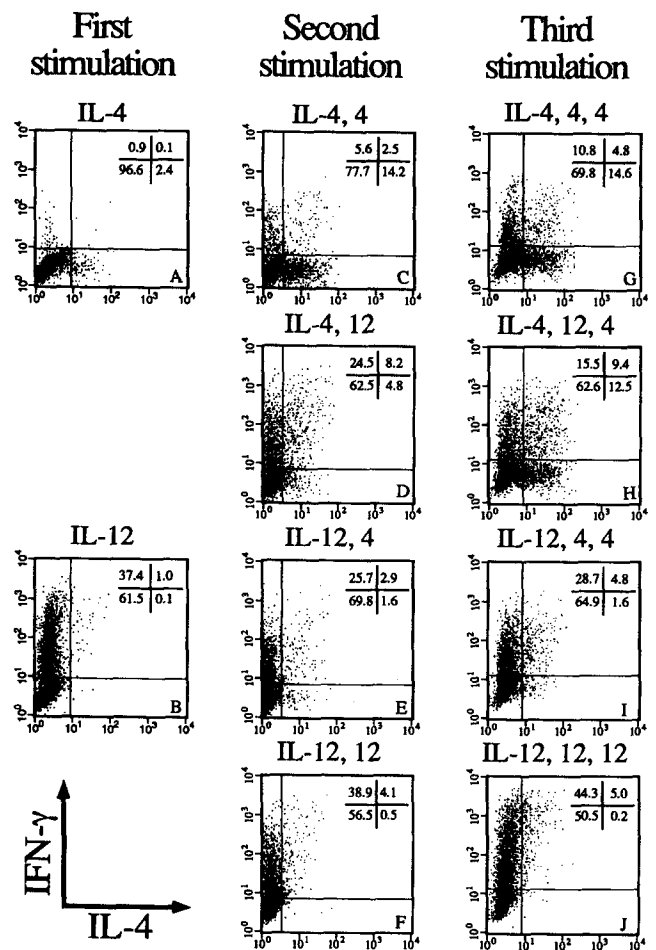


Figure 5. Plasticity of Th1 and Th2 populations after alternating stimulations with oppositely polarizing stimuli. Neonatal CD4⁺ T cells were stimulated for 6 d, with anti-CD3 mAb, L cell transfectants, and rIL-2 in the presence of rIL-4 (A) or rIL-12 (B), respectively. rIL-4-primed cells were restimulated in the presence of rIL-4 (C) or rIL-12 (D) during the second stimulation, and in the presence of rIL-4 during the third stimulation (G and H). rIL-12-primed cells were restimulated, in the presence of rIL-4 (E and I) or rIL-12 (F and J), during the second and the third stimulation. 6 d after the third stimulation, the cells were harvested and the intracellular production of IL-4 and IFN- γ was analyzed by flow cytometry.

of IL-4, IL-5, IL-10, and IL-13, but low levels of IFN- γ . Analysis of cytokine production at the single-cell level revealed that optimal differentiation of neonatal CD4⁺ T cells into IL-4-producing Th2 cells occurs relatively slowly, since this process requires both priming and restimulation in the presence of IL-4. The slow kinetics of Th2 cell differentiation was not due to the initial presence of IFN- γ , since no significant levels of this cytokine could be detected at either the protein level, or the RNA level in the initial population of neonatal CD4⁺ T cells, which is in keeping with reports in the literature (44, 45). Moreover, stimulation of neonatal CD4⁺ T cells in the presence of IL-4 and a neutralizing antibody against IFN- γ did not enhance IL-4 production after the first stimulation (data not shown), suggesting that blocking of IFN- γ does not accelerate Th2 differentiation.

It has been reported that stimulation of murine CD4⁺, CD45RA⁺ naive T cells via CD86 favors differentiation into Th2 cells (46, 47). Therefore, it could be argued that the failure of rIL-4-primed cells to produce IL-4 after a single stimulation is due to the lack of CD86 expression on the L cell transfectants. However, Lanier et al. (48) have reported that stimulation of human peripheral blood T cells with CD86-expressing L cell transfectants induced similar levels of Th1- and Th2-type cytokines. In addition, no differences in cytokine production profiles could be detected between CD45RA⁺ or CD45RA⁻ T cells that were stimulated with transfectants, expressing comparable levels of CD80 or CD86 (49). Finally, stimulation of neonatal CD4⁺ T cells with cross-linked anti-CD3 and cytokines in our culture system yielded similar cytokine production patterns, irrespective of whether the L cells had been transfected with CD80 or CD86 (Sornasse, T., and H. Yssel, unpublished results).

The data reported here are consistent with results reported previously, demonstrating that differentiation of neonatal T cells into effector T cells with a Th2 phenotype requires priming in the presence of IL-4 (14–16, 18, 50). The differentiation of neonatal CD4⁺ T cells into IL-4-, IL-5-, and IL-13-producing cells was completely inhibited by the IL-4 mutant protein IL-4.Y124D, which acts as a potent IL-4R and IL-13R antagonist, underscoring the efficiency of this polypeptide. These results furthermore indicate that the induction of IL-4, IL-5, and IL-13 production depends on the presence of IL-4. Since allergy is a Th2 cell-mediated disease, the inhibitory effects, together with the observation that IL-4.Y124D blocks both IL-4- and IL-13-induced IgE synthesis *in vitro* (42) and ongoing human IgE synthesis in SCID-hu mice (43), suggest that this IL-4 mutant protein may have clinical utility in the treatment of allergic diseases.

Since IL-4 is species specific and therefore does not act on mouse L cells, it can be concluded that its capacity to induce Th2 differentiation is due to a direct effect on T cells. In contrast, IL-13, which shares many biological activities with IL-4, was not effective in this culture system, which is compatible with the observation that IL-13 does not act on T cells, due to the absence of an IL-13R-binding protein on these cells (33, 51). Although IL-13 cannot directly induce Th2 differentiation by direct action on the T cells, it is possible that it may indirectly enhance the generation of Th2 responses through its capacity to downregulate the monocyte- and possibly dendritic cell-derived production of IFN- α and IL-12 (52), both of which are cytokines capable of inducing Th1 differentiation (22, 23).

The low levels of IFN- γ protein detected in cultures of neonatal CD4⁺ T cells stimulated in the presence of rIL-4 were produced by subsets of Th1 cells and, to a lesser extent, by Th0 cells that appear late, at about 4 d after the second stimulation (i.e., 10 d after onset of the culture) in the presence of rIL-4. These results suggest that IL-4 under these conditions can induce the late differentiation of a subset of Th1 cells. On the other hand, it cannot be excluded that the late appearance of these IFN- γ -producing

cells reflects IL-4-mediated outgrowth of a small subpopulation of Th1 cells that was already present in the cultures of neonatal CD4⁺ T cells before the priming of these cells with anti-CD3 mAb. However, this late-appearing Th1 subset seems to be different from the IFN- γ -producing Th1 population, which is induced rapidly and transiently in the presence of IL-12 alone, since the differentiation of the early Th1 population is, unlike the late-appearing Th1 population, suppressed by IL-4.

IL-12-induced differentiation of neonatal CD4⁺ T cells into Th1 cells occurred rapidly, and priming of the cells in the presence of rIL-12 was sufficient to induce the generation of high frequencies of IFN- γ -producing cells after 4 d of culture. Interestingly, in about half of the donors, significant levels of IL-4 and IL-13 protein, but no IL-5 protein, could be detected in culture supernatants of neonatal CD4⁺ T cells that had been repeatedly stimulated in the presence of rIL-12. The capacity of IL-12 to induce the production of IL-4 by CD4⁺ neonatal T cells has been reported before (53). However, here we show that the IL-12-mediated production of IL-4 is derived from a population of Th0 cells that also produces IFN- γ and not from Th2 cells, since no Th2 cell differentiation of CD4⁺ neonatal T cells was observed in the presence of rIL-12.

Repetitive stimulation of neonatal CD4⁺ T cells in the presence of both rIL-4 and rIL-12 resulted in the generation of a population of T cells that produced high levels of IFN- γ and levels of IL-4, IL-5, IL-10, and IL-13 that were about twofold reduced, as compared with the production of these cytokines by neonatal CD4⁺ T cells differentiated in the presence of rIL-4 alone. Analysis of cytokine production at the single-cell level indicated that IL-12 induced a reduction in Th2 differentiation, whereas the percentage of IL-4-producing Th0 cells was increased, which accounted for comparable percentages of IL-4-producing cells under both conditions. However, levels of IL-4 protein produced by T cells differentiated in the presence of rIL-4 and rIL-12 were consistently lower than those generated in rIL-4 alone; this probably implies that IL-4 production by Th0 cells on a per cell basis is most likely lower than that by single IL-4-producing Th2 cells. The induction of IL-4 production in the Th0 population, generated in the presence of rIL-4 and rIL-12, also required repetitive stimulation, suggesting that the requirements for induction of IL-4 production by Th0 and Th2 T cells are similar. Furthermore, these data support the notion that Th0 cells might be considered as a fully differentiated subpopulation of T cells. In addition, these results support recently published data by Kelso et al. (54) which suggest that the induction of IL-4 and IFN- γ production is independently regulated.

Interestingly, Th2 populations derived after two stimulations in the presence of rIL-4 are not stable, since a single restimulation in the presence of rIL-12 gave rise to a population of Th0 and Th1 cells, producing high levels of IFN- γ , in which IL-4-producing Th2 cells could no longer be detected. These results are in keeping with the observation that rIL-12 can rapidly induce high levels of IFN- γ pro-

duction by established allergen-specific Th2 clones (12, 13). In addition, stimulation of allergen-specific Th2 clones with phorbol ester and calcium ionophore (11) via a pathway circumventing triggering of the TCR/CD3 complex, or with anti-signaling lymphocytic activation molecule mAb (55), respectively, was found to induce high levels of IFN- γ production, indicating that the IFN- γ gene is not silent and can be upregulated in differentiated Th2 cells. It is presently not clear whether the rIL-12-induced increase in the frequency of Th1 cells in cultures that had been primed and restimulated in the presence of rIL-4 reflects a complete conversion of IL-4-producing cells into Th1 cells or the differentiation of naive neonatal CD4⁺ T cells still present in these populations, which are not committed to IL-4 or IFN- γ production (Figs. 3–5). Alternatively, we cannot exclude the possibility that these cells produce IL-4 or IFN- γ at levels that are below the sensitivity of the assay.

In contrast to the unstable cytokine production profile of Th2 populations generated in the presence of rIL-4, the cytokine production profile of Th1 neonatal CD4⁺ T cells is stable; repetitive stimulation of these cells in the presence of rIL-4 did not result in the induction of single IL-4-producing Th2 cells. Importantly, however, among the population of Th0 cells generated by priming of neonatal CD4⁺ cells in rIL-4 and subsequent stimulation in rIL-12, single IL-4-producing cells could still be induced after an additional stimulation in the presence of rIL-4. The induction of T cells that produce IL-4 and no IFN- γ under these conditions occurred despite the fact that restimulation of rIL-4-primed cells in the presence of rIL-12 clearly favored the differentiation of IFN- γ -producing Th1 cells and inhibited the differentiation of IL-4-producing Th2 cells. These results underscore the plasticity of IL-4-primed T

cell populations, but, most importantly, they indicate that initial priming with IL-4 is pivotal for efficient Th2 differentiation.

Unlike the ability of human neonatal CD4⁺ Th2 cells to alter their cytokine phenotype, mouse Th2 cells appear to be stable in their cytokine production profile early in their differentiation and either cannot (24) or can only partially (25) be induced to the opposite phenotype by repetitive stimulation in the presence of IL-12 in vitro. The resistance of CD4⁺ murine Th2 cells to the Th1 cell differentiation-inducing effects of IL-12 may be due to a downregulation of IL-12R, resulting in an IL-12-signaling defect in these cells (26). On the other hand, mouse Th1 cells still respond to the Th2 cell differentiation-inducing effects of rIL-4 in vitro (24, 25), whereas human Th1 cells seem to be irreversible in their cytokine production profile. The differences in stability between mouse and human Th subset may be due to the different in vitro conditions and to the frequency of stimulation of Th subsets with differentiation-inducing cytokines. In support of this is the recent study by Murphy et al. (56) in which it was reported that cytokine production profiles of both mouse Th1 and Th2 populations are irreversible in vitro when these cells have been repetitively stimulated in the presence of rIL-12 or rIL-4, respectively. It has to be noted, however, that IL-12 was able to reverse an established lethal Th2 response after chronic infection with *Leishmania major* into a healing Th1 response, when given simultaneously with an antileishmanicidal drug (57), indicating that under certain conditions polarized murine Th2 responses still can be modulated in vivo. The ability to alter the Th phenotype of human T cells by cytokine exposure may similarly be beneficial for treatment of certain human diseases.

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