

Reversibility of T Helper 1 and 2 Populations Is Lost After Long-term Stimulation

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Summary

Commitment of T helper 1 (Th1) or Th2 populations developing during an immune response to a pathogen, or an inappropriate immune response to an allergen or autoantigen, may determine the difference between health and chronic disease. We show that strongly polarized Th1 and Th2 populations assessed by immunoassay are heterogeneous using flow cytometry to detect single cells producing interferon γ (IFN- γ) and interleukin 4 (IL-4). Th1 populations arising after 1 wk of stimulation in IL-12 plus anti-IL-4 antibodies could convert to Th2 cells when restimulated in IL-4. Th2 populations resulting from stimulation for 1 wk in IL-4 could give rise to Th1 cells upon restimulation in IL-12 plus anti-IL-4. In contrast, the cytokine profiles of long-term Th1 and Th2 populations arising originally from repeated stimulation in IL-12 or IL-4 appeared more homogeneous and were not reversible, although IL-4 dramatically reduced the number of IFN- γ -producing Th1 cells. This may explain previous reports that Th1 cells can be converted to Th2 cells.

Development of the appropriate CD4⁺ Th subset during an immune response is critical for eradication of infectious organisms (1–3). These subsets of Th cells may be distinguished by the pattern of cytokines that they produce. Th1 cells produce IFN- γ and lymphotoxin and play a critical role in directing cell-mediated immune responses, important for the clearance of intracellular pathogens. Th2 cells producing IL-4, -5, -10, and -13, have been associated with allergy and are important for humoral responses (1–6). An important role for Th2-type cytokines, such as IL-4 and IL-10, is their ability to regulate Th1 responses (7–11) which are important for the clearance of infectious organisms but may also result in pathology if elicited against self-antigens (12–14).

Cytokines present during the initiation of a CD4⁺ T cell response can determine the development of a particular Th cell phenotype (10, 15–19). Th2 cells develop when naive T cells are stimulated in vitro in the presence of IL-4 (10, 15, 17). In addition, the presence of IL-4 during priming has also been shown to play an important role in vivo, for example, in establishing a Th2 nonhealing response in BALB/c mice to the intracellular parasite *Leishmania major* (20, 21). Conversely, IL-12 is a critical factor driving the development of Th1 cells from antigen-specific naive CD4⁺ T cells (16, 18, 19); in addition, it enhances IFN- γ production by Th1 clones (22) and human Th2/Th0-type

clones and activated Th cells (23, 24). Despite the induction of IFN- γ production by seemingly committed human Th2/Th0 clones (23), such polarizing stimuli as IL-4 or IL-12 have been found to be most effective when delivered at the initiation of the immune response, both in vitro (10, 15–19) and in vivo (20, 21, 25–27).

Recently, ongoing Th2 responses to *L. major* were found to be reverted to a healing Th1 response when BALB/c mice were treated during chronic infection with Pentostam (to reduce the parasite infection) together with IL-12 (28), or by transferring the Th2 populations to *scid* mice under appropriate conditions (29, 30). In addition, polarized Th1 populations obtained from mice infected with *L. major* have been modified to produce Th2-type cytokines by reculture in vitro with specific antigen, APCs, IL-2, and IL-4 (31). These data are reminiscent of studies in human systems that demonstrated that purified protein derivative-specific T cells which normally outgrow with a Th1-like profile, can be selected as Th2-like if IL-4 is added early in bulk cultures (32). In contrast to the in vivo studies discussed above showing the reversal of a Th2 phenotype, recently it has been shown that although the phenotype of early Th1 cells was efficiently reversed in vitro by addition of IL-4, Th2 cells were resistant to phenotype reversal (33, 34). Although in some cases, these studies involve T cell populations that are clonal with respect to their antigen re-

ceptor, they undoubtedly all deal with heterogeneous populations of Th cells with respect to cytokine production (35), and the level of commitment for cytokine production at the single cell level is unclear. To address this, and to obtain information on whether a switch in Th phenotype reflects a true conversion or the suppression of the polarized phenotype by outgrowth and/or differentiation of uncommitted precursors, we have employed flow cytometry to detect single cells producing IFN- γ and IL-4 (35). Using this technology we assessed the plasticity of seemingly polarized Th1 and Th2 populations in an in vitro system using CD4⁺ T cells from TCR-transgenic mice (10, 36), restimulated with antigen under conditions designed to reverse their pattern of cytokine production.

Materials and Methods

Animals. Mice transgenic for the D011.10 TCR- α/β on a BALB/c genetic background (36) were identified at age 4–6 wk by staining peripheral blood leukocytes with the anti-TCR clonotype-specific mAb KJ1-26 (37, 38). All transgenic mice used were heterozygous for the TCR α and β transgenes. Female nontransgenic BALB/c mice between 6 and 10 wk old were purchased from Simonsen Labs (Gilroy, CA).

Th Cell Clones. Stimulation and maintenance of long-term Th clones was as previously described: Th1, HDK1 (BALB/c-derived, KLH specific, I-A^d restricted; 39) and Th2, CDC25 (C3D2/F₁/J-derived, normal rabbit IgG specific, H-2I^d restricted; 40). Short-term Th clones derived from unimmunized DO11.10 TCR- α/β transgenic mice were obtained by sorting single cells on the basis of CD4⁺, leukocyte endothelial cell adhesion molecule (LECAM)¹ 1^{bright}, KJ1-26⁺ into 96-well microtiter wells containing 10⁵ irradiated splenic APCs (3,000 rad) plus OVA (0.3 μ M) under the following conditions: Th1 clones, in the presence of IL-2 (10⁷ U/mg, 330 U/ml), IL-12 (10⁶ U/mg, 100 U/ml; plus small amounts of IL-4, which were required to maintain cell viability), plus TGF- β (2 ng/ml); Th2 clones, in the presence of IL-2 (330 U/ml) and large amounts of IL-4 (10⁷ U/mg, 100 U/ml); and Th0 clones, in the presence of IL-2 (330 U/ml), IL-4 (15 U/ml), and IL-12 (10 U/ml). All the DO11.10-derived clones were 100% positive for the clonotype antibody, KJ1-26 (cells are referred to as clones when they have been derived from a single cell in culture, as opposed to populations described below, which although carrying the same transgene and thus antigen specificity, were not derived from single cells in culture). Clones were restimulated and expanded under the respective conditions every 2 wk. All clones were expanded and maintained in RPMI 1640 (JR Scientific Inc., Woodland, CA) containing 10% FCS (JR Scientific Inc.), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), Hepes buffer (10 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (0.05 mM, Sigma Chemical Co., St. Louis, MO), (complete RPMI), supplemented with mouse rIL-2 (330 U/ml), and the respective initiating conditions were used for growth of the clones. In some cases, clones were stored in liquid nitrogen and cells were thawed into medium containing murine rIL-2 (330 U/ml), as above, 5–7 d before assay.

Rested T cell clones were washed, counted, and resuspended

at 2×10^5 /well. They were restimulated with irradiated (3,000 rad) splenic APCs plus specific antigen or PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h at 37°C, with Brefeldin A (10 μ g/ml; Epicentre Technologies, Madison, WI) added for the last 2 h, for flow cytometric analysis of intracellular cytokine staining. Identical stimulations of T cells were set up in parallel but in the absence of Brefeldin A, for 48 h at 37°C, for analysis of IL-4 and IFN- γ in supernatants by immunoassay as previously described (41–43).

Culture Medium, Cytokines, Antibodies and Antigens, and other Reagents. Cell cultures were maintained in cRPMI as described above, but usually without supplemented cytokines, unless indicated.

Recombinant mouse cytokines were obtained from the following sources: IFN- γ (Schering Research, Bloomfield, NJ), IL-4 (Dr. S. Menon, DNAX Research Institute), TGF- β (R&D Systems, Inc., Minneapolis, MN), and IL-2 (a kind gift from Gerard Zurawski, DNAX Research Institute). Recombinant mouse IL-12 was obtained by transfecting COS7 cells with the cDNA encoding the p35 and p40 cDNA as described previously (44), which was obtained by PCR cloning using published sequences (44). Supernatants from mock-transfected cells were used as a control and showed no effect in the concentration range at which IL-12 was used. The IL-12 content of the supernatants ranged from 2,000 to 10,000 U/ml (22).

Purified rat anti-mouse IL-4 (11B11) and anti-IFN- γ (AN18) antibodies previously described (42, 45), and an isotype-matched control were supplied by J. Abrams (42; DNAX Research Institute). mAbs used for flow cytometric sorting or analysis included anti-mouse CD4-PE and LECAM-1-FITC (both from Pharmingen, San Diego, CA). Additional anticytokine mAbs for immunoassay and flow cytometry, including anti-mouse IL-4 and IFN- γ reagents, were purified from serum-free hybridoma supernatants as previously described (42).

The antigenic OVA-peptide (OVA₃₂₃₋₃₃₉) from chicken OVA was synthesized on a peptide synthesizer (model 430; Applied Biosystems, Lewisville, TX). 100 μ g/ml KLH and 500 μ g/ml rabbit IgG (Sigma Chemical Co.) were used.

Preparation of T Cells and APCs. CD4⁺ T cells were enriched by negative selection using Magnetic Activated Cell Sorting (MACS) with a cocktail of biotinylated anti-mouse CD8 α , I-A^d, B220, and Mac-1 antibodies as previously described (35) (Miltenyi, Sunnyvale, CA). T cells staining positive for CD4 and LECAM-1 were further purified by positive selection using a FACStar^{plus} flow cytometer (Becton Dickinson & Co., San Jose, CA) to achieve 99.8% CD4⁺ T cells. Staining did not alter the function of the T cells (not shown).

Stimulation of Transgenic CD4⁺ T Cells for Cytokine Production. Primary stimulations of CD4⁺ T cells (2.5×10^5 /well) were carried out using OVA peptide (0.3 μ M) and nucleated spleen cells (5×10^6 /well, 3,000 rad) as APCs in a total volume of 2 ml in 24-well plates. In addition, some cultures received cytokines (IL-4 (200 U/ml) or IL-12 [10 U/ml]) or mAbs to block endogenous cytokines (anti-IL 4 [10 μ g/ml]). T cells were expanded and maintained in the same culture conditions for 1 wk. Cells were then harvested on day 6, washed three times, counted, and a portion of them resuspended at 2×10^5 /well, and restimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h at 37°C, with Brefeldin A (10 μ g/ml) added for the last 2 h (to enhance flow cytometric analysis of intracellular cytokine staining). The cells were then fixed and stained for detection of intracellular cytokine synthesis as described below. Identical stimulations of T cells were set up in parallel but in the absence of Brefeldin A, for 48 h

¹Abbreviation used in this paper: LECAM, leukocyte endothelial cell adhesion molecule.

at 37°C, for analysis of IL-4 and IFN- γ in supernatants by immunoassay as described below.

Additionally, naive CD4⁺ T cells were stimulated in primary cultures, under the same conditions as above, but in this case the stimulations were repeated weekly for three consecutive weeks (chronic stimulation) before washing, harvesting, restimulating, and analyzing as above.

For phenotype reversal studies, cultures of Th cells developed from naive CD4⁺ T cells were stimulated under the above conditions either once or repeatedly for three consecutive weeks. Cells were washed and then recultured under the same conditions with APCs and antigen alone, or in the presence of the same polarizing stimulus, or an oppositely polarizing stimulus (IL-4 or IL-12 plus neutralizing anti-IL-4 antibodies), for 1 wk or repeatedly for three consecutive weeks. The cells propagated under all the different conditions and time points were 100% CD4⁺, KJ1.26⁺. Cells were then washed, harvested, and restimulated before analyzing as above.

Cytokine Assays. IFN- γ was detected using a two-site sandwich ELISA (41, 42, 46) which has a lower limit of sensitivity of 125 pg/ml (1 U/ml or 100 pg/ml). The ELISA for IL-4 has been described previously (41, 42) with a lower limit of sensitivity of 100 pg/ml.

Flow Cytometric Analysis of Intracellular IFN- γ and IL-4 Synthesis. Cells were resuspended at 10⁵–10⁶/ml and stimulated with PMA at 50 ng/ml plus ionomycin at 500 ng/ml. 2 h before cell harvest, Brefeldin A was added at 10 μ g/ml, using a stock of 1 mg/ml in ethanol (100%). Cells were harvested, washed, and resuspended in PBS with Brefeldin A before adding an equal volume of 4% formaldehyde fixative (final concentration 2%). After fixing for 20 min at room temperature, cells were either stored at 4°C for up to 2 d, or stained immediately for cytokines using a modified method (35) based on that described by Assenmacher et al. (47) and others (48, 49). For intracellular staining, all reagents and washes contained 1% BSA and 0.5% saponin (Sigma Chemical Co.), and all incubations were at room temperature. Cells were washed and preincubated for 10 min in PBS/BSA/saponin, and incubated with anti-IL-4 (11B11) at 5 μ g/ml or isotype-matched control GL113 (isotype control) at 10 μ g/ml for 30 min. After two washes and blocking with rabbit serum/0.5% saponin, optimal concentrations of FITC anti-rat Ig (antibody raised in rabbit; Vector Laboratories, Inc., Burlingame, CA) were added for 30 min. After two washes, purified rat Ig was added at 300 μ g/ml for 10 min to block residual anti-rat Ig binding. Without washing, anti-IFN- γ (AN18-PE) (or control PE-conjugated rat Ig, Pharmacia, Piscataway, NJ) was added at a 2.5- μ g/ml final concentration. After 20 min, cells were washed twice with PBS/BSA/saponin and then with PBS/BSA without saponin to allow membrane closure. Cells were then stained with the clonotypic antibody KJ1.26 (36) to confirm the presence of 100% OVA-specific TCR-transgenic CD4⁺ T cells. Data are displayed as bivariate dot plots. Samples were analysed on a FACScan[®] flow cytometer (Becton Dickinson & Co.). Thresholds were set on control stains (included for every sample at every time point) to lie on the first percentile (not shown) as previously described (35). Our previous study confirms the specificity of detection of intracellular cytokine synthesis by this method (35). Results were analyzed using Lysis II and Cellquest software.

Results and Discussion

Reversal of the Cytokine Profile of Th1 or Th2 Populations Generated by Stimulation for 1 wk in the Presence of Polarizing Stimuli. We have previously demonstrated that naive CD4⁺

T cells from the DO11.10 OVA-specific TCR- α/β transgenic mouse can be induced to develop into either Th1 or Th2 populations, when stimulated for 1 wk with specific antigen and APCs in the presence of IL-12 or IL-4, respectively (10, 16, 35, 36). Although, these Th populations appeared to be completely polarized as assessed by levels of IFN- γ or IL-4 detected in the supernatants by immunoassay after T cell activation (10, 16, and Figs. 1 A and 2 C), the composition of the population with respect to cytokine production by single cells, or their level of commitment to a particular cytokine profile, was unclear. Using flow cytometry to detect single cells producing IFN- γ and IL-4 (35), we assessed the possible heterogeneity and plasticity of these polarized Th1 and Th2 populations when restimulated with antigen under conditions designed to reverse their pattern of cytokine production (Figs. 1 and 2). To produce polarized Th cell populations, naive CD4⁺, LECAM-1^{bright}, T cells from DO11.10 TCR- α/β -transgenic mice were stimulated with OVA and irradiated splenic APCs for 1 wk, in the presence of either IL-4 to produce Th2 populations, or IL-12 (plus or minus anti-IL-4 mAb) to produce Th1 populations (10, 16).

Naive CD4⁺ T cells stimulated with antigen and APCs in the presence of IL-4 for 1 wk gave rise to Th2 cells since supernatants obtained 48 h after stimulation contained IL-4 but not IFN- γ , as detectable by immunoassay (Figs. 1 A and 2 C). Flow cytometric analysis of these Th2 populations showed that they consisted predominantly of IL-4-producing cells (>50%) (Figs. 1 B and 2 A). A small percentage (4%) of the cells still synthesized IFN- γ , showing that the population was actually heterogeneous, although this cytokine was not detectable in the supernatants (Figs. 1 A and 2 C). Although the flow cytometric analysis of these Th2 populations suggested that ~40% of the cells did not produce IL-4, this may be explained by our previous observations, where we have shown that IL-4 synthesis is relatively transient in Th2 clones (35). This transient production of IL-4 protein correlates with transient expression of IL-4 mRNA (50). Since the cells are not synchronously in cell cycle (35), or possibly not synchronously secreting IL-4, and since IL-4 production is short-lived, it is unlikely that it will be possible to observe 100% of cells within a Th2 population producing this cytokine simultaneously (35). It is also possible that some of these cells staining negative for IFN- γ or IL-4 synthesis represent undifferentiated precursors, which do not produce these cytokines, or produce low levels of IFN- γ and IL-4, which are below the level of detectability. Th2 populations also developed (11% producing IL-4 only), although to a lesser extent, upon stimulation with antigen and APCs alone, reflecting endogenous IL-4 in the cultures (35; Figs. 1 B and 2 A).

We tested the ability of Th2 populations to be converted to a Th1 phenotype after 1 wk of stimulation in the presence of IL-4 (Figs. 1 and 2), by restimulation with antigen for 1 wk (Fig. 1 C) or weekly for 3 wk (Fig. 2 B) under conditions designed to reverse the phenotype. Restimulation of Th2 cells for 1 wk in the presence of IL-12 and anti-IL-4 mAbs initiated a change in phenotype as seen by im-

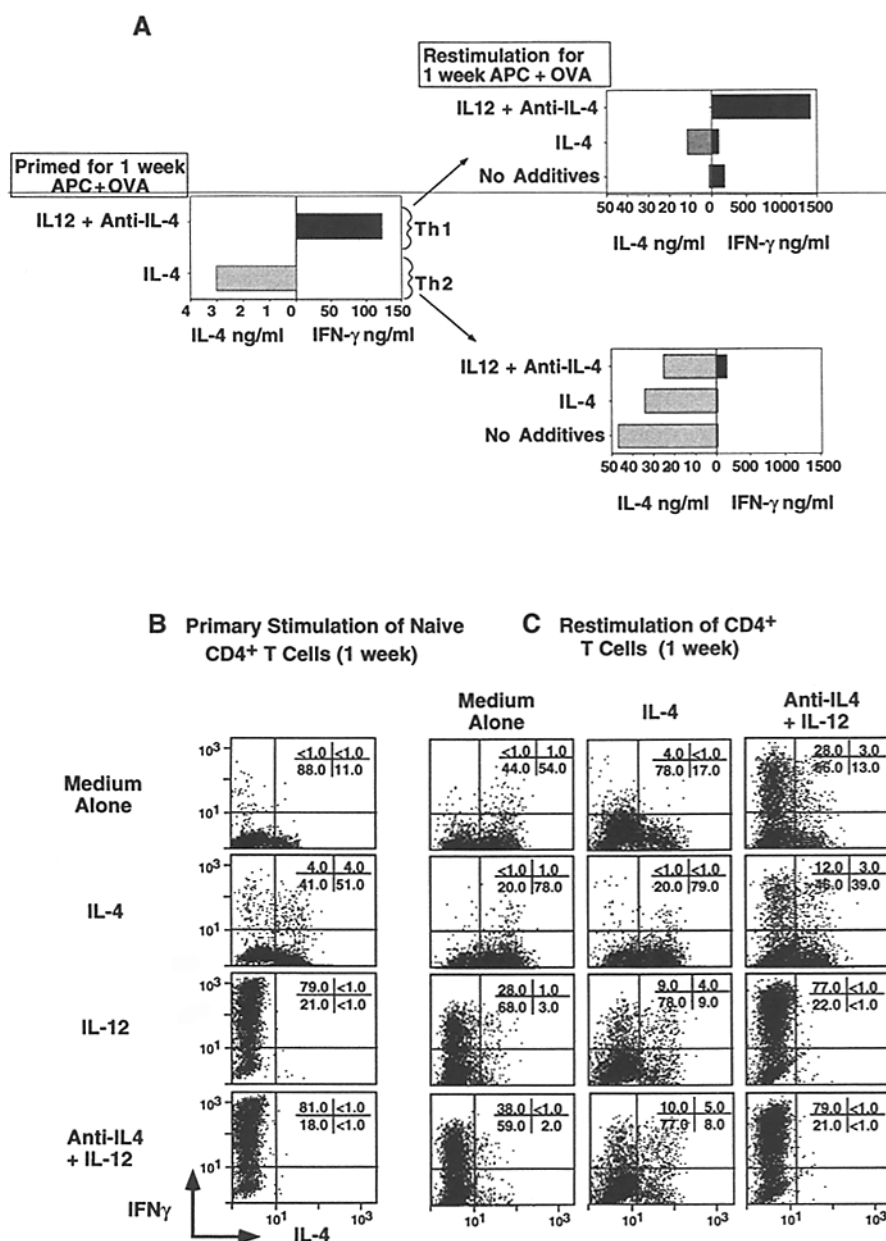


Figure 1. Reversal of cytokine profiles in early Th1 and Th2 populations. LECAM-1^{bright}, CD4⁺ T cells from TCR- α/β DO11.10 transgenic mice were first stimulated for 1 wk with splenic APCs and OVA, in either medium alone, with IL-4, or IL-12 plus or minus anti-IL-4, as described in Materials and Methods. Cells were harvested and a portion of them restimulated for analysis of supernatants for IFN- γ and IL-4 by immunoassay (A), or of cells by flow cytometry (B and C), as described, to determine their Th phenotype. Frequencies of IFN- γ -producing cells are shown on the y-axis and IL-4-producing cells on the x-axis. After 1 wk of antigenic stimulation (as above) the remaining CD4⁺ T cells (100% KJ1-26⁺) propagated from each group were washed and restimulated under conditions designed to achieve phenotype reversal (and controls), for 1 wk as described in Materials and Methods, and supernatants were analyzed by immunoassay or cells were analyzed by flow cytometry for IFN- γ and IL-4 production, as above, after appropriate restimulation.

immunoassay (Fig. 1 A). Analysis at the single cell level showed the emergence of a distinct population of Th1 cells producing IFN- γ only (12%) (Fig. 1 C). The frequency of IL-4-producing cells in Th2 populations restimulated with IL-12 and anti-IL-4 was only slightly diminished (51–39%), whereas restimulation in the presence of exogenous IL-4 enhanced their frequency (51–79%; Fig. 1 C). Distinct populations of IFN- γ -producing cells also appeared when Th2 populations were restimulated repeatedly (for 3 wk) in IL-12 plus anti-IL-4 (Fig. 2 B). In this case, an enhanced frequency of Th1 cells producing IFN- γ (>25%), as well as Th0 cells (51–53) producing both IL-4 and IFN- γ , were seen (7%) (Fig. 2 B). This apparent change from a Th2 to a Th1 phenotype was also observed upon immunoassay of supernatants from these Th cell populations upon restimulation (Fig. 2 C). These Th2 cells, although very similar in their

cytokine profile as measured by immunoassay, are potentially different from those of Szabo et al. (34), since in the present study, endogenous IL-12 was not neutralized during primary stimulation.

We also examined the stability of Th1 populations derived from stimulation for 1 wk with antigen and APCs in the presence of IL-12 plus anti-IL-4 mAbs, which produced high levels of IFN- γ and undetectable IL-4 upon restimulation (Figs. 1 A and 2 C). In agreement with this, flow cytometric analysis of the cells within this population showed that they produced exclusively IFN- γ (>80%) and no IL-4 (Figs. 1 B and 2 A), upon restimulation with PMA and ionomycin. Approximately 20% of cells stained negative for IFN- γ and IL-4 production, suggesting that they may be undifferentiated precursors producing neither cytokine. Alternatively, these cells could be producing low

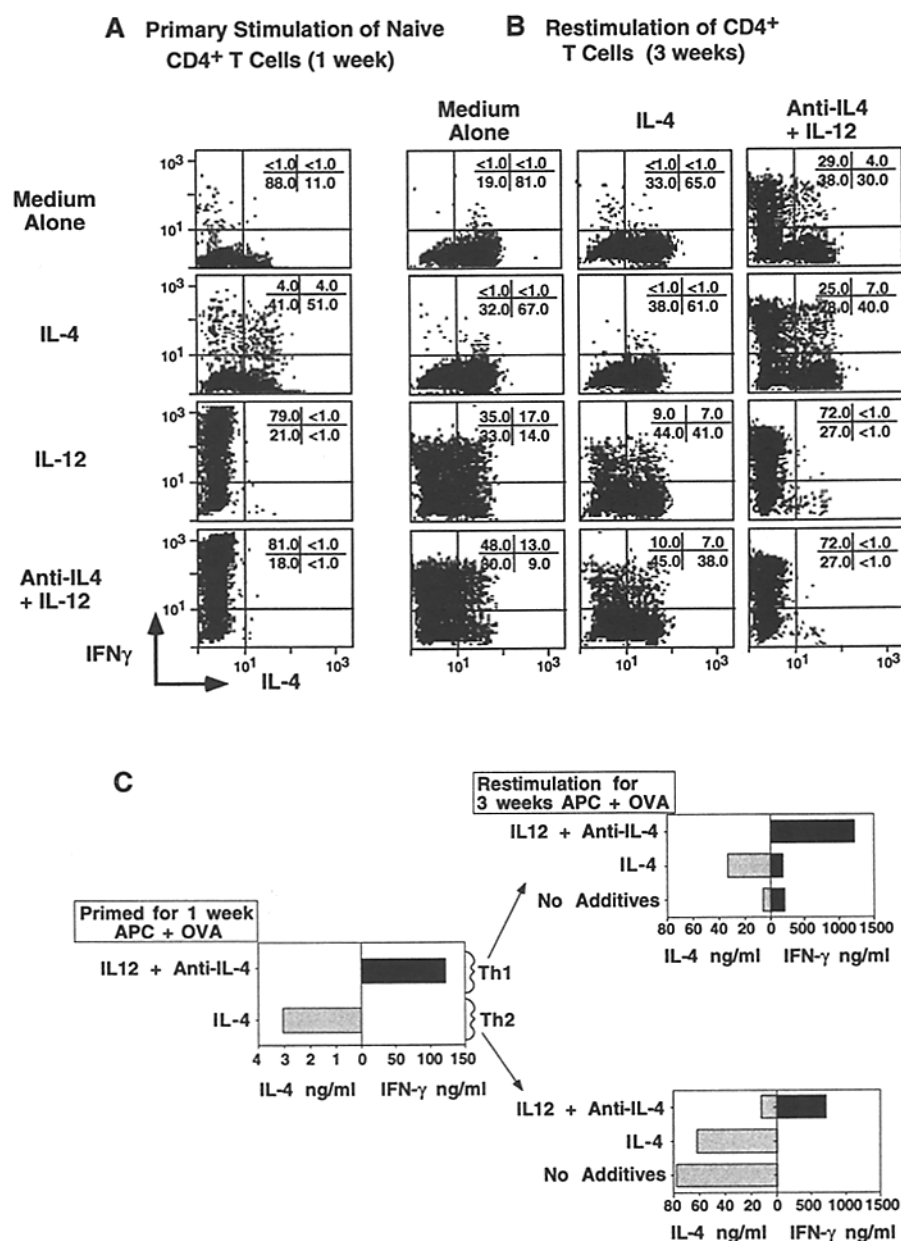


Figure 2. Reversal of phenotype in early Th1 and Th2 populations is more marked after repeated stimulation in oppositely polarizing stimuli. Cells were treated and analyzed as in Fig. 1, except that after the initial 1 wk of stimulation, the cultures were repeatedly stimulated for 3 wk in oppositely polarizing stimuli, to reverse the Th phenotype (and controls) as above. The various populations were analyzed as described in Fig. 1 after stimulation in PMA and ionomycin, for IFN- γ and IL-4 production by immunoassay or flow cytometry.

levels of either cytokine undetectable by the methods used. Restimulation of these strongly polarized Th1 cells with antigen for 1 wk in the presence of IL-4 diminished the IFN- γ levels and enhanced IL-4 levels in the supernatants (Fig. 1 A). Single cell analysis revealed a small number of Th2 cells exclusively producing IL-4 (8%, Fig. 1 C). However, repeated stimulation of the Th1 cells for 3 wk in the presence of IL-4 (Fig. 2 B) generated larger frequencies of cells producing IL-4 (38–41%). This was accompanied by increased levels of IL-4 in the supernatants (Fig. 2 C), suggesting that outgrowth was required to achieve the switch. The changes in the cytokine profiles of both Th1 and Th2 populations were only seen if the conditions required to achieve phenotype reversal were applied during antigenic stimulation, and this was often accompanied by dramatic cell death in the cultures (data not shown).

The most dramatic observation, however, was the ability of added IL-4 to significantly diminish the frequency of Th1 cells producing IFN- γ (81–10%, Figs. 1 and 2) after as little as 1 wk of restimulation (Fig. 1 C). This was also reflected by the decreased levels of IFN- γ detectable in the supernatants of the same cells by ELISA (Figs. 1 A and 2 C). In contrast, the frequencies of IL-4-producing cells in polarized populations of Th2 cells remained relatively stable even when repeatedly stimulated in the presence of IL-12 plus anti-IL-4 mAbs (51–40%, Fig. 2 B).

Thus, although IL-4 and IFN- γ cytokine profiles in the supernatants of stimulated early Th1 and Th2 populations indicated commitment of naive cells towards a strongly polarized Th2 or Th1 phenotype, further stimulation in oppositely polarizing conditions converted the cytokine profile of these populations (Figs. 1 A and 2 C). However,

such analysis of cytokines in the supernatants gives no information on the identity of the cells within the population, or the level of heterogeneity or commitment for cytokine production at the single cell level. Furthermore, immunoassays are limiting since they cannot determine the presence of cells producing both IFN- γ and IL-4 simultaneously, or cells producing neither of these cytokines. It is possible that not all the IFN- γ - or IL-4-producing cells within the early Th1 or Th2 populations are fully committed after a primary stimulation with antigen, but rather retain their ability to respond to signals in their environment (see Fig. 6 A). The flow cytometric analysis of single cells producing IFN- γ and IL-4 shows that early Th1 and Th2 populations are heterogeneous. Taken together, our studies suggest that an apparent reversal of phenotype may reflect clonal outgrowth of a combination of a small number of contaminating Th cells of the opposite phenotype, and differentiating uncommitted precursors, rather than dedifferentiation of Th1 or Th2 cells.

Th1 and Th2 Populations Generated by Chronic Stimulation with Antigen in the Presence of Polarizing Stimuli Are Irreversible. Th1 and Th2 clones have been generally selected from polarized T cell populations derived from hyperimmunized mice, or from mice or humans suffering from chronic infectious disease or allergy (1–4). It has been difficult to reverse the phenotype of such chronic Th1 or Th2 responses in vivo (20, 21, 25–27, 54–56). To simulate polarized Th cell populations isolated in chronic disease, we repeatedly stimulated naive CD4⁺ T cells under polarizing conditions and then assessed the heterogeneity and plasticity of cytokine-producing cells at the single cell level.

CD4⁺ T cells from TCR- α/β DO11.10 transgenic animals, stimulated with splenic APCs and OVA for 3 wk in medium alone or in the presence of IL-4, showed a slightly more dominant Th2 phenotype (>66% cells producing IL-4 only; Fig. 3 A) than Th2 populations arising after 1 wk of stimulation in IL-4 (Figs. 1 B and 2 A). This was reflected by higher levels of IL-4 in the supernatants of these polarized populations upon restimulation (Fig. 3 C). These chronically derived Th2 populations remained relatively stable, even upon repeated restimulation under conditions designed to reverse their cytokine profile, with only the emergence of small subpopulations (<4%) expressing either IFN- γ , or IFN- γ and IL-4 (Fig. 3 B).

Highly polarized Th1 cells developed from repeated stimulation of naive CD4⁺ T cells for 3 wk with antigen and APCs, in the presence of IL-12 and anti-IL-4 (92% produced IFN- γ only; Fig. 3 A). These Th1 populations could not be redirected towards a Th2 phenotype by repeated restimulation in the presence of IL-4 (Fig. 3 B), in contrast to the polarized Th1 populations arising after 1 wk of stimulation under the same conditions (Figs. 1 C and 2 B). Again, the single cell analysis of IFN- γ - and IL-4-producing cells was in keeping with, although again more informative than, standard immunoassay of these cytokines in the supernatants of stimulated Th1 cells (Fig. 3 C). Such resistance to a reversal in their cytokine profile is likely to be due to increased commitment and homogeneity among IL-4- or IFN- γ -

producing cells with time, as well as decreased numbers of precursors, since there is no further source of naive or uncommitted cells in the cultures (see model in Fig. 6 B).

Th1 and Th2 populations arising after chronic stimulation of CD4⁺ T cells in the presence of polarizing conditions greatly resembled those populations arising after short-term stimulation, with a few subtle differences (Figs. 1–3). First, the number of IL-4-producing cells within the Th2 population was slightly increased after repeated stimulation in IL-4 (from 51% after 1 wk, to 66% after 3 wk). This was also the case for the IFN- γ -producing cell number within the Th1 population (from 81% after 1 wk, to 92% after 3 wk). In both situations this was accompanied by a slight decrease in the number of double negative cells producing neither IFN- γ nor IL-4, which could have resulted from their differentiation to IFN- γ - or IL-4-producing cells, or from a lack of survival in the cultures. In addition, in short-term cultures, small percentages of contaminating Th cells of the opposite phenotype were sometimes detectable by flow cytometry (Figs. 1 and 2), although not always by immunoassay (Figs. 1 A and 2 C), and these contaminants often decreased with repeated stimulation in a polarizing stimulus (Fig. 3 A). These subtle differences in the Th1 and Th2 populations arising after 1 wk of stimulation, as compared to those arising from repeated antigenic stimulation in the polarizing stimuli, may account for differences in their level of commitment for production of IFN- γ or IL-4.

It is interesting to note that repeated stimulation of CD4⁺ T cells with IL-12, in the absence of anti-IL-4 mAbs (Fig. 3 A) resulted in the development of large percentages of Th0 cells that produced both IFN- γ and IL-4. This population undoubtedly resulted from repeated stimulation in the presence of endogenous IL-4 and IL-12, and appeared to be relatively unstable, disappearing when there was an alteration in the ratio of IL-4 to IL-12 in the cultures. Whether polarized populations of Th1 and Th2 cells differentiate via an intermediate population producing both IFN- γ and IL-4, as previously proposed, is still unclear (57).

When naive CD4⁺ T cells were stimulated repeatedly in IL-12 alone, a dominant Th1 population emerged, however, these populations also gave rise to 9% of cells producing IL-4 alone, or 5% IFN- γ and IL-4 simultaneously (Fig. 3 A). The increase in IL-4 single positives as well as the IL-4 plus IFN- γ double positives (Fig. 3 A) in the 3- versus 1-wk stimulation (Figs. 1 B and 2 A) may be due to increasing amounts of endogenous levels of IL-4 produced upon repeated stimulation. This is supported by the lack of IL-4-producing cells and dominant Th1 phenotype which emerges from cultures repeatedly stimulated in the presence of IL-12 and anti-IL-4 (Fig. 3 A). Th1 populations derived from 3-wk stimulations in the presence of IL-12 showed no dramatic decrease in IFN- γ -producing cells, although the brightness of the stain was diminished, when repeatedly stimulated in the presence of IL-4. However, anti-IL-4 plus IL-12 markedly increased the frequencies of IFN- γ -producing cells (Fig. 3 B). In addition, cells simultaneously producing IFN- γ and IL-4 appeared to be relatively unstable (Fig. 3, A and B).

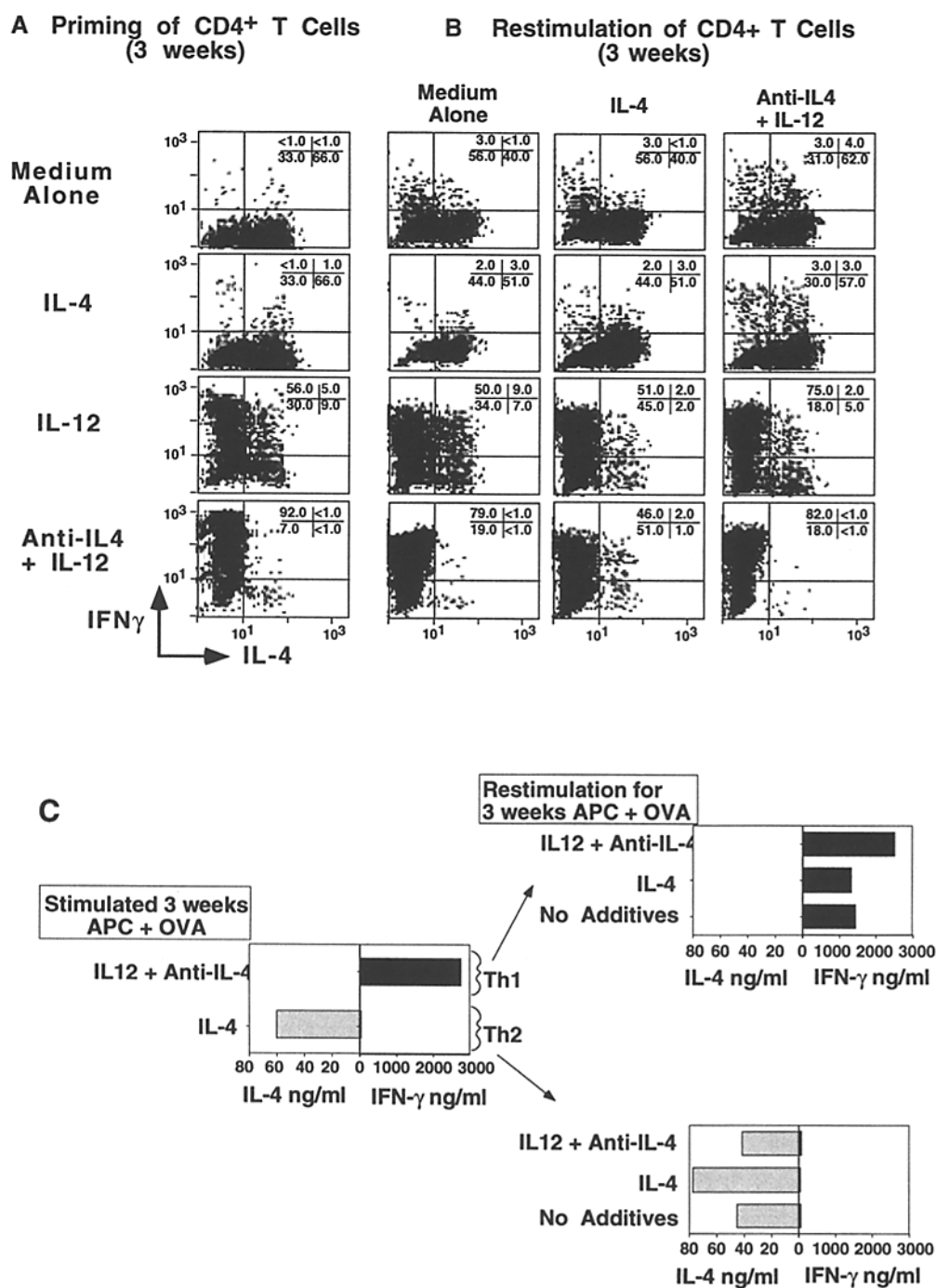


Figure 3. Long-term Th1 and Th2 populations are irreversible. Th effector populations were derived as described in Materials and Methods (Fig. 1), except that in this case, cultures from naive CD4⁺ T cells were stimulated weekly for 3 wk with splenic APCs and OVA in the culture conditions described in Fig. 1. A portion of the cells were harvested after 3 wk, restimulated with PMA and ionomycin, and analyzed for IFN- γ and IL-4 production by immunoassay and by flow cytometry as above (Fig. 1). The remaining CD4⁺ T cells (100% KJ126⁺) were washed and restimulated weekly for 3 wk at 2.5×10^5 /well with irradiated splenic APCs plus OVA in culture conditions designed to reverse the phenotype (and controls) as described above (Fig. 1). Cells were then restimulated with PMA and ionomycin, and IFN- γ and IL-4 production was assessed by immunoassay and by flow cytometry as above.

Although the Th1 and Th2 populations arising from chronically stimulated CD4⁺ T cells were resistant to reversion of phenotype, cultures of polarized Th1 populations restimulated in the presence of IL-4 showed a marked decrease in IFN- γ -producing cells (Fig. 3 B; 92–46%), as with the data obtained with uncommitted Th1 populations, presented in Figs. 1 and 2. In contrast, the frequencies of IL-4-producing cells in polarized Th2 populations did not decrease significantly in the presence of IL-12 and anti-IL-4 (Fig. 3 B), nor increase significantly in the presence of exogenous IL-4.

In summary, Th1 and Th2 populations arising after 1 wk of stimulation, although apparently strongly polarized, are still heterogeneous with respect to differentiation and commitment, possibly as a result of cytokine receptor expression or the engagement of a particular signaling pathway (34). It is likely that repeated stimulation results in a higher degree of homogeneity and renders the population more resistant to change. It is interesting to note that although highly committed Th populations do not appear to acquire the ability to produce cytokines of the opposite Th profile, Th1 cells may be induced to lose their capacity to produce

IFN- γ . In contrast, IL-4-producing cells appear more resistant to the effects of IL-12 and anti-IL-4 mAbs, perhaps reflecting rapid loss of IL-12 receptors for Th1 induction (34). It is possible that this may be specific for the BALB/c genetic background on which this TCR- α/β transgenic mouse was made, since we have reported that mice on this background have a "defect" inasmuch as they default to the Th2 pathway under neutral conditions during secondary stimulation. However, our data may explain previous suggestions that it is possible to reverse the phenotype of early Th1 T cells by addition of IL-4, but not of early Th2 cells by addition of IL-12 (31, 33, 34). Thus the reported apparent reversal of a Th1- to a Th2-type phenotype may result from the disappearance of IFN- γ -producing cells in combination with differentiation and/or clonal outgrowth of Th2 cells. This reversal is more difficult to achieve in Th2 cells, since IL-4-producing cells appear to be much more resistant to downmodulation (33, 34).

Th1 and Th2 Clones Have an Irreversible Phenotype: IL-4 Reduces the Frequency of IFN- γ -producing Cells by Th1 Clones Resulting in Marked Inhibition of IFN- γ Secretion. To assess whether the IL-4-induced reduction in IFN- γ production by long-term Th1 populations reflected a population of still uncommitted cells, a panel of antigen-specific clones derived from single cells were selected from hyperimmunized mice. These clones, which represent a completely differentiated population, were tested similarly by stimulation in

the presence of oppositely polarizing stimuli (Figs. 4 and 5). As expected, resting Th1 and Th2 clones repeatedly stimulated with antigen, in the presence of oppositely polarizing stimuli, did not reverse their cytokine profile, indicating a committed phenotype. However, repeated antigenic stimulation of the Th1 clones in the presence of IL-4 again resulted in a significant reduction of IFN- γ -producing cells or a diminishment in their brightness of staining (Fig. 4), as shown by FACS[®] analysis, and more clearly decreased levels of IFN- γ in supernatants as detected by immunoassay (Fig. 5). Conversely, addition of IL-12 plus (or minus, not shown) anti-IL-4 mAbs resulted in increased percentages of Th1 cells staining brightly for IFN- γ (Fig. 4), which correlated with increased levels of IFN- γ in the supernatants (Fig. 5). IL-4's effects were only seen by immunoassay in chronic stimulations, since in the short term, this cytokine either shows no effect or actually enhances IFN- γ secretion by acting as a growth or viability factor for Th1 clones (7, and Murphy, E., and A. O'Garra, unpublished observations). Again, little effect was observed on IL-4-producing cells in Th2 clones after repeated stimulation in the presence of anti-IL-4 (not shown) or IL-12 and anti-IL-4 mAbs (Fig. 4). The mechanism of IL-4's ability to downregulate IFN- γ -producing cells in differentiated Th1 cells is unclear at this stage. It is possible that IL-4 mediates its effects on Th1 clones by downregulating endogenous levels of IL-12 produced by splenic APCs. However, it is important to note

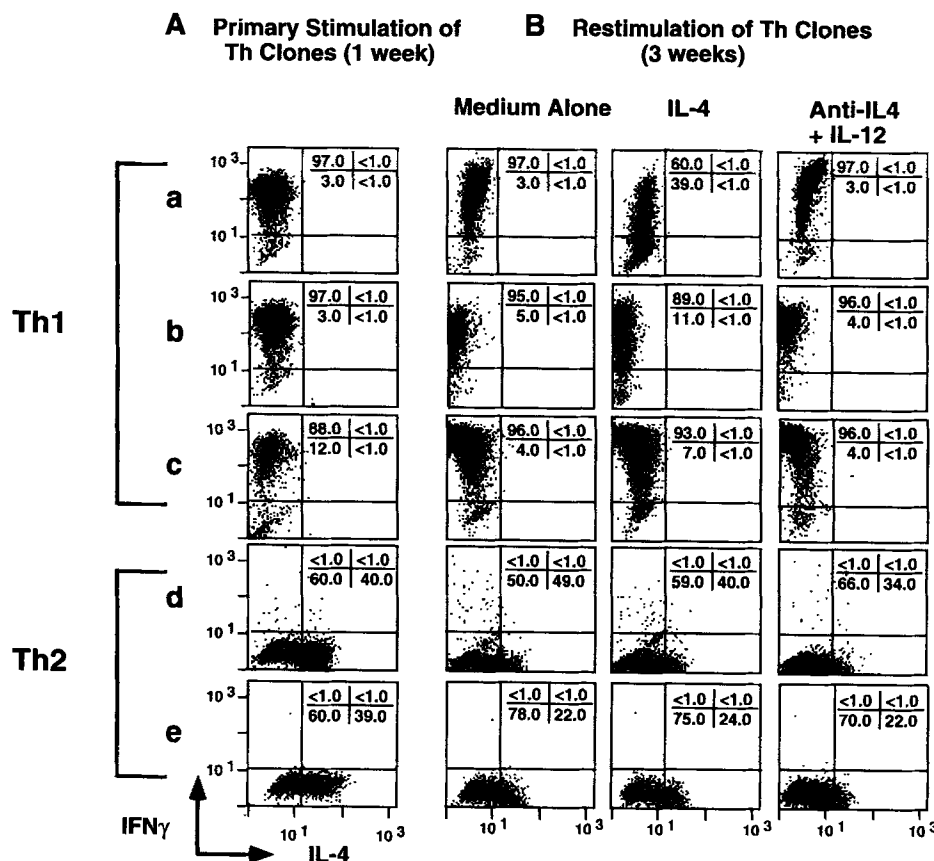


Figure 4. Cytokine phenotype of Th1 and Th2 clones is irreversible: IL-4 downmodulates IFN- γ -producing cells. Long-term established Th1 clones HDK1 (a) and HDK-2 (b) (KLH specific, I-A^d restricted), and Th2 clones, CDC25 (c) (rabbit Ig specific, C3D2/F₁); or short-term Th1 (d) and Th2 (e) clones (CD4⁺ OVA-specific T cells derived from single cell cultures from DO10 TCR- α/β transgenic mice; 35) were tested for their ability to retain their respective phenotypes. Resting T cell clones (2×10^5 /well) were first stimulated with PMA and ionomycin and analyzed for intracellular IFN- γ and IL-4 as above (Fig. 1 A) to assess their basal state (A). T cell clones were then tested for their ability to retain their respective phenotype when stimulated weekly for 3 wk with antigen (KLH, 100 μ g/ml; rabbit Ig, 500 μ g/ml; OVA-peptide, 0.3 μ M) and irradiated splenic APCs (3,000 rad) in the presence of IL-2 (100 U/ml) alone, or in combination with IL-4 (20 ng/ml), or IL-12 (10 U/ml) plus anti-IL-4 (10 μ g/ml). Resulting populations were restimulated with PMA and ionomycin and analyzed for intracellular cytokine synthesis as above (B).

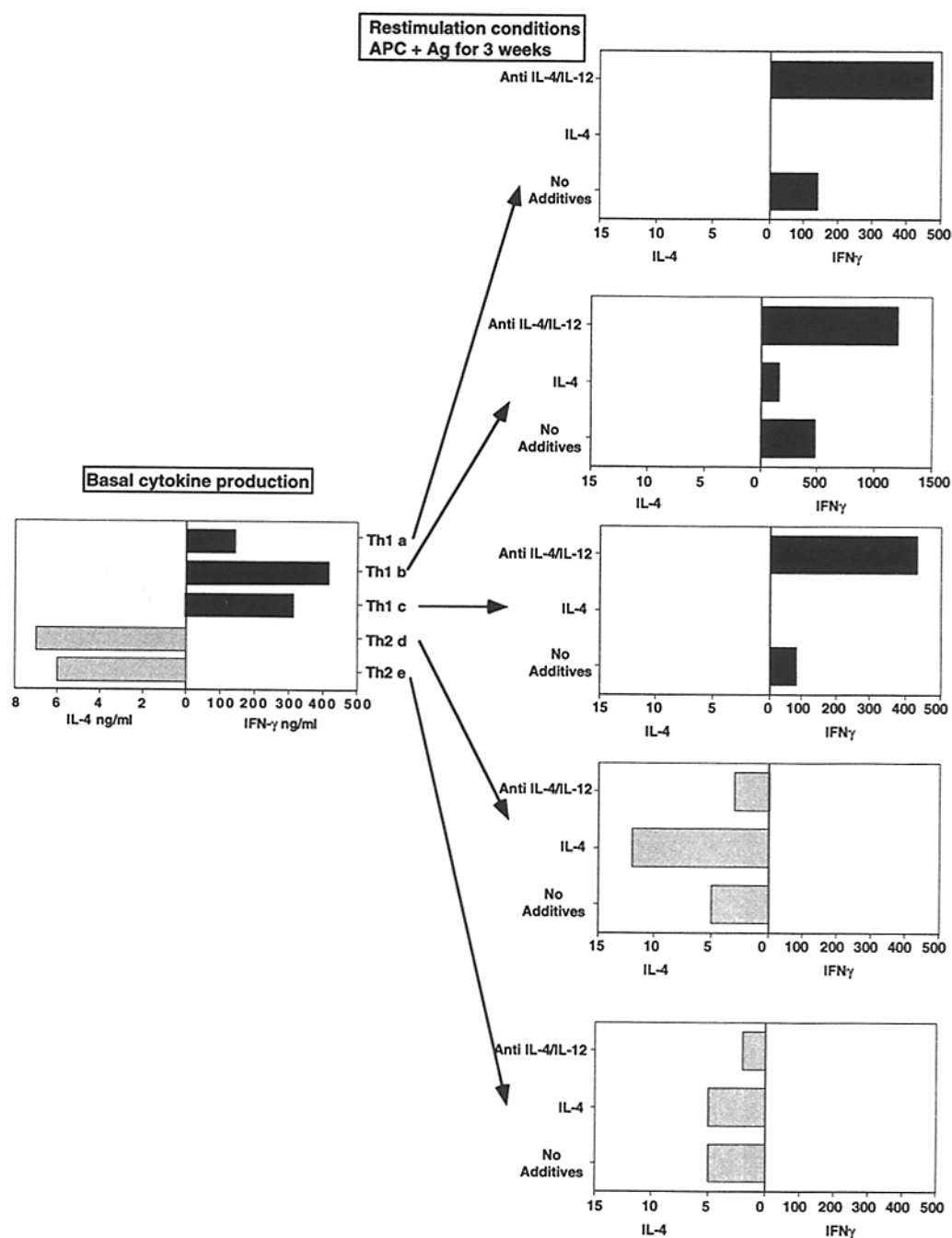


Figure 5. IL-4 significantly downregulates IFN- γ production by Th1 clones. Th clones were treated essentially as in Fig. 4, except that the final restimulation with PMA and ionomycin was for 48 h, after which supernatants were collected for immunoassay of IFN- γ and IL-4 production.

that this effect of IL-4 on IFN- γ production added during repeated antigenic stimulation is still observed upon immediate restimulation of the cells with PMA and ionomycin, in the absence of IL-4 and the splenic APCs.

These findings may explain previous data demonstrating a reduction in IFN- γ levels by IL-4 during a delayed-type hypersensitivity response to *L. major* in vivo (11). Thus, in an in vivo setting, where undoubtedly a heterogeneous population exists, despite the dominant function of extremely

polarized clones obtained from individuals during certain infectious diseases (54, 58–64), or autoimmune pathologies (12, 14, 65–68), the disappearance of IFN- γ -producing cells could suggest an absolute change from a Th1 to a Th2 phenotype.

Implications for Immunotherapy in Chronic Diseases Arising from Infection, Allergy, or T Cell-mediated Autoimmunity. These studies may provide new approaches for therapeutic intervention during chronic disease. It is likely that even

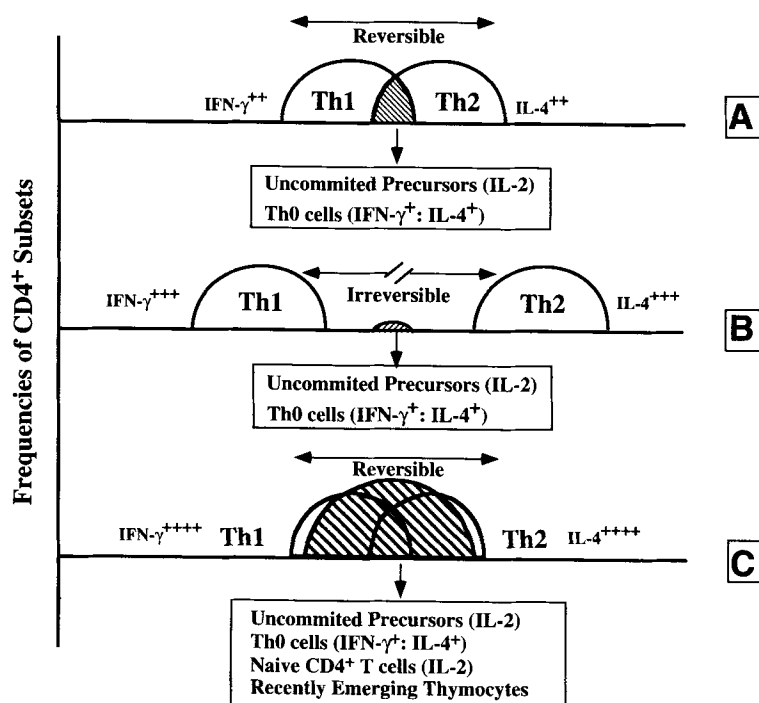


Figure 6. Model for reversal of Th phenotypes. The model for heterogeneity of Th phenotypes and their reversal is based on: (A and B) data obtained from flow cytometric analysis of intracellular cytokine synthesis in polarized Th1 and Th2 populations obtained from TCR- α/β transgenic CD4⁺ T cells stimulated in vitro with APCs plus OVA in the presence of IL-12 plus anti-IL-4 mAbs (Th1) or IL-4 (Th2) for (A) 1 wk or (B) 3 wk, as in Figs. 1–3. (C) The prediction of the heterogeneity of Th phenotypes and their reversal during an immune response in vivo.

during chronic disease, manifested by high levels of Th1- or Th2-type cytokines, that the population of CD4⁺ T cells in a draining lymph node consists of a spectrum of Th cells at different stages of differentiation (Fig. 6 C). Immune regulation in vivo will most likely restrict the frequency of completely differentiated and polarized Th1 and Th2 clones within the population. However, these cells may play an important role in determining the extent of pathology, by their production of high levels of cytokines. Our data suggest that it may be possible to override the controlling influence of such clones producing extreme levels of cytokines. For example, the administration of antigen together with the polarizing conditions will enhance a change in Th phenotype by the recruitment of naive and/or uncommitted cells, which will undoubtedly be present even

during chronic disease. These cells, by differentiation and clonal outgrowth, will suppress the dominant Th phenotype as modeled from our flow cytometric analysis of IFN- γ - and IL-4-producing cells in the in vitro reversal of polarized Th cell populations. The ability of IL-4 to downregulate IFN- γ -producing cells may indicate that it may be more possible to dampen or revert inflammatory or Th1-mediated pathologies, than atopic or chronic Th2-dominated diseases, since IL-4-producing cells were more resistant to change. In these situations it may be necessary to reduce the ongoing immune response as well as to restimulate the populations in the presence of IL-12 plus anti-IL-4 in order to outnumber and suppress dominant clones of a Th2 phenotype.

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