

# Heterogeneity of Intracellular Cytokine Synthesis at the Single-Cell Level in Polarized T Helper 1 and T Helper 2 Populations

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## Summary

CD4<sup>+</sup> T helper (Th) cells can be classified into different types based on their cytokine profile. Cells with these polarized patterns of cytokine production have been termed Th1 and Th2, and can be distinguished functionally by the production of IFN- $\gamma$  and IL-4, respectively. These phenotypes are crucial in determining the type of immune response that develops after antigen priming. There are no surface markers that define them, and cytokine immunoassay or mRNA analysis both have limitations for characterization of single cells. Using immunofluorescent detection of intracellular IFN- $\gamma$  and IL-4, we have studied the emergence of Th1 and Th2 cells in response to antigen exposure and the patterns of cytokine synthesis in established T cell clones. IFN- $\gamma$  production by Th1 clones was detectable in almost all cells by 4 h, and it continued in most cells for >24 h. IL-4 production in Th2 cells peaked at 4 h, but declined rapidly. In Th0 cells containing both cytokines, fewer cells produced IFN- $\gamma$ , which did not appear until IL-4 synthesis declined. Cocultivation of clones showed no such cross-regulation. Antigen stimulation of transgenic T cells expressing an ovalbumin-specific T cell receptor generated Th2 cells, probably as a result of endogenous IL-4 production. Addition of IL-12 and/or anti-IL-4 caused Th1 cells to develop, while some Th0 cells were seen when IL-12 alone was added. These results show that stimulation in the presence of polarizing stimuli results in cells producing either IFN- $\gamma$  or IL-4, but that coproduction can occur in rare cells under defined conditions.

CD4<sup>+</sup> Th cells can be classified into different types that produce characteristic clusters of cytokines, some directing cell-mediated immune responses and others enhancing antibody production by B cells (1–4). Th1 cells produce IL-2, IFN- $\gamma$ , and lymphotoxin, and they promote delayed-type hypersensitivity responses; Th2 cells produce other cytokines, including IL-4, IL-5, IL-10, and IL-13, which direct allergic or antiinflammatory responses, as well as provide help for some B cell responses (2–5). Th cells producing cytokines typical of both Th1 and Th2 clones have also been described in murine and human systems (2–4, 6–8). These have been called Th0 cells, and they may be precursors of the polarized Th1 and Th2 phenotypes (9). Alternatively, Th0 cells may represent a separate, stably differentiated population under some conditions. It is possible, for example, that Th0 cells are instrumental in elimi-

nating some pathogens, where the correct balance of cell-mediated and humoral immunity can clear the organism with minimum immunopathology. By contrast, in many chronic conditions, polarized Th1- or Th2-type immune responses emerge that are mutually exclusive, perhaps in part because of counter-inhibitory effects of cytokines on the reciprocal Th subsets (10–14). The cytokine most characteristic of Th1 responses is IFN- $\gamma$ , a major component of the cell-mediated immune response that is essential for the elimination of *Leishmania major* in mice resistant to infection (15–17). Conversely, IL-4 production can be used to define the Th2 response that renders BALB/c mice susceptible to *L. major* (18, 19).

Polarized populations of CD4<sup>+</sup> Th1- and Th2-type cells can be induced to develop from naive T cells by stimulation in the presence of particular cytokines (20–22). Th2 cells develop when naive T cells are stimulated with either mitogen or specific antigen in the presence of IL-4 (23–26). Conversely, IL-12 is a dominant factor in driving the

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development of Th1 cells from antigen-specific naive CD4<sup>+</sup> T cells (27–29).

To date, most studies of cytokine production by Th cells have used immunoassays or mRNA analysis, neither of which give simultaneous information about production of different cytokines from individual cells. Recently, attempts have been made to analyze cytokine production by mouse T cell clones (30) and by mouse or human peripheral T cells (31–36), in most cases using flow cytometry. We have used this method to examine the patterns of cytokine synthesis in Th1, Th2, and Th0 populations derived from naive CD4<sup>+</sup> T cells stimulated repeatedly with antigen, and we have compared IFN- $\gamma$  and IL-4 production patterns of individual cells with established clones derived from single-cell cultures. This study shows that IL-12 or IL-4 can direct the development of cells producing IFN- $\gamma$  or IL-4 alone, as well as cells producing both cytokines simultaneously, such that the pattern of cytokine production by individual cells in the population resembles that of established Th cell clones.

## Materials and Methods

**Animals.** Mice transgenic for the DO11.10 TCR- $\alpha\beta$  on a BALB/c genetic background (37) were identified at the age of 4–6 wk by staining peripheral blood leukocytes with the anti-TCR clonotype-specific mAb KJ1-26 (38). All transgenic mice used were heterozygous for the TCR- $\alpha$  and - $\beta$  transgenes. Female nontransgenic BALB/c mice between 6–10 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<sup>d</sup> restricted) (39); Th2, CDC25 (C3D2F1/J-derived, normal rabbit IgG-specific, H-2I<sup>d</sup> restricted) (40). Short-term Th clones derived from unimmunized DO11.10 TCR- $\alpha\beta$  transgenic mice were obtained by sorting single cells on the basis of CD4<sup>+</sup>, LECAM-1<sup>bright</sup>, KJ1-26<sup>+</sup> into 96-well microtiter wells containing 10<sup>5</sup> irradiated splenic APC (3,000 rads) plus OVA peptide (0.3  $\mu$ M) under the following conditions: Th1 clones, in the presence of IL-2 (10<sup>7</sup> U/mg, 330 U/ml), IL-12 (10<sup>6</sup> U/mg; 100 U/ml plus small amounts of IL-4 that were required to maintain cell viability) plus TGF- $\beta$  (2 ng/ml); Th2 clones, in the presence of IL-2 (330 U/ml) and large amounts of IL-4 (10<sup>7</sup> U/mg, 100 U/ml); 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 the populations described below, which were not derived from single cells in culture although they carried the same transgene and, thus, antigen specificity). Clones were restimulated and expanded under these respective conditions every 2 wk. All clones were expanded and maintained in [cRPMI 1640 (JR Scientific Inc., Woodland, CA) containing 10% FCS (JR Scientific Inc.), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), Hepes buffer (10 mM), sodium pyruvate (1 mM), 2-ME (0.05 mM; Sigma)], and cRPMI 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 described above, 5–7 d before assay.

Rested T cell clones were washed, counted, and resuspended

at  $2 \times 10^5$  per well, and were restimulated with irradiated BALB/c (3,000 rads) splenic APC plus specific antigen or PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h at 37°C, with Brefeldin A (10  $\mu$ g/ml) 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- $\gamma$  in supernatants by immunoassay as previously described (41, 42).

**Culture Medium, Cytokines, Antibodies, and Antigens and Other Reagents.** The culture medium used was cRPMI, as described above, without supplemented cytokines, unless indicated.

Recombinant mouse cytokines were obtained from the following sources: IFN- $\gamma$ , Schering Research (Bloomfield, NJ); IL-4, Dr S. Menon (DNAX); TGF- $\beta$ , R. & D. Systems, Inc. (Minneapolis, MN), and IL-2, a kind gift from Gerard Zurawski (DNAX). Mouse rIL-12 was obtained by transfecting COS7 cells with the cDNA encoding the p35 and p40 cDNA as described previously (43), which was obtained by PCR cloning using published sequences (43). 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 (44).

Purified rat anti-mouse IL-4 (11B11) and anti-IFN- $\gamma$  (AN18) antibodies have been previously described (44, 45), and an isotype-matched control was supplied by J. Abrams (DNAX) (42). The IL-12-specific mAbs C15.6.7 and C15.1.2 were as described (46). mAbs used for flow cytometric sorting or analysis included anti-mouse CD4-PE and lectin endothelial cell adhesion molecule-1-FITC (both from Pharmingen, San Diego, CA). Additional anticytokine mAbs for immunoassay and flow cytometry, including anti-mouse IL-4 and IFN- $\gamma$  reagents, were purified from serum-free hybridoma supernatants as previously described (42).

The antigenic OVA peptide (OVA<sub>323–339</sub>) from chicken ovalbumin was synthesized on a peptide synthesizer (model 430; Applied Biosystems, Inc., Foster City, CA). KLH and rabbit IgG, both from Sigma, were used at 100 and 500  $\mu$ g/ml, respectively.

Reagents for cell cycle analysis were as follows: 100 $\times$  propidium iodide (PI)<sup>1</sup> stock: 1.0 mg/ml PI in sodium citrate (3.8  $\times$  10<sup>-2</sup> M, pH 7.0, stored at room temperature and protected from light). 40 $\times$  RNase A, 10 mg/ml RNase A (Sigma) in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl boiled for 15 min, cooled to room temperature, and stored at -20°C.

**Preparation of T cells and APC.** CD4<sup>+</sup> T cells were enriched by negative selection using magnetic activated cell sorting (MACS) with a cocktail of biotinylated anti-mouse CD8 $\alpha$ , I-A<sup>d</sup>, B220, and Mac-1 antibodies as previously described (41) (Miltenyi, Sunnyvale, CA). T cells staining positive for CD4 and LECAM-1 (47) were further purified by positive selection using a FACStar<sup>Plus</sup> flow cytometer (Becton Dickinson & Co., Mountain View, CA) to achieve 99.8% CD4<sup>+</sup> T cells. Staining did not alter the function of the T cells (not shown).

**Stimulation of Transgenic CD4<sup>+</sup> T Cells for Cytokine Production.** (a) Primary stimulations of CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  per well) were carried out using OVA peptide (0.3  $\mu$ M) and nucleated spleen cells ( $5 \times 10^6$  per well, 3,000 rads) as APC in a total volume of 2 ml in 24-well plates. In addition, some cultures received cytokines (200 U/ml IL-4 or 10 U/ml IL-12) or mAbs to block endogenous cytokines (10  $\mu$ g/ml anti-IL-4). T cells were ex-

<sup>1</sup>Abbreviations used in this paper: PI, propidium iodide; OVA, antigenic OVA<sub>323–339</sub> peptide.

panded and maintained in the same culture conditions for 1 wk. (b) Cells were then harvested on day 6, washed three times, counted, and resuspended at  $2 \times 10^5$  per well, and restimulated with irradiated BALB/c splenic APC (3,000 rads) plus OVA (0.3  $\mu$ M), or PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h at 37°C, with Brefeldin A (10  $\mu$ g/ml) added for the last 2 h (to enhance 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 IL-4 and IFN- $\gamma$  analysis in supernatants by immunoassay as previously described (41, 42).

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

**Cytokine Assays.** IFN- $\gamma$  was detected using a two-site sandwich ELISA (42, 44), which has a lower limit of sensitivity of 125 pg/ml (1 U/ml = 0.1 ng/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- $\gamma$  and IL-4 Synthesis.** Cells were resuspended at  $10^5$ – $10^6$ /ml and stimulated with PMA at 50 ng/ml plus ionomycin at 500 ng/ml. At various times after stimulation (typically 2 h before cell harvest), Brefeldin A (catalogue no. B905MG; Epicentre Technologies Corp., Madison, WI) was added at 10  $\mu$ g/ml using a stock of 1 mg/ml in ethanol. 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 in PBS at 4°C for up to 2 d, or they were stained immediately for cytokines using a modified method (35, 36) based on that described by Assenmacher et al. (33). For intracellular staining, all reagents and washes contained 1% BSA and 0.5% saponin (S-7900; Sigma), and all incubations were at room temperature. After washing and a 10-min incubation in PBS/BSA/saponin, cells were incubated with anti-IL-4 (11B11) at 5  $\mu$ g/ml or GL113 (isotype control) at 10  $\mu$ g/ml for 30 min. After two washes, optimal concentrations of FITC anti-rat Ig (antibody raised in rabbit, catalogue no. FI4001; Vector Laboratories, Burlingame, CA) were added for 30 min. After two washes, purified rat Ig was added at 300  $\mu$ g/ml for 10 min to block residual anti-rat Ig binding. Without washing, anti-IFN- $\gamma$  (AN18-PE or control PE-conjugated rat IgG, catalogue no. 11025 A; Pharmacia Fine Chemicals, Piscataway, NJ) was added at a final concentration of 2.5  $\mu$ g/ml. After 20 min, cells were washed twice with PBS/BSA/saponin and then with PBS/BSA without saponin to allow membrane closure. Samples were analyzed on a FACScan<sup>®</sup> flow cytometer (Becton Dickinson). Thresholds were set on control stains (included for every sample at every timepoint) to lie on the first percentile. Results were analyzed using Lysis II and Cellquest softwares (Becton Dickinson).

**Cell Cycle Analysis by PI Staining.** Th clones were harvested ( $1$ – $3 \times 10^6$ ) after stimulation with PMA and ionomycin (as above) for various time intervals. Cells were washed twice in PBS and then resuspended in 1 ml PBS. The cell suspension was then added to 5 ml cold ethanol (100%) very slowly (drop by drop) with vortexing to prevent clumping and ensure a single-cell suspension. The cells were fixed in ethanol for 15 min, and can be stored at 4°C for up to 2 wk. After fixation, the cells were washed once with PBS and resuspended in a solution of PI/RNase A (10  $\mu$ g/ml PI, 250  $\mu$ g/ml RNase A in PBS; this solution should be made up from stocks directly before use). The cells were incubated in this solution at 37°C for 30 min and were then analyzed

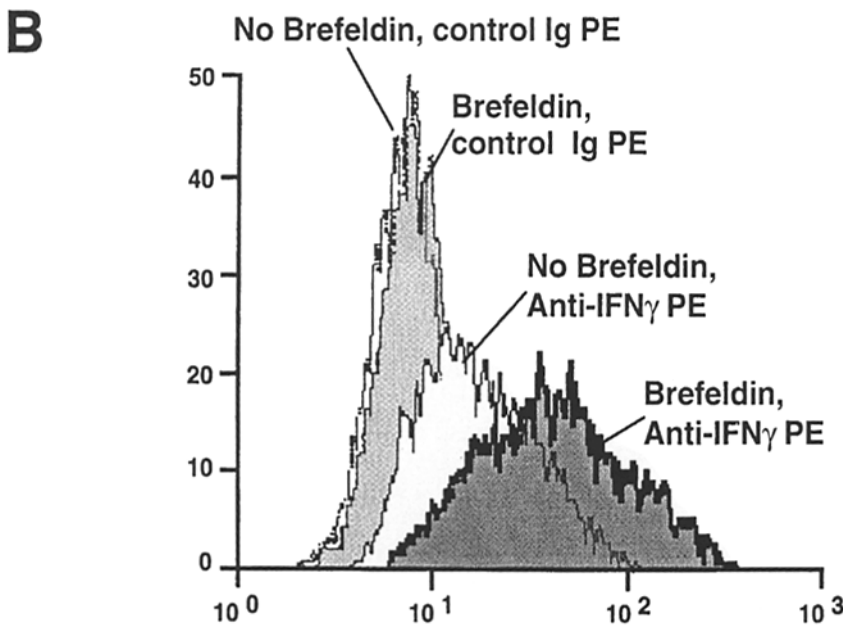
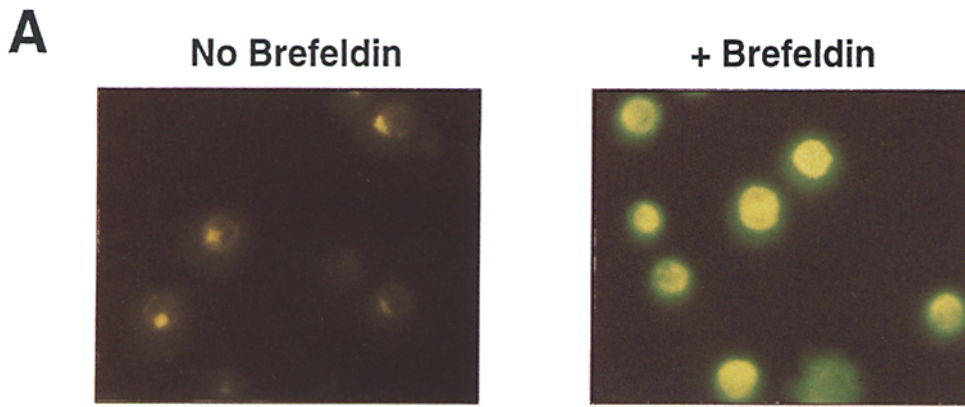
within 24 h on the FACScan<sup>®</sup> flow cytometer. Results were analyzed using Cellquest software (Becton Dickinson).

## Results and Discussion

**Intracellular Synthesis of IFN- $\gamma$  and IL-4 are Mutually Exclusive and Show Different Kinetics in Established Th1 and Th2 Clones.** The aim of this study was to use flow cytometry to simultaneously analyze intracellular synthesis of IFN- $\gamma$  or IL-4 by maturing T cells obtained from naive TCR- $\alpha\beta$  transgenic CD4<sup>+</sup> T cells stimulated with specific antigen in the presence of IL-4 or IL-12. In particular, it is not known whether cells within a Th1 or Th2 population produce IFN- $\gamma$  and IL-4 exclusively, or whether they are capable of simultaneously synthesizing both cytokines. We also address this in Th0 clones known to produce both cytokines by immunoassay. To validate the method of cytokine analysis by flow cytometry, we first studied the pattern of cytokine staining in established Th1 and Th2 clones, known only to produce each cytokine by reverse transcription PCR.

It had been suggested that IL-4 and IFN- $\gamma$  are never co-expressed in individual CD4<sup>+</sup> T cells (33). Since this conclusion may have resulted from limited sensitivity of the methods used, we stimulated cells in the presence of Brefeldin A to disrupt the Golgi apparatus, inhibit protein secretion, and thus increase the sensitivity of cytokine detection (35, 36). Flow cytometric analysis of a long-term Th1 clone showed that disruption of the Golgi complex by Brefeldin A (Fig. 1 A) caused increased sensitivity of IFN- $\gamma$  detection (Fig. 1 B). Brefeldin A also enhanced sensitivity for IL-4 detection in Th2 clones (data not shown). The profile demonstrates a shift in fluorescence of the whole population, relative to control Ig staining, indicating that at least 95% of the cells can synthesize IFN- $\gamma$ . In this case, PMA and ionomycin were used to stimulate the Th1 clone to circumvent heterogeneity in the kinetics of T cell stimulation that might result from processing and presentation of the intact protein by APC.

To assess the frequency of IL-4-expressing cells in Th2 clones relative to that of IFN- $\gamma$  synthesis by Th1 clones, cells were stimulated with PMA and ionomycin for 24 h, with Brefeldin A added for the last 2 h. Th1 clones included (a) a long-term KLH-specific clone, HDK-1 (Fig. 2 A) (39); and (b) two freshly derived short-term Th1 clones obtained from single-cell cultures of CD4<sup>+</sup> T cells from DO11.10 TCR- $\alpha\beta$  transgenic mice (Fig. 2, B and C). Upon stimulation with PMA and ionomycin, 79–96% of the Th1 clones stained positive for IFN- $\gamma$  and negative for IL-4 relative to an isotype control. (Fig. 2 D). Th2 clones included (a) a long-term rabbit Ig-specific Th2 clone, CDC25 (Fig. 2 A) (40); and (b) two recently derived Th2 clones obtained from single-cell cultures of CD4<sup>+</sup> T cells from DO11.10 TCR- $\alpha\beta$  transgenic mice (Fig. 2, B and C). In contrast to the synthesis of IFN- $\gamma$  by Th1 clones, intracellular synthesis of IL-4 could only be detected in 30–33% of the cells; all were negative for IFN- $\gamma$ . The levels of cytokines measured by immunoassay in the supernatants of these Th1 and Th2 clones correlated well with the cyto-

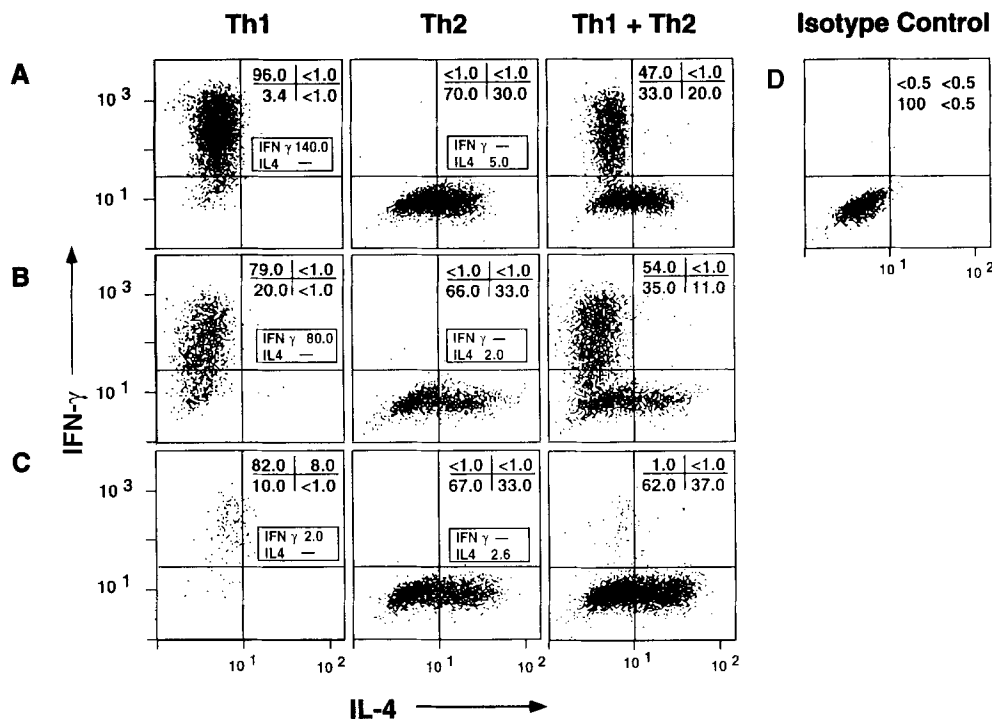


**Figure 1.** Enhancement of intracellular cytokine immunofluorescence by Brefeldin A. Th1 cells (HDK-1) were stimulated for 4 h with PMA and ionomycin, and then fixed and stained for IFN- $\gamma$  synthesis with (A) an anti-IFN- $\gamma$  antibody (AN18) or a control antibody (GL117) followed by an anti-rat FITC or (B) a PE-conjugated anti-IFN- $\gamma$  or control antibody, as described in Materials and Methods. Brefeldin A was added or omitted for the last 2 h of stimulation, and IFN- $\gamma$  was visualized by fluorescence microscopy (A) or flow cytometry (B). Neither Brefeldin-treated nor -untreated cells stained with the control antibody, but Brefeldin enhanced specific staining for IFN- $\gamma$ . Similarly treated CDC25 cells (Th2) showed no specific staining for IFN- $\gamma$  with or without Brefeldin treatment (not shown). Cell fluorescence was measured using a FACScan<sup>®</sup>, and quadrants were set according to the isotype-matched controls. Data were analyzed using Lysis II software.

kine synthesis at the single-cell level (Fig. 2). This result is in agreement with data recently presented about analysis of IFN- $\gamma$  and IL-4 mRNA expression by DO11.10-derived clones, which shows a low frequency of IL-4 mRNA expression by Th2 clones compared to IFN- $\gamma$  expression by Th1 clones (30). Specificity of staining of IFN- $\gamma$  and IL-4 was shown using isotype-matched controls (Fig. 2 D) and by specific appropriate staining of Th1 and Th2 clones (Fig. 2 A–C). These controls were used to set threshold markers on flow cytometric plots as shown (Fig. 2 D). As a further control, Th1 and Th2 clones were mixed at different ratios (Fig. 2 A–C) before stimulation, fixing, and staining for IFN- $\gamma$  and IL-4, to show whether secreted cytokines could bind to the cell surface via specific receptors and give false results when mixed populations were analyzed. Such cell-surface staining would be characterized by cells staining for both IFN- $\gamma$  and IL-4 within mixtures of Th1 or Th2 clones, which are known to express either IFN- $\gamma$  or IL-4

alone. This clearly does not occur (Fig. 2 A–C), demonstrating that the double-positive staining for IFN- $\gamma$  and IL-4 that is sometimes observed (see Figs. 4 and 6) represents true cosynthesis by individual cells. In addition, mixing of different ratios of Th1 and Th2 cells (e.g., Fig. 2 C, Th1/Th2 ratio is 6:94) demonstrates that even when as few as 1% of the cells in a population are expected to stain for IFN- $\gamma$ , they are still detectable by this method.

The observation that synthesis of intracellular IFN- $\gamma$  could be detected in  $\sim$ 100% of the cells within Th1 clones, whereas IL-4 producing cells were always at a lower frequency within Th2 clones, prompted us to investigate the kinetics of synthesis of each of the cytokines as a possible explanation of this phenomenon. Fig. 3 shows representative data obtained from both long- and short-term Th1 and Th2 clones (derived from single cells). Cells were stimulated with PMA and ionomycin at regular intervals for up to 24 h, and Brefeldin A was added for the last 2 h of



**Figure 2.** Specific two-color staining of Th1 and Th2 clones for IFN- $\gamma$  and IL-4. Th1 and Th2 clones were cultured separately or together, as indicated, in IL-2 overnight before stimulation with PMA and ionomycin for 4 h in the presence of Brefeldin A for the last 2 h. Cells were fixed and stained for IFN- $\gamma$  and IL-4 synthesis as described in Materials and Methods. (A) HDK-1 (Th1) and CDC25 (Th2) cells alone or mixed in a 50:50 ratio before overnight culture (Th1 + Th2). (B) DO11.10-derived, OVA-specific Th1 or Th2 clones alone or mixed in a 36:64 ratio (Th1 + Th2) overnight. (C) More DO11.10-derived Th1 or Th2 clones alone or mixed in a 6:94 ratio overnight (Th1 + Th2). In all cases where Th1 and Th2 clones were mixed before stimulation, <1.0% cells stained double positive for both IFN- $\gamma$  and IL-4, demonstrating the sensitivity and specificity of the staining method even under the conditions of cocultivation.

Cell fluorescence was measured using a FACScan<sup>®</sup>, and quadrants were set according to the isotype-matched controls. (D) A representative negative control, CDC25 (Th2), stained for anti-IFN- $\gamma$  and GL113; all other controls were identical (not shown). Data was analyzed using Lysis II software, and they are displayed as bivariate dot plots. Percentages of cells in each quadrant (set according to the negative controls) are shown in the top right-hand corner of each panel. Levels of IFN- $\gamma$  or IL-4 (ng/ml), measured in 48-h supernatants are shown in the boxes contained within each panel.

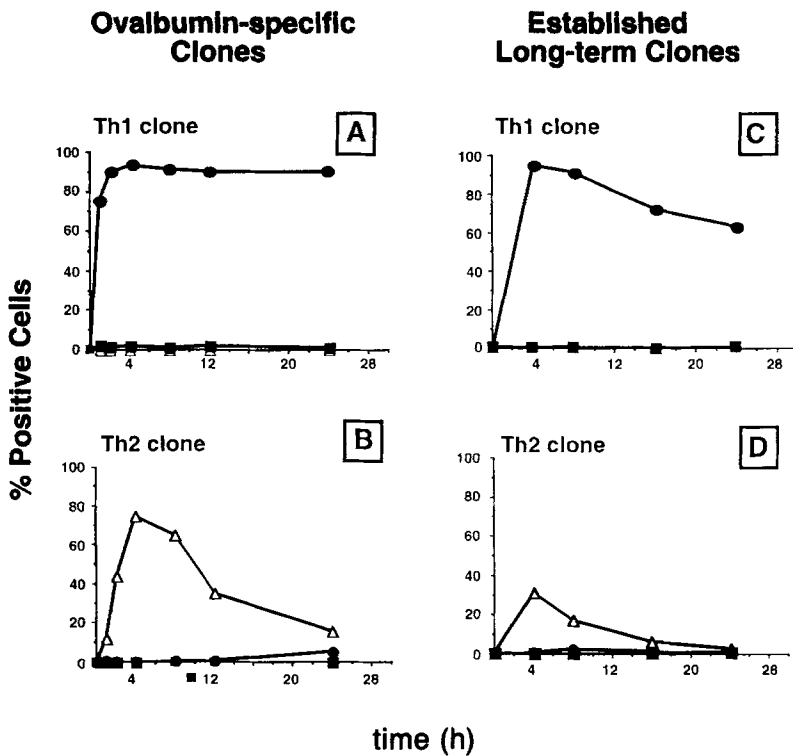
stimulation. In long- and short-term clones, the peak of intracellular synthesis of both cytokines occurred at  $\sim$ 4 h of stimulation (Fig. 3). Whereas IFN- $\gamma$  synthesis by Th1 clones was sustained throughout, IL-4 synthesis was relatively transient, and it declined rapidly straight after synthesis peaked at 4 h. We show in Table 1 that both Th1 and Th2 clones are heterogeneous with respect to their phase in the cell cycle after stimulation with PMA and ionomycin (as above) for various time intervals. Since mammalian cells such as these are not synchronously in cell cycle, it is inevitable that if a cytokine like IL-4 is transiently synthesized, it will never be detectable in 100% of cells simultaneously.

**Analysis of Kinetics of Intracellular Synthesis of IFN- $\gamma$  and IL-4 by Single Cells in Th0 Clones Reveals Cells Simultaneously Producing Both Cytokines.** Mouse and human Th clones that produce both Th1- and Th2-type cytokines have been described (2–4, 6–8, 48, 49). It is not clear, however, what the status of cytokine production by individual cells within a Th0 clone is since few (33) experiments using simultaneous analyses of IFN- $\gamma$  and IL-4 in single cells have been performed. They could consist of a mixture of cells producing either only IFN- $\gamma$ , only IL-4, or cells that are capable of producing both cytokines. We have analyzed a number of short-term Th0 clones obtained from the DO11.10 TCR-transgenic mouse, as described in Materials and Methods. Two representative Th0 clones contained individual cells synthesizing IFN- $\gamma$  or IL-4 exclusively, or both cytokines simultaneously, while the

majority of the cells within the population often produced neither cytokine (Fig. 4). Many of the clones also produced much lower levels of both IFN- $\gamma$  and IL-4 by immunosay (data not shown) than polarized Th1 or Th2 clones, which correlated with the low frequency of cells synthesizing either IFN- $\gamma$  or IL-4 intracellularly, or both cytokines simultaneously (Fig. 4). Interestingly, the IFN- $\gamma$  was synthesized at a later stage in the Th0 clones (Fig. 4) than in the completely polarized Th1 clones (Fig. 3). Furthermore, this later appearance of IFN- $\gamma$ -producing cells correlated with the decline of IL-4 synthesis. This suggests that the synthesis of Th1- and Th2-type cytokines in individual T cells may be mutually exclusive.

**Table 1.** Cell Cycle Analysis of Th1 and Th2 Clones

Stage of cell cycle	Percentage of cells after stimulation (h)					T helper clone
	0	2	4	8	20	
G <sub>0</sub> -G <sub>1</sub>	76	78	75	76	57	Th1
	67	66	63	68	69	Th2
G <sub>2</sub> -M	15	13	19	17	14	Th1
	12	12	14	12	20	Th2
S	10	9	6	7	29	Th1
	21	22	23	20	11	Th2



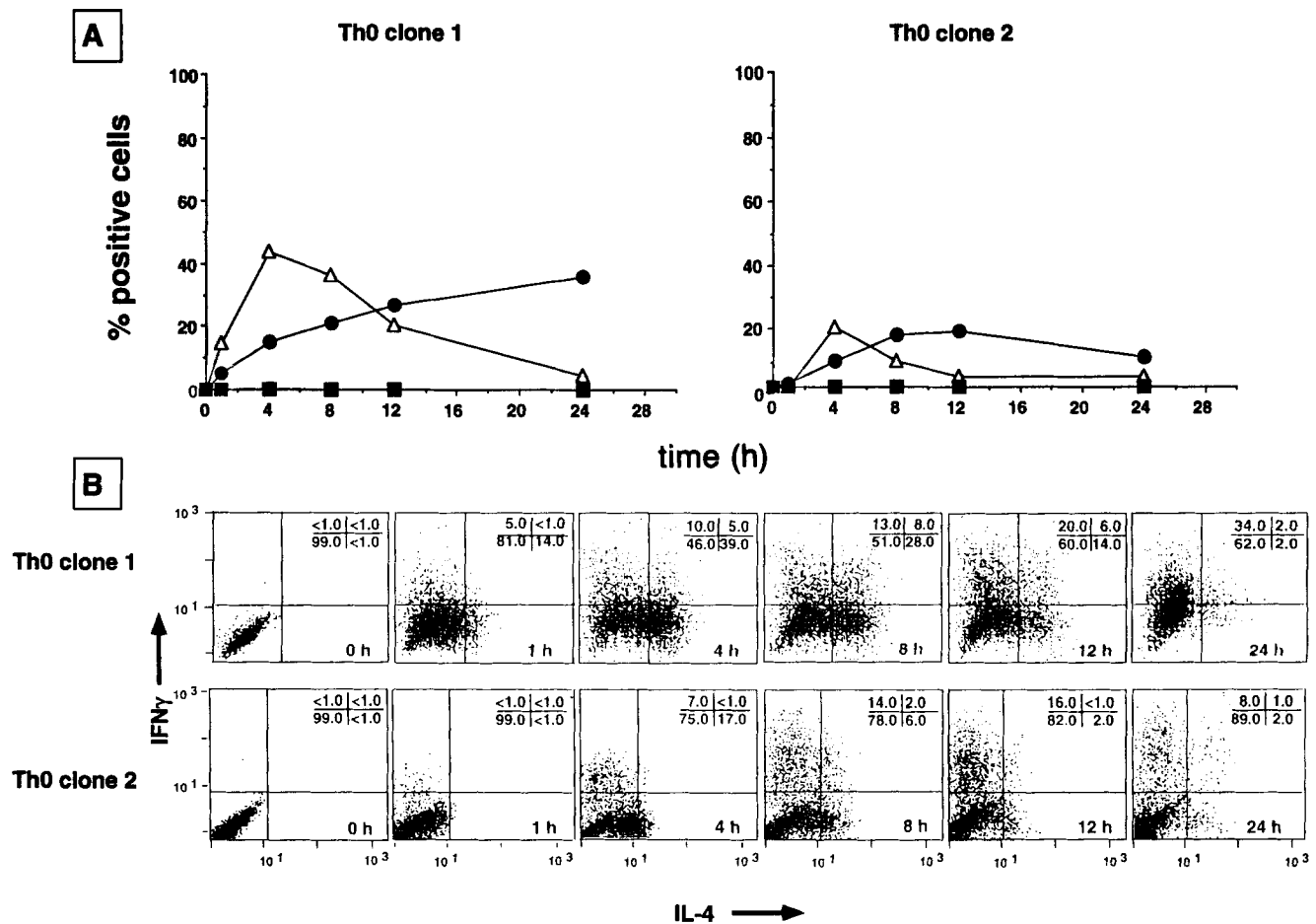
**Figure 3.** Kinetics of intracellular synthesis of IFN- $\gamma$  and IL-4 in clones with polarized cytokine production. T cell clones derived from single-cell cultures were used for experiments 10–14 d after stimulation with antigen and irradiated splenic APC. These resting cells were washed and restimulated with PMA and ionomycin for various times, and Brefeldin A (10  $\mu$ g/ml) was added for the last 2 h. (A and B) DO11.10-derived, OVA-specific, TCR-transgenic Th1 and Th2 clones; (C and D) long-term Th1 (HDK-1, KLH-specific) and Th2 (CDC25, rabbit Ig-specific), respectively. Cells were fixed and stained for intracellular IFN- $\gamma$  and IL-4 synthesis as described in Materials and Methods. Flow cytometric analysis was as described in Fig. 2. ●, IFN- $\gamma$ ;  $\Delta$ , IL-4; ■, control.

*Similar Patterns of Intracellular Synthesis of IFN- $\gamma$  and IL-4 By Th1 and Th2 Clones after Stimulation with Either PMA and Ionomycin or Splenic APC Plus Specific Antigen.* The reason for using PMA and ionomycin rather than specific antigen was to reveal the full cytokine production potential of T cells and try to minimize variability in kinetics resulting from antigen processing and/or presentation. In the case of the DO11.10-derived clones, it could be argued that the use of the mitogen is equivalent to stimulation with the peptide, since all express high levels of the clonotype TCR, as assessed by flow cytometric staining with the anticlonotype antibody (38). Although the aim of this study was to define the full potential of the differentiated Th cell, it was important to assess whether physiological stimulation would yield a response pattern similar to that which is seen after mitogen stimulation. A number of Th1 and Th2 clones derived from the DO11.10 TCR- $\alpha\beta$  transgenic mouse were therefore analyzed after stimulation with antigen and APC or with mitogen. Intracellular cytokine synthesis was similar regardless of the type of stimulation; however, 70% of the Th1 cells stained positive for IFN- $\gamma$  synthesis after stimulation with PMA and ionomycin, compared to only up to 40% of the cells stimulated with splenic APC and peptide antigen (Fig. 5). The staining was marginally brighter after stimulation with the mitogens. In contrast, the frequency of cells synthesizing IL-4 was usually identical after stimulation with specific antigen or mitogen. The difference in IFN- $\gamma$  synthesis may be caused by differences in kinetics resulting from different modes of stimulation, or by endogenous levels of cytokines in splenic APC, which are suppressive for IFN- $\gamma$  synthesis. Thus, in

most cases, experiments were performed in parallel with mitogen or APC and specific antigens, with both stimulations giving broadly similar results.

*Bulk Cultures of Naive CD4<sup>+</sup> T Cells Can Be Induced to Develop into Polarized Th1 and Th2 Populations Containing High Frequencies of IFN- $\gamma$ - or IL-4-producing Cells When Chronically Stimulated with Antigen in the Presence of IL-12 or IL-4.* CD4<sup>+</sup> T cells develop into Th1- or Th2-type populations if stimulated with antigen in a primary response in the presence of IL-12 or IL-4, respectively (24–27, 29, 50, 51). While this has been demonstrated by the analysis of secreted IFN- $\gamma$  or IL-4 (24–27, 29, 50, 51), little information is available regarding the heterogeneity of the populations at the single-cell level. We demonstrate that OVA-specific T cells from the TCR- $\alpha\beta$  transgenic mouse stimulated with splenic APC plus antigen for 1 wk resulted in a population in which 10% of the cells synthesized IL-4 intracellularly, but few cells produced IFN- $\gamma$  (<1%) (Fig. 6). Addition of IL-4 in primary cultures led to a slight increase in IL-4-producing cells (14%). Surprisingly, a small number of IFN- $\gamma$ -producing cells could be detected, half of which produced IL-4 simultaneously (Fig. 6). In accordance with results obtained by measuring cytokine secretion by immunoassay (not shown), CD4<sup>+</sup> T cells stimulated with OVA in the presence of IL-12 gave rise to a high frequency of IFN- $\gamma$ -producing cells (87%), but no IL-4-producing cells. The frequency of IFN- $\gamma$ -producing cells was similar at this stage when anti-IL-4 mAbs were added to IL-12 cultures.

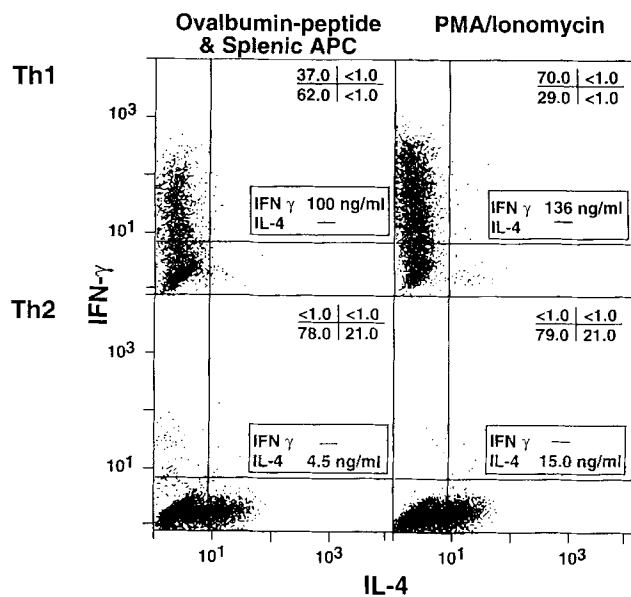
Since many of the original Th1 and Th2 clones described by Mosmann and Coffman (5) were obtained from



**Figure 4.** Kinetics of intracellular synthesis of IFN- $\gamma$  and IL-4 by Th0 clones. Two representative DO11.10-derived, OVA-specific Th0 clones derived from single-cell culture, as described in Materials and Methods, were used for experiments 10–14 d after stimulation with antigen and irradiated splenic APC. Resting cells were washed and restimulated with PMA and ionomycin for various times, and Brefeldin A (10  $\mu$ g/ml) was added for the last 2 h. Cells were fixed and stained for IFN- $\gamma$  and IL-4 synthesis as described in Materials and Methods. Flow cytometric analysis was as described in Fig. 2.  $\bullet$ , IFN- $\gamma$ ;  $\Delta$ , IL-4;  $\blacksquare$ , control.

chronically immunized mice, we attempted to drive the development of a higher frequency of either IFN- $\gamma$ - or IL-4-producing cells by repeated stimulation with antigen and APC for three consecutive weeks in the presence or absence of IL-4, IL-12, or anti-IL-4 plus IL-12. T cell populations stimulated with antigen and APC only contained a high frequency of IL-4-producing cells (83% compared to 10% after the 1st wk). The addition of exogenous IL-4 during the 3 wk of stimulation did not increase the frequency of IL-4-producing cells, and the addition of anti-IL-4 antibodies resulted in T cell populations only producing IFN- $\gamma$ , suggesting that endogenous IL-4 may have caused this initial skewing towards a Th2 phenotype. Since the DO11.10 mice are of a BALB/c genetic background, this may account for the development of a Th2 phenotype resulting from endogenous levels of IL-4 (52), and may not always be the case with TCR transgenic mice of different genetic backgrounds (26). Addition of IL-12 during the repeated stimulation increased the frequency of cells producing only IFN- $\gamma$ , but it also led to the development of cells producing both IFN- $\gamma$  and IL-4 (3%). In some experi-

ments, the number of cells simultaneously synthesizing both IFN- $\gamma$  and IL-4 was higher (5%). The observation that no cells develop producing both IFN- $\gamma$  and IL-4 when cells were chronically stimulated with antigen and APC in the presence of IL-12 plus neutralizing antibodies directed against IL-4 reflects the presence of endogenous IL-4. These data suggest that the development of double-producing cells is determined by the dual action of IL-4 and IL-12 during antigenic stimulation, and that the percentage of these cells developing depends strictly on the relative levels of IL-4 and IL-12 in the cultures. Cultures of CD4<sup>+</sup> T cells containing IL-4 (either endogenous or exogenous) initially proliferated more vigorously than other groups stimulated with antigen and APC. It is not clear whether the differentiation of naive CD4<sup>+</sup> T cells into Th2 cells is linked to this proliferation. However, we have data to support that Th2 cells differentiate from a single cell and do not result from clonal expansion of a subpopulation. Briefly, culturing of single naive CD4<sup>+</sup> T cells obtained by FACS<sup>®</sup> on irradiated splenic APC, plus OVA peptide, plus IL-2 and IL-4 results in up to 80% cloning efficiency, and



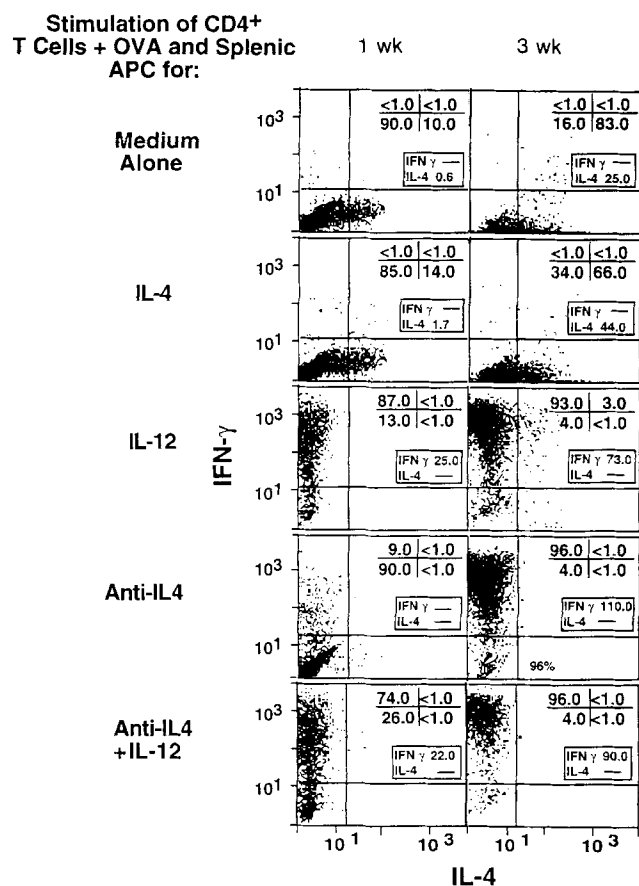
**Figure 5.** Comparison of intracellular synthesis of IFN- $\gamma$  or IL-4 after stimulation with specific antigen or PMA and ionomycin. DO11.10-derived, OVA-specific Th1 (top panel) or Th2 (bottom panel) clones were stimulated with irradiated splenic APC plus OVA (left-hand side) or with PMA and ionomycin for 4 h, with Brefeldin A added for the last 2 h before fixing and staining for IFN- $\gamma$  and IL-4 as described in Materials and Methods. Cell fluorescence was measured using a FACScan<sup>®</sup>, and quadrants were set according to the isotype-matched controls. Data was analyzed using Lysis II software, and they are displayed as bivariate dot plots. Percentages of cells in each quadrant (set according to the negative controls) are shown in the top right-hand corner of each panel. Levels of IFN- $\gamma$  or IL-4 (ng/ml) measured in 48-h supernatants are shown in the boxes contained within each panel.

all the resulting populations are of the Th2 type (Murphy, E., and A. O'Garra, unpublished observation). Although initial stimulation of bulk cultures of naive CD4<sup>+</sup> T cells with antigen and APC in the presence of IL-4 initially shows increased proliferation, repeated stimulation with antigen in the presence of IL-4 leads to a rapid decline in the cell recoveries (Table 2), suggesting that Th2 cells may lose their proliferative capacity as they differentiate. This trend can also be seen with Th1 cells, but this decline in proliferation is more gradual, and initial cultures do not show the dramatic proliferation that is seen in cultures containing IL-4 (Table 2).

In summary, stimulated Th1 clones show prolonged synthesis of IFN- $\gamma$  so that  $\sim 100\%$  of the population is seen to

**Table 2.** Expansion of OVA-specific CD4<sup>+</sup> T Cells

Stimulation	1 wk	2 wk	3 wk
—	57	30	9
IL-4	38	28	4
IL-12	10	25	11
Anti-IL-4 + IL-12	10	22	11
Anti-IL-4 mAb	28	17	5



**Figure 6.** Intracellular synthesis of IFN- $\gamma$  and IL-4 by Th1- and Th2-type populations derived from naive CD4<sup>+</sup> T cells stimulated with antigen and APC in the presence of IL-12 or IL-4. Transgenic CD4<sup>+</sup> T cells ( $2.5 \times 10^5$  per well) prepared from DO11.10 mice were cultured in 2-ml volumes in 24-well plates with irradiated splenic APC ( $2.5 \times 10^6$  per well) and peptide (0.3  $\mu$ M). During primary stimulation, cultures contained the conditions as stated: no additions, IL-4 (200 U/ml), IL-12 (10 U/ml), anti-IL-4 mAb (10  $\mu$ g/ml), or IL-12 (10 U/ml) plus anti IL-4 mAb (10  $\mu$ g/ml). After 7 d (left-hand panels), cells in primary cultures were harvested, washed, and restimulated with PMA and ionomycin for 4 h with Brefeldin A added for the last 2 h. Cells were then fixed and stained for intracellular staining of IFN- $\gamma$  and IL-4 as described in Materials and Methods. Alternatively, cells were stimulated once per week for 3 wk under the above conditions, and then harvested, stimulated, and stained as described above (right-hand panels). Cell fluorescence was measured using a FACScan<sup>®</sup>, and quadrants were set according to the isotype-matched controls. Data was analyzed using Lysis II software, and they are displayed as bivariate dot plots. Percentages of cells in each quadrant (set according to the negative controls) are shown in the top right-hand corner of each panel. Levels of IFN- $\gamma$  or IL-4 (ng/ml) measured in 48-h supernatants are shown in the boxes contained within each panel.

be positive for IFN- $\gamma$  by flow cytometry. In contrast, stimulation of Th2 clones results in transient IL-4 synthesis so that only  $\sim 40\%$  of the cells can be shown to synthesize IL-4 at any one time. Although this could be interpreted as showing heterogeneity of cells within the population, we argue that this could alternatively be interpreted by transient synthesis of IL-4 by nonsynchronously cycling mammalian cells. Th0 cells often show relatively low numbers of cells expressing IFN- $\gamma$  or IL-4 exclusively, as well as low

numbers of cells producing both cytokines simultaneously. Interestingly, IFN- $\gamma$  appears as synthesis of IL-4 diminishes, suggesting that IFN- $\gamma$  synthesis may be regulated by IL-4. It is possible that different Th0 clones may vary in their relative levels of Th1 versus Th2 cytokines, and the respective level of either type will determine whether their role in the outcome of an immune response is to direct more of a cell-mediated-type or a humoral-type response. We show also that chronic stimulation of naive CD4<sup>+</sup> T cells in the presence of polarizing stimuli, can result in high frequencies of Th1- or Th2-type cells (producing exclusively IFN- $\gamma$  or IL-4, respectively) and a reduction in cells that do not produce either IFN- $\gamma$  or IL-4. The presence of

IL-4 together with IL-12 during repeated stimulation, however, also gives rise to cells synthesizing both IFN- $\gamma$  and IL-4. These data confirm at the single-cell level that IL-12 and IL-4 are strong polarizing stimuli for Th1 and Th2 development from naive CD4<sup>+</sup> T cells, and they demonstrate that a large number of cells in the population have the exclusive capacity to produce IFN- $\gamma$  or IL-4 after activation. It is possible that many chronic conditions may cause the emergence of polarized Th1- or Th2-type immune responses that are mutually exclusive, perhaps in part because of counterinhibitory effects of cytokines on the reciprocal Th subset.

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