In Vivo Priming of CD4 T Cells that Produce Interleukin (IL)-2 but not IL-4 or Interferon (IFN)-γ, and Can Subsequently Differentiate into IL-4– or IFN-γ–secreting Cells

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Abstract

The differentiation of antigen-stimulated naive CD4 T cells into T helper (Th)1 or Th2 effector cells can be prevented in vitro by transforming growth factor (TGF)-β and anti–interferon (IFN)-γ. These cells proliferate and synthesize interleukin (IL)-2 but not IFN-γ or IL-4, and can differentiate into either Th1 or Th2 cells. We have now used two-color Elispots to reveal substantial numbers of primed cells producing IL-2 but not IL-4 or IFN-γ during the Th1- or Th2-biased immune responses induced by soluble proteins or with adjuvants. These cells were $CD4^+CD44^{high}$ and were present during immediate and long-term immune responses of normal mice. Naive T cell receptor for antigen (TCR) transgenic (DO11.10) T cells were primed in vivo after adoptive transfer into normal hosts and FACS® cloned under conditions that did not allow further differentiation. After clonal proliferation, aliquots of each clone were cultured in Th1- or Th2-inducing conditions. Many in vivo–primed cells were uncommitted, secreting IL-2 but not IL-4 or IFN-γ at the first cloning step, but secreting either IL-4 or IFN-γ after differentiation in the appropriate conditions. These in vivo-primed, uncommitted, IL-2–producing cells may constitute an expanded pool of antigen-specific cells that provide extra flexibility for immune responses by differentiating into Th1 or Th2 phenotypes later during the same or subsequent immune responses.

Key words: cell differentiation • T lymphocyte subsets • mice • cytokine • immunization

Introduction

$CD4^+$ T cells regulate immune responses by producing various cytokines upon antigen stimulation. Naive $CD4^+$ T cells have limited cytokine responses and secrete only IL-2 before they differentiate into various effector cell types (1–4). In vitro, naive T cells stimulated with antigen in the presence of IL-4 differentiate into Th2 cells secreting IL-4, IL-5, IL-10, and IL-13, whereas IL-12 induces the differentiation of naive cells into Th1 cells secreting IL-2, IFN-γ, and lymphotoxin (5). The choice between Th1 and Th2 responses in vivo can determine the outcome of infections. Th1 cells assist in activating macrophages and $CD8^+$ T cells, which are particularly useful for eliminating intracellular infections; Th2 cells mediate antihelminth and allergic responses (6, 7).

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However, the existence of additional cytokine-secreting T cell effector phenotypes has been suggested by several studies using single-cell analysis methods, including intracellular staining (8–10), reverse transcription PCR (11), in situ hybridization (4), and two-color Elispot assays (12). Although the cytokines expressed by one cell on one occasion may not reflect the full cytokine secretion potential of the cell (4, 12), several lines of evidence confirm the existence of additional cytokine secretion patterns. Th0 clones secrete both Th1 and Th2 cytokines (2, 13–15). Regulatory T cells secreting TGF-β or IL-10 but not IL-4 have been derived from orally tolerized mice (16), or by in vitro culture of mouse and human T cells in the presence of IL-10 (17).

We have previously identified primed T cells producing IL-2 but not IL-4 or IFN-γ during in vitro studies in which TGF-β and anti–IFN-γ were added to prevent T cell differentiation into Th2 or Th1 cells, respectively (18, 19). Although these proliferating precursor T cells (called

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2001/10/1069/12 $5.00
Volume 194, Number 8, October 15, 2001 1069–1080
http://www.jem.org/cgi/content/full/194/8/1069

Thpp cells; reference 19) retained the cytokine secretion pattern of naive cells, they proliferated strongly in response to antigen. Cloning experiments showed that individual IL-2–secreting T cells retained the potential to differentiate into Th2 cells (20) or either Th1 or Th2 cells under appropriate conditions (18). A similar mouse T cell population primed in vitro by brief TCR stimulation secreted only IL-2, and preferentially migrated to LNs when transferred into mice (21). These cells acquired effector function more rapidly than naive T cells and had a lower activation threshold. Thus T cells previously activated by antigen in vitro can retain a cytokine secretion phenotype similar to that of naive cells, i.e., the secretion of IL-2 but not IL-4 or IFN-γ.

Primed cells producing mainly IL-2 may also be present in vivo in both mice and humans. Mouse memory T cells secreted high levels of IL-2 and low levels of IL-4 or IFN-γ when restimulated in vitro (22), and a higher frequency of cells from lymphoid tissues synthesized IL-2 compared with IFN-γ (23). Human T cells expressing high levels of the chemokine receptor CCR7 and low levels of CD45RA mostly expressed IL-2 but not IFN-γ, IL-4, or IL-5 when restimulated, in contrast to the high numbers of $CCR7^-CD45RA^-$ T cells expressing all four cytokines (24). The $CCR7^+CD45RA^-$ population (called central memory cells) included precursors of IFN-γ– and IL-4–secreting cells, although it was not determined whether precursors were uncommitted or separate precursors were present in the $CCR7^+$ population.

We have now investigated the cytokine secretion profiles and differentiation potential of individual mouse CD4 T cells after in vivo immunization. The frequency of each cytokine-secreting cell phenotype was determined immediately ex vivo by restimulating cells with antigen plus APCS and measuring cytokine production by one- and two-color Elispot. In either Th1-polarized or Th2-polarized responses, many antigen-specific cells detected by Elispot secreted IL-2 but not IL-4 or IFN-γ. Primed IL-2–secreting cells expressed high levels of CD44 and could be detected for at least 50 d. To analyze the differentiation potential of in vivo–primed CD4 T cells, TCR transgenic DO11.10 $C\alpha^{-/-}$ CD4 T cells were primed in vivo in an adoptive host and cloned by FACS®. Clone splitting showed that many cells that initially secreted only IL-2 could subsequently differentiate into either IFN-γ– or IL-4–producing cells, demonstrating that a significant number of CD4 T cells remain uncommitted when primed during an in vivo immune response.

Materials and Methods

Mice. Female C57BL/6 were obtained from The Jackson Laboratory. Female Balb/c mice, 6–8 wk old, were obtained from Taconic. Normal female Balb/c mice, 6–8 wk old, carrying the TCR transgene DO11.10, and homozygous for the TCR Cα deletion, were provided by Daniela Metz, University of Rochester, Rochester, NY.

Antibodies and Cell Lines. Purified rat anti–mouse IL-2 (JES6-1A12) antibodies and biotinylated rat anti–mouse IL-2 (JES6-5H4), IL-4 (BVD6-24G2), and IFN-γ (XMG1.2) antibodies were obtained from BD PharMingen. Rat anti–mouse IL-4 and IFN-γ antibodies were purified on a protein G column (Amersham Pharmacia Biotech) from the supernatants of 11B11 (25), XMG1.2 (26), and AN18 (27) hybridoma cell lines. The following PE-, cychrome-, FITC-, and biotin-conjugated antibodies were purchased from BD PharMingen: anti-CD25 (PC61), anti-CD4 (RM4-5), anti-CD44 (IM7), anti-NK1.1 (NKR-P1C), anti-CD45R/B220 (RA3-6B2), anti-CD8 (53-6.7), and anti-CD62L (Mel-14).

Immunization and Cell Preparation. C57BL/6 mice were immunized intraperitoneally with 2.5 or 80 μg KLH (Calbiochem) in 50 μl of PBS; or KLH (2.5 μg in 50 μl PBS) plus 50 μl Ribi adjuvant (Ribi ImmunoChem Research, Inc.). C57Bl/6 mice were also immunized subcutaneously in the neck with KLH (5 μg in 50 μl PBS) plus 50 μl CFA. After 7 or 10 d of immunization, cells from spleen, axillary LNs (ALNs)*, popliteal LNs, or mesenteric LNs (MLNs) were prepared for Elispot analysis. For enrichment of $CD4^+$ T cells, C57BL/6 spleen cells were incubated with streptavidin-Dynabeads (Dynal) coated with biotin-conjugated anti-NK1.1, -B220, and -CD8 antibodies. In some experiments, $CD4^+$ T cells were further purified by sorting on a cell sorter (EPICS Elite; Beckman Coulter) after staining the cells with rat anti–mouse CD4-cychrome, CD44-FITC, and CD25-PE. For $CD4^+$ T cell depletion, whole spleen cells were incubated with streptavidin-Dynabeads coated with biotin-conjugated rat anti–mouse CD4.

Elispot Assay. 96-well plates with a nitrocellulose filter base (Millititer HA; Millipore) were coated with purified anti–IL-2 (2 μg/ml), anti–IL-4 (2 μg/ml), or anti–IFN-γ (2 μg/ml) antibodies. After 2 h of incubation at room temperature, the plates were washed three times with RPMI 1640 medium containing 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 8% FCS (Hyclone Laboratories Ltd. Hornby). Unseparated spleen cells from immunized mice were added to the plates in doubling dilutions from 5×10^5 cells per well in the presence of 80 μg/ml KLH (or as specified in Figure legends) or 5 μg/ml ConA (Amersham Pharmacia Biotech) for restimulation. Spleen cells from a pool of 2–3 normal mice were added to provide additional APCs, so that all wells contained 5×10^5 total spleen cells. For experiments using sorted T cell populations, the first wells in the dilution series contained $1–7 \times 10^4$ sorted cells, and all wells contained 5×10^5 spleen cells from unimmunized mice as APCs. After overnight incubation in a 37°C CO_2 incubator, the plates were washed with PBS with 0.1% Tween (Sigma-Aldrich), and then incubated with biotinylated anti–IL-2 (2 μg/ml), IL-4 (2 μg/ml), or IFN-γ (2 μg/ml) antibodies in PBS plus 0.1% Tween 20 plus 2% BSA (PBSTB), followed by streptavidin-horseradish peroxidase conjugate (SA-HRP; Jackson ImmunoResearch Laboratories) at 1:1,000 in PBSTB, and the AEC substrate kit (Vector Laboratories). The plates were then dried and the filters were removed from the plastic plate by adhesion to thermowell sealers (Costar). The red spots were enumerated using a dissecting microscope. In some experiments (e.g., see Fig. 4), the accuracy of the counting was confirmed by blind counting by another person, and by automated computer counting (Zellnet Consulting, Inc.). Identical conclusions were obtained in each case from all counting methods.

For two-color Elispot assays, the plates were coated with purified anti–IL-2, anti–IL-4, and/or anti–IFN-γ antibodies. Cell

**Abbreviations used in this paper:* ALN, axillary LN; LDA, limiting dilution analysis; MLN, mesenteric LN.

culture was set up as before. After overnight incubation, biotinylated anti–IL-2 antibodies were first added to the plates followed by streptavidin-alkaline phosphatase (SA-AP; Jackson ImmunoResearch Laboratories). The plates were then incubated with RPMI 1640 as a source of free biotin (10 μg/ml) to block the free binding sites of the streptavidin. After 30-min incubation at room temperature, biotinylated anti–IL-4 and/or anti–IFN-γ antibodies were added to the plates followed by SA-HRP. The plates were then incubated with the peroxidase substrate AEC and the phosphatase substrate BCIP/NBT (Biomeda Corp.) sequentially to develop the red and blue color spots. In some experiments, IL-2–producing cells were detected as red spots and IL-4– and/or IFN-γ–producing cells were detected as blue spots.

Elispot Calculations. In two-color Elispot assays, the blue color was dominant, and so in some experiments it was difficult to distinguish blue spots from blue-red spots. Red spots could be counted directly, but comparison of multiple calculations was necessary to reliably enumerate blue and blue-red spots.

In experiments using both single and two-color Elispot assays, the spots were enumerated in the following categories: single-color assays: Bs, Blue (IL-2); Rs, Red (IL-4); and double-color assay: Bd, Blue (IL-2); Rd, Red (IL-4); BR, Blue-Red (IL-2 plus IL-4). From these properties, the number of cells secreting IL-2 and/or IL-4 were calculated as follows: cells secreting IL-4 but not IL-2: Rd; cells secreting IL-2 and IL-4: BR or Rs-Rd; and cells secreting IL-2 but not IL-4: Bd or Bs-(Rs-Rd) or Bs-BR.

Limiting Dilution Cloning. Spleen cells from mice immunized 7 d previously with KLH plus Ribi were centrifuged in Lympholyte-M (Cedarlane Laboratories) to remove RBCs. Lymphocytes were stained with cychrome-anti-CD4 (20 ng/million lymphocytes) and FITC-anti-CD44 (50 ng/million lymphocytes). $CD4^+CD44^{high}$ cells were sorted on a FACS Vantage™ SE sorter (BD PharMingen). Sorted cells were titrated from 2,000 to 0 cells per well and were put into 96-well plate in the following conditions: 2 ng/ml IL-2 (28), 2 ng/ml IL-7 (BD PharMingen), 3% MLR supernatant, 100 μg/ml KLH, 40 μg/ml XMG1.2, 10 μg/ml 11B11, 1 ng/ml TGF-β (GIBCO BRL), and 4×10^5 irradiated whole spleen cells. After 6 d, positive growing wells were picked and split into Th1 conditions with IL-2, IL-7, KLH, IL-12 (0.1% Cos transfection supernatant), and 11B11, or Th2 conditions with IL-2, IL-7, KLH, IL-4 (a gift from Schering Plough, 2 ng/ml), and XMG1.2 for another 4 d. Cells were then washed and rested in medium with only 1 ng/ml IL-2. 2 d later, they were restimulated with 100 μg/ml KLH and APCs (5×10^5 cells per well) in Elispot assay to measure IL-2–, IL-4–, and IFN-γ–producing cell frequencies.

Single Cell Cloning and Split Culturing of In Vivo–primed DO11.10 $C\alpha^{-/-}$CD4 T Cells. Cells from LNs and spleens of naive DO11.10 $C\alpha^{-/-}$ mice were adoptively transferred by intraperitoneal injection of 10^7 lymphocytes into naive Balb/c mice. 1 d later the host mice were immunized subcutaneously in the neck with 25 μg of OVA $peptide_{323–339}$ (P_{OVA}, provided by Daniela Metz) in 50 μl PBS plus 50 μl Ribi adjuvant. 6 d later, ALN cells were stained with cychrome-anti-CD4, biotinylated-KJ1-26 (provided by Daniela Metz), and PE-anti-CD62L antibodies. Single $CD4^+$KJ1-26^+CD62L^{low} lymphocytes were deposited into each well of 96-well plates with the Autoclone attachment on the Coulter Epics Elite FACS®. Sorted cells were stimulated with peptide (2 μg/ml) and APCs (TA3 cells, 10^4 cells per well) in the presence of 1 ng/ml human IL-2 (Chiron), mouse IL-7, 40 μg/ml 11B11, 50 μg/ml XMG1.2, and 1 ng/ml TGF-β. After 7–10 d of culture, the growing clones were split into two aliquots which were restimulated with peptide, APCs, human IL-2, and mouse IL-7 in either Th1 conditions (IL-12 and 11B11) or Th2 conditions (IL-4 and XMG1.2).

Results

KLH Immunization Induced IL-2 and IL-4 Responses. We previously defined in vitro a population of $CD4^+$ T cells that proliferate in the presence of TGF-β and anti–IFN-γ antibody without differentiating into Th2 or Th1 subsets. These cells secrete IL-2 but not IL-4, IL-5, or IFN-γ when restimulated, and have the potential to further differentiate into Th1 and Th2 subsets under appropriate polarizing conditions (18). To look for in vivo evidence of these primed IL-2–producing cells, we immunized mice with a small amount of KLH, without adjuvant. 7 d after in vivo immunization, spleen cells were stimulated directly ex vivo with antigen plus APCs, and the numbers of cells secreting IL-2, IL-4, or IFN-γ were measured by Elispot. Substantial numbers of well-defined IL-2 Elispots were produced by cells from mice immunized with KLH (Fig. 1 a and d) but not from unimmunized mice. The in vitro response was KLH-specific (e.g., Fig. 1 d vs. g).

In addition to the IL-2 Elispots, KLH-specific IL-4 Elispots were also detected, but at a lower frequency (Fig. 1 b, e, and h). However, no KLH-specific IFN-γ–secreting cells were detected (Fig. 1 c and f), although ConA induced high numbers of IFN-γ–secreting cells in the same experiment (Fig. 1 i), confirming that the IFN-γ Elispot assay was effective. Thus, immunization of mice with a small amount of KLH resulted in a population of antigen-specific T cells that secreted IL-2 and/or IL-4, but not IFN-γ when restimulated in vitro. The primed, IL-2–secreting cells were unlikely to be Th1 cells because there was no KLH-specific IFN-γ response, and the higher number of IL-2–secreting cells suggested that at least some primed IL-2–secreting cells did not secrete IL-4.

Many KLH-specific Spleen Cells Secreted IL-2 but not IL-4 when Restimulated. To demonstrate directly that some cells secreted IL-2 but not IL-4, we modified a two-color Elispot method to simultaneously identify IL-2 and IL-4 producers. Spleen cells from mice immunized 7 d previously with KLH were restimulated in vitro with antigen and analyzed by cytokine Elispot for cells secreting IL-2 (blue) and/or IL-4 (red). Three Elispot assays were carried out for each sample, a single-color IL-2 assay and a single-color IL-4 assay, and a double-color assay. In the double-color assay, cells secreting both IL-2 and IL-4 should give blue-red spots. Due to the strong color of the blue spots, it was not always easy to distinguish between blue and blue-red spots, and so multiple methods were used (see Materials and Methods) to calculate the numbers of cells secreting only IL-2, only IL-4, or IL-2 plus IL-4 (Fig. 2).

Additional experiments were carried out with the colors reversed, i.e., red IL-2 spots and blue IL-4 spots (results not shown). In other experiments, three single-color Elispot assays were performed on each sample with anti–IL-2, anti–IL-4, or combined anti–IL-2 and anti–IL-4 antibodies (results not shown). By comparing the individual assays with

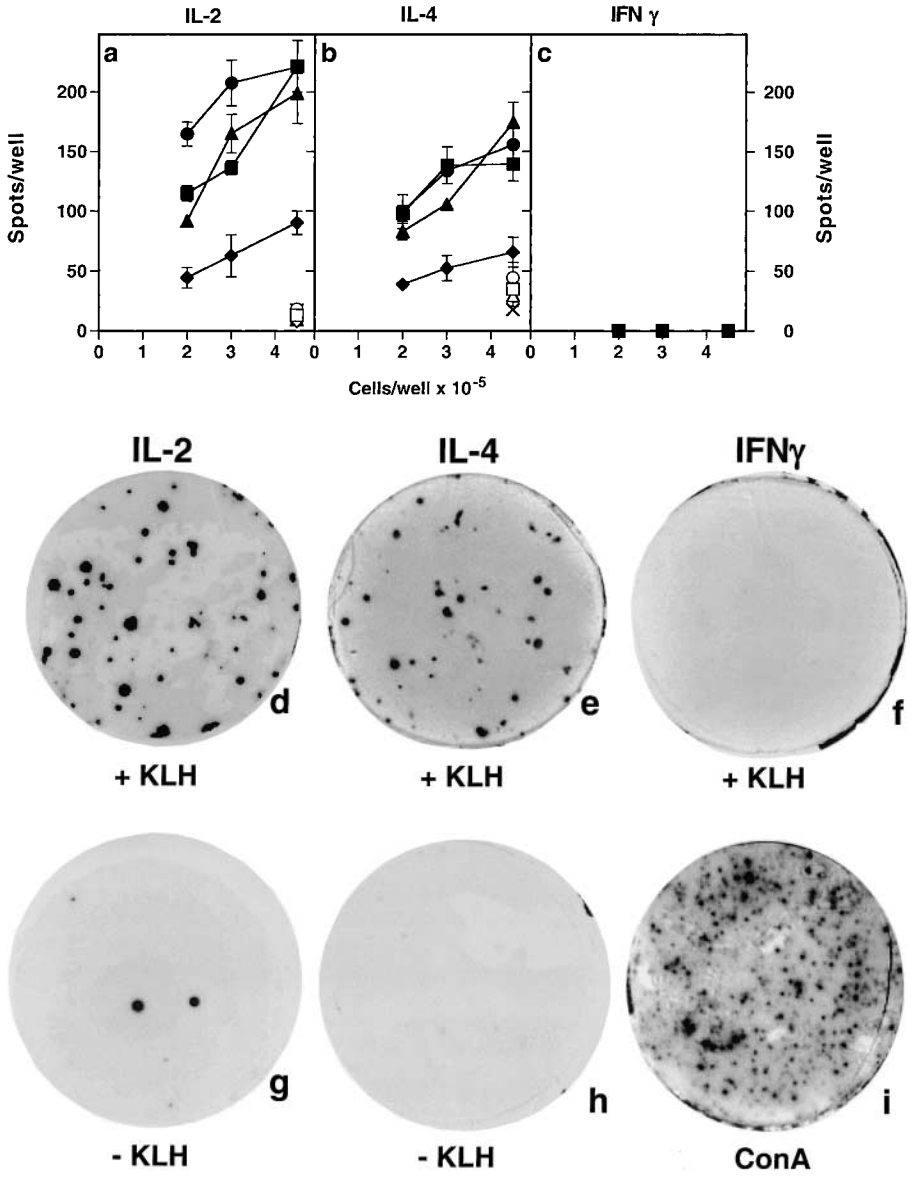

Figure 1. KLH immunization induced IL-2 and IL-4, but not IFN-γ responses. Mice were immunized intraperitoneally with 2.5 μg KLH. 7 d later, spleen cells were restimulated with 80 μg KLH in the Elispot assay. Frequencies of cells secreting IL-2, IL-4, and IFN-γ are expressed as spot numbers per well (a–c). Solid and open symbols represent cells with and without restimulation, respectively. Each symbol represents one of four immunized mice, and X represents a pool of unimmunized mice challenged in vitro. All assays were performed in triplicate, and results are expressed as mean ± SD. IL-2, IL-4, and IFN-γ Elispots of immunized mice (5×10^5 cells per well) with (d–f) or without (g and h) KLH restimulation are shown. IFN-γ Elispots from immunized mice (5×10^5 cells per well) stimulated with ConA are shown in i.

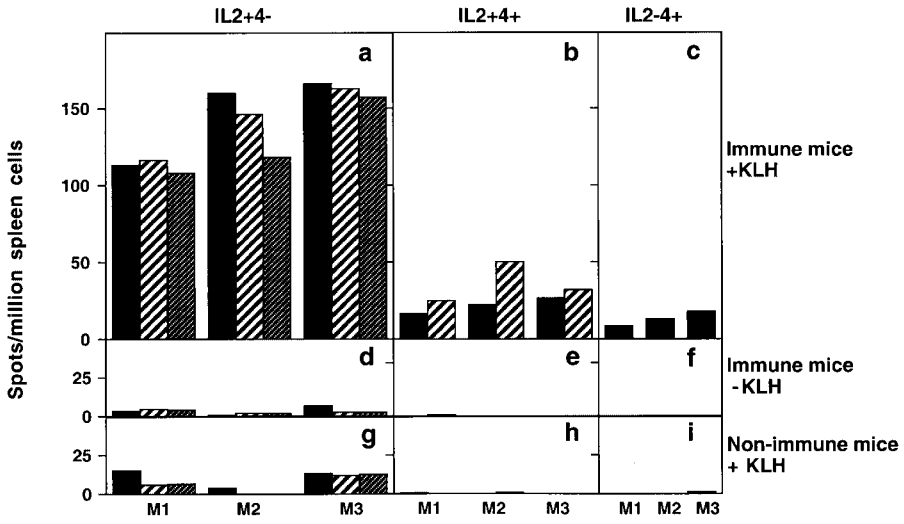

Figure 2. Many KLH-specific spleen cells secrete IL-2 but not IL-4 when restimulated. Mice were immunized intraperitoneally with 2.5 μg KLH. 7 d later, spleen cells were stimulated in Elispot assays with (a–c) or without (d–f) 80 μg/ml KLH. Spleen cells from unimmunized mice were also stimulated with KLH (g–i). Single- and double-color IL-2 and IL-4 Elispot assays were used to determine the frequencies of cells secreting IL-2, IL-4, or both IL-2 and IL-4. M1, M2, and M3 represent three different mice. Frequencies (spots per million spleen cells) were calculated as described in Materials and Methods. (a) Cells secreting only IL-2. Bd, solid bars; Bs-(Rs-Rd), hatched bars; and Bs-BR, stippled bars. (b) Cells secreting both IL-2 and IL-4. BR, solid bars; and Rs-Rd, hatched bars. (c) Cells secreting only IL-4. Rd, solid bars.

the combined assay, the number of single- and double-producing cells could be estimated.

All experiments and calculation methods resulted in the same conclusions. Large numbers of cells produced IL-2 but not IL-4 or IFN-γ, and much smaller numbers of cells secreted either IL-4 or IL-2 plus IL-4. Both IL-2 and IL-4 responses were dependent on immunization with KLH in vivo and restimulation with KLH in vitro. Cells secreting both IL-4 and IL-2, but not IFN-γ, were detected at low frequencies in this and other experiments (e.g., Fig. 3 bottom). The lack of IFN-γ secretion suggests that these were not Th0 cells (2, 15), but could instead be related to previously described allospecific T cell clones secreting IL-2, IL-4, and IL-5 but not IFN-γ (2).

Thus by different calculation methods in the first two-color assay, direct observation of the pure red IL-2 spots in the second two-color assay, or by the combined results of single-color assays, we demonstrated that most of the primed KLH-specific cells secreted IL-2 but not IL-4 or IFN-γ.

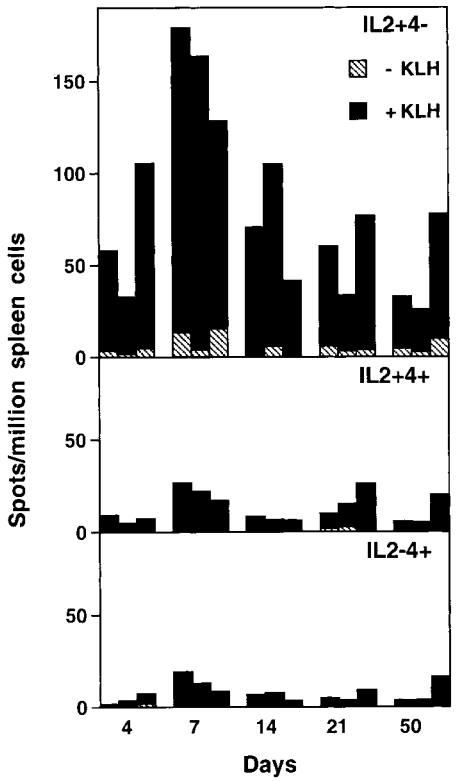

Figure 3. KLH-specific T cells secreting IL-2 but not IL-4 were present for up to 50 d after a single immunization. Spleen cells taken from mice 4, 7, 14, 21, and 50 d after immunization (2.5 μg/mouse, intraperitoneal) were incubated with or without KLH (80 μg/ml) in one- and two-color Elispot assays. IL-2^+IL-4^- cells were determined by Bs-(Rs-Rd); IL-2^+IL-4^+ cells were determined by Rs-Rd; IL-2-IL-4^+ cells were determined by Rd (see Materials and Methods). At each time point, data from each individual mouse is shown. Elispot numbers from unimmunized mice in the presence of KLH stimulation were 3.1, 0.4, and 0 spots per million spleen cells for IL-2^+IL-4^-, IL-2^+IL-4^+, and IL-2^-IL-4^+ cells, respectively.

KLH-specific T Cells Secreting IL-2 but not IL-4 Were Present for up to 50 d after a Single Immunization. As IL-2–secreting CD4 T cells can be precursors of Th1 and Th2 cells (18, 19), we considered the possibility that cells producing only IL-2 were an intermediate stage during CD4 T cell differentiation, and that the response would eventually resolve into Th2 and/or Th1 cells. To study the kinetics of the IL-2–secreting T cell response, we immunized mice with 2.5 μg KLH and carried out two-color Elispot assays 4, 7, 14, 21, or 50 d later to determine IL-2– and IL-4–producing cells. Both IL-2 and IL-4 responses peaked 7 d after immunization (Fig. 3). At all time points, from days 4–50, the majority of KLH-specific cytokine-secreting cells secreted IL-2 but not IL-4 or IFN-γ. In this experiment, the cells secreting only IL-2 made up 75–90% of the total response, and in additional experiments this number ranged from ~50–90%. The number of double-producing (IL-2^+IL-4^+IFN-γ^-) cells was measured indirectly and is therefore less reliable than the numbers for the other two populations. However, low numbers of IL-2^+ IL-4^+ IFN-γ^- cells were consistently detected. Cells producing IL-4 but not IL-2 or IFN-γ were also detected at times up to 50 d. These may represent long-lived, fully differentiated Th2 cells. No significant antigen-specific IFN-γ response was detected (less than two IFN-γ–secreting cells per million spleen cells at all time points). Thus, the cells secreting only IL-2 persist for at least 7 wk after immunization and so do not appear to be transient intermediates in T cell differentiation.

IL-2–secreting Cells Were Induced by Different Doses of Antigen. Antigen dosage may influence T cell activation and differentiation by providing different signaling strength through the T cell receptor (29). To test whether antigen dosage influenced the induction of IL-2–producing cells, we measured the frequency of IL-2–producing cells in mice immunized with 2.5 or 80 μg KLH. 7 d after immunization, pooled axillary and popliteal lymphocytes were restimulated in Elispot assays with 2.5 or 80 μg/ml KLH. Although the frequency of IL-2– or IL-4–producing cells increased as either the in vivo or in vitro KLH amount increased, the ratio of IL-2– to IL-4–producing cells remained fairly constant (Fig. 4). More directly, two-color Elispot assays showed cells producing IL-2 but not IL-4 or IFN-γ (represented by pure red spots) constituted the majority of the IL-2 response in all four combinations of in vivo and in vitro antigen amounts (Fig. 4).

Ribi Adjuvant Increased the Bias towards Cells Secreting IL-2 but not IL-4. To test the possibility that immunization with KLH alone may have caused weak responses that were insufficient to induce T cell differentiation into Th1 or Th2 phenotypes, alternative immunization methods were used. Immunization with KLH-pulsed dendritic cells also induced KLH-specific cells secreting IL-2 or IL-4 but not IFN-γ (results not shown). Mice were immunized with KLH plus Ribi adjuvant, with the expectation that this would induce more T cell differentiation into effector cells, particularly Th2 cells, because Ribi adjuvant induces stronger antibody responses. Addition of Ribi adjuvant to

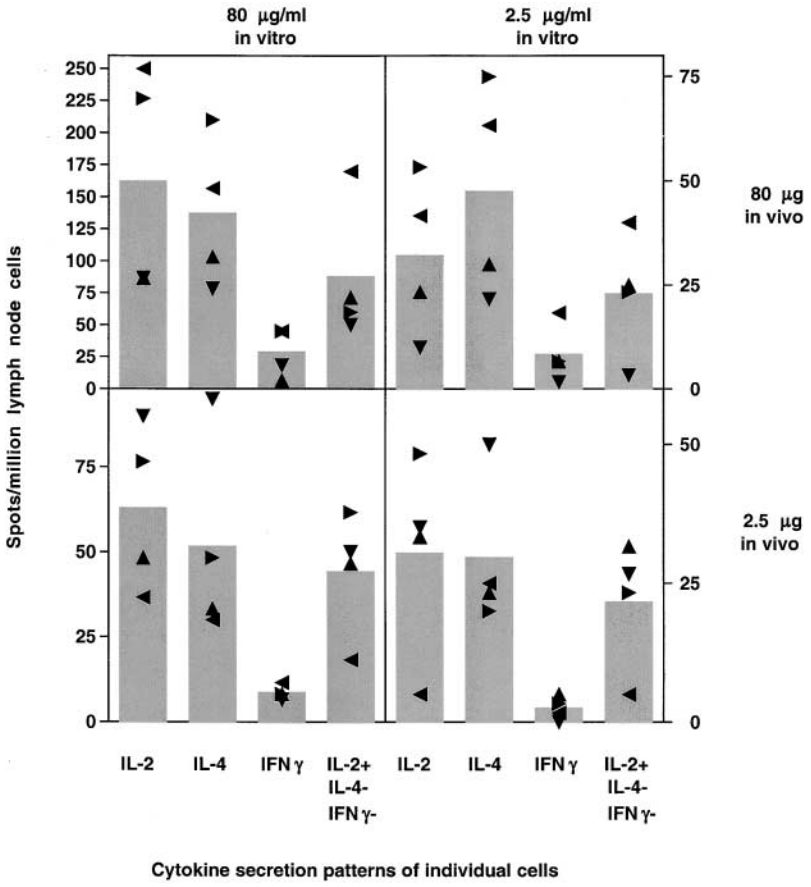

Figure 4. IL-2–secreting cells were induced by different doses of antigen. Mice were immunized intraperitoneally with 2.5 μg or 80 μg KLH. After 7 d, cells from ALNs and popliteal LNs were isolated and restimulated in vitro with 2.5 or 80 μg/ml KLH in one- and two-color Elispot assays. Frequencies of cells secreting IL-2, IL-4, and IFN-γ (one-color assays) or IL-2 but neither IL-4 nor IFN-γ (two-color assay) are expressed as spot numbers per million cells. The bars represent the average spot number of four mice (each represented by a different triangle) in each immunization group. Average background Elispot frequencies without in vitro antigen stimulation were 0.8 (IL-2), 7.9 (IL-4), and 3.3 (IFN-γ) per million cells.

the KLH immunization slightly increased the numbers of IL-2–secreting cells (Fig. 5), but surprisingly, the adjuvant substantially reduced the numbers of IL-4–secreting cells. Low numbers of IFN-γ–secreting cells were detected in only one mouse. Thus Ribi adjuvant increased the proportion of cells secreting IL-2 but not IL-4 or IFN-γ, showing that these cells can be the major antigen-specific population even during a strong immune response.

Cells Producing only IL-2 Were Detected during Th1-like Responses. Soluble KLH induced the production of cells secreting IL-2 or IL-4, but not IFN-γ, suggesting a partially Th2-biased response. We tested whether similar primed IL-2–producing cells were produced during a Th1-biased response by immunizing mice subcutaneously in the neck with KLH in CFA. After 10 d, cells from the draining ALNs, nondraining MLNs, and spleen were analyzed by Elispot. Significant numbers of KLH-specific IFN-γ–producing cells were detected in spleens and ALNs of immunized mice, indicating that a Th1-biased response was induced by this immunization regimen (Fig. 6). IL-2–secreting cells were present in all three sites, and IL-4–secreting cells were present mainly in the spleen. To directly show the presence of cells producing IL-2 in a mixed response that included both IL-4 and IFN-γ production, we performed two-color Elispot assays, in which IL-2–producing cells were identified as red spots and both IL-4 and IFN-γ–producing cells were identified as blue spots. KLH-specific cells producing IL-2 but not IL-4 or IFN-γ were present in ALNs, MLNs, and spleen, and constituted the great majority of KLH-specific cells in nondraining MLNs (Fig. 6). Similar IL-2–secreting T cells were also found in immune responses against the mycobacterial antigen preparation partially puri-

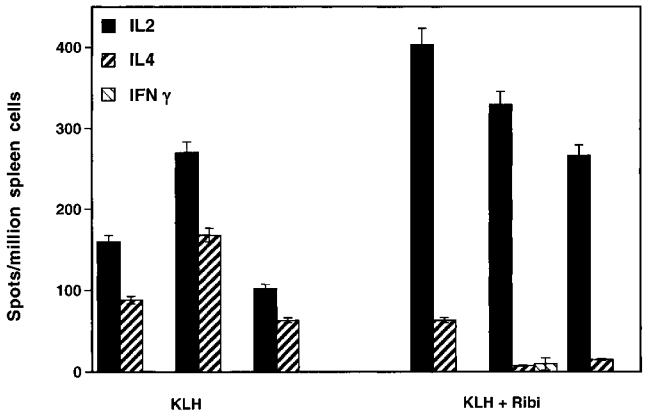

Figure 5. Ribi adjuvant biased the immune response towards IL-2^+4^- cells. Mice were immunized intraperitoneally with either 2.5 μg KLH alone or 2.5 μg KLH plus Ribi adjuvant. 7 d later, spleen cells from three individual mice for each immunization were stimulated with 80 μg/ml KLH in IL-2, IL-4, and IFN-γ Elispot assays. Each group of solid, striped, and hatched bars represents an individual mouse. Background values (spots in the absence of antigen) were <23 spots per million spleen cells and have been subtracted from each value.

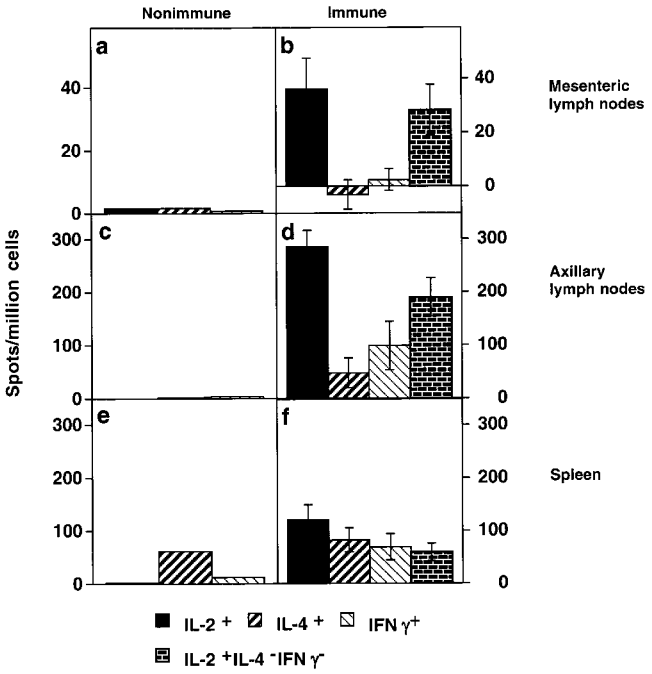

Figure 6. IL-2–producing cells were detected in Th1 responses. Mice were immunized with 5 μg KLH plus CFA subcutaneously in the neck. After 10 d, (a and b) MLNs (2×10^5 cells per well), (c and d) ALNs (2×10^5cells per well), and (e and f) spleen cells (4×10^5 per well) from one unimmunized (a, c, and e) and five immunized (b, d, and f) mice were stimulated with 80 μg/ml KLH in Elispot assays. Frequencies of cells producing IL-2, IL-4, and IFN-γ were determined in single-color Elispot assays. Cells producing IL-2 but not IL-4 or IFN-γ were represented by the frequencies of pure red spots determined in the two-color Elispot assays, in which IL-2–producing cells were detected as red spots and both IL-4– and IFN-γ–producing cells were detected as blue spots.

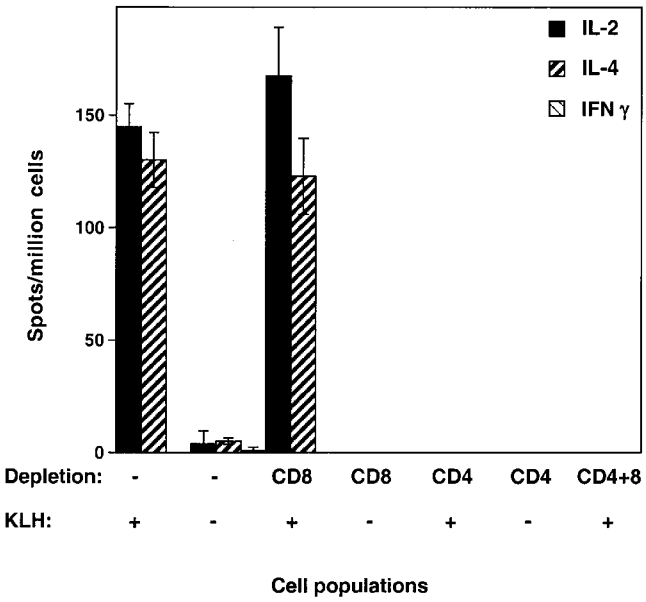

Figure 7. CD4 T cells were responsible for the KLH-specific IL-2 and IL-4 responses. Mice were immunized intraperitoneally and/or $CD8^+$ T cells were restimulated with or without 80 μg/ml KLH in IL-2, IL-4, and IFN-γ Elispot assays. Background Elispot values from unimmunized mice with KLH stimulation ranged from 2–10 spots per million spleen cells.

fied derivative of *Mycobacterium tuberculosis* (results not shown). Thus T cells secreting IL-2 but not IFN-γ were found in responses with a significant Th1 component.

$CD4^+$ T Cells Were Responsible for the KLH-specific IL-2 and IL-4 Responses. Although IL-2 production in response to exogenous protein immunization is normally due to CD4 cells, it is also possible to prime CD8 T cells to exogenous protein antigens (30), and CD8 T cells can also secrete IL-2 at moderate levels. To determine the type of cells producing IL-2 in our experiments, spleen cells from KLH-immunized mice were depleted of $CD4^+$ or $CD8^+$ T cells by magnetic bead separations. KLH-specific IL-2- and IL-4-producing cells were detected in unseparated and CD8-depleted spleen cells, but not in either CD4-depleted or CD4/CD8-depleted cells (Fig. 7). Thus $CD4^+$ but not $CD8^+$ cells were required for the IL-2–secreting cell response. Sorting of $CD4^+$ T cells (e.g., Fig. 8) directly demonstrated that the IL-2–producing cells were $CD4^+$ cells.

The IL-2–producing Cells Expressed High Levels of CD44 but not CD25. During an immune response, naive antigen-specific $CD4^+$ T cells are activated and induced to differentiate into effectors. Various cell surface markers distinguish naive from effector and memory cells. CD44 expression is low on naive T cells and high on activated and memory T cells (31, 32). CD25 (IL-2Rα) is expressed on T cells only transiently after antigen activation (33, 34). Because the KLH-specific IL-2–producing cells were primed T cells, we tested whether they had a recently activated/memory cell phenotype. 7 d after KLH immunization, spleen cells were depleted of $CD8^+$, $B220^+$, and $NK1.1^+$ cells, and sorted into three $CD4^+$ populations expressing low, medium, or high levels of CD44. The highest frequency of IL-2– or IL-4–secreting cells was found in the $CD4^+CD44^{hi}$ population (Fig. 8 a). There were nearly 10,000 IL-2–secreting cells per million $CD4^+CD44^{hi}$ cells, and as this cell population was 5.0% of the unsorted, CD4-enriched population, this corresponds to 500 IL-2–secreting cells per million unsorted cells, i.e., ~50% recovery of the activity in the original cells (Fig. 8 b). Negligible numbers of KLH-specific cells secreting IL-2 or IL-4 were found in the $CD44^{med}$ or $CD44^{low}$ populations. In other experiments, CD44 expression was also high on IL-2–secreting cells taken 6, 8 d, or 3 mo after immunization with KLH, or 7 d after immunization with KLH in Ribi adjuvant (results not shown).

To study CD25 expression, spleen cells from KLH immunized mice were depleted of $CD8^+$, $B220^+$, and $NK1.1^+$ cells using magnetic beads, and sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations. 5 d after immunization, IL-2–secreting cells were detected at a higher frequency in the $CD4^+CD25^+$ population than the $CD4^+CD25^-$ population (Fig. 9 a). However, $CD25^+$ cells constituted only 9.2% of the $CD4^+$ population, and so if the Elispot numbers are expressed as a fraction of the unsorted cells (Fig. 9 b) the $CD4^+CD25^+$ cells accounted for

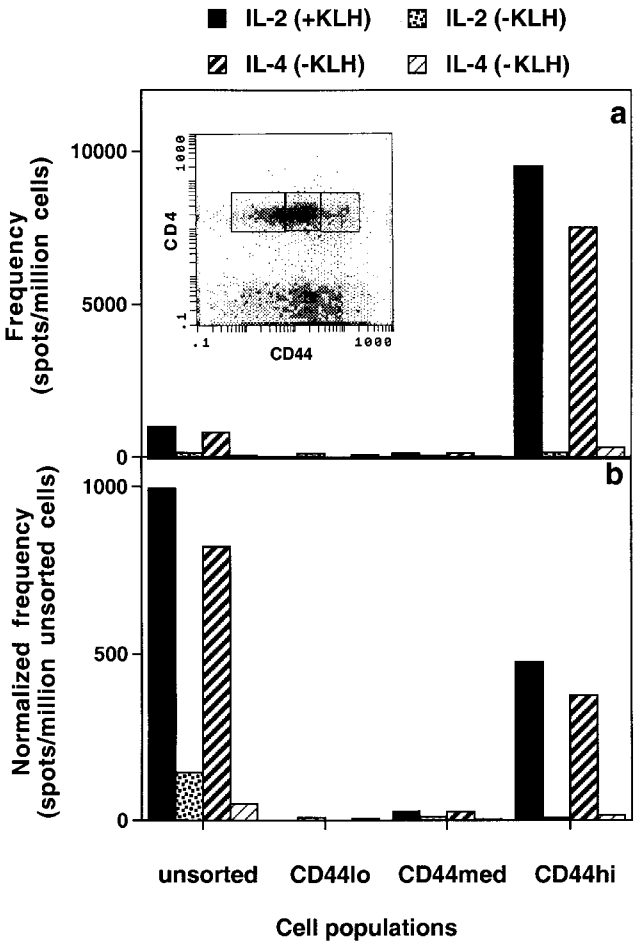

Figure 8. The IL-2–producing cells express high levels of CD44. Mice were immunized intraperitoneally with 2.5 μg KLH for 7 d. Spleen cells were depleted of cells expressing CD8, B220, or NK1.1. The remaining cells were stained with cychrome-anti-CD4, FITC-anti-CD44, and PE-anti-CD25 and sorted into $CD4^+CD25^-$ populations expressing high, medium, or low levels of CD44 (insert), representing 5.0, 19.4, and 7.5% of the unsorted cells, respectively. The sorted cells were stimulated with 80 μg/ml KLH in Elispot assays. Frequencies of KLH-specific IL-2, IL-4, and IFN-γ–secreting cells are expressed as spots per million cells (a) or per million unsorted cells (b). The background from unimmunized mice in the presence of KLH was 13 IL-2– and 7 IL-4–secreting cells per million spleen cells.

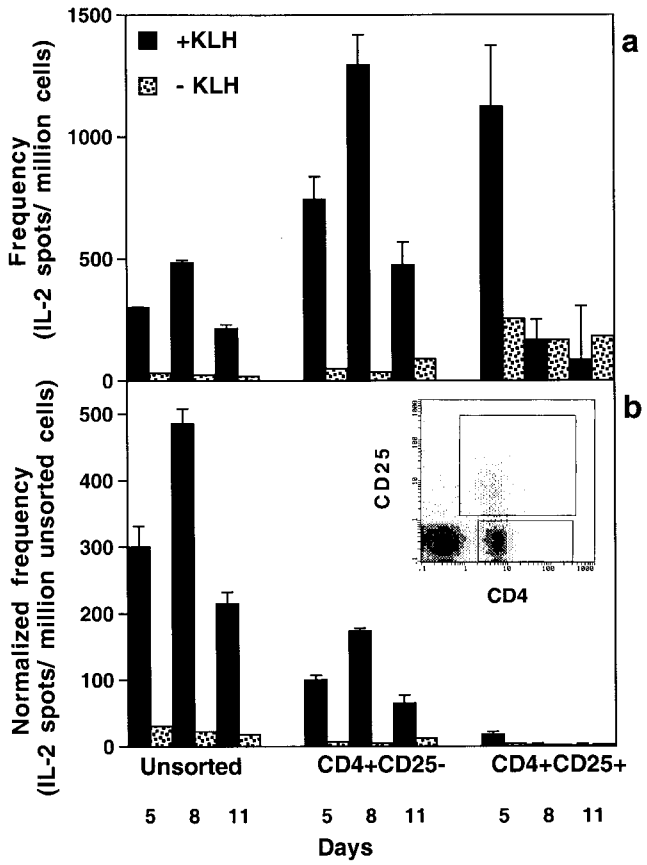

Figure 9. CD25 expression on cells secreting IL-2. Mice were immunized intraperitoneally with 2.5 μg KLH for 5, 8, or 11 d. Spleen cells were depleted of $CD8^+$, $B220^+$, and $NK1.1^+$ cells, stained with cychrome-anti-CD4 and PE-anti-CD25, and sorted into two populations: $CD4^+CD25^+$ and $CD4^+CD25^-$ (insert). The sorted cells were stimulated with 80 μg/ml KLH in Elispot assays. The frequencies of KLH-specific IL-2–producing cells are expressed as spots per million cells (a) or per million unsorted cells (b). The background from unimmunized mice in the presence of KLH was 1.7 IL-2–secreting cells per million spleen cells.

only a small part of the response. After 8 or 11 d, the IL-2–secreting cells were almost entirely $CD25^-$ consistent with other studies (33, 34).

In Vivo–primed CD4 T Cells Can Differentiate In Vitro into IL-4– or IFN-γ–producing Cells. The ability of in vitro–derived IL-2–producing cells to differentiate into either Th1 or Th2 cells suggested that if the in vivo–primed IL-2–producing cells represent a similar phenotype, they might also be uncommitted precursors of Th1 and Th2 cells. Therefore, we tested the differentiation potential of the in vivo–primed IL-2–producing cells by split cloning. $CD4^+CD44^{high}$ spleen cells from mice immunized with KLH plus Ribi were sorted and stimulated with KLH and irradiated syngenic spleen cells in limiting dilution analysis (LDA) conditions that included anti–IFN-γ, anti–IL-4, and TGF-β to prevent further differentiation. After 5 d, the wells with growing cells were tested for cytokine secretion in response to KLH by Elispot. Each well that was >90–95% likely to be clonal (according to the Poisson equation), and contained cells secreting mainly IL-2, was split into two aliquots that were restimulated with KLH in either Th1 or Th2 differentiation conditions. After a further 5 d, the differentiation status of the daughter cells from each split well was determined in IL-2, IL-4, and IFN-γ Elispot assays. A few clones that secreted IL-2 but not IL-4 or IFN-γ after the primary LDA cloning retained their KLH specificity and became IFN-γ–producing cells in Th1 conditions and IL-4–producing cells in Th2 conditions (Fig. 10). This suggests that some in vivo–primed KLH-specific cells were uncommitted, i.e., they did not produce IL-4 or IFN-γ after the first cycle of growth in vitro and could differentiate into either Th1- or Th2-like cells in the second cycle.

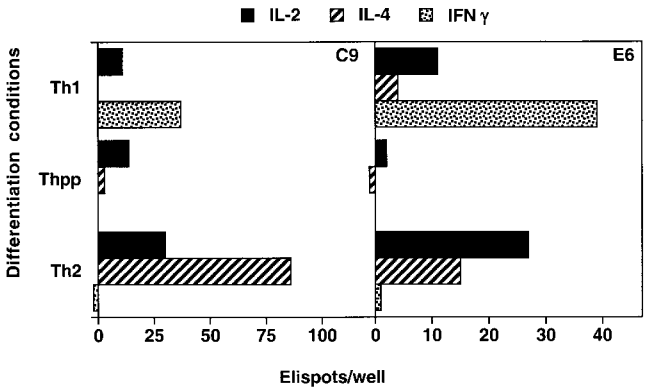

Figure 10. Uncommitted KLH-specific IL-2–producing cells revealed by LDA. C57Bl/6 mice were immunized intraperitoneally with 2.5 μg KLH plus Ribi adjuvant. 7 d later, spleen cells were stained with cychrome-anti-CD4, FITC-anti-CD44, PE-anti-CD8, and PE-anti B220. $CD4^+CD44^{high}CD8^-B220^-$ cells were sorted and stimulated with 100 μg/ml KLH and APCs in LDA plates, in the presence of anti–IFN-γ, anti–IL-4, TGF-β, IL-7, and IL-2. After 6 d, positive growing wells were picked and restimulated with KLH in Th1 conditions with IL-2, IL-7, IL-12, and 11B11, or Th2 conditions with IL-2, IL-7, IL-4, and XMG1.2 for another 4 d. Cells were then washed and rested in medium with only IL-2. 2 d later, they were restimulated with KLH in an Elispot assay to measure the frequencies of IL-2–, IL-4–, and IFN-γ–producing cells. The probability that each well initially contained a single KLH-specific T cell was 90% (well C9) and 95% (well E6).

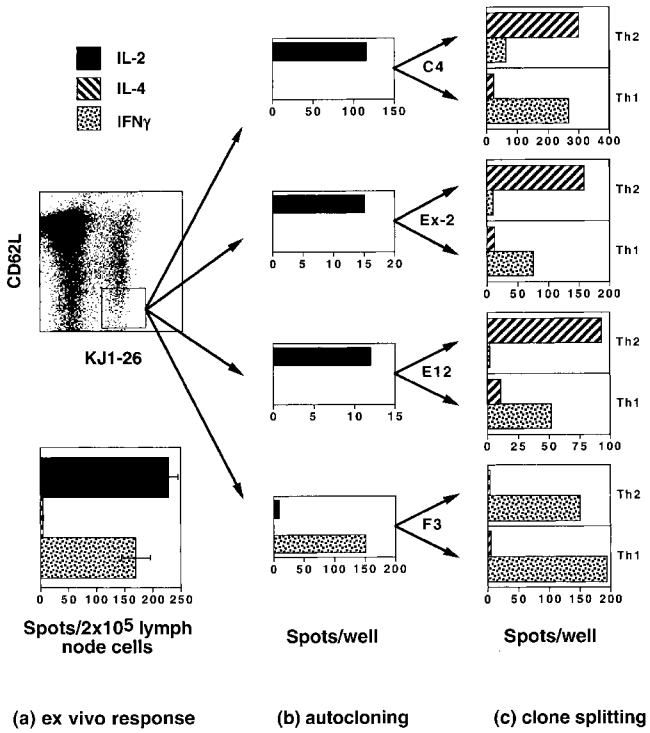

Figure 11. In vivo–primed DO11.10 cells secreting only IL-2 can differentiate into IL-4 and IFN-γ–producing cells. Balb/c mice were immunized with 25 μg OVA peptide plus Ribi adjuvant subcutaneously in the neck. 6 d later, cells from the ALNs were restimulated with 2 μg/ml OVA peptide in IL-2, IL-4, and IFN-γ Elispot assays (a). The cells were also stained with cychrome-anti-CD4, biotinylated-KJ1-26, PE-anti-CD62L, and FITC-streptavidin for autoclone sorting. Single $CD4^+CD62^{low}KJ1\text{-}26^+$ cells were stimulated with 2 μg/ml OVA peptide and 10^4 TA3 cells in the presence of IL-2, IL-7, anti–IFN-γ, and anti–IL-4. 7 d later, IL-2–, IL-4–, and IFN-γ–producing cell frequencies were determined in Elispot assays by stimulating with 2 μg/ml OVA peptide and 4×10^5 Balb/c whole spleen cells per well (b). Aliquots of cells from each growing clone were then restimulated in Th1 or Th2 conditions with 2 μg/ml OVA peptide and 10^4 TA3 cells for 5 d, followed by IL-4 and IFN-γ Elispot assays of the split clones (c).

Many TCR Transgenic Cells Primed In Vivo Are Uncommitted and Can Differentiate into either IL-4– or IFN-γ–producing Cells. Although the split-cloning LDA experiments demonstrated that IL-4– or IFN-γ–producing cells were derived from in vivo–primed cells that did not initially produce either cytokine in vitro, the uncertainty of clonality meant that these experiments could not unambiguously prove that both Th1- and Th2-like cells were derived from a single primed precursor. To resolve this uncertainty, the differentiation potential of IL-2–producing cells was further investigated using DO11.10 $C\alpha^{-/-}$ TCR transgenic mice. Using the clonotypic antibody KJ1-26, individual DO11.10 cells could be directly isolated for cloning by direct deposition using a Coulter Elite FACS®, i.e., each well contained one cell with no statistical uncertainty.

Pooled spleen and LN cells of naive DO11.10 $C\alpha^{-/-}$ TCR transgenic mice were adoptively transferred into naive Balb/c mice, which were then immunized subcutaneously in the neck with the OVA $peptide_{323–339}$ (P_{OVA}) recognized by the DO11.10 transgenic TCR plus Ribi adjuvant. In preliminary experiments, immunization increased the frequency of $KJ1\text{-}26^+$ transgenic CD4 T cells in the ALNs (7.0 versus 1.0% in unimmunized mice), and the mean fluorescence intensity of CD62L expression on the transgenic T cells decreased from 678 to 93, suggesting that the transgenic T cells had been efficiently primed by immunization. Fig. 11 a shows that 6 d DO11.10-injected mice were immunized with P_{OVA}, many P_{OVA}–specific cells in the ALNs secreted IL-2 or IFN-γ but not IL-4, suggesting that a Th1-biased response was induced. Single $CD62L^-$ $KJ1\text{-}26^+$ $CD4^+$ cells from the ALNs of immunized host mice were sorted into 96-well plates and stimulated with antigen and APCs in the presence of anti–IFN-γ, anti–IL-4, and TGF-β, conditions that maintain an uncommitted phenotype during in vitro culture (18). After 10 d, aliquots of cells from each growing clone were restimulated in IL-2, IL-4, and IFN-γ Elispot assays before splitting to determine the differentiation status of the clones (Fig. 11 b). Of the 146 clones derived from the first cloning, 111 clones contained a significant number of cytokine-producing cells (at least 10 IL-2, IL-4, or IFN-γ spots). 60 of these 111 clones produced IL-2 but not IL-4 or IFN-γ.

Each clone was then split into two aliquots, which were restimulated in either Th1 or Th2 differentiation conditions. After 5 d, the cytokine secretion phenotype of each split clone was determined by Elispot assay (Fig. 11 c). All clones that produced IL-2 and IFN-γ after the initial cycle in vitro (i.e., committed Th1 clones) also produced IFN-γ after the second cycle, regardless of whether they were cultured in Th1- or Th2-inducing conditions (e.g., Fig. 11 c,

clone F3). Of the 54 IL-2–producing cell clones that grew in the second cycle, 27 of these (e.g., Fig. 11 c, clones C4, Ex-2, E12) were judged to be initially uncommitted because these clones secreted only IL-2 after the first cycle of growth in vitro; the majority of the spot-forming cells secreted IFN-γ after growth in Th1 conditions; and the majority secreted IL-4 after growth in Th2 conditions. This demonstrated that in vivo priming induced a substantial proportion of IL-2–producing cells that were uncommitted precursors that could further differentiate into Th1 and Th2 effector cells in the appropriate conditions.

Discussion

When first stimulated by antigen in tissue culture, naive T cells normally differentiate into effector phenotypes such as Th1 or Th2, particularly with the addition of IL-12 or IL-4. However, in the current studies we examined the phenotypes of T cells produced during an in vivo response and found that many antigen-primed T cells are uncommitted precursor cells that secreted IL-2 but not IL-4 or IFN-γ. This phenotype is similar to the proliferating precursor cells derived in vitro by stimulating naive mouse CD4 T cells with alloantigens in the presence of TGF-β and anti–IFN-γ (18), although the exact equivalence of the in vitro and in vivo primed precursor cells has not yet been established.

Several lines of evidence suggest that the primed IL-2–secreting cells are not a transient intermediate stage during T cell differentiation. Previous studies have shown that memory T cells produce relatively high levels of IL-2 and lower levels of IL-4 or IFN-γ (2, 32). In our experiments, the frequency of cells secreting IL-2 remained at a relatively high level even 50 d after immunization with a low amount of soluble antigen. Although we expected that Ribi adjuvant would induce a stronger response and therefore cause more T cells to fully differentiate into effector cells (e.g., Th2), this adjuvant actually increased the proportion of cells secreting IL-2 but not IL-4 or IFN-γ. Immunization of mice with antigen plus CFA induced a Th1-biased response with both IL-2- and IFN-γ–producing cells in the spleen and draining LNs. Although cells producing only IL-2 could have been Th1 cells that failed to produce IFN-γ on that particular occasion (12, 35), the IL-2–secreting cells were much more abundant than IFN-γ–secreting cells in the nondraining LNs, and the cloning experiments with TCR transgenic T cells (Fig. 11) confirmed that uncommitted IL-2–secreting cells could be induced during Th1-biased responses. Taken together, these results suggest that the IL-2–producing cells are not a transient T cell population, but can survive for a long time and constitute an important part of the memory T cell reservoir.

Do the IL-2–producing cells secrete any other cytokines? The two-color Elispot results, and the Ribi adjuvant experiments, showed clearly that the majority of IL-2–secreting cells did not secrete either IL-4 or IFN-γ. Although some single-cell methods analyzing cytokine production at one time point (in situ hybridization, intracellular staining) have suggested that even T cell clones do not always express cytokines coordinately (8, 35), the Elispot method detects cytokine secretion over a longer period and so should be less subject to this reservation. Also, no IFN-γ–secreting cells were detected in most KLH-immunized populations, so the large excess of IL-2–secreting cells in all of our experiments strongly suggests that the IL-2–secreting cells are not just Th1 cells that did not secrete IFN-γ on that particular occasion. Even after immunization with partially purified derivative of *Mycobacterium tuberculosis* or KLH plus Ribi adjuvant, which induced low numbers of IFN-γ–secreting cells, the great majority of the antigen-specific cytokine-secreting cells were IL-2$^+$IL-4$^-$ IFN-g$^-$. In preliminary experiments, we have also tested the antigen-specific secretion of IL-3, IL-5, and IL-6, and found that most of the IL-2–secreting cells did not secrete any of these cytokines (results not shown). Thus, the IL-2–secreting cells appear to secrete few if any of the characteristic Th1 or Th2 cytokines.

We have shown that IL-2–secreting cells primed in vitro (18) or in vivo (this study) are uncommitted, and can differentiate into either Th1 or Th2 cells. An analysis of human peripheral blood T cells revealed a subpopulation that may be the human equivalent (24) of the long-term primed IL-2–secreting mouse T cells that we have identified. These human T cells were CCR7^{high} CD45RA^{low} and secreted high levels of IL-2 and low levels of IFN-γ when stimulated. After 10 d of stimulation in vitro, a substantial number of cells secreted IFN-γ and had lost expression of CCR7, suggesting that some of the CCR7$^+$ cells could differentiate or selectively proliferate. The human CCR7$^+$ IL-2–producing cells were found at long times after immunization, consistent with our data from mice immunized for 50 d.

Primed, IL-2–producing cells show different distribution patterns from effector cells. The expression of CCR7 and CD62L on the human IL-2–producing memory cells suggests that they home preferentially to LNs (24). A LN population of mouse memory CD4 T cells generated in vivo by antigen plus LPS immunization secreted only IL-2, in contrast to the secretion of both IL-2 and IFN-γ by T cells from nonlymphoid tissue (23). In addition, mouse IL-2–producing cells generated in vitro by a short antigenic stimulation (21) home mainly to LNs but not to spleen and other peripheral tissues, in contrast to polarized Th1 and Th2 cells. Together with our observation that the in vivo–primed IL-2–producing cells were enriched in nondraining LNs (Fig. 6), this suggests that the primed, uncommitted cells circulate preferentially through LNs, whereas effector T cells mainly enter the spleen and nonlymphoid tissues.

What conditions are required in vivo for the maintenance of these IL-2–producing cells? TGF-β (or anti–IL-4) and anti–IFN-γ are required for the maintenance of similar IL-2–producing cells in vitro, (18, 19). TGF-β is produced by several cell types (36) and so may also influence T cell differentiation in vivo, and the cytokine antagonist functions may be supplied by soluble receptors instead of anti-

bodies. Alternatively, IL-4, IL-12, and IFN-γ may exist at such low concentrations in some locations that T cells do not receive sufficient signals to induce differentiation and therefore remain as IL-2–secreting cells. Brief antigen stimulation also induced a similar population of primed, IL-2–producing cells (21). Finally, it is possible that there are additional costimulatory or cytokine signals that maintain primed cells in an undifferentiated state.

The primed IL-2–producing CD4 T cells comprise the majority of some immune responses, even with strong adjuvants. The IL-2 may cause optimal proliferation of these cells, resulting in rapid expansion of the antigen-specific population. These primed, bipotential CD4 T cells might provide an expanded population of uncommitted antigen-specific precursor T cells that could include both recently activated and memory cells, as IL-2–producing T cells comprised the majority of KLH-specific cells for at least 50 d after immunization. Preferential migration of these cells into LNs during an immune response should provide an ideal environment for future reeducation and differentiation according to APC signals. Even if an appropriate immune response was mounted during the priming infection, the effector phenotype required during a secondary response might be different if the secondary infection involved a cross-reactive pathogen that was best attacked by different effector mechanisms. Thus, the induction of a substantial population of primed, uncommitted cells would give more flexibility for subsequent differentiation into Th1, Th2, or other phenotypes, allowing a more appropriate reaction to future infections.

The first part of this work was performed in the Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada. The authors acknowledge the technical help of Rita Marcotte and Dorota Rutkowski. We also thank Alexandra Livingstone for helpful discussion and advice on dendritic cells, and Dave Topham and Ben Segal for comments on the manuscript.

This work was supported by the Medical Research Council (Canada), the Howard Hughes Medical Institute International Research Scholar Program, and the University of Rochester Medical Center Strategic Plan.

Submitted: 9 December 1999
Revised: 8 August 2001
Accepted: 20 August 2001

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