CD4^{pos}, NK1.1^{pos} T Cells Promptly Produce Interleukin 4 in Response to In Vivo Challenge with Anti-CD3

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Summary

Injection of anti-CD3 antibodies causes prompt expression of interleukin (IL)-4, IL-2, and interferon γ (IFN-γ) mRNA among spleen cells. The optimal dose of anti-CD3 for such induction was 1.33 μg/animal; lymphokine mRNA was first observed at 30 min, peaked at 90 min, and was undetectable (for IL-4) or had declined markedly by 4 h. Cells harvested from spleens of mice injected with anti-CD3 90 min earlier secreted IL-4, IL-2, and IFN-γ without further stimulation. By contrast, in vitro stimulation with anti-CD3 of spleen cell suspensions or splenic fragments from noninjected donors failed to cause prompt production of IL-4 and, even after 24 h of stimulation, the amount of IL-4 produced in such cells was substantially less than that secreted within 1 h by spleen cell suspensions or splenic fragments from mice injected with anti-CD3 90 min earlier. Production of IL-4 by spleen cells from anti-CD3–injected mice was not inhibited by pretreatment with anti-IL-4 antibody or with IFN-γ or tumor growth factor β nor enhanced by treatment with IL-4. By contrast, CTLA-4 immunoglobulin (Ig) treatment clearly diminished IL-4 production in response to in vivo anti-CD3, indicating that cellular interactions involving CD28 (or related molecules) were important in stimulation. Cell sorting analysis indicated that the cells that produced IL-4 in response to in vivo injection of anti-CD3 were highly enriched in CD4^{pos} cells with the phenotype leukocyte cell adhesion molecule-1 (LECAM-1)^{dull}, CD44^{bright}, CD45RB^{dull}, NK1.1^{pos}. Indeed, the small population of CD4^{pos}, NK1.1^{pos} cells had the great majority of the IL-4–producing activity of this population. Injection with *Staphylococcal* enterotoxin B also caused prompt induction of IL-4 mRNA; the cells that were principally responsible for production also had the phenotype of CD4^{pos}, NK1.1^{pos}. These results suggest that possibility that this rare population of T cells may be capable of secreting IL-4 at the outset of immune responses and thus may act to regulate the pattern of priming of naive T cells, by providing a source of IL-4 to favor the development of T cell helper 2–like IL-4–producing cells.

Immune responses of CD4^{pos} T cells are often dominated by the production of IFN-γ or of IL-4. Indeed, the choice of lymphokine produced by CD4^{pos} T cells may have a profound effect on the protective value of that response (1–3). Recent in vitro studies have analyzed the factors that determine whether naive CD4^{pos} T cells develop into IFN-γ or IL-4 producers (4–8). These studies have revealed a dominant role for IL-4 itself. Thus, priming T cells in the presence of IL-4 leads to the appearance of cells that produce large amounts of IL-4 upon rechallenge but little or no IFN-γ (6, 7). By contrast, priming cells in the presence of anti-IL-4 antibody results in primed cell populations that produce little or no IL-4 but are good IFN-γ producers. For IL-4 to have a major effect on priming for IL-4 production, it must be present early in the culture.

IL-4 also has a major effect on in vivo priming of CD4^{pos} T cells to become IL-4–producing cells (9–12). Treatment of mice with anti-IL-4 at the time of priming with hemocyanin diminishes the frequency of T cells that produce IL-4 in response to in vitro challenge with hemocyanin (11). Similarly, treatment of BALB/c mice with anti-IL-4 at the time of infection with *Leishmania major* (9) or *Candida albicans* (10) enhances their production of IFN-γ and suppresses priming for IL-4 production in response to specific challenge, but only if anti-IL-4 is injected very early in the course of the infection. Treating C57BL/6 mice with IL-4 at the time of infection with *L. major* increases the appearance of T cells that produce IL-4 upon subsequent in vitro challenge with leishmanial antigens (12). CD4^{pos} T cells from mice in which the IL-4 gene has been disrupted by gene targeting produce substantially less IL-5 as a result of infection with *Nippostrongylus brasiliensis* (13). Since IL-5 and IL-4 are both products of Th2-type T cell clones (14) and their production by normal T cells is often coregulated (5), these results further support

the concept that IL-4 plays a critical role in the in vivo acquisition of the lymphokine-producing phenotype. Taken together, these studies indicate that IL-4 produced early in an immune response is important in determining the pathway of differentiation of naive $CD4^{pos}$ T cells.

The central role played by IL-4 in in vivo priming leads us to ask what the source of IL-4 might be for determining that naive $CD4^{pos}$ T cells will develop into IL-4 producers. Although T cells themselves are an obvious possibility, the very modest production of IL-4 by T cell populations from naive donors when stimulated in vitro has cast some doubt on their potential importance and raised the possibility that other cell types, such as basophils and/or mast cells, might be the source of such IL-4 (15–18). Nonetheless, both Scott et al. (19) and Flamand et al. (20) have observed that intravenous injection of soluble anti-CD3 antibody results in rapid but transient expression of IL-4 mRNA and protein in the spleen. In this report, we confirm this observation and show that IL-4 protein is induced as early as 30 min after the injection of anti-CD3 antibody. It is interesting to note that in vitro treatment of spleen cells with anti-CD3 in either suspension or fragment cultured led to induction of modest amounts of IL-4 protein with much slower kinetics indicating that in vivo and in vitro regulation of IL-4 production may be quite different.

We show that the cells responsible for IL-4 production in response to intravenous anti-CD3 are largely $CD4^{pos}$ T cells. The $CD4^{pos}$ T cells that produce IL-4 in response to intravenous injection of anti-CD3 are leukocyte cell adhesion molecule (LECAM)^{1}-1^{dull},$CD44^{bright}$,$CD45RB^{dull}$. IL-4–producing cells are enriched among $CD4^{pos}$, $NK1.1^{pos}$ cells suggesting that the major producers are the small population of $CD4^{pos}$, $NK1.1^{pos}$ T cells that have been previously implicated as important lymphokine-producing cells in the thymus and bone marrow (21). The production of IL-4 by this cell population is independent of the need for IL-4 since anti-IL-4 treatment does not inhibit it. Such production of IL-4 is also mediated by intravenous injection of *Staphylococcal* enterotoxin B (SEB) suggesting an in vivo mechanism through which superantigens may elicit a source of IL-4 that could influence priming to associated conventional antigens.

Materials and Methods

Animals. Virus-free C57BL/6 and BALB/c female mice, 8–12 wk of age, were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Culture Medium. RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% FCS (Inovar Biologicals, Inc., Gaithersburg, MD), 2-ME (50 μM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and sodium pyruvate (1 mM) was used as culture medium.

Recombinant Lymphokines. Recombinant mouse IL-4 was obtained from a recombinant baculovirus (AcMNPV.IL-4) prepared by Cynthia Watson (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]). 1 U of IL-4 is equal to ~0.5 pg. Human recombinant IL-2 was a gift Cetus Corporation (Emeryville, CA). 1 U of IL-2, defined as a "Cetus unit", is equal to 6 IU and to ~0.3 ng. Purified mouse IFN-γ and TGF-β were purchased from Genzyme Corporation (Boston, MA).

Reagents. Anti-CD3 (2C11) (22) and control hamster monoclonal IgG antibody (HH16) (established by Carol Kinzer, Laboratory of Immunology, NIAID, NIH) were purified from tissue culture supernatants. Purified rat anti-mouse IL-4 (11B11) (23) was prepared by Verax Corporation (Lebanon, NH). Rat anti-mouse Fcγ receptor antibody (2.4G2) (24) was purified by Jane Hu-Li (Laboratory of Immunology, NIAID, NIH). Rat anti-mouse IFN-γ (XMG 1.2) (25), FITC-rat anti-mouse LECAM-1 (MEL-14) (26), FITC-rat anti-mouse CD44 (PgP-1) (27), FITC-rat anti-mouse CD45RB (16A) (28), FITC-rat anti-mouse B220 (RA3-6B2) (29), FITC-rat anti-mouse I-A^d (AMS 32.1) (30), PE-rat anti-mouse CD4 (RM 4-5) (31), FITC-rat anti-mouse CD8 (53-6.7) (32), and biotinylated anti-mouse NK1.1 (PK 136) (33) were purchased from PharMingen (San Diego, CA). SEB was purchased from Sigma Chemical Co. (St. Louis, MO). A soluble IgCγ1 chimera of CTLA-4 (CTLA-4Ig) (34) and its control human–mouse chimeric mAb (Chi-L6) (35) were provided by Dr. Peter S. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA).

In Vivo Treatment of Mice. Mice were injected intravenously with a single dose of anti-CD3 (0.44–12 μg), or SEB (25–800 μg). Control mice were injected with same amount of hamster IgG or HBSS. Spleens were removed at the specified times after injection for RNA extraction and cell culture. For some experiments, mice were treated with lymphokines, anti-IL-4 or CTLA-4Ig before injection with anti-CD3. For the stimulation of popliteal lymph node cells, 10 μg of anti-CD3 in 50 μl of HBSS was injected into both hind foot pads of C57BL/6 mice. The draining popliteal lymph nodes were removed for RNA extraction at various times after injection.

Cell Preparation. Spleens were removed at various times after injection with anti-CD3 or SEB. Cell suspensions were washed twice with HBSS. Except where indicated, 5×10^6 spleen cells/well were cultured in 24-well plates with or without 3 μg/ml of anti-CD3 for 1–24 h and supernatants harvested to measure lymphokine content. In some experiments, spleens from mice injected with or without anti-CD3 were cut into cubes with a volume of ~1 mm^3. Individual cubes were placed in wells of 96-well plates and cultured with or without anti-CD3 for 1–24 h.

For the preparation of $CD4^{pos}$ splenic T cells, cells were suspended at a concentration of 2×10^7/ml in RPMI 1640 containing 5 mM EDTA (NIH Media Unit) and 5% fetal FCS. The cell suspension was incubated with 10 μg/ml each of FITC anti-B220; FITC anti-I-A^d, and FITC anti-CD8 antibodies for 30 min at 4°C on a turning wheel. The cells were then washed twice and resuspended with magnetic beads coated with sheep anti-FITC antibodies (Advanced Magnetics Inc., Cambridge, MA). Cells that had bound antibodies were depleted by two rounds of exposure to magnetic field. The residual cells were collected and washed twice.

Fluorescence Analysis and Cell Sorting. Fluorescence staining was performed at 4°C in 100 μl containing 10^6 CD4-enriched spleen cells and PE anti-CD4 in combination with FITC anti-LECAM-1, FITC anti-CD44, or FITC anti-CD45RB in PBS containing 3% FCS and 0.5% NaN_3. For detection of $NK1.1^{pos}$ cells, biotinylated anti-NK1.1 and FITC-labeled avidin were used. Fluorescence analysis was carried out with a FACScan® Flow Cytometer (Becton Dickinson & Co., Mountain View, CA). For sorting experiments, a FACStar® Plus Flow Cytometer (Becton Dickinson & Co.) was used. Cells were maintained at 4°C during the sorting process.

Lymphokine Assays. CT.4S, an IL-4–dependent cell line, and

[1] *Abbreviations used in this paper:* LECAM, leukocyte cell adhesion molecule; RT, reverse transcriptase; SEB, *Staphylococcal* enterotoxin B.

CT.EV, an IL-2–dependent cell line (36), were used to measure IL-4 and IL-2 content, respectively, using serial dilutions of supernatants and comparing responses to those elicited by known amounts of murine IL-4 and human IL-2 as standards. IFN-γ was assayed with a specific two-site ELISA (37, 38), with reference standard curves prepared using known amounts of rIFN-γ.

Analysis of Expression of IL-4, IFN-γ, and IL-2 mRNA. Total spleen RNA was prepared using the Guanidinium Method (39). In brief, after making spleen cell suspensions, cells were washed in ice-cold PBS and lysed in 4 ml lysis solution, containing 4 M guanidine-thiocyanate (Fluka Chemika-BioChemika, Ronkonkoma, NY), 25 mM sodium citrate (pH 7.0), 0.5% *N*-lauroyl sarcosine (Sigma Chemical Co.), and 100 mM 2-ME. Lysates were vortexed and stored at −70°C until further processing. After thawing, 400 μl of 2 M sodium acetate (pH 4.0), 4.4. ml of water-saturated phenol (AMRESCO, Solon, OH), and 800 μl of chloroform-iso-amyl alcohol (49:1) were added to the lysates with thorough vortexing after each addition. The mixture was then chilled on ice for 15 min and spun at 10,000 *g* for 15 min at 4°C. The aqueous phase was recovered and RNA was precipitated with an equal volume of 2-propanol at −20°C for at least 1 h. The precipitated RNA was resuspended with 600 μl of lysis solution and an equal volume of 2-propanol, and precipitated at −20°C for 1 h. Precipitates were pelleted at 4°C, washed twice in 70% ethanol, and repelleted at 4°C at 10,000 *g* for 20 min. Vacuum-dried pellets were resuspended in 50 μl of diethylpyrocarbonate-treated double-distilled water (DEPC-ddH_2O) and total RNA concentration was measured. As positive controls, mRNAs extracted from the IL-4–producing cell line, LT-1 (40) and from purified T cells stimulated with APC plus anti-CD3 and anti-IL-4 for 24 h (8) were used.

For RNA preparation from sorted cells, we used the modified method of Chomczynski and Sacchi (39) and resuspended pellets in 10–20 μl DEPC-ddH_2O.

For Northern hybridization, 10 μg of RNA from each sample were separated by electrophoresis in a 1% agarose/formaldehyde gel and blotted onto a nitrocellulose-Nytran membrane (Schleicher and Schuell, Inc., Keene, NH). cDNA probes specific for mouse IL-4, IFN-γ and IL-2 (a 373 EcoRI/HindIII fragment for IL-4; a 643 bp Pst-1 fragment for IFN-γ; and a 600 bp Pst-1 fragment for IL-2) were ^{32}P-labeled by the random primer method to a specific activity of 5×10^8–2×10^9 cpm/μg. After baking, the filters were prehybridized at 42°C for 1 h and then hybridized with labeled probe for 18 h. The filters were washed twice with 2 × SSC and 0.1% SDS at room temperature and twice at 60°C with 0.1 × SSC and 0.1% SDS.

For analysis of expression of IL-4, IFN-γ, and IL-2 mRNA from sorted cells and in other selected experiments, mRNAs were amplified by a modified standard reverse transcription (RT)-PCR amplification procedure (41). Primers specific for murine IL-4, IL-2, IFN-γ, and β-actin were prepared by Cynthia Watson. Primer sequences were as follows: IL-4: 5′ primer, GAATGTACCAGCAGCCATATC, and 3′ primer, CTCAGTACTACGAGTAATCCA; IL-2: 5′ primer, ACTTCAAGCTCCACTTCAAGC, and 3′ primer, GCTTTGAGAAAGGGCTATCCA; IFN-γ: 5′ primer, AACGCTTACACACTGCATCTTGG, and 3′ primer, GACTTCAAAGAGTCTGAGG; β-actin: 5′ primer, GATGACGATATCGCTGCGCTG, and 3′ primer, GTACGACCAGAGGCATACAGG. Initially, 2 μl (1 μg) RNA were added to 18 μl of reverse transcription mixture containing 5 mM $MgCl_2$, 1× PCR buffer II, 1 mM of each dNTP (all from Perkin-Elmer Cetus, Norwalk, CT), 2,000 U/ml RNase inhibitor (Promega, Madison, WI), 2.5 U/ml Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD), and 0.75 μM specific 3′ oligo primer for each lymphokine. Tubes were then placed in a thermal cycler (GenAmp PCR System 9600; Perkin-Elmer Cetus) and incubated for 60 min at 37°C, followed by 5 min at 99°C, and then 5 min at 5°C.

After reverse transcription, 80 μl of the PCR mix were added to each tube to give a final concentration of 2.5 U Taq DNA polymerase (Perkin-Elmer Cetus), 0.15 μM 5′ primer, 0.15 μM 3′ primer, 2 mM $MgCl_2$, and 1× PCR buffer II/100 μl. cDNAs were amplified for 30 cycles, each composed of 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min. At the end of 30 cycles, samples were stored at 4°C until analyzed. After amplification, PCR products were separated by electrophoresis in 8% acrylamide gels and visualized by UV light illumination after ethidium bromide staining. Some PCR products were analyzed by Southern blotting, using the probes described above.

Results

Expression of Lymphokine mRNA In Vivo. C57BL/6 mice were injected intravenously with purified anti-CD3 (2C11) or control hamster antibody (HH16). Spleens were removed at various times after injection for RNA extraction. mRNAs for IL-4, IFN-γ, and IL-2 were measured by Northern analysis. After injection with 4 μg of anti-CD3, IL-4 mRNA was first detectable at 30 min, reached a higher level at 1 h, and was markedly diminished at 4 h. (Fig. 1 *A*). To determine the optimal dose of anti-CD3 for this induction of IL-4 mRNA, Northern analysis was carried out 1 h after injection with varying amounts of anti-CD3; 1.33 μg of anti-CD3 per mouse was found to maximally induce IL-4 mRNA

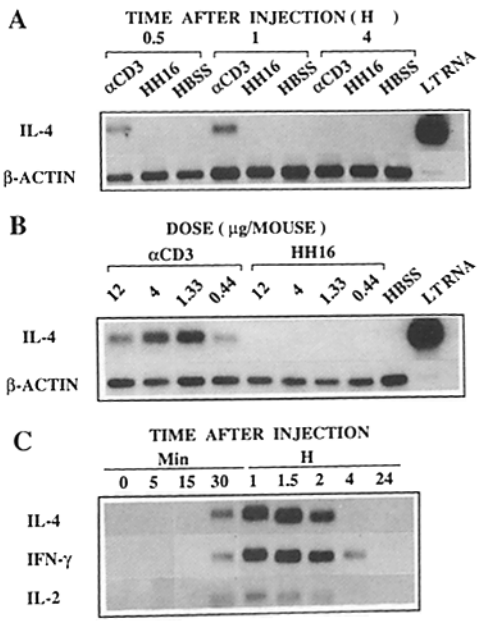

Figure 1. Anti-CD3 induces the expression of lymphokine mRNAs in vivo. C57BL/6 mice were injected with (*A*) 4 μg; (*B*) 0.44–12 μg; and (*C*) 1.33 μg of purified anti-CD3 (2C11), control hamster Ig (HH16), or HBSS, and spleens were harvested at the indicated time for RNA extraction. RNA from IL-4–secreting LT-1 cells (*LTRNA*) is included as a positive control for IL-4 mRNA.

expression (Fig. 1 *B*). In these experiments, the control hamster mAb did not induce detectable IL-4 mRNA.

To further examine the critical time for maximal induction of IL-4 mRNA, spleens were removed as early as 5 min after injection of 1.33 μg of anti-CD3. By Northern analysis, IL-4 mRNA was first detected at 30 min, reached its highest level at 1.5 h, and declined rapidly thereafter (Fig. 1 *C*). IFN-γ and IL-2 mRNAs were also initially expressed at 30 min and, although detectable at 4 h, had diminished considerably from their peak at 1.5 h. Based on these experiments, we used 1.33 μg of anti-CD3 per mouse and removed the spleen at 1.5 h after injection in the following experiments.

Production of Lymphokines by Spleen Cells from Mice Treated with Anti-CD3. To determine whether the expression of lymphokine mRNAs led to production of lymphokine, 5×10^6 spleen cells from anti-CD3-injected mice were cultured for 1 h without additional stimulus. As shown in Fig. 2, a substantial amount of lymphokine was present in these supernatants; the time after in vivo injection to gain and then to lose the capacity to produce each of the lymphokines in short-term culture was the same as that for expression of mRNA at the time of harvest from the donor. Spleen cells removed 1.5 h after in vivo treatment produced maximal amounts of IL-4, IFN-γ, and IL-2. We measured lymphokine secretory activity as well as lymphokine mRNA in most of the following experiments.

Organ Distribution of IL-4 mRNA Expression from Mice Injected with Anti-CD3. In contrast to the prompt expression of IL-4 mRNA in spleen cells from mice injected intravenously with anti-CD3, little or no IL-4 mRNA was detected in thymocytes or peripheral blood cells of these mice (Fig. 3). Bone marrow cells and mesenteric lymph node cells showed modest amounts of IL-4 mRNA in response to injection with anti-CD3, but considerably less than was found in spleen cells.

Analysis of penetration of anti-CD3 into spleen, lymph nodes, and thymus was done by staining cells with FITC anti-hamster IgG and comparing the frequency of positive cells with that obtained by staining cells with anti-CD3 plus FITC anti-hamster IgG. Half of splenic T cells were stained within 15 min of injection of anti-CD3; neither the fraction nor the degree of staining increased for up to 90 min after injection (data not shown). By contrast, <10% of lymph node

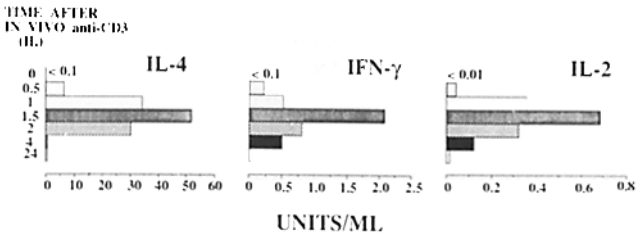

Figure 2. Production of lymphokines by spleen cells from mice treated with anti-CD3. Spleen cells from mice injected previously (as indicated) with 1.33 μg of anti-CD3 were cultured in 24-well plates at 5×10^6/ml for 1 h without additional stimulation. Culture supernatants were harvested and tested for production of IL-4, IFN-γ, and IL-2.

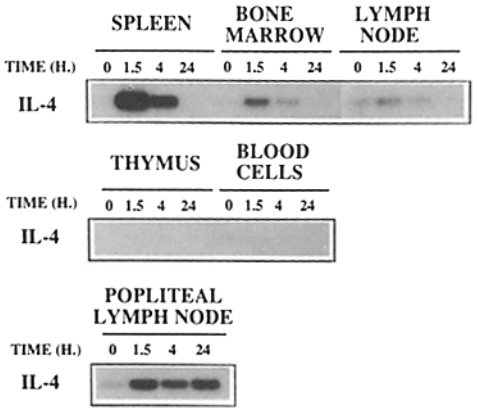

Figure 3. Organ distribution of IL-4 mRNA expression from mice injected with anti-CD3. RNA was extracted from spleen cells, bone marrow cells, mesenteric lymph node cells, thymocytes, and peripheral blood cells at various times after injection of 1.33 μg of anti-CD3. Popliteal lymph node cells were removed after injection of 10 μg of anti-CD3 in 50 μl of HBSS into hind foot pads. IL-4 mRNA levels were determined by RT-PCR with Southern blot analysis.

T cells and virtually none of the $CD3^{pos}$ thymocytes had bound anti-CD3 at 90 min after injection. This suggests that limited (or absent) IL-4 production in these organs is due to poor penetration of injected anti-CD3.

Interestingly, popliteal lymph nodes from mice injected with anti-CD3 in the footpads displayed considerable IL-4 mRNA; peak levels were reached within 1.5 h but, in contrast to the spleen cells after intravenous treatment with anti-CD3, IL-4 mRNA among popliteal lymph nodes cells remained elevated for 24 h after footpad injection of anti-CD3.

Comparison of IL-4 Production by Spleen Cells in Response to In Vivo and In Vitro Stimulation with Anti-CD3. Spleen cells from mice treated with anti-CD3 for 1.5 h produce large amounts of IL-4 without further in vitro stimulation. By contrast, spleen cells from uninjected mice produced little or no IL-4 without in vitro stimulation and failed to produce substantial amounts of IL-4 in response to in vitro exposure to anti-CD3 until 24 h of culture (Fig. 4 *A*). Even at 24 h, the amount of IL-4 in culture supernatants of cells from uninjected donors cultured in vitro with anti-CD3 was less than the amounts of IL-4 in 1-h culture supernatants of spleen cells from mice injected with anti-CD3 1.5 h earlier and cultured without any further stimulant.

We also cultured splenic fragments from mice injected with anti-CD3 1.5 h earlier and from uninjected mice with or without additional anti-CD3 in vitro (Fig. 4 *B*). Fragments from the "1.5-h injected" mice produced substantial amounts of IL-4 within 1 h without the need for the addition of anti-CD3. Although splenic fragments from uninjected mice produced detectable amounts of IL-4 in response to culture with anti-CD3 somewhat earlier than was observed in spleen cell suspensions from these mice, this production was less in magnitude and still much delayed in comparison to the IL-4 production by splenic fragments from mice injected with anti-CD3 but not further cultured with anti-CD3. These results

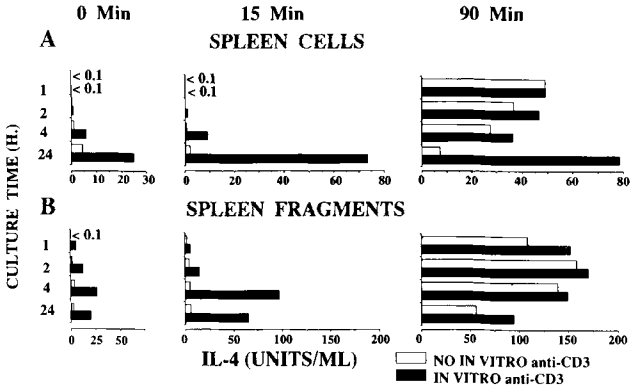

Figure 4. IL-4 production by spleen cells or splenic fragments in response to in vivo and in vitro stimulation with anti-CD3. Spleen cells (*A*) or spleen fragments (*B*) from uninjected mice (*left*) or from mice injected with anti-CD3 15 min (*middle*) or 90 min (*right*) earlier were cultured with (■) or without (□) 3 μg/ml of anti-CD3 for 1–24 h. Spleen cells suspensions were cultured at 2 × 10^5 cells in 0.2 ml of culture medium in 96-well plates; single spleen fragments with a volume of ~1 mm^3 were cultured in 0.2 ml in individual wells of 96-well plates. Supernatants were harvested and tested for production of IL-4.

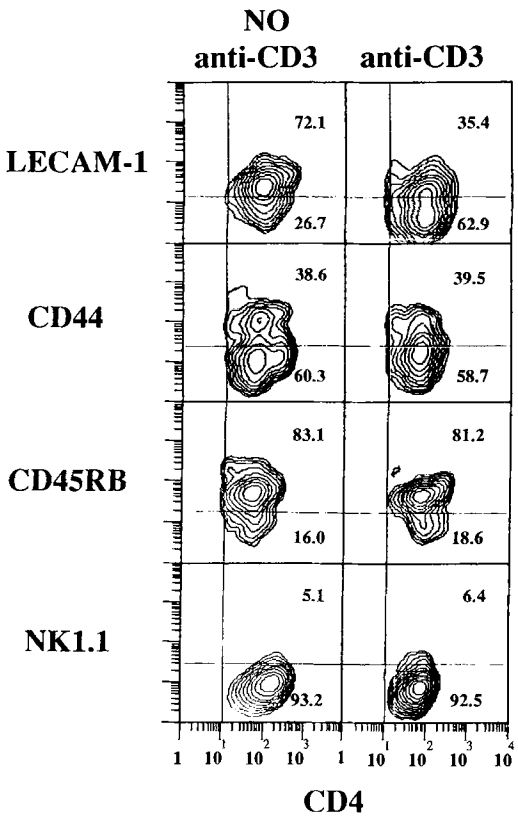

Figure 5. The frequency of $CD4^{pos}$,$LECAM\text{-}1^{dull}$ spleen cells is increased in response to anti-CD3 injection. CD4-enriched spleen T cells from uninjected mice or mice injected with anti-CD3 1.5 h earlier were stained with a mixture of PE anti-CD4 and FITC anti-LECAM-1, FITC anti-CD44, FITC CD45RB or biotinylated anti-NK1.1 and FITC-avidin. The percentages shown represent the proportion of bright and dull (LECAM-1, CD44, and CD45RB), or positive and negative (NK1.1) cells among the $CD4^{pos}$ T cells. The frequency of $NK1.1^{pos}$ cells in the experiment illustrated here was determined in the absence of a blocking anti-FcγRII/III antibody (2.4G2). However, in another experiment in which unlabeled 2.4G2 was added, there was no difference in the percent of $NK1.1^{pos}$ cells among $CD4^{pos}$ cells in uninjected and injected mice although the absolute percentages (4.5 and 4.8) were slightly lower than in the experiment shown here.

suggest that IL-4 production in response to injection with anti-CD3 is not mimicked by in vitro exposure to anti-CD3 even when the microenvironment of the interaction is preserved by the use of splenic fragments. One possibility that might account for the failure of in vitro anti-CD3 to promptly elicit IL-4 production in splenic fragments from uninjected mice is that the anti-CD3 might penetrate the fragment relatively slowly. To rule out this possibility, we prepared splenic fragments from mice that had been injected with anti-CD3 15 min before sacrifice. As noted above, anti-CD3 binds to T cells by this time in amounts as great as at 1.5 h after injection. Nonetheless, these fragments, even when cultured in anti-CD3, which should ensure a maintenance of anti-CD3 concentrations, fail to produce IL-4 in substantial amounts until 4 h of culture. These results further support the concept that the splenic fragment culture does not mimic the in vivo interaction of anti-CD3 with T cells that leads to prompt production of IL-4.

Surface Phenotype of Cells That Produce IL-4 in Response to Injection of Anti-CD3. We next wished to determine the phenotype of the spleen cells that produce IL-4 after in vivo treatment with anti-CD3. We first examined the composition of the spleen cells in uninjected mice and in mice injected with anti-CD3 1.5 h earlier. Fig. 5 shows that among the $CD4^{pos}$ T cells, there is a considerable increase in the frequency of $LECAM\text{-}1^{dull}$ cells in the spleens of anti-CD3–injected mice. No differences in the frequency of $CD44^{bright}$, $CD45RB^{dull}$, or $NK1.1^{pos}$ cells were noted, nor were there any obvious differences in the expression of these markers on $CD4^{neg}$ cells (data not shown).

Spleen cells from mice injected with anti-CD3 1.5 h earlier were separated into $CD4^{pos}$ and $CD8^{pos}$ populations and into a series of subpopulations of $CD4^{pos}$ cells. Using RT-PCR to analyze mRNA expression, IL-4–producing cells were found in the $CD4^{pos}$ but not the $CD8^{pos}$ group (Fig. 6 *A*). In a separate experiment, $CD4^{neg}$,$CD8^{neg}$ cells from anti-CD3–injected mice showed little or no IL-4 mRNA (data not shown). Among $CD4^{pos}$ cells, the IL-4 producers were in the group that were $LECAM\text{-}1^{dull}$, $CD44^{bright}$, and $CD45RB^{dull}$, consistent with the expression of an activated or memory phenotype (42). $CD8^{pos}$ T cells expressed more IFN-γ mRNA than $CD4^{pos}$ T cells. Nonetheless, among the $CD4^{pos}$ T cells, those that expressed IFN-γ mRNA had the same phenotype as those that expressed IL-4 mRNA. IL-2 mRNA was observed in both $CD4^{pos}$ and $CD8^{pos}$ T cells. Among the $CD4^{pos}$ T cells, the dominant phenotype of the IL-2 producers was also $LECAM\text{-}1^{dull}$, $CD44^{bright}$ and $CD45RB^{dull}$.

In separate experiments, spleen cells from C57BL/6 donors injected 1.5 h earlier with anti-CD3 were sorted in $CD4^{pos}$, $NK1.1^{pos}$ and $CD4^{pos}$,$NK1.1^{neg}$ populations. The $CD4^{pos}$,

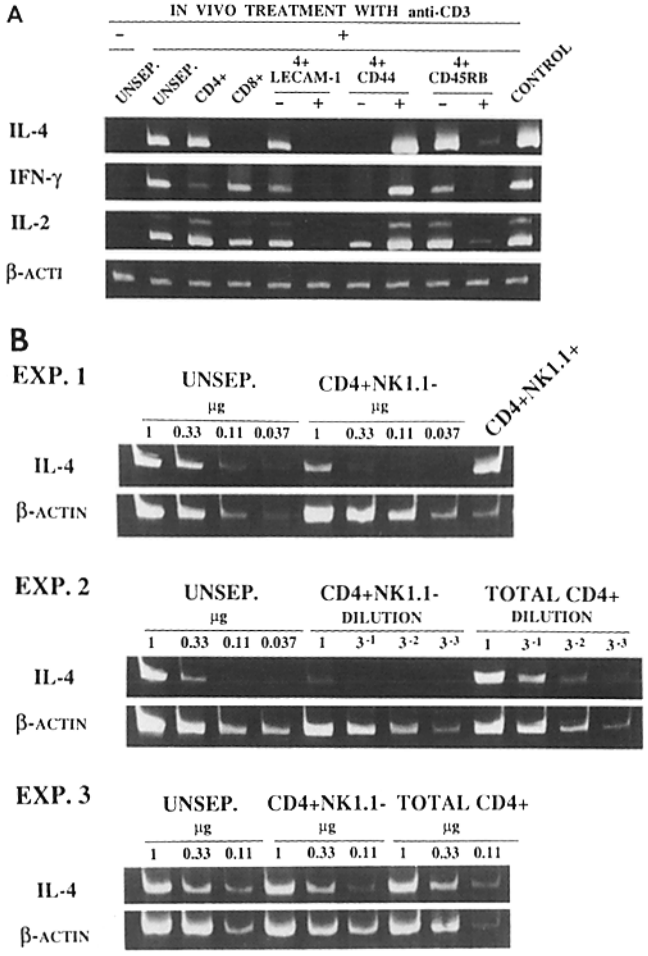

Figure 6. Expression of lymphokine mRNAs by T cell subsets from mice treated with anti-CD3. (*A*) Spleen cells from C57BL/6 mice injected with anti-CD3 1.5 h earlier were separated into $CD4^{pos}$ and $CD8^{pos}$ populations and into a series of subpopulations of $CD4^{pos}$ cells ($LECAM\text{-}1^{dull/bright}$,$CD44^{dull/bright}$ and $CD45RB^{dull/bright}$). During the sorting, spleen cells were kept at 4°C to insure stability of lymphokine mRNAs. Isolated mRNA was analyzed for expression of IL-4, IFN-γ, IL-2, and β-actin by using RT-PCR as described in Materials and Methods. As positive controls, mRNAs extracted from LT-1 cells and from purified T cells, stimulated with APC, anti-CD3 plus anti-IL-4 in vitro for 24 h were used for IL-4 and IFN-γ/IL-2, respectively. (*B*) Spleen cells from C57BL/6 mice injected with anti-CD3 1.5 h earlier were sorted into $CD4^{pos}$,$NK1.1^{pos}$ and $CD4^{pos}$,$NK1.1^{neg}$ populations (Exp. *1*). Spleen cells from C57BL/6 (Exp. *2*) or BALB/c (Exp. *3*) mice were sorted into total $CD4^{pos}$ and $CD4^{pos}$, $NK1.1^{neg}$ populations. A series of threefold diluted RNA samples were analyzed for expression of IL-4 and β-actin mRNA with RT-PCR. Where known, the amount of RNA samples used is reported in μg. UNSEP, total spleen cells before sorting.

$NK1.1^{pos}$ population, being relatively infrequent, yielded substantially less mRNA as judged by the RT-PCR amplification of actin mRNA. Despite this, IL-4 mRNA in this population was much greater than in the $CD4^{pos}$,$NK1.1^{neg}$ population (Fig. 6 *B*, exp. *1*). Although this result illustrates that $CD4^{pos}$,$NK1.1^{pos}$ T cells produce substantial amounts of IL-4 mRNA, they do not allow a direct determination of the total amounts of IL-4 mRNA in the $NK1.1^{pos}$ and $NK1.1^{neg}$ portions of the $CD4^{pos}$ cell population since the latter are much more numerous than the former. To test the magnitude of the contribution of $CD4^{pos}$,$NK1.1^{pos}$ cells to the production of IL-4 mRNA by the $CD4^{pos}$ cell population, we sorted C57BL/6 cells into total $CD4^{pos}$ and into $CD4^{pos}$,$NK1.1^{neg}$ subpopulations and compared the amounts of IL-4 mRNA by semiquantitative RT-PCR, using actin mRNA as a control. As seen in Fig. 6 *B* (exp. *2*), $CD4^{pos}$, $NK1.1^{neg}$ cells express approximately ninefold less IL-4 mRNA than do total $CD4^{pos}$ cells; this conclusion was verified by quantitative densitometric comparison of intensities IL-4 and β-actin RT-PCR products (data not shown). To control for specificity of staining with NK1.1, we separated BALB/c spleen cells into $CD4^{pos}$,$NK1.1^{neg}$ cells and total $CD4^{pos}$ cells. Since BALB/c cells do not express NK1.1, no difference in IL-4 mRNA between these populations would be expected. Indeed, no difference was observed (Fig. 6 *B*, exp. *3*). These results imply that the small population of $CD4^{pos}$,$NK1.1^{pos}$ cells produces the great majority of the IL-4 mRNA expressed by $CD4^{pos}$ cells from mice injected with anti-CD3. The data indicate that IL-4 production in the T cell population derives principally from $CD4^{pos}$,$LECAM\text{-}1^{dull}$, $CD44^{bright}$,$CD45RB^{dull}$,$NK1.1^{pos}$ cells.

IL-4 Is Not Required for IL-4 Production in Response to Anti-CD3 Injection. As noted in the beginning, IL-4 production in vitro requires or is markedly enhanced by the presence of IL-4 during priming (6, 7). Furthermore, anti-IL-4 treatment of mice at the time of immunization or infection diminishes subsequent IL-4 production upon rechallenge (9–11). To determine whether acute production of IL-4 in response to injection of anti-CD3 was similarly regulated, mice were treated with anti-IL-4 or with IL-4, IL-2, IFN-γ, or TGF-β beginning at 16 h before injection with anti-CD3. These mice showed no change in their expression of IL-4 mRNA in response to injection of anti-CD3 (Fig. 7 *A*). These results indicate that the factors that appear to regulate priming of T cells to become IL-4 producers do not control the acute production of IL-4 in response to injection of anti-CD3 antibody, thus suggesting that such cells could be a source of IL-4 for the regulation of priming of naive antigen-specific T cells.

Although production of IL-4 mRNA is not influenced by the lymphokines that normally regulate priming of IL-4-producing T cells, in vivo anti-CD3 stimulation does depend upon the interaction of T cells with cells expressing accessory ligands since CTLA-4Ig, which blocks the interaction of ligands for CD28 and CTLA-4 with these accessory receptors (35), substantially diminishes the IL-4 response to injection of anti-CD3 (Fig. 7 *B*).

IL-4 Production Can Be Promptly Induced by Injection of SEB. The capacity of anti-CD3 to rapidly induce IL-4, IFN-γ, and IL-2 production in spleen cells indicates that a potential source for the prompt production of these lymphokines exists that could be of importance in the process through which naive T cells develop into IL-4 or IFN-γ producers. However, anti-CD3 itself is not a physiologic stimulant and the frequency of T cells that could respond to conventional antigens is probably too low for the production of amounts of lymphokine sufficient to influence priming of naive T cells. We consid-

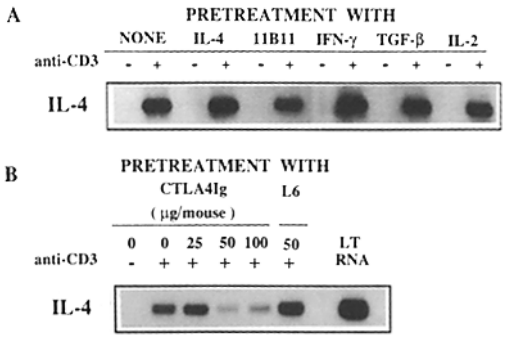

Figure 7. IL-4 is not required for IL-4 production in response to anti-CD3 injection but production is inhibited by CTLA-4Ig. (*A*) Mice were pretreated intraperitoneally with 9 mg of anti-IL-4 (11B4) or intravenously with 5×10^6 U of IL-4, 1 μg of IL-2, 1 μg of IFN-γ, or 2 μg of TGF-β 16 h and 1 h before injection of anti-CD3 or HBSS. (*B*) Mice were pretreated with CTLA-4Ig (0, 25, 50, 100 μg/mouse) or control human–mouse chimeric mAb, Chi-L6 (50 μg/mouse) intravenously 1 h before injection of anti-CD3. Spleens were removed at 1.5 h after injection of anti-CD3 for RNA extraction and IL-4 mRNA was measured by Northern blot analysis.

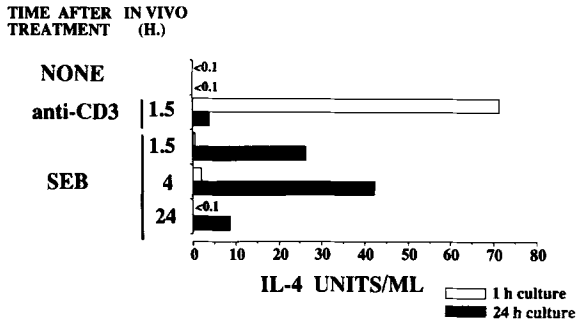

Figure 9. Production of IL-4 by T cells from SEB-injected mice. Five million spleen cells from mice injected with 1.33 μg of anti-CD3 (1.5 h earlier) or 100 μg of SEB (1.5, 4, 24 h earlier) were cultured in individual wells of 24-well plates for 1 h and 24 h without additional stimulus. Culture supernatants were harvested and tested for production of IL-4.

ered the possibility that superantigens might function like anti-CD3 since a response to these molecules could possibly make a substantial contribution to the lymphokine environment at the time of priming. Injection of SEB into C57BL/6 mice caused the appearance of IL-4 mRNA in the spleen within 1.5 h (Fig. 8 *A*). 200 μg of SEB caused a peak response; IL-4 mRNA could be detected by RT-PCR in response to 25-50 μg of SEB (Fig. 8 *B*). The amount of IL-4 mRNA and of IL-4 protein was less than in response to anti-CD3 and the time course of expression was somewhat prolonged, since mRNA and activity could still be detected in cells harvested 4 h after injection. However, by 24 h after injection, no IL-4 mRNA or activity was observed in spleen cells from mice injected with SEB or anti-CD3, suggesting that the overall response might be quite similar. Interestingly, T cells from SEB-injected mice required a more prolonged period of culture to produce IL-4 than did cells from anti-CD3–injected mice; more IL-4 was found in culture supernatants at 24 h of culture, without further stimulant, than after 1 h of culture (Fig. 9).

$CD4^{pos}$ T cells from mice injected with SEB were sorted into total $CD4^{pos}$ and $CD4^{pos}$,$NK1.1^{neg}$ subpopulations. IL-4 mRNA was substantially diminished in the $CD4^{pos}$, $NK1.1^{neg}$ cells in comparison with the total $CD4^{pos}$ subpopulation. Densitometric analysis of Southern-blotted PCR products indicated a threefold diminution, in comparison with total $CD4^{pos}$ cells in one experiment and a failure of $CD4^{pos}$, $NK1.1^{neg}$ to produce IL-4 in a second experiment (Fig. 10). These results indicate that the cells producing IL-4 in response to anti-CD3 and to SEB were of the same phenotype.

Discussion

Primed $CD4^{pos}$ T cells, both in vitro and in vivo, generally exhibit either a Th1- or Th2-like phenotype (2, 43–48). Recently, the mechanisms by which a particular T cell lin-

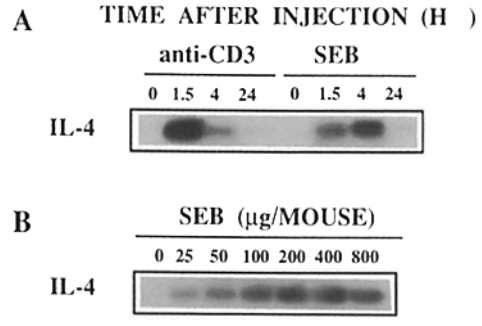

Figure 8. In vivo challenge with SEB can stimulate prompt production of IL-4. (*A*) C57BL/6 mice were challenged intravenously with 1.33 μg of anti-CD3 or 100 μg of SEB and spleens were removed for RNA extraction at indicated times. (*B*) C57BL/6 mice were injected with various doses of SEB as indicated and sacrificed at 4 h after injection to prepare total spleen RNA. IL-4 mRNA levels were analyzed by RT-PCR with Southern blot analysis.

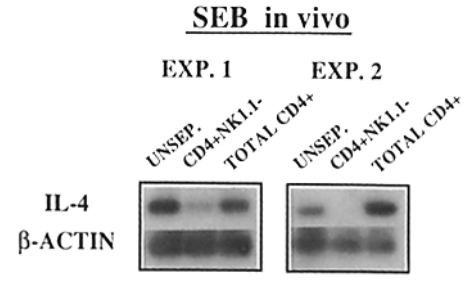

Figure 10. $CD4^{pos}$,$NK1.1^{pos}$ T cells promptly produce IL-4 in response to in vivo challenge with SEB. Spleen cells from C57BL/6 mice injected with 200 μg of SEB 4 h earlier were sorted into total $CD4^{pos}$ and $CD4^{pos}$, $NK1.1^{neg}$ populations. Expression of IL-4 and β-actin mRNA was measured by RT-PCR with Southern blot analysis. Two experiments are shown. In the first, 1 μg of RNA prepared from each group was used; in the second, 0.33 μg of RNA was used.

eage is steered down the path toward these distinctive phenotypes have been clarified both by studies of in vitro priming requirements (6–8) and by examining in vivo responses to infectious agents and to conventional antigens (9–12). These studies have emphasized the importance of cytokines themselves in the determination of a lymphokine-producing phenotype. IL-4 has been shown to be critical for priming cells to become IL-4 producers (6, 7) and IL-12 enhances priming for IFN-γ production (49, 50). In addition, IL-2, IFN-γ, and TGF-β have been shown to have effects on the priming process (4, 51–54). If we limit ourselves to the two lymphokines that have a dominant role, IL-4 and IL-12, a potential source of one of these, IL-12, available at the time of priming and produced under conditions that are appropriate for priming cells to become IFN-γ-producers, has been identified (49, 50). That is, macrophages infected with intracellular microorganisms or even exposed to heat-killed *Listeria monocytogenes* produce substantial amounts of IL-12 (49, 55). Since IFN-γ, produced by T cells primed in the presence of IL-12, enhances the capacity of macrophages to destroy intracellular pathogens, a logical link between production of the inducer and the effect of the product is established.

For IL-4, the situation is more complex. First, the inducer and the product are the same (6, 7). Second, IL-4 is known to be made mainly by activated T cells and by FcεRI^{pos} cells, mainly cells with the morphology of basophils (17, 18). Neither of these cell populations appears ideally suited to be a physiologic source of IL-4 that could be used in the priming process to drive naive cells to become IL-4 producers. For the activated CD4^{pos} T cells to do so would require that the inducing antigen could lead to their activation. Unless the inducing antigen had polyclonal stimulating capacities, IL-4 production in response to it would imply that the response was secondary, not primary, and would thus not solve the problem of how primary IL-4–dominated responses were induced. The difficulty about FcεRI^{pos} cells being the principal source of IL-4 available at the time of priming is that the only established physiologic pathway through which these cells are stimulated to produce IL-4 is by cross-linkage of FcεRI or FcγRII/III (16, 17). In both cases, the cross-linkage would normally be dependent on antibodies of the IgE or IgG classes. Since the production of IgE (56), mouse IgG1 (57), and human IgG4 (58) is IL-4 dependent, an FcR-mediated stimulation of lymphokine production would appear to be likely only after the production of IL-4 had occurred. It is, of course, possible that non-FcR–mediated stimulation of basophils and/or mast cells stimulates IL-4 production; studies addressing this possibility are underway. Nonetheless, these arguments have led us to more seriously consider the possibility that cell types other than basophils and mast cells might be responsible for the initial burst of IL-4 production that plays a central role in the priming process.

We have confirmed the prior observations that spleen cells can produce large amounts of IL-4 mRNA in response to acute in vivo treatment with anti-CD3 (19, 20). Our studies reveal several quite interesting features of this response. First, in response to intravenous injection, IL-4 production appears to be mainly a property of spleen cells, although popliteal lymph nodes do show substantial IL-4 mRNA in response to foot pad injection of anti-CD3. Even more striking is the fact that rapid induction of IL-4 mRNA and secretion of IL-4 in response to anti-CD3 is not mimicked by in vitro exposure of spleen cell suspensions or of spleen cell fragments to anti-CD3. Although this might still be explained by a critical microenvironmental requirement for stimulation of T cells to produce IL-4 promptly, it is equally consistent with the possibility that the cell that is responsible for lymphokine production is not in the spleen at the time of injection of anti-CD3 but only migrates there after stimulation. However, we have cultured blood cells in vitro with anti-CD3 and did not observe prompt production of IL-4 (data not shown) suggesting that homing of CD4^{pos} peripheral blood T cells to the spleen after activation does not explain these results. For the moment, the difference between in vivo and in vitro responses to anti-CD3 are unexplained.

Our results implicate an unusual T cell population as the major producer of IL-4 in response to in vivo injection with anti-CD3. Thus, we observed that CD4^{pos} T cells were the major cell population that expressed IL-4 mRNA in response to anti-CD3. Among the CD4^{pos} cells, it was cells that expressed low levels of LECAM-1, high levels of CD44, and low levels of CD45RB that had virtually all the IL-4–producing capacity (Fig. 6 *A*). Although this phenotype is consistent with the IL-4–producing cell being a recently activated T cell (42), the observation that the great majority of IL-4 was made by the relatively small number of NK1.1^{pos} cells in that population, suggests that prompt IL-4 production in vivo may be mediated by a specialized cell population. Indeed, cells with this phenotype and related phenotypes have already been implicated as the major source of IL-4 among thymocytes (21, 59, 60). Thymic migrants within the spleen retain substantial IL-4–producing activity in response to in vitro treatment with anti-CD3, but only for a few days (60). It has been shown that a population of CD4^{pos}, NK1.1^{pos} cells is present within the bone marrow (21). Although we found only modest amounts of IL-4 mRNA in bone marrow cells in response to injection of anti-CD3, it is possible that these cells only gain high level responsiveness in vivo upon redistribution to the periphery. However, we have not excluded the possibility that normal CD4^{pos} T cells that become primed for IL-4 production in vivo acquire the expression of NK1.1, just as they become LECAM-1^{dull}, CD44^{pos}, and CD45RB^{dull}.

The alternative possibilities (i.e., a specialized cell population or a population of recently primed cells) are consistent with the observed lack of requirement of IL-4 at the time of stimulation for production of IL-4 in response to in vivo anti-CD3. Similarly, the finding that the production of IL-4 in response to anti-CD3 is inhibited by prior treatment of mice with CTLA-4Ig (Fig. 7 *B*) while indicating an important requirement for a costimulatory signal (34), does not distinguish between these two alternatives. It has been reported that the CD4^{pos},NK1.1^{pos} cell population displays a skewed expression of TCR-β chains, with Vβ8, Vβ7, and Vβ2

dominating (61). We are in the process of determining whether Vβ8 is also more highly represented among the IL-4 producing cells derived from in vivo challenge with anti-CD3.

The possibility that the $CD4^{pos}$,$NK1.1^{pos}$ cells described here make an important contribution to the production of the IL-4 that biases T cell responses to the Th2-like pathway would depend very greatly on the likelihood that such cells could be induced to produce IL-4 in response to natural immunization with agents that normally induce IL-4–dominated responses. We think it unlikely that responses to conventional T cell epitopes on these immunogens could recruit a sufficiently large fraction of the potential IL-4 producers to cause the secretion of enough IL-4 to affect a true primary response. However, the finding that SEB can induce a response rather like that mediated by anti-CD3, although of lower magnitude (Figs. 8 and 9), raises the possibility that immunogens that have associated superantigens might be able to stimulate a sufficient number of $CD4^{pos}$,$NK1.1^{pos}$ so that the local concentration of IL-4 available at the time of priming might be adequate for the differentiation of naive, antigen-specific T cells to develop into IL-4 producers. This would predict that agents such as nematodes and allergens would have associated superantigens.

Despite the many unresolved issues raised by these experiments, the demonstration of a cell population that promptly expresses IL-4 mRNA in vivo in response to receptor-ligation provides an important advance in the effort to determine the physiologic mechanisms through which immunization leads to $CD4^{pos}$ T cell responses dominated by Th1-like cells or Th2-like cells.

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