

Cellular and Molecular Mechanisms for Reduced Interleukin 4 and Interferon- γ Production by Neonatal T Cells

David B. Lewis,* Charles C. Yu,* Jeff Meyer,* B. Keith English,* Stuart J. Kahn,* and Christopher B. Wilson*[‡]

*Divisions of Immunology/Rheumatology and Infectious Diseases, Departments of Pediatrics and Immunology,[§] University of Washington and Children's Hospital and Medical Center, Seattle, Washington 98105; and [‡]Immunex Corporation, Seattle, Washington 98101

Abstract

The mechanisms by which T lymphocytes acquire the capacity to produce interleukin 4 (IL-4) and other lymphokines during intrathymic and extrathymic development are poorly understood. To gain insight into this process, we determined the capacity of human neonatal and adult T lineage cell populations to produce IL-4 after polyclonal activation. IL-2 and interferon- γ (IFN- γ) production were studied in parallel, since their production by neonatal T cells is known to be similar or diminished, respectively, compared to adult T cells. Production of IL-4 by neonatal CD4⁺ T cells and IFN- γ by neonatal CD4⁺ and CD8⁺ T cells was markedly lower compared with analogous adult cell populations, whereas IL-2 production was similar. Transcription of IL-4, as determined by nuclear run-on assays, and IL-4 mRNA-containing cells, as determined by in situ hybridization, were undetectable in neonatal T cells, whereas both were detectable in adult T cells. IFN- γ transcription and IFN- γ mRNA-containing cells were reduced in neonatal T cells compared with adult T cells. Reduced lymphokine production by neonatal T cells correlated with their lack of a CD45R⁻ (putative memory T cell) population; cells with this surface phenotype comprised 30–40% of the adult CD4⁺ T cells and were highly enriched for IL-4 and IFN- γ , but not IL-2 production. IL-4, IFN- γ , and IL-2 mRNA expression by neonatal CD4⁺CD8⁻ thymocytes was similar to that found in circulating neonatal CD4⁺ T cells. Taken together, these findings suggest that the extrathymic generation of memory T cells during postnatal life may result in an increased capacity for IL-4 and IFN- γ gene expression. In addition, IFN- γ and IL-2 mRNA were significantly more abundant than IL-4 mRNA in activated neonatal CD4⁺CD8⁻ thymocytes and CD4⁺ T cells, as well as adult CD4⁺ CD45R⁻ T cells. Therefore, the capacity of T lineage cells to express the IL-4 gene may be more restricted compared to other lymphokine genes beginning in intrathymic development. This restricted capacity appears to persist during postnatal extrathymic maturation of T cells. (*J. Clin. Invest.* 1991. 87:194–202.). Key words: CD45 • interferon- γ • interleukin 4 • memory T cells • neonatal T cells

Introduction

Secretion of lymphokines such as the interleukins (IL) IL-2 and IL-4 and interferon- γ (IFN- γ) by activated T cells is an essen-

tial component of antigen-specific immunity (1–3). How T cells acquire, during their intrathymic and extrathymic maturation, the capacity to produce particular lymphokines remains largely unknown. Recently we have shown that virtually all IL-4 and most IFN- γ produced by polyclonally activated adult CD4⁺ T cells is mediated by a CD45R⁻ subset, whereas CD45R⁺ and CD45R⁻ CD4⁺ T cells produce comparable amounts of IL-2 (4); the CD45R⁻ subset, which is enriched in functional memory cells and is UCHL1⁺, appears to be derived from CD45R⁺ (virgin) T cells which have been activated in vivo, presumably by suitably presented cognate antigen (5–9). We reasoned that if previous in vivo activation is a major determinant of the capacity for T cells to produce IL-4 and IFN- γ , then neonatal T cells, which presumably have had a minimal exposure to exogenous antigens, should exhibit a profile of lymphokine production similar to that of adult virgin T cells. In agreement with this prediction, reduced IFN- γ production by neonatal T cells compared to adult T cells has been noted by us and other workers (10–14). We now report that such a selective reduction in both IL-4 as well as IFN- γ gene expression by activated neonatal T cells does occur. This pattern of lymphokine production correlates with a uniformly CD45R⁺ surface phenotype on neonatal T cells, and is largely transcriptionally regulated. A similar selective reduction in IL-4 and IFN- γ compared with IL-2 gene expression was observed in CD4⁺, CD8⁻ thymocytes, suggesting that the results with neonatal T cells may reflect in part their antigenically naive status.

Methods

Monoclonal antibodies (MAb). The following murine anti-human MAb were used for either cell purification or flow cytometric analysis: 9.6, anti-CD2, IgG2a (15); OKT4, anti-CD4, IgG2b (16); 66.1, anti-CD4, IgM (17); OKT8, anti-CD8, IgG2a (18); FC-1, anti-CD16, IgM (19); 3AC5, anti-CD45R (200-, 220-kD isoforms), IgG2a (20, 21); and UCHL1, anti-CD45 (180-kD isoform), IgG2a (21, 22). The 9.6 and 66.1 MAb were provided by Dr. Paul Martin, Fred Hutchinson Cancer Research Center, Seattle, WA; FC-1 and 3AC5 MAb by Dr. Edward Clark, University of Washington, Seattle, WA; and UCHL1 MAb by Dr. Peter C. L. Beverley, Imperial Cancer Research Fund–Human Tumour Immunology Unit, London, UK. The OKT4 and OKT8 hybridomas were purchased from the American Type Culture Collection, Rockville, MD. RPC-5 (IgG2a) and MOPC-195 (IgG2b) murine MAb, which served as isotype-specific negative controls for flow cytometric analysis were purchased from Litton Bionetics, Kensington, MD as affinity-purified preparations. All other MAb were used in the form of sterile ascites except for UCHL1 which was used as a sterile hybridoma culture supernatant.

Cell preparations. Peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of adult volunteers or umbilical cord blood of healthy term neonates using Ficoll-Hypaque density gradient centrifugation (23), and T cells were prepared by treatment of PBMC with T cell Lymphokine, (One Lambda, Los Angeles, CA) as previously described (4). T cell preparations were routinely $\geq 97\%$

Address reprint requests to Dr. Lewis, Division of Immunology/Rheumatology, Children's Hospital and Medical Center, P.O. Box C-5371, Seattle, WA 98105.

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CD2-positive as assessed by flow cytometry after indirect immunofluorescent staining with MAb 9.6. CD4⁺ and CD8⁺ T cell subsets were purified by negative selection using MAb and complement: T cells were incubated with saturating concentrations of either OKT8 or 66.1 MAb, respectively, for 30 min at 4°C in RPMI-1640 medium (Mediatech, Washington, DC) containing 2% FCS (Hyclone Laboratories, Logan, UT). The anti-CD16 MAb, FC-1 was included in these incubations to deplete residual NK cells. After washing at 4°C, the cells were resuspended in RPMI medium with 20% (vol/vol) HLA-typing grade rabbit complement (Pel-Freez Biologicals, Brown Deer, WI) and incubated at 37°C for 1 h with gentle agitation. Remaining viable cells were collected by Ficoll-Hypaque density gradient centrifugation and washed twice in RPMI medium with 2% fetal calf serum (FCS) before use. Adult CD4⁺ T cells were further fractionated into CD45R⁺ and CD45R⁻ populations by incubation with MAb UCHL1 or 3AC5, respectively, followed by negative selection with indirect panning (24) using plastic petri dishes (Baxter Scientific Products, McGaw Park, IL) previously coated with affinity-purified goat anti-mouse IgG (Tago Inc., Burlingame, CA). Human thymus samples were obtained from neonates undergoing cardiac surgery for congenital heart disease who were free of other significant congenital anomalies. After removal of fibrous capsular tissue and grossly visible blood vessels, samples were finely minced and pressed through sieves, and the viable thymocytes were collected by Ficoll-Hypaque centrifugation. CD4⁺ CD8⁻ single-positive thymocytes were prepared by incubating unfractionated thymocytes with MAb OKT8 for 30 min at 4°C in RPMI medium with 2% FCS. The cells binding MAb were lysed using rabbit complement and the remaining viable cells subsequently isolated as described above for the purification of the CD4⁺ and CD8⁺ peripheral T cells. The purity of all T cell and thymocyte subsets was > 95% as determined by immunofluorescent flow cytometry after indirect staining with appropriate MAb.

T cell and thymocyte activation. Cells (5×10^6 /ml) were incubated with either 0.5 μ M ionomycin (Calbiochem-Behring Corp., San Diego, CA), 25 μ g/ml concanavalin A (Con A; Pharmacia Fine Chemicals, Piscataway, NJ), or OKT3 (anti-CD3) MAb (18) (1:25 [vol/vol] of sterile azide-free ascites) in combination with 50 ng/ml phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO); these concentrations were found to be optimal for lymphokine induction in preliminary experiments. RPMI 1640 medium containing 5% (vol/vol) human AB serum was used in all cases except for experiments assaying IL-4 and IFN- γ protein in cell culture supernatants, for which AIM-V, a commercial serum-free medium (Gibco Laboratories, Grand Island, NY), was employed. Both media were supplemented with 2 mM L-glutamine, 50 U/ml penicillin G, and 50 μ g/ml streptomycin.

IL-4 and IFN- γ protein assay. T cell culture supernatants were collected after 24 h of ionomycin and PMA treatment and concentrated using a Centricon-10 apparatus (Amicon Corp., Danvers, MA)

as previously described (4). The IL-4 and IFN- γ content of concentrated supernatants was determined by radioimmunoassay (RIA) as described (4, 25). The final IL-4 and IFN- γ concentrations reported are calculated for cell culture supernatants before concentration, assuming 100% recovery. The recovery of samples spiked with recombinant human IL-4 or IFN- γ and concentrated was ~ 70–80%. The results for IL-4 and IFN- γ protein concentrations for three of these adult T cell supernatants have been reported previously (4). For both IL-4 and IFN- γ RIA determinations, neonatal and adult T cell samples were analyzed in parallel.

In vitro transcription with isolated nuclei. Transcription assays were performed as previously described (26), using nuclei isolated from T cells after 1.5 or 3 h of incubation with ionomycin and PMA.

RNA blot analysis. Total cellular RNA was isolated by the guanidinium thiocyanate/CsCl method (27), electrophoresed in 2.2 M formaldehyde, 1% agarose gels, blotted, hybridized, and washed as previously described (4, 14). ³²P-labeled RNA probes were synthesized from human IL-4 (28), IFN- γ (29), and IL-2 (30) cDNAs subcloned into transcription vectors, using a commercially available kit (Promega Biotech, Madison, WI). The human elongation factor 1- α (EF)¹ cDNA probe (provided by R. Perlmutter, University of Washington) was ³²P-labeled by the random hexamer primer method (31). The cDNAs were all full length except for IFN- γ for which a 0.4-kb internal fragment was used (14).

In situ hybridization. In situ hybridization was performed exactly according to the method of Pardoll et al. (32), using ³⁵S-labeled single-stranded anti-sense or sense RNA probes prepared from the IL-4, IFN- γ , and IL-2 cDNAs as previously described (4). After counter staining with Diff-Quik reagent according to the manufacturer's instructions (Dade Diagnostics, Aguada, Puerto Rico) slides were permanently mounted and coded. For each slide, the number of grains over 300 individual cells was determined by counting 75 cells in each of four randomly selected fields free of obvious artifacts. Cells were considered positive for a mRNA species if their grain counts after hybridization with the antisense probe exceeded that of 99% of cells hybridized with the control sense probe.

Indirect immunofluorescent staining and flow cytometric analysis. Unless otherwise indicated, PBS (pH 7.4) containing 1% BSA and 0.1% sodium azide was used to dilute all reagents and for cell washes. Cells were washed once and then incubated with saturating concentrations of diluted sterile MAb for 30 min at 4°C. After two washes, the cells were incubated with affinity purified fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG or IgM F(ab')₂ fragments (Tago Inc.) diluted 1:40 (vol/vol). The cells were subsequently washed three times and fixed in 2% paraformaldehyde/PBS for 5 min at room temperature, and then transferred to PBS. At least 3×10^3 cells per sample were analyzed using a flow cytometer (Epics C, Coulter Electronics, Inc., Hialeah, FL) equipped with a 2-W argon laser.

Results

Decreased production of IL-4 and IFN- γ by neonatal T cells.

After treatment with ionomycin and PMA, which induces maximal IL-4 and IFN- γ production by adult T cells (4), IL-4 concentrations exceeded 10 pM in cell culture supernatants from three different adult donors but were below the 3.5 pM limit of detectability in all neonatal samples (Table I). Unlike IL-4, IFN- γ was detectable in all neonatal T cell culture supernatants, but in concentrations (25–30 pM) that were uniformly < 10% of those measured in adult T cell supernatants (Table I), as reported previously (4, 13, 14). Nevertheless, IFN- γ production by neonatal T cells was significantly greater than IL-4 pro-

Table I. IL-4 and IFN- γ Protein Concentrations in Supernatants of Activated Adult and Neonatal T Cell Cultures

Cell type	IL-4	IFN- γ
	pM	
Adult		
A	14.6	1762
B	34.6	4164
C	26.8	4766
D	46.0	ND
Neonate		
A	<3.5	30.0
B	<3.5	29.5
C	<3.5	26.5
D	<3.5	ND

1. Abbreviations used in this paper: EF, elongation factor 1- α ; LCA, leukocyte common antigen; PKC, protein kinase C.

duction on a molar basis, as previously reported for adult T cells (4).

Decreased IL-4 and IFN- γ production reflects diminished gene transcription and mRNA accumulation. The production of IFN- γ and IL-2 by T cells appears to be primarily regulated by the rate at which these lymphokine genes are transcribed (33–37). To determine whether decreased IL-4 and IFN- γ protein production by neonatal T cells was also regulated in this manner, the transcription rates of the IL-4 and IFN- γ genes were compared in cell nuclei purified from activated adult and neonatal T cells (Fig. 1). The IL-2 gene, whose product is expressed in similar amounts by adult and neonatal T cells (12–14), and the EF gene, whose transcript comprises ~0.5% of mRNA in most types of mammalian cells (4), were transcribed at similar rates by nuclei from adult and neonatal T cells and served as positive controls in these and subsequent experiments. IL-4 gene transcription by activated adult T cells was detectable as shown by the greater signal with the slot-blotted IL-4 cDNA plasmid than the negative control plasmid (Fig. 1 B). However, the IL-4 gene appeared to be transcribed in adult T cells at a markedly lower rate than the IFN- γ , IL-2, or EF genes, and, unlike these other genes, required high concentrations of labelled nuclear RNA to detect its transcription (Fig. 1, A vs. B). In contrast, IL-4 gene transcription by neonatal T cells was not clearly detectable (as shown by a signal essentially equal to that of the negative control) even when high concentrations of labelled transcripts were used for hybridization (Fig. 1 B), and the rate of IFN- γ gene transcription, although measurable, was much lower than in adult T cells (Fig. 1 A). Thus, the

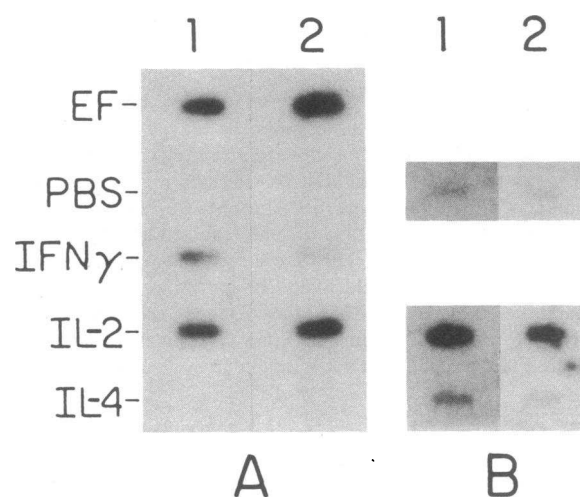


Figure 1. Transcription rates of the IL-4, IFN- γ , IL-2, and EF genes in nuclei from activated adult and neonatal T cells; PBS refers to the negative control, the plasmid, pBluescribe. (A) Nuclei from adult (lane 1) or neonatal T cells (lane 2) incubated with ionomycin and PMA for 3 h. Both filters were hybridized with 1.0×10^6 dpm/ml of 32 P-labelled transcripts. (B) Nuclei from adult (lane 1) or neonatal T cells (lane 2) incubated with ionomycin and PMA for 1.5 h. Both filters were hybridized with 2.5×10^6 dpm/ml of 32 P-labelled transcripts. Integrated densitometry of the bands done with a densitometer (Visage 60, BioImage, Ann Arbor, MI) after subtraction of the values for the PBS negative control, gave the following results (arbitrary units): experiment A—adult (lane 1) EF 7.0, IFN- γ 2.3, IL-2 5.8, IL-4 —; neonatal (lane 2) EF 10.1, IFN- γ 0.5, IL-2 6.5, IL-4 —; experiment B—adult (lane 1) IL-4 1.03, IL-2 11.93; neonatal (lane 2) IL-4 0.16, IL-2 6.35.

transcription rates of the IL-4 and IFN- γ genes in adult and neonatal T cells corresponded well with the amounts of IL-4 and IFN- γ protein these cells secreted, suggesting that production of these lymphokines was largely regulated at a transcriptional level in both cell types.

The decreased transcription of the IL-4 and IFN- γ genes by neonatal T cells was associated with diminished steady-state levels of these lymphokine mRNAs (Fig. 2 A). Adult T cells contained markedly more IL-4 mRNA than did neonatal cells, in which such transcripts were in some cases completely undetectable (Fig. 2, A and B). IFN- γ mRNA was routinely measurable after a shorter period of exposure of autoradiographs than was necessary for the detection of IL-4 mRNA, consistent with the relative concentrations of these lymphokines in culture supernatants. Although T cells from different individuals varied in their levels of IL-2 mRNA, in general, the amounts of these transcripts in adult and neonatal T cells were comparable as previously reported (4, 13, 14). Similar results for IL-4, IFN- γ , and IL-2 mRNA accumulation were also observed using T cells from an additional six adult and six neonatal subjects (data not shown), and when other stimuli were used (either Con A and PMA or anti-CD3 mAb and PMA—see Fig. 2, B and C), indicating that these differences were not unique to particular individuals or the conditions used for cell activation. Because these other stimuli were generally less effective than ionomycin and PMA, in subsequent experiments cells were stimulated with ionomycin and PMA to maximize detection of the IL-4 and IFN- γ gene products.

The markedly reduced levels of IL-4 and IFN- γ mRNA in neonatal T cells were not attributable to kinetic differences between adult and neonatal cells in the accumulation of these transcripts (Fig. 2 C). In both cell types, lymphokine mRNA was undetectable before *in vitro* activation (lanes 1 and 8) and peak levels of IL-4, IFN- γ , and IL-2 mRNA were achieved by ~6 h of incubation with ionomycin and PMA or Con A and PMA. In neonatal T cells, IL-4 mRNA, if measurable, was only observed at 6 h of incubation and not at later times (data not shown). After hybridization with a full-length IL-2 cDNA probe as shown in Fig. 2 C, transcripts were observed at 24 h of incubation in neonatal and adult T cells, which had a lower apparent molecular weight than those which accumulated by 6 h of incubation (lanes 4, 7, and 11). However, these late-appearing transcripts were undetectable after hybridization with an IL-2 cDNA probe which lacked the last 100 bp of the 3' untranslated region but included the entire coding sequence (30), whereas the earlier-appearing transcripts were still present (data not shown). Thus, these late-appearing transcripts presumably did not encode IL-2, but cross-hybridized with a portion of the 3' untranslated region of the IL-2 gene. EF mRNA was present in activated neonatal and adult T cells in similar amounts, indicating that the differences observed in lymphokine mRNA accumulation were not attributable to differences in the amount of RNA analyzed in each lane.

Expression of IL-4, IFN- γ , and IL-2 mRNA by T cell subsets. Because IL-4 mRNA was measurable by RNA blotting in at least some activated neonatal T cell samples under conditions in which IL-4 gene transcription or secreted IL-4 protein was undetectable, we used this more sensitive technique to compare lymphokine gene expression by adult and neonatal CD4⁺ and CD8⁺ T cells after ionomycin and PMA activation (Fig. 3). In agreement with previous observations (4), among adult T cells, the CD4⁺ subset was enriched for IL-2 and IL-4

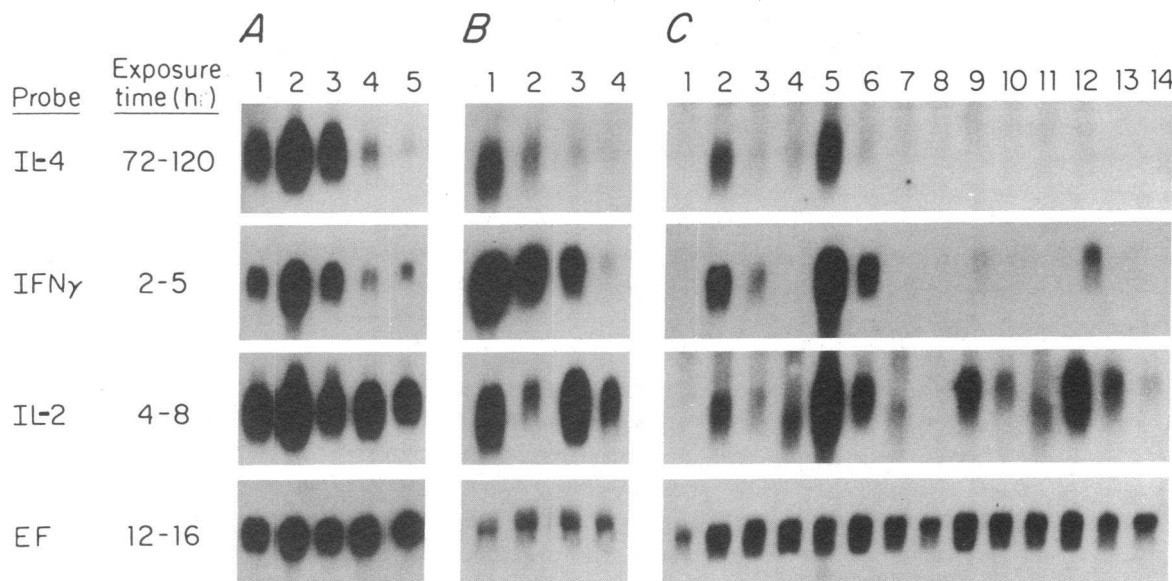


Figure 2. Total RNA samples hybridized with IL-4, IFN- γ , IL-2, and EF probes. All blots are representative of at least three different experiments. (A) T cells lymphokine mRNA accumulation after ionomycin and PMA incubation for 6 h. Lanes 1-3, adult T cells from three different individuals. Lanes 4 and 5, neonatal T cells from two different individuals. (B) Lymphokine mRNA accumulation in adult vs. neonatal T cells under different conditions of activation. Lanes 1 and 2, adult T cells incubated for 6 h with ionomycin and PMA or OKT3 and PMA, respectively. Lanes 3 and 4, neonatal T cells incubated for 6 h with ionomycin and PMA or OKT3 and PMA, respectively. (C) Kinetics of lymphokine mRNA accumulation in adult vs. neonatal T cells. Lanes 1-4, adult T cells incubated for 0, 6, 12, and 24 h, respectively, with Con A and PMA. Lanes 5-7, adult T cells incubated for 6, 12, and 24 h, respectively, with ionomycin and PMA. Lanes 8-11, neonatal T cells incubated for 0, 6, 12, and 24 h, respectively, with Con A and PMA. Lanes 12-14, neonatal T cells incubated for 6, 12, and 24 h, respectively, with ionomycin and PMA. All lanes were loaded with 5 μ g of total RNA, except for lanes 3 and 4 of B in which 10 μ g of total RNA was loaded.

mRNA, whereas the CD8 $^{+}$ subset was enriched for IFN- γ transcripts (lanes 1 and 2). The marked reduction in IL-4 mRNA, but similar amounts of IL-2 mRNA observed in unfraction-

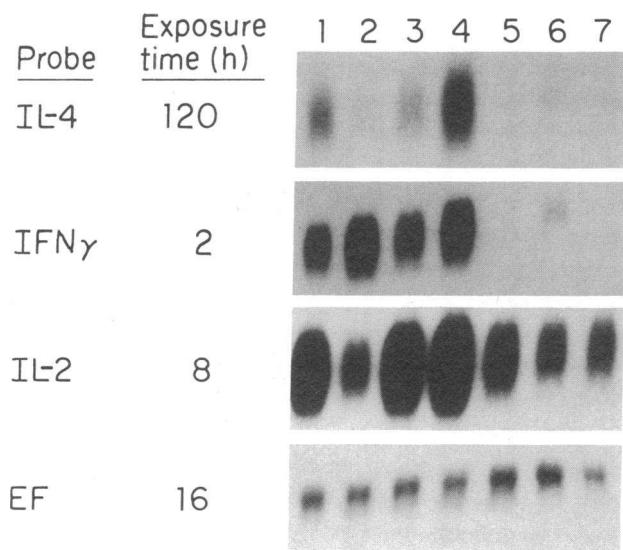


Figure 3. Total RNA samples from subsets of adult and neonatal T cells and neonatal thymocytes after incubation with ionomycin and PMA for 6 h, hybridized with IL-4, IFN- γ , IL-2, and EF probes. This blot is representative of two separate experiments. Lanes 1 and 2, adult CD4 $^{+}$ and CD8 $^{+}$ T cells, respectively. Lanes 3 and 4, adult CD4 $^{+}$ CD45R $^{+}$ and CD4 $^{+}$ CD45R $^{-}$ T cells, respectively. Lanes 5 and 6, neonatal CD4 $^{+}$ and CD8 $^{+}$ T cells, respectively. Lane 7, neonatal CD4 $^{+}$ CD8 $^{-}$ thymocytes. All lanes were loaded with 5 μ g of total RNA.

ated neonatal T cells compared to adult cells, was also found in purified neonatal CD4 $^{+}$ T cells (lane 1 vs. lane 5). Like adult T cells, the CD8 $^{+}$ subset of neonatal T cells was enriched in IFN- γ mRNA and the CD4 $^{+}$ subset was enriched for IL-2 mRNA, but differences between CD4 $^{+}$ and CD8 $^{+}$ neonatal T cells and between CD8 $^{+}$ adult and neonatal T cells in IL-4 mRNA levels could not be evaluated because of their very low to undetectable amounts of IL-4 transcripts.

The relatively low levels of IL-4 and IFN- γ mRNA in activated neonatal T cells could be explained by either a reduced number of cells expressing these mRNAs or a reduced level of IL-4 or IFN- γ transcripts per cell or both. To distinguish between these possibilities, we performed *in situ* hybridization. Results representative of one of three such experiments are shown in Fig. 4 and Table II. Among activated adult cells, IFN- γ and IL-2 mRNA was expressed by \sim 40% of T cells, and in a greater percentage of CD8 $^{+}$ and CD4 $^{+}$ T cells respectively, whereas IL-4 mRNA expression appeared restricted to a small fraction (\leq 5%) of CD4 $^{+}$ T cells, as previously described (4). In striking contrast, IL-4 mRNA was undetectable in all neonatal T cell fractions (Fig. 4 and Table I), even after autoradiographic exposure of slides for up to 5 wk (data not shown). All neonatal T cell fractions also had markedly fewer cells with detectable IFN- γ mRNA. In those neonatal CD4 $^{+}$ or CD8 $^{+}$ T cells which were positive for IFN- γ transcripts, the average amount of IFN- γ mRNA per cell, as assessed by grain counts, was lower than in the adult cell population (Fig. 4, data not shown). Like adult cells, neonatal CD4 $^{+}$ and CD8 $^{+}$ T cells were enriched for IL-2 and IFN- γ transcripts respectively, compared to unfractionated T cells, in agreement with the results obtained by RNA blotting.

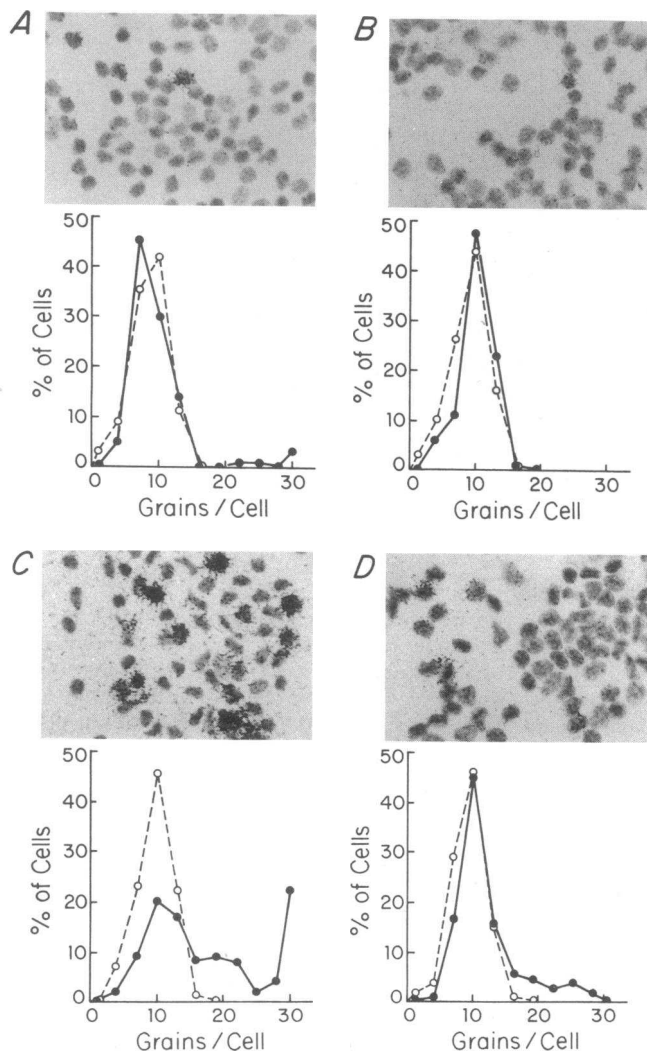


Figure 4. Adult or neonatal T cells after incubation with ionomycin and PMA for 6 h, hybridized in situ for IL-4 and IFN- γ mRNA. Photomicrographs ($\times 24$) of adult (A and C) or neonatal (B and D) T cells hybridized with an anti-sense probe for IL-4 (A and B) or IFN- γ (C and D). Directly below each photomicrograph is the corresponding histogram of the number of grains per cell after hybridization with an antisense probe (●) for the slide depicted, or with a control sense probe (○) by use of a duplicate slide processed in parallel. All slides were exposed for 7 d before development.

Expression of IL-4, IFN- γ , and IL-2 mRNA by CD45R⁺ or CD45R⁻CD4⁺ T cells. Because IL-4 production by adult T cells appeared to be almost exclusively limited to a small fraction of CD4⁺ T cells which was undetectable in the peripheral blood of neonates, we sought to further define this population. Previous studies have shown that CD4⁺ T cells that augment B cell immunoglobulin production are enriched in proliferative responses to recall antigens and express the 180-kD isoform of the leukocyte common antigen (LCA) (recognized by MAb UCHL1), whereas the mutually exclusive subset of CD4⁺ cells that expresses the 200- and 220-kD isoforms of the LCA, CD45R (recognized by MAb 3AC5), are ineffective in providing such help and are not enriched in functional memory cells (6–9, 38, 39). These two LCA isoforms do not appear to identify distinct lineages but rather T cells which have (CD45R⁻,

Table II. IL-4, IFN- γ , and IL-2 mRNA Expression in Adult or Neonatal T Cells and T Cell Subsets after Activation with Ionomycin and PMA for 6 h

Cell type	Cells positive for mRNA		
	IL-4	IFN- γ	IL-2
	%		
Unfractionated T cells			
Adult	3	41	42
Neonate	<1	3	35
CD4 ⁺ T cells			
Adult	4	34	51
Neonate	<1	2	45
CD8 ⁺ T cells			
Adult	1	58	20
Neonate	<1	4	16

The results presented are representative of three (unfractionated T cells) or two (CD4⁺ and CD8⁺ T cells) separate experiments. Slides hybridized with the IL-4 probe were exposed for 14 d, whereas those hybridized with the IFN- γ and IL-2 probes were exposed for 7 d.

UCHL1⁺) or have not (CD45R⁺, UCHL1⁻) been previously primed by their exposure to cognate antigen in vivo (7–9, 40, 41), although this interpretation has been disputed by others (42). The CD45R⁻ subpopulation of adult CD4⁺ T cells is enriched in the production of IL-4 as well as IFN- γ (4). Recently others have found that nearly all neonatal T cells are CD45R⁺, UCHL1⁻ (5, 43, 44). By immunofluorescent flow cytometry we confirmed that both CD4⁺ and CD8⁺ neonatal T cells were almost all CD45R (3AC5)-positive and UCHL1-negative or dull; in contrast, similar populations of adult T cells demonstrated the previously described (7–9, 40, 41) bimodal pattern of surface staining: ~40% of cells expressed UCHL1 and ~60% of cells expressed CD45R in a mutually exclusive manner (data not shown).

Thus, to directly compare their lymphokine mRNA expression with that by neonatal CD4⁺ T cells, adult CD4⁺ T cells were fractionated into CD45R⁺ (UCHL1⁻) and CD45R⁻ (UCHL1⁺) subsets. Cell purification was accomplished by negative selection using MAb 3AC5 and UCHL1, since the activation characteristics of T cells can be altered by treatment with MAb directed against LCA (19). IL-4 mRNA expression in adult CD4⁺ T cells was virtually limited to the CD45R⁻ fraction and IFN- γ mRNA expression was also highly enriched in this fraction (Fig. 3, lanes 1 and 4). Activated neonatal CD4⁺ and adult CD4⁺ CD45R⁺ T cells contained similarly low to undetectable amounts of IL-4 mRNA and markedly lower levels of IFN- γ mRNA (lanes 3 and 5) compared to the CD45R⁻ fraction of adult CD4⁺ T cells (lane 4). Similar amounts of IL-2 mRNA were found in the CD45R⁺ and CD45R⁻ fractions of adult CD4⁺ T cells, as well as in neonatal CD4⁺ T cells.

Production of IL-4, IFN- γ , and IL-2 mRNA by CD4⁺ CD8⁻ thymocytes. These results suggested that diminished production of IL-4 and IFN- γ by neonatal CD4⁺ and adult CD4⁺ CD45R⁺ T cells might reflect their antigenically naive or virgin status. If this were so, then a similar pattern of lymphokine expression might be expected for CD4⁺ CD8⁻ thymocytes, a relatively mature population which includes CD4⁺ T lineage

cells destined to emigrate into the circulation (45). This was largely true, in that CD4⁺ CD8⁻ neonatal thymocytes completely lacked detectable IL-4 mRNA and had low amounts of IFN- γ mRNA similar to those of neonatal CD4⁺ T cells. In addition, this thymocyte subset had slightly reduced levels of IL-2 and EF mRNA compared with adult or neonatal CD4⁺ T cells (Fig. 3, lanes 5 and 7). The staining of this filter with methylene blue after hybridizations were completed indicated that equivalent amounts of ribosomal RNA were present in all lanes (data not shown). Similar results were obtained using CD4⁺ CD8⁻ thymocytes from older children (data not shown).

Discussion

We found that IL-4 production by unfractionated and CD4⁺ neonatal T cells was markedly and consistently reduced compared to adult cells after polyclonal activation. We also found that IFN- γ production both by CD4⁺ and CD8⁺ neonatal T cells was diminished compared to adult cells, as previously reported for unfractionated T cells (4, 13, 14). The decreased IL-4 and IFN- γ production is selective in that production by neonatal and adult T cells of IL-2 (this report and references 4, and 12–14) and of tumor necrosis factor- α and - β and the IL-2 receptor p55 chain (13, 46) are comparable. IL-4 production by neonatal T cells was usually undetectable, apparently because of the complete absence of a relatively rare subpopulation present in circulating adult T cells that produced this lymphokine. IFN- γ -producing neonatal T cells, although detectable, were decreased in number and produced lower amounts of IFN- γ per cell compared to adult T cells. Decreased IL-4 and IFN- γ production by neonatal CD4⁺ and CD8⁺ T cells correlated with the complete absence of CD45R⁻ T cells which are highly enriched for IL-4 and IFN- γ -producing cells (4). The results obtained for lymphokine production by neonatal CD4⁺ CD8⁻ thymocytes paralleled those for circulating neonatal CD4⁺ T cells, and suggested that absent IL-4 production and reduced IFN- γ production by neonatal T cells was a functional phenotype established during intrathymic development. The undetectable amount of IL-4 and reduced amount of IFN- γ protein secreted by neonatal T cells was paralleled by proportional decreases in the transcription rates of the IL-4 and IFN- γ genes as well as accumulation of their cognate mRNA, indicating that this decreased lymphokine production was primarily transcriptionally mediated.

In previous reports, reduced IFN- γ production by mitogen activated neonatal leukocytes or T cells has been variously attributed to suppression mediated by radiosensitive CD4⁺ or CD8⁺ T cell populations (47, 48). However, our studies of IFN- γ mRNA expression in bulk cultures or at the individual cell level did not corroborate these findings in that purified CD4⁺ or CD8⁺ neonatal T cells did not accumulate substantially more IFN- γ or IL-4 mRNA than did unfractionated neonatal T cells. Although our results also do not exclude the possibility of suppression of neonatal T cell IFN- γ and IL-4 production by a population found in both the CD4⁺ and CD8⁺ subsets, a more direct explanation is that reduced expression of these lymphokines is a feature intrinsic to naive T cells or, at least, T cells at this stage of development.

There is increasing evidence that memory T cells, defined as those which have previously been activated or "primed" by antigen, are capable of increased levels of lymphokine production, compared to antigenically naive, virgin T cells. T cell

fractionation studies making use of the mutually exclusive expression of CD45R and the 180-kD LCA isoform (recognized by MAb UCHL1 in humans), or markers coexpressed with the 180-kD isoform, such as 4B4 (CDw29), LFA-3, and in the mouse, PgP-1 (CD44), have demonstrated that postimmunization proliferative recall responses to soluble antigens, viruses, and alloantigens are mediated by CD45R⁻ T cell populations (6–9, 38, 49, 50). In humans, this memory T cell-enriched CD45R⁻ subset accounts for virtually all IL-4 and most IFN- γ produced by polyclonally activated adult CD4⁺ T cells (4), and IL-4 production by adult CD8⁺ T cells also appears to be restricted to a CD45R⁻ subpopulation (D. B. Lewis and C. B. Wilson, unpublished observations). Similarly in mice, CD4⁺ or CD8⁺ T cells with high surface levels of PgP-1 are markedly enriched in IFN- γ production compared to PgP-1 dull or unfractionated T cells (51). This enhanced lymphokine production is selective in that CD45R⁺ and CD45R⁻, or PgP-1 dull and PgP-1 bright T cell populations produce similar amounts of IL-2 (4, 9, 50). The observation originally made by Tedder et al. (5) and confirmed by others (43, 44), that human neonatal T cells are uniformly CD45R⁺ whereas circulating adult T cells are ~ 60% CD45R⁺, led them to propose that CD45R⁺ T cells are converted to a CD45R⁻ surface phenotype by a post-thymic maturational process involving, at least in part, T cell activation by cognate antigen (6). The present study confirmed that both CD4⁺ and CD8⁺ neonatal T cells had a CD45R⁺ UCHL1⁻ phenotype, consistent with their presumed antigenically naive status. This model is also supported by the fact that human neonatal or adult CD45R⁺ T cells polyclonally activated *in vitro* acquire a CD45R⁻ UCHL1⁺ surface phenotype (7, 9, 40, 41) and by recent adoptive transfer studies in the rat directly demonstrating that CD45R⁺ T cells are the precursors of the antigen specific CD45R⁻ memory population (52). Taken together, these findings strongly suggest that the similar amount of IL-2, but markedly diminished amounts of IL-4 and IFN- γ produced by neonatal T cells compared to adult T cells is due to the absence of an antigenically primed, or at least previously activated, memory T cell subpopulation.

Studies of postnatal T cell maturation also support a model in which increased lymphokine production is induced by antigenic priming. Mitogen-induced IFN- γ production by peripheral leukocytes increases with postnatal age, as does the number of circulating CD45R⁻ T cells (43, 53), although a direct connection between these events has not been established. Additional evidence comes from longitudinal studies of IFN- γ production in humans after neonatal or adult infection with herpes simplex virus (HSV): peripheral blood mononuclear cells from 2-mo-old infants who were infected as neonates, produced as much IFN- γ in response to HSV antigen as cells from adults 2 mo after primary HSV infection, suggesting that once neonates developed T cells responsive to recall HSV antigens, the capacity of these cells to secrete IFN- γ was similar to that of T cells responding to the same antigen from infected adults (Burchett, S. K., K. Mohan, J. Westall, R. Ashley, L. Corey, and C. B. Wilson, manuscript submitted for publication). In cells from these infants, IFN- γ production after HSV antigen stimulation was equivalent to that observed after polyclonal activation with Con A, consistent with the notion that virtually all T cell-mediated IFN- γ production was by memory cells. In contrast, adult T cells produced much larger amounts of IFN- γ after Con A treatment than after HSV antigen stimulation, presumably because Con A activated significant num-

bers of memory T cells directed against antigens other than HSV that would be expected to be present in adults but not in neonates. Whether antigen-specific IFN- γ production after neonatal or adult infection is predominantly mediated by CD45R⁻ T cells, and antigen-specific IL-4 production parallels that for IFN- γ remains to be determined.

Although the above studies suggest that humans can develop functional memory T cells as early as 2 mo of age, they do not exclude the possibility that this ability is impaired at birth but rapidly matures during postnatal development. Several observations are consistent with this idea. First, in cells from infants infected with HSV as neonates, peak IFN- γ production by memory T cells does not occur until \sim 6 wk after infection, whereas adult cells achieve peak responses after only 2 weeks (Burchett, S. K., K. Mohan, J. Westall, R. Ashley, L. Corey, and C. B. Wilson, manuscript submitted for publication). Whether this delay reflects differences in events occurring proximal to T cell activation, such as in antigen-processing and presentation, or reflects a decrease in antigen-specific T cells or their ability to be clonally expanded, remains to be determined. In previous studies, the antigen-presenting ability of neonatal and adult mononuclear cells in vitro has been comparable (54). Secondly, immunization of full-term infants with T-dependent antigens is often ineffective during the first 2 wk of life, but results in significant antibody responses when performed at 2 mo of age (55). Although decreased neonatal B cell function or interference by maternal antibodies has been proposed to account for these results, a maturational defect in cognate help by T cells is plausible. Finally, neonatal T cells obtained at birth express uniformly high levels of the CD38 antigen (recognized by MAb OKT10), which is also found on virtually all thymocytes but only very small numbers of circulating adult CD45R⁺ T cells (56, 57, and D. B. Lewis and C. B. Wilson, unpublished observations), suggesting that they are, in part, an immature, transitional population. The kinetics of disappearance for circulating CD38⁺ T cells postnatally, and whether this population acquires a CD38⁻ phenotype, or is replaced by a new population of CD38⁻ T cells, remains to be determined. Because surface expression of the CD38 antigen is also increased after T cell activation (58), this may limit its usefulness as a marker with which to follow the extrathymic maturation of neonatal T cells.

A direct comparison of the ability of neonatal and adult CD45R⁺ T cells to develop into functional memory T cells would, therefore, be of interest. One approach is to compare lymphokine production by neonatal and adult CD45R⁺ populations after in vitro priming. Priming of adult murine T cells using antigens, mitogens, or anti-CD3 MAb increases IL-4 and IFN- γ , but not IL-2 production; this appears to mimic the in vivo process by which antigen activation selectively enhances lymphokine production (59–62). These studies are in progress.

Stimulated neonatal CD4⁺ CD8⁻ thymocytes contained undetectable amounts of IL-4 mRNA, and low levels of IFN- γ mRNA compared to adult CD45R⁻ CD4⁺ T cells, a pattern of lymphokine mRNA expression similar to that of circulating neonatal CD4⁺ or adult CD45R⁺ CD4⁺ T cells. In addition, the greater capacity for IL-2 production by CD4⁺ and for IFN- γ production by CD8⁺ circulating neonatal T cells, is also observed for the CD4⁺ CD8⁻ and CD4⁻ CD8⁺ subsets of human thymocytes, respectively (D. B. Lewis, unpublished observations). Although these observations suggest that the capacity for lymphokine gene expression by circulating virgin T cell

populations is largely determined during intrathymic differentiation, CD4⁺ CD8⁻ thymocytes also had modestly reduced levels of IL-2 mRNA compared to circulating CD45R⁺ CD4⁺ T cells. This may reflect the known heterogeneity within CD4⁺ CD8⁻ thymocytes (41, 45, 63) or additional immaturity in lymphokine production by this population.

The molecular mechanism for decreased IL-4 and IFN- γ gene transcription and protein production by neonatal or adult CD45R⁺ T cells compared to the adult CD45R⁻ T cell subset remains to be defined. The intracellular events involved in the induction of lymphokine gene transcription in T cells have been best characterized for the IL-2 gene, and suggest that two signals—an elevation in the intracellular concentration of calcium [Ca²⁺] and activation of protein kinase C (PKC)—are required (64), although a PKC-independent pathway may also exist (65). Neonatal and adult T cells appeared to share similar requirements for the induction of all three of these lymphokine transcripts: in the presence of PMA, a direct PKC activator, the calcium ionophore ionomycin was consistently superior to Con A, which, in turn, was more efficient than anti-CD3 MAb for inducing IL-2, IFN- γ , and IL-4 mRNA in either adult or neonatal T cells. These results suggest that the initial signal transduction pathways for IL-2, IL-4, and IFN- γ gene expression are similar in neonatal and adult T cells. Because IL-2 mRNA expression was comparable in neonatal and adult T cells under the conditions of activation we employed, and because reduced IL-4 and IFN- γ gene transcription was seen with stimuli that would bypass early steps in this signal pathway, the differences observed may be mediated by alternative signal transduction events or events occurring after elevation of [Ca²⁺] and PKC activation. It is also possible that reduced transcription might result from alterations in the interaction of *trans*-acting factors with regulatory regions of the IL-4 and IFN- γ genes. These possibilities are not mutually exclusive and are avenues for future investigation.

We can only speculate as to the potential advantage to the immune system of limiting most IFN- γ production and virtually all IL-4 production to a memory T cell population. Possibly, this might focus the effects of these lymphokines onto suitably responsive effector cells, such as B cells or cytotoxic T cells which have also been previously primed by antigen. It is interesting in this regard that exogenous IL-4 can inhibit B cell proliferation and the generation of T cell-mediated cytotoxicity when added to in vitro lymphocyte cultures at early times, while later addition of IL-4 is actually stimulatory; in contrast, IL-2 addition does not have this time-dependent inhibitory effect (28, 66, 67). Expression of high amounts of IL-4 or IFN- γ by T cells during the primary immune response or during intrathymic development could have other deleterious consequences. For example, IL-4 and IFN- γ by virtue of their ability to increase class II MHC expression on a variety of hematopoietic cell types (1, 2), could predispose to autoimmune reactivity at a critical stage of immunological development. Future in vivo experiments in which IL-4 or IFN- γ are overexpressed by T lineage cells during early ontogeny may provide insight as to the physiologic significance of restricting most production of these lymphokines to secondary T cell responses.

Whereas both IL-4 and IFN- γ production by peripheral T cells appear dependent on whether these cells have been previously activated in vivo, our results also indicate that the capacity for IL-4 production was remarkably restricted compared to IFN- γ throughout T cell ontogeny. IL-4 mRNA was

undetectable or markedly less abundant than IFN- γ or IL-2 mRNA in activated CD4⁺ CD8⁻ thymocytes, as well as circulating neonatal and adult T cells, and previous studies have demonstrated that this is also true for lymphoid tissue-associated T cells from mice or humans (4, 68). Based on the results of in situ hybridization for lymphokine mRNA, this low level of production was due to the rarity of IL-4 producing cells. These findings suggest that during intrathymic as well as extrathymic T cell maturation, the capacity for T cells to produce IL-4 is relatively restricted, indicating that IL-4 may play a more specialized role in mediating T cell effector functions than does IFN- γ or IL-2. It remains to be determined if particular T cells are permanently committed to produce IL-4 and/or IFN- γ and the extent to which production of these lymphokines by primary T cells is mutually exclusive, a situation commonly observed among long-term murine CD4⁺ T cell clones, but not among short-term murine or most human CD4⁺ T cell clones (69–73). Recently, mutually exclusive production of IL-4 and IL-2 has been demonstrated for primary murine CD4⁺ splenic T cells, fractionated on the basis of reactivity with mAb against two uncharacterized T lineage determinants (74, 75). It will be of interest to determine which of these CD4⁺ splenocyte populations secrete IFN- γ , and whether analogous surface markers will also identify human primary T cells with specific patterns of lymphokine production.

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