Cytokine Transcriptional Events during Helper T Cell Subset Differentiation

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Summary

The molecular basis for changes in cytokine expression during T helper (Th) cell subset differentiation is not well understood. We have characterized transcriptional events related to cytokine gene expression in populations of naive T cell receptor–transgenic T cells as they are driven in vitro toward Th1 or Th2 phenotypes by interleukin (IL)-12 or IL-4 treatment, respectively. Quantitative reverse transcriptase–polymerase chain reaction analysis of cytokine transcripts indicates that interferon (IFN) γ, IL-4, and IL-2 mRNA are expressed with distinct kinetics after naive T cells are stimulated with antigen and either IL-4 or IL-12. IFN-γ mRNA appears as early as 6 h in IL-12–treated cultures, IL-4 appears only after 48 h in IL-4–treated cultures, and IL-2 is equivalently expressed in both types of cultures. Analyses were performed to determine if there were any differences in activation of IL-2 or IL-4 transcription factors that accompanied Th1 versus Th2 differentiation. These studies demonstrated that signal transducer and activator of transcription 6 (STAT6) binds to a sequence in the IL-4 promoter and that this STAT6-binding site can support IL-4–dependent transcription of a linked heterologous promoter. Prolonged activation of STAT6 is characteristic of populations undergoing Th2 differentiation. Furthermore, STAT6 is activated in an autocrine manner when differentiated Th2 populations are stimulated by antigen receptor ligation. Th1 populations derived from IL-12 plus antigen treatment of naive T cells remain responsive to IL-4 as indicated by induction of STAT6 and IL-4 mRNA. These data indicate that Th1 and Th2 differentiation represents the combination of different, apparently independently regulated transcriptional events. Furthermore, among transcription factors that bind to the IL-4 or IL-2 promoters, STAT6 is the one whose activation distinguishes Th2 versus Th1 development.

The development of effector populations of CD4$^+$ Th cells that produce distinct cytokines is of central importance in infectious, allergic and autoimmune diseases (1, 2). Th1 cells, which secrete IL-2 and IFN-γ, are responsible for phagocyte-dependent protective immunity and tissue injury in many organ-specific autoimmune diseases. Th2 cells, which produce IL-4, IL-5, and IL-10, are involved in the development of allergies and in defense against helminthic parasites. In addition, the cytokines produced by Th2 cells, especially IL-4 and IL-10, may be critical for down-regulating or suppressing inflammation associated with Th1-mediated immune responses. Experimental evidence indicates that these polarized subsets of differentiated Th cells arise from a common precursor, which is a naive CD4$^+$ T cell that produces small amounts of IL-2 and no detectable IFN-γ or IL-4 when stimulated for the first time

in vitro (3, 4). The presence of exogenous cytokines at the time of primary antigenic stimulation profoundly influences which differentiation pathway a naive T cell will follow. Thus, IL-4 is required to drive differentiation of naive cells into Th2 effectors. IL-12 and IFN-γ are the principal cytokines that induce Th1 differentiation. There is some evidence that these cytokines, particularly IL-4 and IL-12, act directly on the T cells and not on APCs. It is clear that IL-4 has a dominant effect over IL-12, causing Th2 differentiation even when both cytokines are present (2, 5).

Very little is known about the molecular events that underlie T cell subset differentiation. Studies with T cell lines indicate that production of cytokines such as IL-2 and IL-4 is controlled primarily by transient activation of transcription induced by the binding of nuclear factors to 5'-regulatory regions of the cytokine genes (6–9). The regulation of IFN-γ expression may be more complex, involving methylation of DNA and posttranscriptional events (10, 11). We have previously shown that in Th1 and Th2 clones, the ac-

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tivity of exogenously introduced IL-2 and IL-4 promoter constructs correlates with IL-2 and IL-4 production (6). Furthermore, we found that TCR-inducible nuclear translocation of the p50/p65 heterodimer of nuclear factor-κB (NF–κB)[1] occurs in IL-2–producing Th1 and Th0 clones, but not in Th2 clones. It is, therefore, important to establish if bulk populations of differentiated Th1 and Th2 cells show distinct patterns of cytokine gene transcription, and whether this correlates with the activation and nuclear translocation of specific transcription factors.

In this paper we describe our initial studies to determine changes in cytokine gene transcription and transcription factors during the differentiation of naive CD4+ T cells to Th1 and Th2 populations. The model system we have used is the in vitro antigen plus cytokine stimulation of naive T cells from a TCR-transgenic mouse line. Such T cells have been shown to develop into populations of Th1 and Th2 cells by stimulation with antigen and IL-12 or IL-4, respectively (12–14). Our experiments show that patterns of cytokine gene expression are clearly different within 48 h of stimulation, and changes in IL-2, IL-4, and IFN-γ gene expression occur at distinct time points. Furthermore, we have identified a functionally active site in the IL-4 promoter that specifically binds the IL-4–induced transcription factor signal transducer and activator of transcription 6 (STAT6). STAT6 activation consistently and uniquely correlates with IL-4–induced Th2 differentiation.

Materials and Methods

Animals. TCR-transgenic mice expressing the AND TCR specific for pigeon cytochrome c (PCC) peptide 81-104 in association with I-E^k (15) were bred in our virus-free animal facility. All animals were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW Publication No. [NIH] FS-23). Transgenic TCR expression was screened by PCR as previously described (5).

Antigen, Cytokines, and Antibodies. The antigen used in all studies was the COOH-terminal 81-104 peptide of PCC and was obtained from the peptide synthesis facility of the Center for Neurologic Diseases, Brigham and Women's Hospital. Supernatant containing recombinant murine IL-4 was generated from the I3L6 cell line transfected with a constitutively expressed murine IL-4 cDNA (a gift of Dr. Robert Tepper, Massachusetts General Hospital, Boston, MA). Recombinant murine IL-12 was a gift of Dr. Stan Wolf (Genetics Institute, Cambridge, MA). Supernatant containing recombinant murine IL-2 was obtained from the IL-2 gene–transfected X63 cell line (16). The hybridoma producing anti-IL-4 mAb, 11B11, obtained from Dr. William Paul (National Institutes of Health, Bethesda, MD) was used to produce ascites in nude mice.

Culture Conditions. CD4+ T cells were purified from pooled lymph nodes and spleens of TCR-transgenic mice by complement-mediated lysis of MHC class II–, CD8- and J11D-expressing cells, followed by depletion of remaining B cells by panning on anti-Ig–coated dishes, as previously described (5). These T cell populations were >95% CD3+CD4+. T cell differentiation was induced by culturing 2 × 10^5 purified TCR-transgenic T cells with 2 × 10^6 APCs, 1 μM PCC peptide 81-104, and either IL-12 (10 ng/ml) or IL-4 (1,000 U/ml) in 1 ml of medium. APCs were splenic B cells purified from littermate B10.BR mice by anti-Ig panning and were treated with 50 μg/ml mitomycin C at 37°C for 30 min, as described (5). All cultures were carried out in RPMI 1640 supplemented with 1 mM L-glutamine, sodium pyruvate, nonessential amino acids, 5 × 10^{-5} 2-mer and 10% heat-inactivated FCS (all from GIBCO BRL, Gaithersburg, MD).

ELISA Determination of Secreted IL-2, IL-4, and IFN-γ. Culture supernatants were assayed for the presence of IL-2, IL-4, and IFN-γ using ELISA reagents from PharMingen (San Diego, CA).

Quantitation of Cytokine Transcripts by Reverse Transcriptase-PCR. After various times in culture, cells were lysed with Ultraspec RNA extraction solution (Biotecx, Houston, TX), RNA was precipitated with isopropanol, washed, and reverse transcribed using oligo d(T)12-18 by standard methods (17). Quantitative PCR was performed by using a competitive fragment (CF) that contains various cytokine cDNA sequences that yield different size PCR-amplification products than do endogenous cytokine cDNAs (18). The β-actin cDNA concentration of each sample was first determined using β-actin–specific primers by keeping the cDNA concentration constant and adding serial dilutions of CF. Amplified products were separated on an agarose gel and stained with ethidium bromide to determine the serial dilution of CF that gave amplified products from the CF and cDNA of equal intensity. The cDNA samples were normalized to equal β-actin cDNA concentrations, amplified with IL-2–, IFN-γ– and IL-4–specific primers, and products were separated on agarose gels. The cDNA concentrations were then determined from the dilution of CF that yielded an equal amount of amplified product as had the cDNA. The cDNA concentrations were calculated as attomoles of cDNA transcripts divided by femtomoles of input β-actin cDNA.

Electrophoretic Gel Mobility Shift Assays. Electrophoretic gel mobility shift assays (EMSA) for transcription factors were performed as previously described (6). Briefly, oligonucleotides that correspond to the consensus DNA-binding elements in the murine IL-2 promoter (NFIL-2A, AP-1, NF-κB, nuclear factor of activated T cells [NFAT]) and IL-4 promoter (STAT6, IL-4 NFAT) were synthesized for use as probes in EMSA using a DNA synthesizer (model 381A; Applied Biosystems, Inc., Foster City, CA) or they were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). These oligonucleotides included:

NFIL-2A	5'-TATGTGTAATATGTAAAA-3'
AP-1	5'-AATTCCAGAGAGTCATCAGA-3'
NF-κB	5'-ACCAAGAGGGATTTCACCTAAATC-3'
NFAT	5'-AAGAGGAAAATTTGTTTCATACAG-3'
STAT6	5'-TGATTTCACAGGAAAATT-3'
IL-4 NFAT	5'-ATAAAATTTTCCAATGTAAA-3'

The complementary single-stranded oligonucleotides were also synthesized and annealed to the above listed oligonucleotides, and the double-stranded oligonucleotides were end-labeled with γ-^{32}P-ATP (DuPont NEN Research Products, Wilmington, DE) using T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) for use as probes in EMSA. Labeled oligonucleotide was separated from unincorporated γ-^{32}P-ATP using spin-

10 columns (Clontech, Palo Alto, CA). Nuclear protein extracts were prepared as described (6). Nuclear extract (5 μg protein) was incubated for 20 min at room temperature in binding buffer (10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 2 mM dithiothreitol, 5% glycerol, and 100 mM KCl) with ∼10,000 cpm of ^{32}P-labeled oligonucleotides. The binding reactions were then mixed with 6X gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol), and protein–DNA complexes were separated from free oligonucleotide probes by electrophoresis through a 9% nondenaturing polyacrylamide gel. After electrophoresis, the gels were dried under vacuum and prepared for autoradiography. Control experiments using 100-fold molar excess unlabeled oligonucleotide probes demonstrated that the shifted protein–DNA complexes were specific for each respective oligonucleotide probe used in this study. For supershifts, normal rabbit serum or antisera specific for murine STAT4 or STAT6 were added to the binding reactions.

Functional Analysis of the IL-4 Promoter STAT6 Site by Transient Transfection. Upper and lower strand oligonucleotides containing the IL-4 promoter STAT6 site sequence with 5′-phosphorylated TCGA overhangs were purchased from Integrated DNA Technologies, Inc., annealed, ligated to SalI-cut pBLCAT2 reporter construct (19), and used to transform *Escherichia coli*. Plasmid miniprep DNA from transformants were screened by hybridization to a ^{32}P-labeled STAT6 oligonucleotide probe, and positive clones were sequenced. The 10g and 14g clones identified in this manner contain one and two tandem copies of the STAT6-binding site, respectively. These constructs or the pBLCAT2 parent plasmid were transfected into the TA3 B-lymphoblastoid line (20) by electroporation using a Gene Pulsar instrument (Bio-Rad Laboratories, Richmond, CA). Transfection was performed with 40 μg of DNA and 20 × 10^{7} cells as described previously (6). Electroporated cells were cultured in 5 ml culture medium for 48 h. IL-4 (500 U/ml) was added to some cells for the final 24 h. Cytoplasmic lysates were prepared and chloramphenicol acetyl transferase (CAT) assays were performed as described (6).

Results

Kinetics of IL-2, IFN-γ, and IL-4 Gene Expression during Th1 and Th2 Cell Differentiation. In the initial set of experiments, we wished to determine when during in vitro differentiation T cells acquired cytokine transcription profiles characteristic of Th1 and Th2 subsets. To first establish that cytokine secretion correlated with mRNA levels, we examined TCR-transgenic CD4^{+} T cells that had been cultured with antigen, splenic B cell APCs, and either IL-12 or IL-4 for 6 d, in order to induce differentiation. As shown in Table 1, restimulation of cells from 6-d IL-12–treated primary cultures resulted in IL-2 and IFN-γ production, but no detectable IL-4 production. In contrast, cells from IL-4–treated primary cultures produced IL-4, but no detectable IFN-γ and very little IL-2 upon restimulation with antigen. Thus, as expected, IL-12 and IL-4 induce distinct patterns of Th1/Th2 differentiation. Because some experiments addressing the induction of IL-2 and IL-4 transcription factors required the use of anti-CD3 as a stimulus, we also needed to examine the phenotype of IL-12– and IL-4–treated T cell populations when restimulated with anti-CD3. Table 1 shows that anti-CD3–induced IL-4 production was observed only in IL-4–treated cells. Surprisingly, anti-CD3 restimulation induced significant levels of IFN-γ protein in T cells from IL-4–treated primary cultures. This was seen in three separate experiments. Furthermore, there was some IL-2 detectable in anti-CD3–restimulated T cells from both IL-12– and IL-4–treated primary cultures.

Cytokine transcript levels as determined by quantitative reverse transcriptase (RT)-PCR using competitive PCR-MIMICs were also distinctly different in these Th1 and Th2 populations. Upon restimulation, Th1 cells induced

Table 1. *Cytokine Expression After Restimulation*

| Cytokine added in 1° culture	Restimulation conditions	Cytokine			Cytokine mRNA expression (attomoles/femtomole β-actin mRNA)		
		IL-2	IFN-γ	IL-4	IL-2	IFN-γ	IL-4
		U/ml					
IL-12	APCs	0	0	0	0.33	100	n.d.
	APCs + PCC peptide	133	878	0	3.3	10,000	n.d.
	None	0	0	0	n.d.	<1	n.d.
	Anti-CD3	34	664	0	3.5	100	n.d.
IL-4	APCs	0	0	0	n.d.	n.d.	n.d.
	APCs + PCC peptide	2	0	60	0.33	1	33
	None	0	0	0	n.d.	<1	n.d.
	Anti-CD3	5	466	51	3.5	10	100

Cytokine secretion and mRNA expression in Th1 and Th2 populations of TCR-transgenic T cells. Naive T cells from AND mice were cultured with splenic B cell APCs, PCC (81-104) peptide (1 μM), and either IL-4 (1,000 U/ml) or IL-12 (10 ng/ml) as indicated, for 6 d. T cells were then harvested, washed, and recultured in microwells with splenic B cell APCs minus or plus PCC peptide, no stimulus, or plate-bound anti-CD3. RNA was extracted after 18 h for RT-PCR analysis of cytokine transcripts. Quantities of cytokine mRNA were calculataed from competitive PCR shown in Fig. 1. Identical cultures were left for 48 h, after which supernatants were collected and analyzed for cytokine protein by ELISA.
n.d., none detected.

by priming with antigen, APCs, and IL-12 had low amounts of IL-4 gene transcripts and high levels of IL-2 and IFN-γ, whereas the IL-4–treated T cell population had high levels of IL-4 gene expression and lower levels of IL-2 and IFN-γ transcripts (Fig. 1 and summarized in Table 1). Somewhat surprisingly, cells primed in the presence of IL-4 and re-stimulated with antigen plus APCs did express low IFN-γ mRNA although no IFN-γ was detectable by ELISA. Thus, bulk populations of Th1 and Th2 TCR-transgenic T cells produced significantly different levels of IL-2, IFN-γ,

and IL-4 gene transcripts. Moreover, at the level of cyto-kine gene transcription, IFN-γ and IL-4 appear to most clearly distinguish Th1 and Th2 populations.

We next wanted to define when during their differentia-tion T cells became committed to distinct cytokine patterns. This issue can only be addressed by measuring cytokine mRNA expression, because exogenous cytokines are present during in vitro differentiation. Therefore, we used quanti-tative RT-PCR analysis of IL-2, IFN-γ, and IL-4 mRNA to determine the kinetics of differential cytokine gene ex-
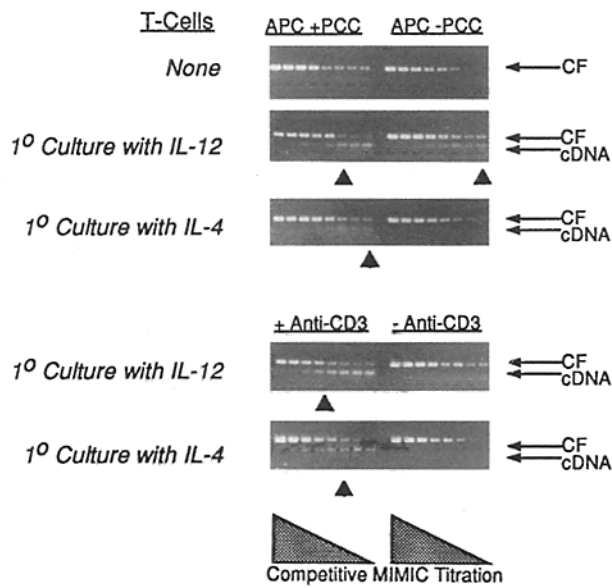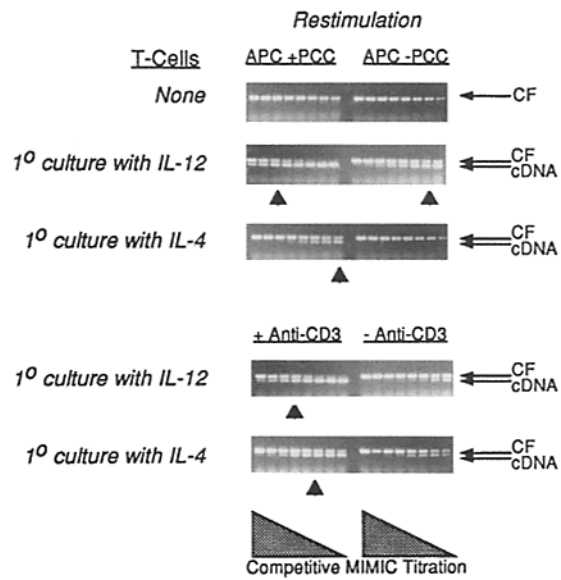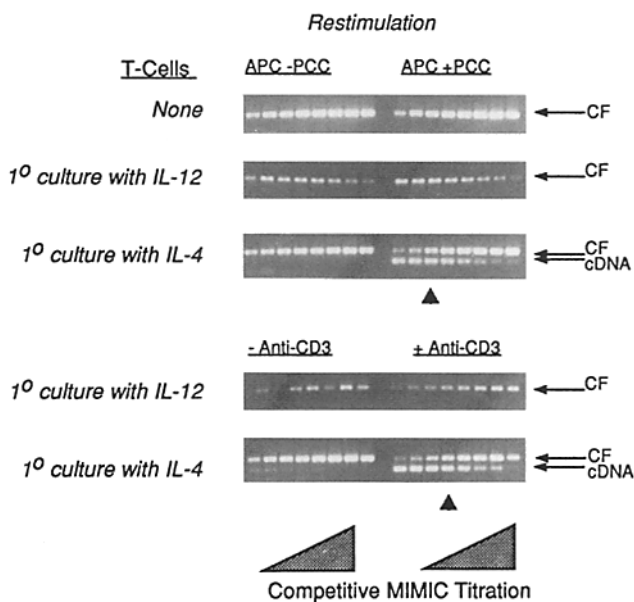

Figure 1. Cytokine-driven Th1 and Th2 differentiation of naive TCR transgenic T cells: determination by quantitative RT-PCR. Naive AND CD4+ T cells were stimulated in vitro with PCC (81-104) peptide, B cell APCs, and either IL-4 or IL-12, as described in Materials and Methods. After 6 d, cells were harvested, washed, and restimulated in microwells with splenic B cell APCs and 1 μM PCC peptide, or plate-bound anti-CD3, as indicated. Restimulation cultures were terminated at 18 h by Ul-traspec RNA extraction solution, and amounts of IL-2 (A), IFN-γ (B), and IL-4 (C) mRNAs were determined by quantitative RT-PCR using β-actin cDNA concentrations as internal standards. Competitive MIMICs (CF) were diluted in half-log increments. (Arrows) Points of equivalent intensity of ethidium bromide–stained bands between cDNA and CF dilution.

pression in differentiating Th populations (Fig. 2). The results show that IL-12 enhanced IFN-γ mRNA expression as early as 6 h after initial stimulation, and that IFN-γ gene expression remained significantly higher in the IL-12–treated cultures than in the untreated or IL-4–treated cultures throughout the differentiation process. A striking observation was that IL-4 gene expression was not detected until 48 h after stimulation of naive T cells with antigen and IL-4. The expression of IL-2 was not significantly affected by IL-12 and IL-4 treatment, even at 48 h when IL-4 gene expression was the highest in the IL-4–treated stimulation cultures.

These results indicate that changes in expression of IL-2, IL-4, and IFN-γ genes during subset differentiation are temporally distinct events, suggesting that they may be regulated in different ways. In vitro differentiation of naive T cells is associated with clearly polarized patterns of IL-4 and IFN-γ gene transcription within 48 h. Early transcription of IL-2 is not distinct in these differentiating T cells, and the diminished expression of the IL-2 gene in Th2 populations is only observed after restimulating cells that have differentiated in the presence of IL-4.

Analysis of IL-2 and IL-4 Promoter-specific Transcription Factors in Th1 and Th2 Cell Populations. The expression of cytokine genes is controlled, in part, by sets of transcription factors that bind to cytokine promoters. We conducted EMSA analyses in order to determine if the distinct patterns of cytokine gene transcription in T cell populations undergoing Th1 versus Th2 differentiation could be attributed to distinct patterns of transcription factor activation. The well characterized regulatory elements within the IL-2 gene promoter include AP-1, NFIL-2A (Oct-1/OAP-40), NFAT,

and NF-κB (9). The IL-4 promoter contains multiple NFAT sites that have been shown to be involved in regulation of IL-4 gene expression (7, 21). Recently, several groups have described a transcription factor that becomes tyrosine phosphorylated and rapidly translocates from the cytoplasm to the nucleus in response to IL-4–induced signals. This factor, called STAT6 or IL-4STAT, binds to sites in the 5′ regulatory regions of several IL-4–responsive genes that have an inverted repeat spaced by three nucleotides that is characteristic of several described cytokine response– or STAT factor–binding sites (22–24). Comparison of these sequences with the murine IL-4 promoter sequences revealed a potential binding site located at -153 to -167 from the transcriptional initiation site within the murine IL-4 promoter. This site partially overlaps a previously identified NFAT-binding site (7). Thus, for the EMSA assays in these studies, we used oligonucleotide probes corresponding to the AP-1–, NFIL-2A (Oct-1/OAP-40)–, NFAT-, and NF-κB–binding sequences of the murine IL-2 gene promoter, and the NFAT- and STAT6-binding sites from the murine IL-4 promoter.

When we compared cells from IL-4– and IL-12–treated cultures, we observed no significant difference in the nuclear presence of IL-2 promoter–binding factors, including AP-1, NFAT, NFIL-2A, and NF-κB. This was true early during T cell differentiation, i.e., 48 h after stimulation of naive T cells with IL-12 or IL-4 (Fig. 3), and even in cells that had been differentiated for 6 d and were restimulated (Fig. 4). Parallel assays for cytokine secretion by 6-d differentiated populations showed that Th1 and Th2 differentiation had occurred in these IL-12– and IL-4–treated cultures, respectively (data not shown). Although we have
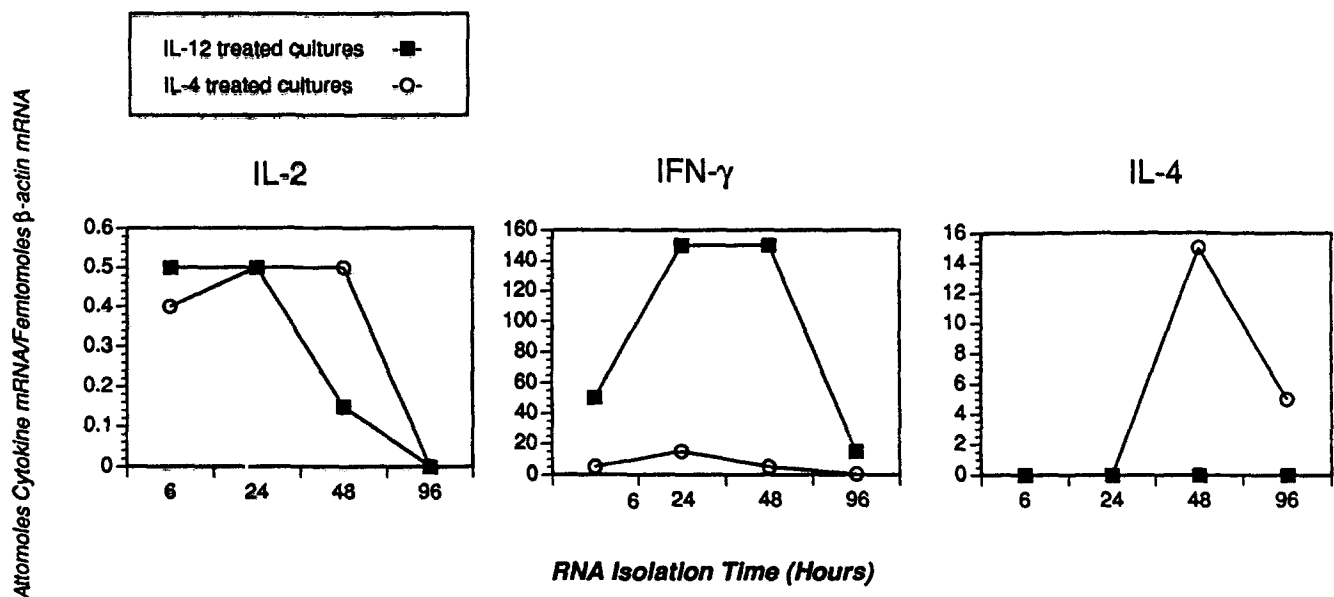

Figure 2. Kinetics of cytokine mRNA expression in differentiating CD4+ T cells: quantitative RT-PCR analysis. Differentiation cultures were set up as described in Fig. 1. RNA was isolated from the cultures at the indicated time points and reverse transcribed IL-2, IFN-γ, and IL-4 mRNA was analyzed by quantitative RT-PCR.
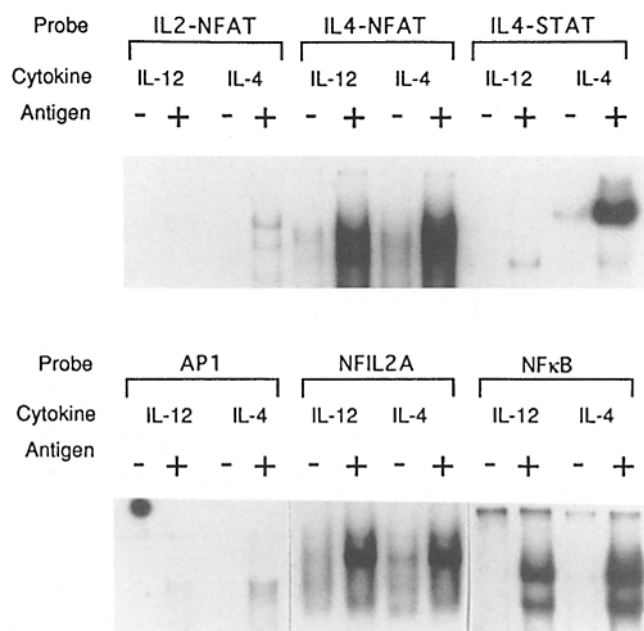
Probe IL2-NFAT IL4-NFAT IL4-STAT

Cytokine IL-12 IL-4 IL-12 IL-4 IL-12 IL-4

Antigen - + - + - + - + - + - +

Probe AP1 NFIL2A NFκB

Cytokine IL-12 IL-4 IL-12 IL-4 IL-12 IL-4

Antigen - + - + - + - + - + - +

Figure 3. Nuclear transcription factors in differentiating Th1 and Th2 populations. TCR transgenic T cells were cultured with splenic B cell APCs, antigen, and either IL-12 or IL-4, as described in Materials and Methods. After 48 h of culture, nuclear extracts were prepared and analyzed by EMSA for the presence of DNA-binding proteins using oligonucleotide probes containing binding sites for the indicated factors.

Probe IL4-NFAT AP-1 IL4-STAT

Cytokine during 1° culture IL-12 IL-4 IL-12 IL-4 IL-12 IL-4

Restimulation with anti-CD3 - + - + - + - + - + - +

Probe NFκB NFIL2A IL2-NFAT

Cytokine during 1° culture IL-12 IL-4 IL-12 IL-4 IL-12 IL-4

Restimulation with anti-CD3 - + - + - + - + - + - +

Figure 4. Nuclear transcription factors in restimulated Th1 and Th2 cells. Identical cultures to those described in Fig. 3 were continued for 6 d, at which time cells were harvested and restimulated with anti-CD3. Nuclear extracts were prepared from these restimulation cultures 3 h after initiation, and were analyzed by EMSA.

previously observed a lack of NF-κB activation in TCR-stimulated Th2 clones that do not transcribe the IL-2 gene, the finding here that NF-κB activation does occur in stimulated Th2-like populations is consistent with the fact that these cells do express IL-2 transcripts (Fig. 1 *A*). The lack of TCR-inducible NF-κB activation may be a phenotype of cells that have undergone multiple rounds of differentiation.

Among the IL-4 promoter–binding proteins, we found no difference in nuclear proteins that bind to an IL-4 NFAT site, but there was a distinct difference in EMSA patterns using an oligonucleotide probe corresponding to the putative STAT6-binding sequence in the mouse IL-4-promoter. STAT6 protein was detected in the nuclei of IL-4–treated T cells at 48 h after initiation of cultures, the time point during differentiation when we first detect IL-4 mRNA (Fig. 2). Supershift analysis using a murine STAT6-specific antibody indicated that STAT6 was in fact present in the IL-4–induced gel-shift complex detected with this probe. The anti-STAT6 serum, but not anti-STAT4 or preimmune sera, supershifted complexes of the probe and nuclear proteins from IL-4–treated TCR-transgenic T cells (Fig. 5), as well as complexes formed with nuclear extracts from anti-CD3–treated TCR-transgenic Th2 populations (not shown). Kinetic analysis showed that nuclear STAT6 was also detectable as early as 24 h after culture initiation (Fig. 6), indicating that STAT6 activation precedes IL-4 gene transcription. Thus, antigen stimulation of a naive population of T cells in the presence of IL-4 leads to the induction of STAT6 proteins before and during the time of
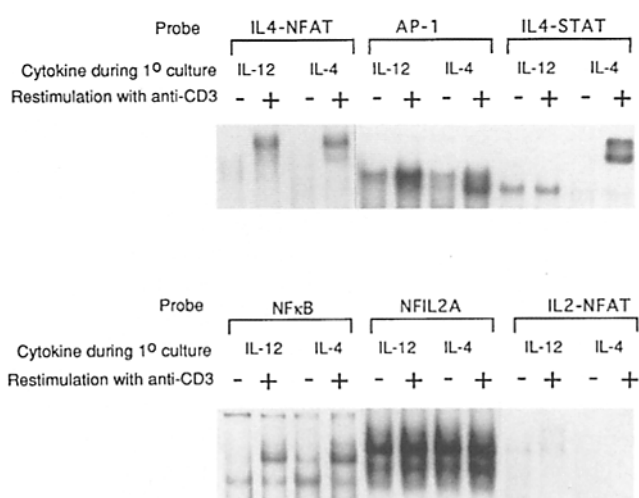
differentiation when IL-4 gene transcript levels peak. IL-4 induction of STAT6 is generally a transient event with nuclear STAT6 waning after 2 h (24, and data not shown). Interestingly, the presence of nuclear STAT6 at either 24 or 48 h appeared to be dependent on both the presence of IL-4 and antigen stimulation, suggesting that other signals besides those induced by IL-4 were required for sustained activation of STAT6 (Fig. 3 and 6). No STAT6 was detectable in cells from IL-12–treated primary cultures. Nuclear STAT6 was also induced by anti-CD3 restimulation of cells from IL-4–treated primary cultures, but not from IL-12–treated primary cultures (Fig. 4).
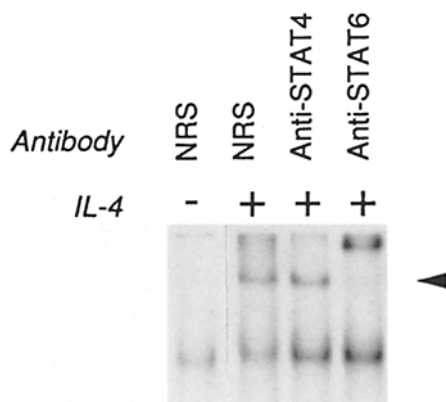
Antibody NRS NRS Anti-STAT4 Anti-STAT6

IL-4 - + + +

Figure 5. Identification of STAT6 in gel–shift complexes using a probe with a murine IL-4 promoter sequence. TCR-transgenic T cells were cultured with splenic B cell APCs, antigen, and IL-4 for 6 d, as described in Figs. 3 and 4, and then restimulated with 500 U/ml IL-4 for 30 min followed by nuclear extract preparation. EMSA analyses were performed using these extracts and the STAT6 probe corresponding to a sequence in the murine IL-4 promoter (see Materials and Methods). Antisera specific for normal rabbit serum, murine STAT4 (negative controls), or murine STAT6 were added to the binding reactions, as indicated. (*arrowhead*) Location of the STAT6-containing complexes.
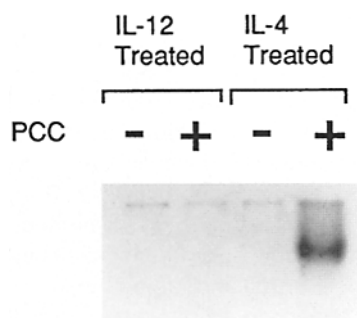
Figure 6. Nuclear STAT6 in 24-h Th2-differentiating cultures. Naive TCR-transgenic T cells were stimulated with APCs, antigen, and cytokines as described in previous figures. Nuclear lysates were prepared at 24 h after culture initiation and were analyzed by EMSA for the presence of factors binding to the IL-4 promoter STAT6 site.

STAT6, like other cytokine-inducible STAT proteins, is activated and translocated to the nucleus after exposure of cells to IL-4. Since we observed STAT6 in differentiated Th2 cells stimulated with antigen or anti-CD3 (in the absence of added IL-4), this raised the possibility that STAT6 was induced by the autocrine effect of secreted IL-4. To test this, differentiated Th2 cells were stimulated with anti-CD3 in the presence of anti-IL-4 antibody, and nuclear STAT6 was assayed by EMSA. Anti-IL-4 antibody partially blocked the appearance of nuclear STAT6 in restimulated Th2 populations (Fig. 7), indicating that autocrine IL-4 was responsible for at least part of the induction of STAT6 in these cultures. The failure to completely block STAT6 induction with anti-IL-4 antibody may reflect the high sensitivity of this signaling pathway to small amounts of IL-4, or direct effects of TCR signaling on STAT6 activation that are independent of IL-4.

Reversibility of Th1 and Th2 Populations and Correlation with STAT6. Recently, it has been shown that bulk populations of Th1 cells can be converted to IL-4 producers by culture with IL-4, but Th2 populations appear to be irreversibly committed (5, 25, 26). The stability of Th2 cells
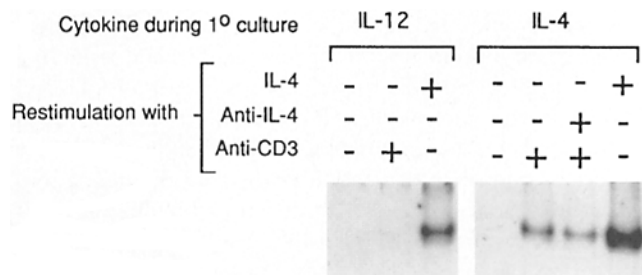

Figure 7. Exogenous IL-4 activation of STAT6 in Th1 cells and autocrine activation of STAT6 in Th2 cells. TCR-transgenic T cells were differentiated in the presence of B cell APCs, antigen, and either IL-12 or IL-4 for 6 d and were then recultured with no added stimulus, anti-CD3, IL-4, or anti-CD3 plus anti-IL-4 (1/1,000 dilution of 11B11 ascites) as indicated. Nuclear lysates were prepared after 3 h of restimulation and analyzed for the presence of STAT6, as described in previous figures.

may in part be explained by the fact that these cells are unable to transduce IL-12–induced signals, including the activation of STAT3 and STAT4 (26). If STAT6 is important in inducing Th2 differentiation, then both Th1 and Th2 populations should be able to activate STAT6. To test this hypothesis, Th1 and Th2 populations generated by 6-d cultures of naive T cells with IL-12 or IL-4 were restimulated with anti-CD3 alone or with anti-CD3 plus IL-4, and nuclear STAT6 was assessed by EMSA. We found that both IL-12–pretreated Th1 populations and IL-4–pretreated Th2 populations translocated STAT6 to the nucleus in response to IL-4 (Fig. 7). This correlated with the enhanced expression of IL-4 mRNA in Th1 populations by 48 h after restimulation with anti-CD3 plus IL-4 (Table 2). Th1 populations also expressed abundant IFN-γ mRNA without antigen stimulation, and this was suppressed by antigen stimulation in the presence of IL-4. In contrast to the primary cultures, IL-2 mRNA was no longer detectable in either Th1 or Th2 populations at 48 h after restimulation.

Functional Analysis of the Murine IL-4 Promoter STAT6-binding Site. To demonstrate that the STAT6-binding site identified in the IL-4 promoter can function to enhance transcription, we prepared reporter gene constructs containing one or two copies of this site linked to promoter- CAT sequences and introduced these DNAs into the efficiently transfectable TA3 lymphoblastoid line. EMSA analyses in-

Table 2. *Cytokine mRNA Expression in Th1 and Th2 Cell Populations Restimulated in the Presence of IL-4 or IL-12*

Cytokine added in 1° culture	Restimulation conditions	Cytokine mRNA expression (attomoles/ femtomole β-actin mRNA)		
		IL-2	IFN-γ	IL-4
IL-12	APCs	<3	10	<3
	APCs + PCC peptide	<3	30	<3
	APCs + IL-12	<3	100	<3
	APCs + PCC peptide + IL-12	<3	100	<3
	APCs + IL-4	<3	100	<3
	APCs + PCC peptide + IL-4	<3	10	10
IL-4	APCs	<3	<3	3
	APCs + PCC peptide	<3	<3	30
	APCs + IL-12	<3	30	3
	APCs + PCC peptide + IL-12	<3	<3	30
	APCs + IL-4	<3	<3	3
	APCs + PCC peptide + IL-4	<3	<3	100

Th1 and Th2 populations were derived in vitro as described in Table 1, and cells were restimulated with B cell APCs plus PCC peptide in the presence of no cytokine, IL-4 (1,000/ml), or IL-12 (10 ng/ml). Cultures were terminated at 48 h and cytokine mRNA expression was determined by quantitative RT-PCR as described in Fig. 1.

Table 3. *Functional analysis of the IL-4 Gene 5'
STAT6-binding Site*

Plasmid	Number of STAT6 sites	Fold induction by IL-4
pBLCAT2	None	0.6
10g	1	1.0
14g	2	4.0

TA3 cells were transfected with the indicated CAT-reporter plasmids by electroporation. The cells were stimulated with 1,000 U/ml IL-4 at 24 h, and CAT activity was assayed by phase extraction liquid scintillation (see Materials and Methods) in cell lysates prepared at 48 h. Fold induction was calculated as counts per minute from IL-4–treated cells divided by Counts per minute from untreated cells. A representative experiment is shown. IL-4 induction of CAT activity in cells transfected with the 14g plasmid was observed in three independent experiments.

dicated that IL-4 treatment leads to STAT6 activation in these cells (data not shown). The data in Table 3 indicate that the IL-4 promoter STAT6 site does support IL-4–enhanced transcription of a linked reporter gene.

Discussion

These studies were undertaken to characterize changes in transcriptional regulation of cytokine genes during Th cell subset differentiation. The kinetics of IL-4, IL-2, and IFN-γ mRNA expression that we observed in differentiating cultures of CD4+ T cells indicates temporally independent regulation of the expression of different cytokine genes. IFN-γ transcripts appear early, within 24 h, in populations differentiating toward a Th1 phenotype. In contrast, IL-4 transcripts do not appear until 48 h in cells differentiating towards a Th2 phenotype. IL-2 transcripts are detectable at 6 h and no difference is seen in the appearance of IL-2 transcripts in primary cultures differentiating toward either Th1 or Th2 phenotypes. This observation is not surprising since it is known that IL-2 is required for both differentiation pathways (27). Thus, there does not appear to be a single switch event during subset differentiation that affects multiple cytokine genes at the same time.

The only clear difference we observed in nuclear transcription factors between cultures of differentiating CD4+ cells was the presence of STAT6 in cells driven toward the Th2 phenotype. STAT6 has been identified as a protein that binds to DNA sequences found in the promoters of IL-4–responsive genes (22, 24) A cDNA encoding a protein that binds to the IL-4 response element in the human FcγR1 gene has been cloned and sequenced (14). The predicted protein product of this cDNA shares features with other STAT family members and it contains regions that can apparently bind to the cytoplasmic tail of IL-4R. STAT6 activation is thought to involve association of latent monomers with the IL-4R, tyrosine phosphorylation, dimerization, and translocation to the nucleus, where the dimers bind to response elements in the promoters of vari-

ous genes. Recently, it has been reported that mice with targeted disruptions of the STAT6 gene are incapable of mounting Th2 responses (28–30). These studies confirm the essential role of IL-4 signaling in promoting Th2 differentiation, but they do not indicate how IL-4 may induce T cells to become competent at transcribing their own IL-4 genes. The identification of a functional STAT6-binding site in the IL-4 promoter reported here, and the correlation of STAT6 activation with Th2 differentiation, implicate that STAT6 is involved directly with IL-4 transcriptional activity during Th2 differentiation.

Gel shift assays using nuclear extracts from IL-4–treated THP-1 cells and a sequence from the human FcγR1 gene demonstrated rapid indcution of STAT6 activity, within 5 min, and a rapid decay, within 2 h (24). We have seen similar kinetics in mouse T cells, using an oligonucleotide probe with a murine IL-4 promoter sequence (Lederer, J.A., and A.H. Lichtman, unpublished observation). A longer half-life for nuclear STAT6 was reported in HepG2 cells using an interferon response factor 1 γ activation site (GAS) probe, which binds a variety of STAT family members (11). We found that STAT6 was present in the nuclei of IL-4–treated cells at 24 and 48 h as well as in restimulated Th2 cells derived from 6-d IL-4–treated cultures. Thus, STAT6 remains consistently activated before and during the onset of IL-4 gene transcription in these cultures. The sustained presence of nuclear STAT6 days after the initial exposure to IL-4, as well as the antigen-dependence of STAT6 activation after 24 h, suggests that other signals besides those provided by the exogenously added IL-4 are responsible for maintaining STAT6 activation. Clearly, autocrine IL-4 production can induce STAT6 activation, as we observed in restimulated Th2 cells. Thus, it is possible that autocrine IL-4 can contribute to the transcriptional regulation of the IL-4 gene after the effects of exogenous sources of IL-4 have waned.

A recently reported study with TCR-transgenic T cells has demonstrated that there is an extinction of IL-12 signaling in populations differentiating toward the Th2 phenotype (16). IL-12–signaling pathways involve tyrosine phosphorylation of STAT3 and STAT4. T cells undergoing Th2 differentiation may fail to generate the active forms of these transcription factors at the same time that they generate active STAT6 in response to exogenous and autocrine IL-4. Thus, it is possible that an abundance of STAT3 and STAT4 in the nucleus of a T cell at the time of antigen stimulation favors commitment to a Th1 phenotype, whereas a relative abundance of STAT6 favors Th2 differentiation. Since the presence of STAT6 correlates with Th2 differentiation, it is important to determine the functional significance of STAT6 binding to the IL-4 promoter. We are currently using mutational analyses and reporter gene transfections to formally test the role of STAT6 in transcriptional activation of the IL-4 gene. To date, there is no evidence indicating that STAT3 or STAT4 bind to regulatory sequences of the IFN-γ or IL-2 genes and regulate their transcription.

The lack of correlation of subset differentiation with

other transcription factors, including AP-1, NF-AT, and NFIL-2A, indicates that these factors may not be key determinants of early commitment towards a Th1 or Th2 phenotype. This finding is consistent with the observation that IL-2 gene transcription is not modulated early during the T cell differentiation process and that upon restimula-tion, both Th1 and Th2 populations have the capacity to express IL-2. Perhaps multiple rounds of stimulation by chronic antigen exposure lead to a more pronounced segregation of IL-2 expression in Th cells similar to what has been observed in T cell clones and in Th1 and Th2 populations after multiple rounds of restimulation (6, 31).

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