T Cell Priming Enhances IL-4 Gene Expression by Increasing Nuclear Factor of Activated T Cells

Randy Q. Cron, Susan J. Bort, Yunxia Wang, Mark W. Brunvand and David B. Lewis

*J Immunol* 1999; 162:860-870; ;
http://www.jimmunol.org/content/162/2/860

References
This article cites 83 articles, 53 of which you can access for free at:
http://www.jimmunol.org/content/162/2/860.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
T Cell Priming Enhances IL-4 Gene Expression by Increasing Nuclear Factor of Activated T Cells

Randy Q. Cron,* Susan J. Bort,* Yunxia Wang,* Mark W. Brunvand,† and David B. Lewis2*

The repetitive activation of T cells (priming) enhances the expression of many cytokines, such as IL-4, but not others, such as IL-2. Molecular mechanisms underlying selective expression of cytokines by T cells remain poorly understood. Here we show that priming of CD4 T cells selectively enhances IL-4 expression relative to IL-2 expression by a transcriptional mechanism involving nuclear factor of activated T cells (NFAT) proteins. As detected by in vivo footprinting, priming markedly increases the activation-dependent engagement of the P0 and P1 NFAT-binding elements of the IL-4 promoter. Moreover, each proximal P element is essential for optimal IL-4 promoter activity. Activated primed CD4 T cells contain more NFAT1 and support greater NFAT-directed transcription than unprimed CD4 T cells, while activator protein 1 binding and activator protein 1-mediated transcription by both cell types is similar. Increased expression of wild-type NFAT1 substantially increases IL-4 promoter activity in unprimed CD4 T cells, suggesting NFAT1 may be limiting for IL-4 gene expression in this cell type. Furthermore, a truncated form of NFAT1 acts as a dominant-negative, reducing IL-4 promoter activity in primed CD4 T cells and confirming the importance of endogenous NFAT to increased IL-4 gene expression by effector T cells. NFAT1 appears to be the major NFAT family member responsible for the initial increased expression of IL-4 by primed CD4 T cells.

Copyright © 1999 by The American Association of Immunologists 0022-1767/99/$02.00

\[PDF\] Downloaded from http://www.jimmunol.org by guest on July 25, 2017
their subsequent shuttling into the nucleus (12, 19). The role of NFAT proteins and P elements in regulating IL-4 gene transcription during the extrathymic differentiation of antigenically naive T cells into effector or memory T cells remains to be defined.

In this study, we demonstrate that increased IL-4 production by primed CD4 T cells is due, at least in part, to enhanced transcription directed by the immediate 5′ flank promoter of the IL-4 gene. We further show that both proximal P elements of the IL-4 promoter, P0 and P1, are occupied in vivo during IL-4 transcription in normal human T cells, and that both are independently required for optimal IL-4 reporter gene activity in this cell type. An analysis of nuclear protein complexes formed in vitro with an IL-4 P1 element oligonucleotide demonstrates that NFAT1 is the major NFAT protein forming a specific complex in fresh and primed CD4 T cells, and that there is a striking and direct correlation between the abundance of NFAT1 in the nucleus and IL-4 gene expression. Importantly, expression of wild-type and truncated NFAT1 constructs demonstrates that NFAT1 is a limiting component for IL-4 gene transcription by fresh CD4 T cells and also plays a role in the enhanced level of IL-4 gene expression by primed T cells. Western blot analysis and IL-4 promoter-directed reporter gene studies involving transient overexpression of various NFAT family members both indicate that NFAT1, rather than other NFAT family members, contributes to increased IL-4 gene expression by primed human CD4 T cells. Together, these results suggest that an increase in NFAT1 in primed CD4 T cells is an important mechanism by which priming of T cells enhances the expression of IL-4. This mechanism, or a similar one, may also contribute to the increased expression of other cytokines by primed T cells, a critical event in T cell-mediated immunity.

Materials and Methods

Cells and priming in vitro

Whole blood mononuclear cells were isolated from the peripheral blood of normal adult volunteer donors, and circulating T cells or CD4 T cells were obtained by treatment of whole mononuclear cells with T-cell Lymphok-wik or T-Helper Kwik, respectively (One Lambda, Los Angeles, CA), as described (20). Flow cytometric (FCF) analysis with appropriate directly conjugated mouse anti-human mAbs revealed that the isolated CD4 T cell populations were routinely >98% CD3+, >1% CD8+, and 85–98% CD4+. T cells or CD4 T cells (1–2 × 10⁶/ml) were primed in vitro by incubation in complete medium (CM; Ref. 20) containing 10 ng/ml of human rIL-2 (Proleukin-2; Promega, Madison, WI), 3 μg/ml of Con A (Pharmacia, Piscataway, NJ), 1 ng/ml of PMA (Sigma, St. Louis, MO), and an equal number of irradiated (3300 rad) syngeneic whole mononuclear cells in 12-well flat-bottom plastic tissue culture dishes (Corning, Corning, NY). Medium was replaced every 2–3 days with fresh CM containing IL-2. Primed CD4 T cells were harvested after 10–30 days in culture, and viable cells were obtained by centrifugation over a Ficoll/Hypaque gradient before experimentation.

Northern blot analysis

Primed or fresh CD4 T cells were stimulated in CM with 1.5 μM ionomycin (Calbiochem, San Diego, CA) and 50 ng/ml of PMA for 5–6 h, under conditions previously determined to be optimal for maximal IL-4 mRNA accumulation (7). Total RNA was isolated by the method of Chomczynski and Sacchi (21) using a commercial kit (Trizagent; Molecular Research Center, Cincinnati, OH). RNA (10 μg per sample) was analyzed for IL-2, IL-4, and elongation factor-1α (EF-1α) transcripts by RNA blotting as previously described (7).

Intracellular cytokine detection

CD4 T cells were cultured for 6 h in vitro with CM containing 1.5 μM ionomycin, 50 ng/ml of PMA, and 2 μM monensin (Calbiochem) as previously described (22). The cells were washed twice in ice-cold PBS (pH 7.4) and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5 min at 37°C. Fixed cells were washed in ice-cold PBS with 0.1% (w/v) BSA and permeabilized by incubation overnight at 4°C in PBS-S (PBS [pH 7.4] with 0.01 M HEPES, 0.1% [w/v] BSA, 0.1% [w/v] saponin (Fluka, Ronkonkoma, NY), and 10% human type AB serum). Aliquots of cells (2 × 10⁵) were incubated with 100 ng of phycoerythrin (PE)-conjugated 8D4-8 mAb (murine IgG1 anti-human IL-4; Pharmingen, San Diego, CA) or PE-conjugated M61-17H12 (murine IgG2a anti-human IL-2; Pharmingen) for 30 min on ice. FITC-conjugated murine IgG2a anti-human CD3 mAb (S4.1; Caltag Laboratories, San Francisco, CA) or a FITC-conjugated IgG2a control (Caltag Laboratories) were simultaneously added to detect antigen-specific cytokine mAbs. The cells were serially washed with PBS-S and PBS alone before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Preincubation of anti-IL-4 with 100 ng of human rIL-4 (provided by Dr. Ken Grabstein, Immunex, Seattle, WA) or anti-IL-2 with 100 ng of human rIL-2 (Proleukin-2) for 1 h on ice before addition to the cell resulted in >95% inhibition of staining with anti-IL-4 mAbs (data not shown). Debris were removed from the analysis by gating, using side-scatter vs forward-scatter plots. FCF analysis was performed using the Lysys II software program (Becton Dickinson) and plotted with the program Reproman (True Facts Software, Seattle, WA).

Dimethyl sulfate in vivo footprinting

In vivo footprinting was performed as previously described (23). Primed or fresh CD4 T cells were stimulated at 5 × 10⁶ cells/ml in CM, or CM with 1.5 μM ionomycin, and 50 ng/ml of PMA for 2 h at 37°C. The cells were centrifuged and resuspended in 2 ml of CM at 23°C, and 2.5 μl of dimethyl sulfate (Aldrich, Milwaukee, WI) was added to methylate the N-7 position of the guanidines of the N-3 position of A residues. After gentle mixing for 30 s, the cells were immediately transferred to 15 ml of ice-cold PBS (pH 7.4) with 2 ml of 2-ME. The cells were washed in ice-cold PBS and resuspended and lysed in SDS as described (23). Genomic DNA was isolated from the cell lysate by digestion with 1 mg/ml of proteinase K (Life Technologies, Gaithersburg, MD) for 1 h at 37°C, followed by repeated phenol/ chloroform extraction and precipitation with sodium acetate and ethanol (24). The DNA was dissolved in 0.017 M MCl and incubated for 15 min on ice to open the ring structure of the methylated bases. Strand cleavage was effected at each methylated base by piperidine (Aldrich) (25). The cleaved DNA was used as a substrate for ligation-mediated PCR amplification (26). The nested primers used to visualize the noncoding strand of the human IL-4 proximal promoter were primer 315 (5′-AAGGTTTCCATTACCTATTGGTC) and primer 316 (5′-GGTCGTAGTTCCACAGGACATTTACCTGT and primer 317 (5′-GGTCGTAGTTCCACAGGACATTTACCTGGTTGTGAG). Primers 315, 316, and 317 were annealed in 3 mM MgCl₂ at 54°C, 57°C, and 62°C, respectively. Primer 317 was 32P-labeled, and the footprints were detected by analysis on sequencing gels using autoradiography as described (26). In vitro dimethyl sulfate modification of genomic DNA was conducted as previously described (23).

Reporter gene and NFAT expression constructs

pIL-4-Luc was created by PCR amplification of the human IL-4 promoter (~745 to +60 with respect to the transcriptionsal start site (TSS)) using standard methods (27) and cloned into the multiple cloning site (MCS) of the pGL2-Basic luciferase reporter gene vector (Promega). pIL-2-Luc was made by subcloning the human HindIII/HindIII IL-2 promoter segment (~568 to +50 with respect to the TSS) (28) into pGL2-Basic. pCMV-Luc, provided by Dr. Marianne Sweeter (University of Washington, Seattle, WA), was created by inserting a 1150-bp Smal/HindIII digested fragment of the promoter of the immediate-early CMV gene (29) into pGL2-Basic. The pPA1-Luc and pNFAT-Luc multimer-driven luciferase constructs (30) were generously provided by Dr. Charles Zacharchuk (National Cancer Institute, National Institutes of Health, Bethesda, MD). NFATI/pREP-4, containing a full-length human NFATp cDNA segment driven by the human TGF-βresponsive Rous sarcoma virus (RSV) promoter in the pREP-4 expression vector (InVitrogen, San Diego, CA). A dominant-negative NFAT1 expression vector, NFATDNH2/pREP-4, which lacks the amino terminal transcriptional activation domain, was created by subcloning a 2.8-kb Xhol/BamHI fragment containing a full-length human NFATc cDNA segment (13) (generously provided by Dr. Gerald Crabtree, Stanford University, Palo Alto, CA) immediately downstream of the Rous sarcoma virus promoter in the pREP-4 expression vector (InVitrogen, San Diego, CA). A dominant-negative NFAT1 expression vector, NFATΔNH2/pREP-4, which lacks the amino terminal transcriptional activation domain, was created by subcloning a 5.7-kb XhoI/KpnI truncated fragment of the full-length NFAT1-coding sequence into the pREP-4 expression vector. Multiple independent preparations of plasmids used for transfection were isolated by banding in cesium chloride (24) or by using a commercial affinity column following the manufacturer’s instructions (Qiagen, Chatsworth, CA).
Site-directed mutagenesis

Mutant luciferase constructs of the human IL-4 promoter, P0m-Luc and P1m-Luc, were generated by a standard protocol for single-stranded DNA site-directed mutagenesis, using Escherichia coli C1236, M13K07 helper phage, and a commercially available kit (Bio-Rad, Hercules, CA) (31). The human IL-4 P0 site (5'- end beginning at -58) was mutated from ATTTCCTC to ATTTGAGCT, and the human IL-4 P1 site (5'- end beginning at -76) was changed from AATTTCCTAA to AATTGCAGAA. Mutant plasmid constructs were confirmed by DNA sequencing and restriction enzyme digestion, since the designed mutations created new unique restriction sites.

Transient transfections

T cells were transiently transfected with plasmid DNA using a modified protocol (32, 33). In brief, freshly isolated or in vitro-primed CD4 T cells (5 x 10^6 cells) were electroporated with 10 μg of luciferase reporter plasmids as previously described (27, 32). For cotransfections, 5–20 μg of luciferase reporter plasmids using an Oligo 1000 DNA Synthesizer (Beckman, Palo Alto, CA). The TCA (coding strand) (40). A mutant human IL-4 P1 oligonucleotide (5'-ATTGGAAATTTTCGTTA (noncoding strand) (39); and a human mutant human IL-4 P0 oligonucleotide (5'-ACGTGACCTCAGCGCGC (coding strand)) was used as a nonspecific competitor, with the mutated residues indicated in bold.

Western blot analysis

Total cell protein preparations from freshly isolated or in vitro-primed CD4 T cells were prepared as described (41). One hundred million cells per group were washed in ice-cold PBS and then lysed in 1 ml of Tris-EDTA-NaCl lysis buffer (50 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl, 10 μM dichloroisouccumin (Sigma), 0.5 ng/ml of leupeptin (Boehringer Mannheim, Indianapolis, IN), 0.5 ng/ml of pepstain A (Calbiochem), 0.5 ng/ml of aprotinin (Boehringer Mannheim), 10 μM chymostatin (Sigma), 0.5 μM pepfabc (Boehringer Mannheim), 1 mM DTT (Sigma), and 1 mM sodium orthovanadate) for 5 min on ice. Cellular debris was centrifuged for 5 min at 7,500 × g, and supernatants containing total cellular proteins were stored at -80°C until analyzed. Protein concentration was determined for an aliquot of each protein preparation by the Bradford assay using a commercially available kit (Pierce Chemical, Rockford, IL). Total or nuclear protein (20 μg/sample) was loaded per lane in SDS-containing loading buffer onto a vertical SDS-PAGE gel with a 4.6% acrylamide stacking gel and a 10% acrylamide separating gel, and electrophoresed for 16 h at 50 V/h in SDS-containing running buffer. The proteins were then transferred to nitrocellulose (Amerham, Arlington Heights, IL) by electrophoretic blotting at 50 V/h for 12 h. The blot was blocked for 1 h with PBS (pH 7.4) and 0.1% (v/v) Tween 20 (PBS-Tween), containing 3% (w/v) BSA, and then immunohybridized for 2 h with anti-NFAT1-specific rabbit antisera, 67.1, at a concentration of 1:2,000 in PBS-Tween. After three consecutive washes in PBS-Tween, the blot was reacted for 2 h with a 1:2,000 dilution of donkey anti-rabbit Ig-horseradish peroxidase (Amerham) in PBS-Tween, followed by three washes with PBS-Tween. In the experiments shown in Fig. 7, nuclear protein (15 μg per lane) from freshly isolated or primed CD4 T cells was analyzed using 4–15% polyacrylamide precast gels (Bio-Rad). In Fig. 7, NFAT1 was detected with a 1:500 dilution of a commercially available NFAT1-specific mouse mAb (Transduction Laboratories, Lexington, KY); NFAT2 with a 1:2,500 dilution of a NFAT2-specific mouse mAb, 7A6 (42), generously provided by Drs. Luika Timmerman and Gerald Crabtree (Stanford University, Palo Alto, CA), and NFAT4 with a 1:1,000 dilution of an anti-NFAT4-specific rabbit antisera, provided by some of the investigators (42). Mouse mAbs were detected with 1:500 dilutions goat anti-mouse alkaline phosphatase-conjugated Abs, and the rabbit antisera was detected with a 1:500 dilution of goat anti-rabbit alkaline phosphatase-conjugated antiserum, as per the manufacturer’s instructions (Bio-Rad). Nuclear extracts were determined to be free of cytosolic contamination, since c-Rel was only detected after activation (data not shown). Multicolored m.w. protein standards (Novex, San Diego, CA; or Amerham) were run in parallel for comparison.

Results

Primed CD4 T cells have greater expression of IL-4 transcripts and more IL-4 protein per cell than fresh CD4 T cells

Our previous in situ hybridization experiments using polyclonally activated adult human CD4 T cells have shown a markedly lower frequency of cells expressing IL-4 mRNA than IL-2 transcripts (7). The bulk of these IL-4-producing CD4 T cells express a CD45R0+CD45RAlow memory/effector cell surface phenotype (7, 43), while IL-2 is produced at a similar frequency by effector and antigenically naive (CD45R0lowCD45RAhigh) cells. Since freshly isolated T cells activated and cultured in vitro for several days to weeks (primed) 1) have an increased ability to produce most T cell-derived cytokines, with the exception of IL-2 (8, 9), and 2) tend to lose expression of CD45RA and gain expression of CD45RO (44), priming appears to mimic the process by which T cells in vivo acquire an enhanced ability to produce cytokines during their differentiation from antigenically naive to memory/effector T cells.

In agreement with these earlier results, we found here that the amount of mRNA for IL-4 relative to that for EF-1α, a constitutively expressed housekeeping gene (7, 20), was substantially higher in primed CD4 T cells than freshly isolated circulating CD4 T cells after polyclonal activation (Fig. 1A). The small amount of IL-4 transcripts detected in fresh CD4 T cells is most likely produced by a subset of circulating memory CD45RO+CD45RAlow CD4 T cells (7). In contrast, fresh CD4 T cells produced either more or equivalent amounts of IL-2 mRNA, relative to EF-1α transcripts, compared with primed/effector CD4 T cells (Fig. 1A).

Primed CD4 T cells always produced more IL-4 on a per cell basis than fresh CD4 T cells, although there was substantial variability among donors for IL-4 production by fresh CD4 T cells. Results of an experiment, in which IL-4 production was analyzed in fresh and primed CD4 T cells by intracellular cytokine staining, and where the donor source of the fresh and primed CD4 T cells was identical, are shown in Fig. 1B. After a 6-h stimulation with ionomycin and PMA, 38% of the primed CD4 T cells accumulated IL-4 protein, while only 13% (typically only 2–5% for most donors) of fresh CD4 T cells had detectable IL-4. Furthermore, relatively high
levels of IL-4 in individual T cells were only detected among the primed CD4 T cell population.

In contrast, the vast majority of both primed and fresh CD4 T cells made IL-2 (78% and 72%, respectively), and most IL-2-expressing fresh CD4 T cells contained more IL-2 on a per cell basis than primed CD4 T cells (Fig. 1B). Memory/effector cell markers, such as CD29 (β1 integrin chain), were present at high levels on usually <15% of fresh CD4 T cells, whereas almost all (>95%) primed CD4 T cells expressed these markers at high levels (data not shown), in agreement with previous reports (44). Together, these results indicated that the increase in IL-4 production by priming was due both to an increase in the frequency of the IL-4-producing cells, as well as the amount of IL-4 produced per cell. It seems unlikely that we were selectively expanding a subset of IL-4-producing CD4 T cells that do not also make IL-2, since other investigators have shown that approximately half of the IL-4-producing CD4 T cells also expressed IL-2 (45, 46). Thus, while fresh and primed bulk CD4 T cell populations are polyclonal and display some heterogeneity of cytokine production at the single cell level, these two cell populations provide a useful model for molecular analysis of how priming selectively increases the expression of IL-4 relative to IL-2.

**IL-4 promoter activity is greater in primed CD4 T cells than fresh CD4 T cells**

The recent development of a protocol for transient transfection of normal human T cells allowed us to analyze IL-4 promoter-driven reporter gene constructs in nontransformed bulk populations of T cells (27, 33). Primed and fresh CD4 T cells were compared for their abilities to transcribe, after transient transfection, a reporter gene construct (pIL-4-Luc, containing 0.8 kb of the 5' flank human IL-4 promoter). This determined whether the increased IL-4 mRNA and protein levels in primed CD4 T cells were due to an increased level of transcription conferred by the 5' flank of the IL-4 promoter. For comparison, both cell types were also analyzed for their ability to transcribe a corresponding reporter plasmid containing the proximal human IL-2 promoter (pIL-2-Luc). The ratio of luciferase detected using the pIL-4-Luc construct between the primed and fresh CD4 T cells was substantially greater (17-fold) than the ratio obtained with pIL-2-Luc construct (3.4-fold) (Fig. 2A).

To control for transfection efficiency between the two cell types, fresh and primed CD4 T cells were analyzed for expression of GFP and luciferase reporter genes under the control of the immediate-early CMV promoter. In three separate FCF analyses, 0.57 ± 0.16% of fresh CD4 T cells and 1.54 ± 0.64% of primed CD4 T cells expressed GFP, indicating that the transfection efficiency of primed cells was typically about 3-fold greater than that of fresh cells. In these experiments, GFP and luciferase were determined in parallel, and the ratio of pCMV-Luc activity of primed to fresh CD4 T cells was virtually identical to the ratio of primed to fresh cells which were GFP+.

The correlation coefficient (R) between the absolute values of arbitrary luciferase light units and the percentages of GFP-positive cells for

![FIGURE 1](image1.png) Primed/effector CD4 T cells accumulate more IL-4 mRNA and protein than freshly isolated CD4 T cells. A, Northern blot analysis of 10 μg of total RNA from fresh and primed CD4 T cells before (−) and 5 h after (+) treatment with PMA and ionomycin. The blot was probed for IL-4 and IL-2, and, to control for equivalence of loading, with EF-1α. B, Intracellular cytokine staining of saponin-permeabilized fresh and primed CD4 T cells after 6 h of stimulation with PMA and ionomycin. Cell surface staining for CD3ε is depicted along the x-axis, with cytokine staining for IL-4 (top panels) and IL-2 (bottom panels) on the y-axis.

![FIGURE 2](image2.png) Increased levels of IL-4 by primed CD4 T cells are controlled at the level of transcription. A, Fresh and primed CD4 T cells were transiently transfected with luciferase reporter plasmids and stimulated with PMA and ionomycin for 5 h. Arbitrary light units from fresh (filled bars) and primed (cross-hatched) CD4 T cells transfected with pIL-4-Luc (left) or pIL-2-Luc (right) are presented with SEs of the means for one representative experiment. The background from the control vector, pGL2-Basic, was subtracted for all values. B, Arbitrary light units for primed CD4 T cells from A were corrected for transfection efficiency. Absolute values were divided by the ratio of pCMV-Luc activity obtained with primed and fresh CD4 T cells. C, Ratio of pIL-4-Luc to pIL-2-Luc arbitrary light units from fresh (left) and primed (right) CD4 T cells plotted as individual experiments, and as means ± SDs.
both populations was 0.966 \( (p = 0.002) \). Therefore, the ratio of CMV promoter-directed luciferase activity between the primed and fresh T cells was used to correct for their relative transfection efficiency in subsequent experiments.

IL-2 promoter activity was similar in fresh and primed CD4 T cells when the luciferase light units were corrected for transfection efficiency (Fig. 2B). By contrast, IL-4 promoter activity was approximately 3.5-fold greater in primed CD4 T cells compared with fresh cells. We also determined the ratio of pIL-4-Luc to pIL-2-Luc luciferase activity for either primed effector or fresh T cells, a value that is not influenced by differences in transfection efficiency for these two cell types. The ratio of pIL-4-Luc to pIL-2-Luc in multiple experiments was 0.35 ± 0.09 for the primed CD4 T cells, and 0.09 ± 0.06 for the fresh CD4 T cells (Fig. 2C; \( p = 0.002 \), by the two-tailed, unpaired Student’s \( t \) test). Taken together, these results suggested that increased IL-4 mRNA and protein expression by primed T cells were due, at least in part, to an enhanced level of IL-4 gene transcription directed by the elements within the 0.8-kb segment of the proximal IL-4 promoter. They also indicated that within the context of the reporter plasmid, pGL2-Basic, the transcriptional activity of the IL-2 promoter was stronger than that of the IL-4 promoter in both fresh and primed CD4 T cells. Thus, the immediate 5' flank promoter regions of the IL-4 and IL-2 genes mediated, at least in part, differences in their transcription by primed vs fresh CD4 T cells.

The two P elements most proximal to the TSS are engaged in vivo after cell activation in primed CD4 T cells

Since the region immediately 5' of the IL-4 gene TSS appears critical for activation-induced expression of the IL-4 gene in T cells (47–52), a plausible mechanism for the increased activity of the IL-4 promoter in primed, compared with fresh, CD4 T cells would be increased binding of transcriptional activator proteins to this region in primed CD4 T cells. To test this possibility, we used in vivo footprinting to compare engagement of this region by proteins in these two cell types before and after polyclonal activation for 2 h with PMA and ionomycin. A striking finding was that footprints were detected over both the P0 and P1 elements of the IL-4 gene in primed CD4 T cells following activation (Fig. 3A), indicating protein binding to these sites in vivo. Previous in vitro studies using nuclear protein extracts from activated T cells have indicated that both of these sites bind proteins of the NFAT transcription factor family (53, 54). However, no such footprints were detectable in multiple experiments using polyclonally activated fresh CD4 T cells. This is most likely due to the low percentage of cells in this population expressing IL-4 and the semiquantitative nature of the PCR amplification process used during in vivo footprinting (Fig. 3A). Such footprints were also not observed in unstimulated fresh or primed CD4 T cells, in that the pattern of bands in this region were similar to that obtained with purified human genomic DNA exhaustively methylated in vitro. Clear footprints in response to activation were also detectable at the 5' end of the putative c-maf response element, a site implicated in expression of the IL-4 gene by Th2 cells (55), as well as immediately 3' of this site in primed but not fresh CD4 T cells (Fig. 3A).

In contrast, the octamer-binding site (Oct) located between P0 and P1 was footprinted in primed CD4 T cells before activation (Fig. 3A), but became hypersensitive relative to unstimulated primed CD4 T cells after activation (Fig. 3A), suggesting that activation resulted in the release of protein(s) bound to this site. Moreover, mutation of the Oct site within the context of an otherwise unperturbed pIL-4-Luc reporter gene resulted in increased transcriptional activity (data not shown). Similar repression of transcription by octamer engagement preventing binding of a nearby transcriptional activator, before cell activation, has been observed for the IL-8 promoter (56), but a recent report suggests that octamer binding, in conjunction with P0 element engagement, may actually increase IL-4 promoter activity in a T cell clone (57). This discrepancy may reflect differences between T cells that have been propagated in vitro for short vs long periods of time. Nonetheless, increased human IL-4 promoter activity by primed human CD4 T cells correlated with increased engagement of P0, P1, and the putative c-maf response element, and decreased engagement of the Oct cis-element.

In addition to being bound in vivo after activation in primed CD4 T cells (Fig. 3A), previous work using murine Th2 clones has suggested that P0 and P1 are each critical for IL-4 promoter activity in T cells in response to cell activation (53, 54). To test the functional importance of these elements in primed CD4 T cells, the critical purine residues of P0 and P1 required for NFAT binding (53, 58) were mutated by substitution in an otherwise unaltered pIL-4-Luc construct to create P0m-Luc and P1m-Luc, respectively. Each mutant construct decreased IL-4 expression by >75% in multiple experiments (Fig. 3B), indicating that each of these NFAT binding sites was important for optimal transcription of the IL-4 gene in nontransformed human T cells.

**Primed T cell nuclei are enriched for NFAT1 capable of binding to the IL-4 P1 element**

Since the proximal NFAT-binding sites appeared critical for IL-4 gene expression, we determined if specific binding of nuclear protein, from primed vs fresh CD4 T cells, to P elements in vitro correlated with IL-4 promoter activity and gene expression. Primed CD4 T cells, rested for 3 days by incubation in medium without exogenous recombinant IL-2, contained significant levels

**FIGURE 3.** The proximal NFAT-binding sites (P elements) of the IL-4 promoter are bound in vivo after activation and are critical for optimal IL-4 expression. A. The noncoding strand of the proximal IL-4 promoter was analyzed by in vivo footprinting of primed (above) and fresh (below) CD4 T cells before (Unstim.) and after activation (Stim.) with PMA and ionomycin for 2 h. Sites of protein binding (footprints) are depicted by arrows, and hypermethylated sites are represented by diamonds. The A/G cleavage pattern for genomic DNA (in vitro) is presented for comparison (top). The data are representative of more than four independent experiments for each cell type. In some experiments the hypermethylated band in P1 (far left diamond) was footprinted in primed CD4 T cells. P1 and P0 represent the most proximal NFAT binding sites; Oct is a putative octamer binding site; Maf is a putative binding site for c-maf; TATA is the TATA box region of the IL-4 promoter. B. Primed CD4 T cells were transiently transfected and activated for 5 h, as in Fig. 2, with pIL-4-Luc or the identical plasmid mutated at the P0 or P1 sites. Arbitrary light units, minus the background from pGL2-Basic (means ± the SEs), are shown that are representative of more than five independent experiments.
Primed CD4 T cells have increased levels of NFAT1 in the nucleus capable of binding the human IL-4 P1 element. A. Nuclear protein from primed T cells, before (−) or after (+) polyclonal activation for 2 h, was reacted with an IL-4 P1 oligonucleotide and analyzed by EMSA. Lanes 2 and 4 represent nuclear protein preincubated with an excess of unlabeled P1 oligonucleotide. The migration of free (unbound) labeled probe is indicated at the bottom of the gel. B. EMSA using nuclear protein extracts from activated primed (left lanes) and fresh (right lanes) T cells, and the IL-4 P1 element as a probe. The specificity was confirmed in lane 5 by competition with a 50-fold excess of the identical unlabeled probe. For lanes 2, 3, and 6, the nuclear extracts were preincubated with antisera specific for NFAT1. The specificity of the antisera-induced supershifts was confirmed in lane 3 by preincubating the antisera with the peptide against which it was generated. C. Nuclear protein from activated primed (left) and fresh (right) T cells was analyzed by EMSA using an AP-1 oligonucleotide probe. The specificity of the complex was confirmed by inhibition of complex formation by preincubation of the protein extracts with the identical (lanes 2 and 3), but not an unrelated, nonspecific (ns; lanes 3 and 6), unlabeled competitor oligonucleotide. D. A Western blot of nuclear (left lanes) and total protein (right lanes) from fresh (Fr) and primed (Pr) CD4 T cells before (−) and after (+) activation, probed with antisera to NFAT1. The migratory positions of standard m.w. markers are indicated in kDa to the left of the gel.

To determine whether the observed increased NFAT1 binding to the P1 element in primed T cells was due to quantitative differences, nuclear and total protein from stimulated and unstimulated fresh and primed CD4 T cells was analyzed by Western blot using an NFAT1-specific antisem. NFAT1 was barely detectable, as either a 110- or 130-kDa protein, in nuclear extracts of fresh CD4 T cells (Fig. 4D, lane 1). A small increase in the lower m.w. form was noticeable after activation, and this may represent a dephosphorylated form of NFAT1 (Fig. 4D, lane 2) (62). In contrast, significant levels of NFAT1 were present in the nucleus of unstimulated primed CD4 T cells, primarily as a 130-kDa protein (Fig. 4D, lane 3). After polyclonal activation of the primed CD4 T cells, an increase in the amount of the 130-kDa form of NFAT1 was observed, as well as the appearance of a dominant form of ~120 kDa (Fig. 4D, lane 4). Analysis of metabolically labeled nuclear protein (63) from primed CD4 T cells confirmed that the protein binding to the P1 oligonucleotide was ~115 kDa in size (data not shown). By comparison, significant levels of the higher m.w. form of NFAT1 were detected from total protein preparations of fresh and primed CD4 T cells (Fig. 4D, lanes 5 and 6). However, there was more total NFAT1 detected in the primed CD4 T cells, including low levels of the 110- and 120-kDa forms, possibly derived from the nucleus. Thus, these results suggested that quantitative and, possibly, qualitative differences in nuclear NFAT1 binding (Fig. 4B, lanes 1 and 4) there were roughly equivalent levels of AP-1 protein binding in the nuclei of primed and fresh human T cells after polyclonal activation (Fig. 4C, lanes 1 and 4). The specificity of binding to the AP-1 element was shown by inhibition of complex formation with unlabeled self oligonucleotide (Fig. 4C, lanes 2 and 5), but not by a nonspecific competitor (lanes 3 and 6).
representative of at least seven similar, independent experiments for each cell type. B and cotransfected with $10^6$ (means of pIL-4-Luc and cotransfected with $10^6$), it was significantly higher in primed CD4 T cell types (Fig. 5 A). Although the NFAT multimer had promoter activity in both or the human IL-2 AP-1 site (a multimer of an AP-1 binding site, NFAT site (a multimer of a P element and an adjacent AP-1 site) constructs driven by multimers of either the distal human IL-2 NFAT site (a multimer of a P element and an adjacent AP-1 site) or the human IL-2 AP-1 site (a multimer of an AP-1 binding site, alone). Although the NFAT multimer had promoter activity in both cell types (Fig. 5A), it was significantly higher in primed CD4 T cells compared with fresh CD4 T cells, even when promoter activities were corrected for transfection efficiency (Fig. 5B). Similar results were obtained using a murine IL-4 promoter-derivated NFAT multimer construct site (59) that includes the P1 binding site and an adjacent AP-1 (data not shown). In contrast, both cell types contained similar levels of luciferase activity after transfection with the pAP-1-Luc construct when normalized for transfection efficiency (Fig. 5B). This finding suggested that the increased activity of the NFAT multimer compared with the AP-1 multimer in primed CD4 T cells was due to the presence of the P element component. The mean ratios of the pNFAT-Luc to the pAP-1-Luc activity in fresh and primed CD4 T cells were $1.5 \pm 0.16$ and $9.3 \pm 0.88$, respectively (Fig. 5C). This highly significant difference ($p < 0.001$ by the two-tailed, unpaired Student’s t test) supports the idea that priming preferentially increased P element-directed transcription relative to that directed by AP-1 elements.

**NFAT is critical for optimal IL-4 gene transcription**

To determine whether increased NFAT expression could augment IL-4 promoter activity in fresh and primed CD4 T cells, an expression vector for NFAT1 (NFAT1/pREP-4) was cotransfected with pIL-4-Luc into these cell types. Transfection of fresh CD4 T cells with increasing amounts of NFAT1/pREP-4 markedly increased IL-4 promoter activity in a dose-dependent fashion, achieving levels of activity that were ~3.5-fold greater than basal levels (Fig. 6A). Although transfection of increasing amounts of NFAT1/pREP-4 plasmid into primed CD4 T cells also progressively increased IL-4 promoter activity, the maximal enhancement of promoter activity was relatively modest compared with the basal level (~1.5-fold). Together, these results suggested that the low endogenous levels of NFAT proteins in fresh CD4 T cells were limiting for IL-4 promoter activity, while these levels were

**FIGURE 5.** Priming of CD4 T cells increases their functional NFAT activity. A, Fresh (filled bars) and primed (cross-hatched) CD4 T cells were transfected with pAP-1-Luc (left) and pNFAT-Luc (right) multimer plasmids and activated for 5 h. Arbitrary light units (means ± SEs) are presented for one representative experiment of three. B, Values from A for primed CD4 T cells corrected for transfection efficiency as per Fig. 2. C, Ratio of pNFAT-Luc to pAP-1-Luc light units for fresh (left) or primed (right) CD4 T cells. Values for individual experiments, and means ± SDs are shown.

(translational or posttranslational) may have contributed to the increased binding of the human IL-4 P1 element by EMSA (Fig. 4B).

**Primed/defector CD4 T cells have more functional NFAT than fresh CD4 T cells**

To determine whether increased expression of NFAT protein by primed T cells correlated with an increased capacity for these cells to direct transcription via NFAT-binding cis-elements, fresh and primed CD4 T cells were transfected with luciferase reporter gene constructs driven by multimers of either the distal human IL-2 NFAT site (a multimer of a P element and an adjacent AP-1 site) or the human IL-2 AP-1 site (a multimer of an AP-1 binding site, alone). Although the NFAT multimer had promoter activity in both cell types (Fig. 5A), it was significantly higher in primed CD4 T cells compared with fresh CD4 T cells, even when promoter activities were corrected for transfection efficiency (Fig. 5B). Similar results were obtained using a murine IL-4 promoter-derivated NFAT multimer construct site (59) that includes the P1 binding site and an adjacent AP-1 (data not shown). In contrast, both cell types contained similar levels of luciferase activity after transfection with the pAP-1-Luc construct when normalized for transfection efficiency (Fig. 5B). This finding suggested that the increased activity of the NFAT multimer compared with the AP-1 multimer in fresh (filled bars) and primed (cross-hatched) CD4 T cells transiently transfected with pIL-4-Luc and stimulated as in Fig. 2. An expression plasmid for wild-type NFAT1 (NFAT1/pREP-4) was cotransfected at various concentrations of plasmid (0, 5, 10, or 20 μg) along with 10 μg of pIL-4-Luc. The parent expression plasmid, pREP-4, was added to NFAT1/pREP-4 so that the total quantity of transfected plasmid DNA was equal to 30 μg in all cases. Data are presented as arbitrary luciferase light units (means ± SEs), representative of at least seven similar, independent experiments for each cell type. B, Primed CD4 T cells transiently transfected with 10 μg of pIL-4-Luc and cotransfected with 10 μg of pREP-4 (control), NFAT1/pREP-4 (NFAT1), or NFAT1ΔNH2/pREP-4 (NFAT1 dom. nea.). Arbitrary luciferase light units (means ± SEs) are presented for one experiment representative of five performed. C, Freshly isolated CD4 T cells were transiently transfected with 10 μg of pIL-4-Luc and cotransfected with 10 μg of pREP-4 (control), NFAT2/pREP-4 (NFAT2), or NFAT4/pREP-4 (NFAT4). Arbitrary luciferase light units (means ± SE) are presented for one experiment representative of four performed.

**FIGURE 6.** Levels of wild-type NFAT are critical to IL-4 expression in normal human T cells. A, Fresh (left) and primed (right) CD4 T cells transiently transfected with pIL-4-Luc and stimulated as in Fig. 2. An expression plasmid for wild-type NFAT1 (NFAT1/pREP-4) was cotransfected at various concentrations of plasmid (0, 5, 10, or 20 μg) along with 10 μg of pIL-4-Luc. The parent expression plasmid, pREP-4, was added to NFAT1/pREP-4 so that the total quantity of transfected plasmid DNA was equal to 30 μg in all cases. Data are presented as arbitrary luciferase light units (means ± SEs), representative of at least seven similar, independent experiments for each cell type. B, Primed CD4 T cells transiently transfected with 10 μg of pIL-4-Luc and cotransfected with 10 μg of pREP-4 (control), NFAT1/pREP-4 (NFAT1), or NFAT1ΔNH2/pREP-4 (NFAT1 dom. nea.). Arbitrary luciferase light units (means ± SEs) are presented for one experiment representative of five performed. C, Freshly isolated CD4 T cells were transiently transfected with 10 μg of pIL-4-Luc and cotransfected with 10 μg of pREP-4 (control), NFAT2/pREP-4 (NFAT2), or NFAT4/pREP-4 (NFAT4). Arbitrary luciferase light units (means ± SE) are presented for one experiment representative of four performed.
close to optimal for directing IL-4 promoter activity in primed CD4 T cells. To determine whether NFAT proteins binding to P elements are essential for IL-4 promoter activity by primed CD4 T cells, we generated an expression construct for an amino-terminal-truncated form of NFAT1 (NFAT1ΔNH2/pREP-4). This truncated form of NFAT1 should act as a dominant-negative since it lacks a region containing an important transcriptional activation domain, while retaining the domain required for binding to the P element (64). As seen in Fig. 6B, cotransfection of this construct into primed CD4 T cells substantially decreased pIL-4-Luc activity, in contrast to the full-length NFAT1 plasmid, which slightly enhanced luciferase production. The NFAT1ΔNH2/pREP-4 inhibitory activity was specific, in that it did not inhibit a reporter plasmid (pCMV-Luc) lacking P elements in the promoter (data not shown). These results strongly suggest that NFAT proteins play a critical role in the transcription of the IL-4 gene by primed T cells.

Although the vast majority of NFAT protein bound to the IL-4 P1 element in vitro appeared to be NFAT1 (Fig. 4B), and because NFAT2 and NFAT4 have been detected in cells of the immune system (17, 42, 61), it remained formally possible that either NFAT2 or NFAT4 was contributing to the increased IL-4 gene transcription in primed CD4 T cells. To address this possibility, we analyzed nuclear protein extracts from freshly isolated and in vitro-primed human peripheral blood CD4 T cells, before and after polyclonal activation in vitro, for NFAT2 and NFAT4 expression by Western blot analysis. It was first confirmed that NFAT1 was markedly increased in CD4 T cells by in vitro priming, as detected by an NFAT1-specific mAb directed against a unique epitope from the antisera used in Fig. 4D (Fig. 7A). By comparison, NFAT2 levels appeared to be decreased in the primed CD4 T cells (Fig. 7B). However, NFAT4 also appeared to be increased with priming, although to a lesser degree than NFAT1 (Fig. 7C). To test the functional significance of these results, freshly isolated CD4 T cells were transiently transfected with the pIL-4-Luc reporter gene and cotransfected with 100 ng of pREP-4 control, NFAT2/pREP-4, or NFAT4/pREP-4 expression vectors. Like NFAT1/pREP-4 (Fig. 6A), NFAT2/pREP-4 was able to substantially (~4.5-fold) augment IL-4 promoter-directed transcription in freshly isolated CD4 T cells relative to the pREP-4 control (Fig. 6C). In contrast, NFAT4/pREP-4 was consistently unable to augment IL-4 promoter-directed transcription (Fig. 6C), while capable of increasing IL-2 promoter-directed transcription (data not shown). This may reflect the reported poor binding of NFAT4 to the IL-4 P1 element in vitro (14). Since NFAT2 can be substantially increased in response to T cell activation, it is possible that this protein may play a role in augmenting IL-4 transcription after NFAT1 acts initially (13). Thus, it appears as if NFAT1 is largely responsible for the initial NFAT-dependent increase in IL-4 expression by primed CD4 T cells from peripheral blood.

Discussion

The IL-4 gene serves as a useful model for the alterations in cytokine gene expression during extrathymic T cell differentiation, since its expression by T cells is dramatically increased by priming both in vitro (8) and in vivo (65). Moreover, the events leading to increased IL-4 production by T cell priming are of considerable interest, since IL-4 potentially regulates the outcome of the effector T cell immune response by its ability to promote Th2 differentiation and inhibit Th1 differentiation (reviewed by Abbas et al. in Ref. 66). The acquisition of an enhanced ability for cytokine gene transcription and production during T cell priming is critical for normal effector T cell function, although the mechanisms underlying this are poorly understood.

In this study we have focused on defining how priming increases IL-4 expression by human T cells, comparing molecular events involved in IL-4 gene transcription in freshly isolated peripheral blood CD4 T cells with those occurring when these cells have been primed in vitro. We determined that the IL-4 gene 5′ flank promoter segment was significantly more active in primed than fresh CD4 T cells, whereas the proximal human IL-2 gene promoter was equally active in both cell types. In vivo footprint analysis of the proximal IL-4 promoter demonstrated activation-dependent engagement of the two most proximal NFAT-binding sites in primed but not fresh CD4 T cells. In parallel, primed CD4 T cells contained more NFAT1 and directed more NFAT-driven transcription than fresh CD4 T cells. Moreover, exogenous NFAT1, achieved via transfection of expression plasmids, dramatically enhanced IL-4-mediated transcription by fresh CD4 T cells, but only modestly augmented IL-4 transcription in primed CD4 T cells. This demonstrated that NFAT1 levels were limiting for IL-4 transcription by fresh CD4 T cells but adequate for IL-4 transcription by primed CD4 T cells. Thus, the regulation of cellular NFAT levels appears to be a powerful, yet elegant, mechanism by which to control the selective ability of primed effector CD4 T cells to transcribe IL-4 and probably most other T cell-derived cytokines whose promoters possess NFAT-binding cis-elements.

The notable exception to this dictum is IL-2. The IL-2 promoter appears to be less selective in which NFAT proteins it can bind, allowing for transcription (16). Thus, the lack of NFAT1 in freshly isolated T cells may have little to no effect on IL-2 production, if, for example, NFAT2 or NFAT4 is available. Furthermore, the IL-2 promoter may rely more on other transcription factors, such as NF-kB, for activation-dependent transcription (67, 68), while these same factors in the same cellular context may actually inhibit IL-4 promoter activity (69). Finally, it is possible that the IL-2 promoter may simply require lower levels of NFAT for binding and transactivation than the IL-4 promoter.
Previously, sites of DNA-protein interactions involved in transcrip-
tion of the proximal IL-2 promoter have been examined by in
vivo footprinting (23, 70). However, to the best of our knowledge,
no in vivo footprinting of cytokine promoters has been performed
with nontransformed T cells, nor has there been any previous re-
ports of in vivo footprinting analysis of the proximal IL-4 pro-
moter. Our in vivo footprinting analysis of the two P elements
closest to the IL-4 TSS, P0 and P1, revealed that after polyclonal
activation both sites were clearly engaged in the primed CD4 T
cell population. Consistent with the DNA residues found to inter-
act with NFAT based on the recently derived solution (NFAT2)
(71, 72) and crystal (NFAT1) (73) structures of NFAT, we found
the identical three purine residues (GGA) of the P0 and P1 ele-
ments were most consistently footprinted after activation. In ad-
dition, both P0 and P1 were shown to be independently critical for
optimal IL-4 promoter activity by reporter gene analysis, similar to
what has been previously shown for proximal P elements of the
human CD40-ligand promoter (27) and for corresponding sites in
the murine IL-4 promoter (74). Thus, engagement of the two most
proximal NFAT-binding P elements is critical for, and correlates
with, IL-4 gene transcription by CD4 T cells. The importance of
the P1 element in IL-4 gene expression by effector T cells is also
supported by recent murine experiments in which a P1 multimer-
driven reporter transgene was active in effector T cells but not
freshly isolated T cells (75).

Previously, we and others have found the bulk of NFAT protein
in peripheral T cells capable of binding P elements was NFAT1
(17, 20, 27, 42) and the remainder was NFAT2 (41, 42, 76). In
agreement with these results, supershift assays using the IL-4 P1
element oligonucleotide indicated that the vast majority of the
complex formed with nuclear protein from fresh or primed T cells
contained NFAT1 (Fig. 4B). Since NFAT proteins have been
shown to bind to DNA in vitro as monomers (14, 72, 73), this
suggested that NFAT1 was the predominant NFAT species in-
volved in binding to the proximal IL-4 P elements in vitro and, by
inference, in vivo. Moreover, primed T cells clearly possessed
more protein binding activity for the IL-4 P1 element than did
fresh T cells, whereas both cell populations had similar amounts
of AP-1 protein binding activity. This quantitative difference in
NFAT1 may account for the ability to detect in vivo footprints of
the proximal P elements in primed but not fresh CD4 T cells.

In addition to the quantitative differences in nuclear NFAT1
protein in the two cell types, qualitative differences were noted
between nuclear NFAT1 protein from fresh and primed CD4 T
cells, such that a significant portion of the nuclear NFAT1 from the
primed CD4 T cells migrated at an apparent lower m.w. Others
have shown that lower m.w. forms of NFAT detected after acti-
vation represent less phosphorylated proteins (19, 62). The lower
m.w. NFAT1 protein detected in the nucleus of primed CD4 T
cells most likely reflects a requirement for dephosphorylation of
NFAT1 for its nuclear translocation and trans-activation, similar to
NFAT4 (19). A requirement for dephosphorylation may also ex-
plain the lack of in vivo footprints in primed CD4 T cells before
activation when higher m.w. NFAT proteins are present in the
nucleus. The presence of NFAT proteins in the nucleus before
activation has also recently been observed in murine T cells fol-
lowing priming with mitogen and exogenous IL-4 (60), indicating
that this observation is not unique to human T cells or the partic-
ular priming conditions we employed. The presence of NFAT in
the nucleus most likely reflects a balance between the phosphatase
activity of calcineurin and nuclear kinases which enhance nuclear
export of NFAT (19, 77). Therefore, although priming of CD4 T
cells results in quantitative differences in NFAT, qualitative alter-
ations in NFAT may also be required for NFAT to bind to, and
promote transcription of, the IL-4 promoter and gene, respectively.

Wild-type NFAT protein appears critical for optimal IL-4 trans-
scription by primed CD4 T cells, in that expression of a form of
NFAT1 lacking the amino-terminal trans-activation domain de-
creased IL-4 promoter activity. Since this truncated NFAT1 pro-
tein retained its central DNA-binding domain (64), it is likely that
this protein interfered with IL-4 promoter activity by competing
for binding to P elements, although its involvement at other steps
of NFAT activation and/or nuclear translocation cannot be ex-
cluded. Thus, binding of wild-type NFAT protein to the proximal
P elements of the IL-4 promoter contributes to the ability of
primed CD4 T cells to transcribe IL-4 mRNA. This suggests that
priming acts to increase IL-4 gene transcription by a NFAT-de-
pendent mechanism.

Our results also clearly show that fresh CD4 T cells are rela-
tively deficient in nuclear NFAT proteins after activation, and that
this cell type’s limited ability for IL-4 promoter-directed transcrip-
tion can be overcome by increased amounts of wild-type NFAT1
protein. This finding suggests that NFAT1 may be critical for lim-
iting expression of certain cytokines, such as IL-4, to effector or
memory T cell populations. Our results suggest that there may be
a threshold level of NFAT1, lacking in fresh CD4 T cells but
present in primed CD4 T cells, that is required for NFAT binding
to the proximal P elements of the IL-4 promoter to initiate tran-
scription. If NFAT binding to P elements is competed by binding
of neighboring inhibitory factors in fresh CD4 T cells, then a
greater level of nuclear NFAT might be required in this cell type
for sustained binding. It is also possible that other transcription
factors, scaffolding proteins, or modifiers of transcription factors
or chromatin accessibility may also be required for P element bind-
ing by NFAT to the IL-4 promoter. These proteins might be lacking in
the nuclei of fresh CD4 T cells and might be preferentially in-
creased in their activity or abundance by priming. The recently
identified NFAT-interacting protein (NIP45) (78) or GATA-3 (79)
may be such proteins.

Recently, three laboratories have independently generated mice
that lack NFAT1 as a result of selective gene disruption (80–82),
but the effect of this complete NFAT1 deficiency on memory and
effector T cell development has not been well characterized.
The expression of cytokines by T cells from two of these lines is
reminiscent of the cytokine profile of fresh CD4 T cells. In NFAT1−/−
mice challenged in vivo with a polyclonal stimulus (anti-CD3e
mAb), the T cells demonstrated early defective mRNA production
of most all T cell-derived cytokines, except IL-2 (80), and in sim-
lar NFAT1−/− mice, splenocytes polyclonally activated in vitro
for up to 2 days also demonstrated substantial defects in most
cytokine mRNA while expressing normal levels of IL-2 message
(82). This included the almost complete loss of transcripts for IL-4,
as well as substantial reductions in IL-5, IL-13, GM-CSF, TNF-α,
CD40-ligand, and Fas-ligand mRNA (80, 82). These cytokine
genes contain one or more NFAT binding sites within their en-
hancers or 5′ flanking promoters (11), and, in most cases, expression
of these cytokines is increased by T cell priming (8, 9, 20, 83). In
contrast, IL-2 transcript levels were only minimally reduced (80,
82). These results are consistent with a model in which NFAT1 is
limiting for most early cytokine gene expression, with the excep-
tion of IL-2. However, IL-4 gene transcription by polyclonally
activated primary T cells was reported to be unaffected early in a
third set of NFAT1−/− mice, and actually increased at later time
points (81, 84). It remains unclear whether these markedly differ-
ent results are attributable to differences in experimental design,
the nature of the gene disruption used and its effects on intrathymic
or extrathymic T cell maturation, or differences in compensatory
mechanisms for cytokine gene transcription among the three different knockout strains. Clearly, under conditions of chronic stimulation, such as allergic challenge, NFAT1 is not essential for high levels of IL-4 production in vivo (81, 85). However, all three independently generated NFAT1−/− mouse strains have evidence of abnormally increased accumulation of peripheral T cells with age (80–82), and, at least one strain, increased expression of cell surface activation markers (86). This raises the possibility that T cell homeostasis is altered in these animals, perhaps due to the decreased expression of Fas-ligand (80) by NFAT1−/− T cells. Decreased Fas-ligand or other alterations leading to decreased apoptosis of T cells in vivo (86) might perturb their priming for increased cytokine gene expression. These results can be reconciled with our data with human CD4 T cells, assuming that NFAT1 has an early important role in the initiation of IL-4 gene transcription and a later role that directly or indirectly inhibits Th2 development in vivo.

In summary, priming of CD4 T cells increases IL-4 promoter-directed transcription, and significantly increases NFAT1 capable of binding the proximal P elements of the IL-4 promoter in vivo. Addition of exogenous wild-type NFAT1 can partially overcome the comparative defect in IL-4 production by unprimed CD4 T cells, which are relatively NFAT1-deficient. Furthermore, dominant-negative experiments show the importance of wild-type NFAT for optimal IL-4 transcription in primed CD4 T cells. Lastly, Western blot analysis reveals that priming increases NFAT1 and NFAT4, but not NFAT2, levels in CD4 T cells, and NFAT4 does not appear capable of substantially increasing IL-4 promoter-directed gene transcription in normal human CD4 T cells. Together with the results observed in NFAT1−/− mice (80, 82), these results suggest that the increase in NFAT1 with priming may not only contribute to increased IL-4 production, but may also contribute globally to the enhanced production of most T cell-derived cytokines by effector CD4 T cells.

Acknowledgments
We thank Dr. David Coder and Kathryn Allen for help with FCF; Dr. Seymour Klebanoff (University of Washington, Seattle, WA) for help with lymphoperoxidase measurements; Dr. Chris Hughes (University of California at Irvine, CA) for the electroporation protocol; Dr. Calman Prussin (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD) for the intracellular cytokine staining protocol; Drs. Anjana Rao (Center for Blood Research, Harvard Medical School, Boston, MA), Timothy Hoey (Tulakir, South San Francisco, CA), Kenneth Murphy (Washington University, St. Louis, MO), Charles Zacharchuk (National Cancer Institute, National Institutes of Health, Bethesda, MD), Marianne Sweetser (University of Washington, Seattle, WA), Luika Timmerman, Gerald Crabtree (Stanford University, Palo Alto, CA), and Ken Grinstein (Immunex, Seattle, WA) for providing reagents; Lisa Schubert for reading of the manuscript; and Pam Stepick-Biek and Dr. Lisa Zhou for technical assistance.

References


