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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.

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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 Lymphokine- or T-Helper Kwik, respectively (One Lambda, Los Angeles, CA), as described (20). Flow cytometric analysis of surface markers was performed with BD Cytoflex or FACSCalibur flow cytometers (Becton Dickinson, Mountain View, CA).

In vivo footprinting

In vivo footprinting was performed as previously described (23). Primed or fresh CD4 T cells were cultured for 6 h in vitro with 1.5 μM ionomycin, 50 ng/ml of PMA, 2 mM ionic calcium (Calbiochem, San Diego, CA), 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 N6 position of the N3 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 HCl and incubated for 15 min on ice to open the ring structure of the methylated bases. Strand cleavage was affected 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′-AAGGGTTTCTCACTCATATTGGTC-3′), primer 316 (5′-GGTCTGATTTCCACAGGAACATTCTACCTG-3′), and primer 317 (5′-GGTCTGATTTCCACAGGAACATTCTACATGGATTG-3′). 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 autoradiography as described (26). In vitro dimethyl sulfate modification of genomic DNA was conducted as previously described (23).

Reporter gene and NFAT expression constructs

pLl-Luc was created by PCR amplification of the human IL-4 promoter (~745 to +60 with respect to the transcriptional start site (TSS)) using standard methods (27) and the human IL-4 genomic clone, 10.2, as a template (20). The PCR primers included Pyl and HindIII sites in their 5′ region to facilitate subcloning into the polylinker of the promoterless pGL2-Basic luciferase reporter gene vector (Promega). pLl-2-Luc was made by subcloning the human HindIII/HindIII IL-2 promoter fragment (~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 Small/HindIII-digested fragment of the promoter of the immediate-early CMV gene (29) into pGL2-Basic. The pAP1-Luc and pNFAT-Luc multimter-driven luciferase constructs (30) were generously provided by Dr. Charles Zacharchuk (National Cancer Institute, National Institutes of Health, Bethesda, MD). NFAT1/pREP-4, containing a full-length human NFATp cDNA segment driven by the Rous sarcoma virus promoter (14), and a similar construct, NFAT2/pREP-4, were generously provided by Dr. Timothy Hoey (Tularik, South San Francisco, CA). NFAT2/pREP-4 was generated 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, NFAT1ΔNH2/pREP-4, which lacks the amino terminal transcriptional activation domain, was created by subcloning a 5.7-kb Xhol/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) 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 ATTTTCCCT to ATTTTGAGCT, and the human IL-4 P1 site (5' end beginning at −76) was changed from AATTTCGCAA to AATTCCGAAA. 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 × 10^6 cells) were electroporated with 10 μg of luciferase reporter plasmids as previously described (27, 32). For cotransfections, 5–20 μg of each expression plasmid was included in the electroporation. All time constants generated during electroporations were within 10% of the mean time constant for any particular experiment. Cell viability, as assayed by trypan blue exclusion at 1 h postelectroporation, was >80% for each individual transfection. One million live cells per well were stimulated and analyzed for luciferase activity as previously described (27). Statistical analyses, including Student’s t tests for two group comparisons, were performed using Sigma Stat 2.0 software (Jandel Scientific, San Rafael, CA). In some experiments, transfection efficiency was analyzed by FCF using a plasmid, pGreen Lantern-1 (Life Technologies), encoding green fluorescent protein (GFP) from Aequorea victoria jellyfish (34) under the control of the intact immediate-early CMV promoter. Primed and fresh CD4 T cells (5 × 10^6 cells) were electroporated with 15 μg of pGreen Lantern-1 or pEQ-176 (LucZ driven by the immediate-early CMV promoter, as a control), and cultured in CM for 5 h at 37°C. The cells were washed in PBS with 0.1% (v/v) BSA, incubated with PE-conjugated anti-CD4 (IgG2a, clone S3.5; Caltag Laboratories) or a PE-conjugated IgG2a control mAb (Caltag Laboratories) for 30 min on ice, and then washed sequentially in PBS with 0.1% (v/v) BSA, and in PBS before analysis by FACS. GFP was detected using FITC filters (475-nm excitation peak, 485-nm barrier peak, 490-nm emission peak) as described (35).

Nuclear protein preparations and electrophoretic mobility shift assays (EMSAs)

Fresh and primed whole T cell and CD4 T cell populations were stimulated for 2 h with Con A (25 μg/ml), or ionomycin (1.5 μM) (see Fig. 7), and PMA (25 ng/ml), and nuclear proteins were extracted as described (36). All nuclear preparations analyzed were free of significant contamination by cytoplasmic protein based on the lack of detectable signal for lactate dehydrogenase protein by chemiluminescent Western blot analysis (data not shown). EMSAs were performed as previously described (20). The NFAT1-specific antisense and peptide, 67.1, against which it was made (37), were generously provided by Dr. Anjana Rao (Dana-Farber Cancer Research Center, Boston, MA). This antisense recognizes the amino-terminal portion of NFAT1 (AISPSGLAYFVDVLGYGL) that is not conserved in NFAT2 (37) or NFAT4 (38). Double-stranded oligonucleotides used as 32P-labeled probes or as unlabeled competitors were synthesized using an Oligo 1000 DNA Synthesizer (Beckman, Palo Alto, CA). The oligonucleotides (the sequence of only one strand is shown) included the human IL-4 promoter P1 element (−66 to −82 with respect to the TSS), 5′-ATTGGAAATTTCCGTTA (noncoding strand) (39); and a human metallothionein promoter AP-1 binding site, 5′-ACGTTGACATCGCGGC (coding strand) (40). A mutant human IL-4 P1 oligonucleotide (5′-ATTGTCATTCTTCGTTA) 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 wash buffer (50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 μM dichloroisocoumarin (Sigma), 0.5 ng/ml of leupeptin (Boehringer Mannheim, Indianapolis, IN), 0.5 ng/ml of pepstatin A (Boehringer, Mannheim), 10 μM pefabloc (Boehringer Mannheim), 0.5 μM pefabloc (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 com- mercially 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 (Amersham, Arlington Heights, IL) by electroblotting 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 antiserum, 67.1, at a concentration of 1:2000 in PBS-Tween. After three consecutive washes in PBS-Tween, the blot was reacted for 2 h with a 1:2000 dilution of donkey anti-rabbit Ig-horseradish peroxidase (Amersham) 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 1:2500 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 antiserum, provided by some investigators (42). Mouse mAb were detected with a 1:500 dilutions goat anti-mouse alkaline phosphatase-conjugated Abs, and the rabbit antiserum 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 cytotoxic contamination, as c-Rel was only detected after activation (data not shown). Multicolored m.w. protein standards (Novex, San Diego, CA; or Amersham) 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 CD45R0lowCD45RAlow memory/effector cell surface phenotype (7, 43), while IL-2 is produced at a similar frequency by memory/ effector and antigendriven 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 CD45ROlowCD45RAlow 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. 1A). 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. 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. 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′ flanking 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

![Image](http://www.jimmunol.org/)
of nuclear protein that bound in vitro to the human IL-4 P1 element (Fig. 4A, lane 1), similar to what was found by Wenner et al. (59) and Rincon and Flavell (60). Polyclonal activation of the primed CD4 T cells for 2 h further increased this level of protein binding (Fig. 4A, lane 3). This binding was specific in that addition of a 50-fold molar excess of unlabeled self-oligonucleotide competed for binding (Fig. 4A, lanes 2 and 4). Furthermore, we found no evidence for contaminating cytoplasmic protein since the same nuclear extract had no detectable lactate dehydrogenase (a strictly cytosolic protein) by Western blot analysis, whereas lactate dehydrogenase was easily detected in whole cell extracts from the same cell population (data not shown).

Antiserum to NFAT1 was used during EMSA to determine the identity of the nuclear proteins from activated primed T cells bound to the human IL-4 P1 element. As seen in Fig. 4B, the vast majority of the complex bound to the human IL-4 P1 was supershifted with NFAT1-specific antiserum (lane 2). The supershift was specific since preincubation of the antiserum with the peptide against which it was generated. Furthermore, we found no evidence for contaminating cytoplasmic protein since the same nuclear extract had no detectable lactate dehydrogenase (a strictly cytosolic protein) by Western blot analysis, whereas lactate dehydrogenase was easily detected in whole cell extracts from the same cell population (data not shown).

NFAT1 binding (Fig. 4B, lanes 1 and 4) were those that bound 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 by preincubation of the protein extracts with the identical (lanes 2 and 5), but not an unrelated, nonspecific (ns; lanes 3 and 6), unlabeled competitor oligonucleotide. 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 antiserum 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 antiserum. 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). The small amount detected, however, was specific (lane 5) and predominantly supershifted by the NFAT1-specific antiserum (lane 6), similar to recent results by Lykah et al. (61) and Timmerman et al. (42).
representative of at least seven similar, independent experiments for each cell type. Bm and cotransfected with 10 μg of pIL-4-Luc and cotransfected with 10 μg of pAP-1-Luc. (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.

Primed/effector 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 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 ± 0.16 and 9.3 ± 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 increased by priming.

FIGURE 6. Levels of wild-type NFAT are critical to IL-4 expression in normal human T cells. A. Freshly isolated 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 formed normally 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 10 μg 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 promotor 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 elegantly simple, 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-κB, 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 transcription 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 reports of in vivo footprinting analysis of the proximal IL-4 promoter. 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 interact 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 elements were most consistently footprinted after activation. In addition, 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 involved 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 activation 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 explain 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 following priming with mitogen and exogenous IL-4 (60), indicating that this observation is not unique to human T cells or the particular 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 alterations 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 transcription by primed CD4 T cells, in that expression of a form of NFAT1 lacking the amino-terminal trans-activation domain decreased IL-4 promoter activity. Since this truncated NFAT1 protein 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 excluded. 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-dependent mechanism.

Our results also clearly show that fresh CD4 T cells are relatively deficient in nuclear NFAT proteins after activation, and that this cell type’s limited ability for IL-4 promoter-directed transcription can be overcome by increased amounts of wild-type NFAT1 protein. This finding suggests that NFAT1 may be critical for limiting 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 transcription. 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 binding by NFAT to the IL-4 promoter. These proteins might be lacking in the nuclei of fresh CD4 T cells and might be preferentially increased 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-CD3ε mAb), the T cells demonstrated early defective mRNA production of most all T cell-derived cytokines, except IL-2 (80), and in similar 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 enhancers 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 exception 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 different 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.

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References

Nfat Enhances Il-4 in Primed Cd4 T Cells


