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Integration of Distinct Intracellular Signaling Pathways at Distal Regulatory Elements Directs T-bet Expression in Human CD4\(^+\) T Cells\(^1\)

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T-bet is a key regulator controlling Th1 cell development. This factor is not expressed in naive CD4\(^+\) T cells, and the mechanisms controlling expression of T-bet are incompletely understood. In this study, we defined regulatory elements at the human T-bet locus and determined how signals originating at the TCR and at cytokine receptors are integrated to induce chromatin modifications and expression of this gene during human Th1 cell differentiation. We found that T cell activation induced two strong DNase I-hypersensitive sites (HS) and rapid histone acetylation at these elements in CD4\(^+\) T cells. Histone acetylation and T-bet expression were strongly inhibited by cyclosporine A, and we detected binding of NF-AT to a HS in vivo. IL-12 and IFN-\(\gamma\) signaling alone were not sufficient to induce T-bet expression in naive CD4\(^+\) T cells, but enhanced T-bet expression in TCR/CD28-stimulated cells. We detected a third HS 12 kb upstream of the mRNA start site only in developing Th1 cells, which was bound by IL-12-induced STAT4. Our data suggest that T-bet locus remodeling and gene expression are initiated by TCR-induced NF-AT recruitment and amplified by IL-12-mediated STAT4 binding to distinct distal regulatory elements during human Th1 cell differentiation.


Functional differences in subsets of CD4\(^+\) Th cells are essential to orchestrate efficient immune responses against microbial pathogens. These subsets were primarily defined on the basis of the secretion of specific sets of cytokines that act on other immune cells or cells of nonhematopoietic origin. The development of CD4\(^+\) T cell subsets from naive precursors has been intensely studied because of their essential role for host defenses and because they were shown to be involved in the pathogenesis of various immune-mediated inflammatory diseases. In particular, Th1 or Th17 cells have been associated with chronic inflammatory and autoimmune diseases, whereas Th2 cells have been linked to allergy and asthma.

Th1 cells play critical roles in cell-mediated immune responses, mainly by secretion of their signature cytokine IFN-\(\gamma\). Th1 cell differentiation is initiated by triggering of the TCR on naive CD4\(^+\) T cells and is directed by IL-12. IL-12 binds and signals to a specific receptor expressed on the surface of activated Th1, CD8\(^+\), and NK cells. Naive CD4\(^+\) T cells, however, are unable to respond to IL-12 because they do not express the signaling subunit of the IL-12R, IL-12R\(\beta2\). We have recently shown that TCR signaling is necessary and sufficient to induce chromatin remodeling by the SWI/SNF-like Brahma-associated factor (BAF)\(^5\) complex at two regulatory elements at the human IL-12R\(\beta2\) locus, and to induce low-level expression of the IL-12R\(\beta2\) gene. This initial IL-12R\(\beta2\) expression is then strongly amplified by IL-12 or IFN-\(\gamma\)-\(\alpha\)-mediated activation and binding of STAT4 to an enhancer element (1).

Commitment to the Th1 lineage is controlled by the transcription factor T-bet (T-box expressed in T cells; also called Tbx21) (2). T-bet is not expressed in naive CD4\(^+\) T cells, but is induced during Th1 cell development. However, little is known about the regulatory elements controlling T-bet expression during Th1 cell differentiation.

Evidence that T-bet is a key regulator of Th1 cell development stems from the analysis of T-bet-deficient mice. T-bet\(^-/-\) mice show strongly impaired IFN-\(\gamma\) production by CD4\(^+\) T cells, fail to resolve Leishmania major infection, and spontaneously develop airway hyperreactivity, consistent with a marked propensity to develop Th2 responses in vivo (3–5). T-bet appears to directly activate the IFN-\(\gamma\) gene by binding to regulatory elements (6). Work in the mouse system initially proposed that expression of T-bet was induced by signals via the IL-12/STAT4 axis (2). Subsequently, it was shown that T-bet expression and Th1 development could occur in STAT4-deficient mice and that retrovirus-mediated expression of T-bet in STAT4\(^-/-\) T cells induced IFN-\(\gamma\) production, chromatin remodeling of the IFN-\(\gamma\) locus, expression of IL-12R\(\beta2\), and up-regulation of endogenous T-bet (7), suggesting that T-bet initiates Th1 differentiation independently from IL-12.

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5 Abbreviations used in this paper: BAF, Brahma-associated factor; ChIP, chromatin immunoprecipitation; Ca\(\alpha\), cyclosporin A; CT, cycle threshold; GAS, IFN-\(\gamma\) activation site; HS, hypersensitive site; QCP, quantitative chromatin profiling; qRT-PCR, quantitative RT-PCR; RHO, rhodopsine gene; sRNA, small interfering RNA; sS-TAT, small interfering STAT.
STAT4 signaling. Further studies reported that T-bet expression is strongly induced by IFN-γ signaling via STAT1 activation (8–11). More recent studies have invoked a role for Notch signaling and the transcription factors Sp1 and STAT1/STAT4 in the regulation of T-bet expression in the mouse (10, 12, 13). T-bet has also been shown to play an important role in human Th1 cell differentiation, as has been demonstrated by retroviral transduction (14–16). In this study, we have investigated how T-bet expression is controlled at early time points following stimulation of naive human CD4+ T cells. Using a combination of biochemical and bioinformatics approaches, we have identified cis-regulatory elements at the human T-bet locus. Mapping of DNase I-hypersensitive sites (HS) revealed a weak HS proximal to the mRNA start site in naive CD4+ T cells. TCRR/CD28 signaling induced two strong HS in developing Th1 and Th2 cells (HS2 and 3). A third HS (HS1) was detected 12 kb upstream of the mRNA start site only in developing Th1 cells. T cell activation induced rapid histone H4 acetylation at these elements and binding of NF-AT to HS2 in vivo. H4 acetylation, NF-AT binding, and T-bet expression were strongly reduced when cells were stimulated in the presence of cyclosporin A (CsA), suggesting that TCR-induced NF-AT activation and binding to the T-bet locus were required to initiate T-bet expression in naive CD4+ T cells. IFN-γ and IL-12 signaling alone were not sufficient to induce T-bet expression in naive CD4+ T cells, but cytokine signaling enhanced T-bet expression in TCR/CD28-stimulated cells. We found that IL-12 signaling induced binding of STAT4 to a specific sequence at HS1 and showed that small interfering RNA (siRNA)-mediated knockdown of STAT4 expression was sufficient to induce T-bet expression in naive CD4+ T cells, but that cytokine signaling enhanced T-bet expression in TCR/CD28-stimulated cells. Further studies reported that T-bet expression is associated with the Th1 phenotype.

Collectively, our data suggest that epigenetic modifications and gene expression at the T-bet locus are controlled at least in part by two distal regulatory elements that serve as platforms to integrate TCR and cytokine signaling during human CD4+ T cell differentiation.

Materials and Methods

**Purification of naive CD4 + and CD8 + T cells**

Human mononuclear cells were isolated from peripheral blood or cord blood by Ficoll-Paque (GE Healthcare) density gradient centrifugation. CD4+ T cells were purified from peripheral blood leukocytes by negative selection on magnetic columns using the human naive CD4+ T cell isolation kit. CD4+ T and CD8+ T cells from cord blood leukocytes were isolated by positive selection on magnetic columns using CD4 or CD8 microbeads according to the protocol provided by the manufacturer (Miltenyi Biotec).

**T cell stimulation**

Purified naive CD4+ or CD8+ T cells were stained using anti-CD3 (1 μg/ml, 1XE; CLB) and anti-CD28 Abs (1 μg/ml; BD Biosciences) in the presence or absence of IL-12 (2.5 ng/ml; Roche), IFN-γ (1000 U/ml; Roche) and IL-4 (1 ng/ml; BD Biosciences), or in the presence of neutralizing anti-IFN-γ (1 μg/ml; BD Biosciences), anti-IL-12 (Roche), or anti-IL-4 Abs (1 μg/ml; BD Biosciences), as described (1), or by stimulating CD8+ T cell-depleted cord blood leukocytes with PHA (2 μg/ml; Sigma-Aldrich) in the presence of IL-12 and anti-IL-4 mAb or IL-4 and anti-IL-12 and anti-IFN-γ Abs, as described (17).

**NF-AT inhibition with CsA**

Naive T cells from cord blood were incubated with or without CsA (1 μg/ml; Sigma-Aldrich) for 45 min and stimulated with plate-bound anti-CD3 (TR66) and anti-CD28 mAbs, as described (1).

**RNA interference**

RNA interference experiments were performed exactly as described (1). Briefly, siRNA oligos (Qiagen) were resuspended in siRNA suspension buffer (provided by Qiagen; composition not indicated) at a final concentration of 20 μM. Oligos were heated to 90°C and then incubated 1 h at 37°C. Naive CD4+ T cells were resuspended in human T cell Nucleofector solution (Amaxa) at a concentration of 30–40 × 10^6 cells/ml, and 95 μl of cell suspension was mixed with 5 μl of the oligo (1 μM final concentration). Transfection was performed, as described in the protocol from the manufacturer, using the program U-14. Immediately after nucleofection, cells were transferred with prewarmed medium to a 24-well plate and incubated overnight. Cells were stimulated the next morning with anti-CD3/CD28 in the absence or presence of IL-12 and neutralizing anti-IFN-γ Abs. Efficiency of the knockdown was determined by RT-PCR using TaqMan probes 24 and 48 h after stimulation. The sequences (sense strand) of siRNA duplexes are given as follows: small interfering control, UUCUCGAACGUGACAGCUTdTdT; small interfering RNA targeting STAT (siSTAT4), GGAAGGUCUUCAGGG AAAAdTdT; siSTAT1, AUCUUCGUACGAAUAAdTdT.

**RNA extraction and quantification of mRNA level**

Total RNA was purified with RNaseasy columns (Qiagen), cDNA was reverse transcribed from 1 μg of total RNA using the SuperScript II kit (Invitrogen) and random hexamers (Roche). RT-PCR was performed using the 7300 RT-PCR System (Applied Biosystems) with TaqMan probes purchased from Applied Biosystems or MWG Biotec for the following genes: IL-2, IL-12Rβ2, T-bet, and IFN-γ, as described (1). Normalization was performed using hypoxanthine phosphoribosyltransferase mRNA or 18S RNA.

**Western blot**

Western blotting was performed on cord blood-derived CD4+ T cells stimulated overnight, as described above, in the presence of the indicated cytokines or anti-cytokine Abs. Cell lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (GE Healthcare), and probed with mouse anti-T-bet mAbs (sc-21749; Santa Cruz Biotechnology) or rabbit polyclonal anti-actin Abs (Santa Cruz Biotechnology) as a loading control. Proteins were visualized by ECL (Pierce).

**ELISA**

CD4+ T cells from cord blood were stimulated for Western blotting, and IFN-γ production was measured in supernatants using a protocol provided by the manufacturer (Biolegend).

**Mapping of DNase I HS**

Freshly purified naive CD4+ T cells or Th1 and Th2 cells (1 × 10^6, harvested 6 days after stimulation) were washed with PBS, resuspended in 2 ml of buffer A (10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 1.5 mM MgCl2), and disrupted by dounce homogenization. Nuclei were resuspended in DNase I digest buffer B (0.15 M sucrose, 80 mM KCl, 35 mM HEPES/KOH (pH 7.4), 5 mM KH2PO4 (pH 7.4), 5 mM MgCl2, and 2 mM CaCl2) divided in five aliquots, and treated with increasing concentrations of DNase I (none, 1 U, 2 U, 5 U, and 20 U) for 2 min at 25°C. After extraction (Qiaprep Spin tips), genomic DNA (10 μg) was digested with AscI or Spel restriction enzymes, separated on agarose gels, transferred to a membrane (Hybond N+; GE Healthcare), and hybridized with a probe located 5 kb upstream of the mRNA start site.

**Mapping of DNase I HS by quantitative chromatin profiling (QCP)**

Naive CD4+ T cells from cord blood were stimulated overnight in Th1 or Th2 conditions. Cells were harvested and washed in 5 ml of buffer A (15 mM Tris-Cl (pH 8), 15 mM NaCl, 60 mM KCl, 1 mM EDTA (pH 8), 0.5 mM EGTA (pH 8), 0.5 mM spermine, and 0.15 mM spermidine). Cells were resuspended in 5 ml of buffer A; 5 ml of Nonidet P-40 buffer (0.08% buffer A) was added dropwise, and the cells were incubated 10 min on ice to release nuclei. Nuclei were then washed, collected, and resuspended in buffer A to a concentration of ~10^6 nuclei/ml. 10X digestion buffer (60 mM CaCl2, and 750 mM NaCl) was added. Digestions were conducted at 37°C with different concentrations of DNase I (none, 200 U, 400 U, and 800 U) for 3 min after being stopped by the addition of an equal volume of stop buffer (200 mM NaCl, 200 mM Tris Cl (pH 8), 25 mM EDTA (pH 8), and 0.5% SDS). Samples were treated with proteinase K overnight and DNA recovered after extraction with phenol/chloroform and precipitation in ethanol.

To study DNase I sensitivity, we performed RT-PCR on the DNA samples using the 7300 RT-PCR System (Applied Biosystems) with TaqMan probes covering the T-bet locus every 1.5 kb up to 13.5 kb upstream of the mRNA start site. The amount of template DNA was standardized by co-amplification of the rhodopsine gene (RHO), which is DNase I insensitive in T cells, as described (18, 19).
**Chromatin immunoprecipitation (ChIP) assay**

The histone modification state at the HS sites of the T-bet locus and the NF-AT and STAT4 binding to HS2 and HS1, respectively, of the T-bet gene and GAPDH promoter (control) were analyzed by ChIP assays, according to the following protocol. Cross-linking was performed by incubating cells in medium containing formaldehyde (1%) for 10 min at room temperature. The fixation reaction was stopped by incubating cells in glycine stop-fix for 5 min at room temperature. Cells were washed twice with PBS with protease inhibitors (Roche), resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris (pH 8.1)), and incubated for 10 min on ice. Each lysate was sonicated for 5 cycles of 10 s on and 30 s off. The sonicated lysate was then diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl). A total of 20 μl of diluted lysate was taken to normalize the results, which is referred to as the input sample. The samples were incubated overnight at 4°C with anti-acetylated histone H4 Abs (2 μg; Upstate Biotechnology), rabbit IgG (2 μg, Upstate Biotechnology) as a negative control, anti-NF-AT2 Abs (2 μg, 7A6; Santa Cruz Biotechnology), and anti-STAT4 Abs (2 μg, C20; Santa Cruz Biotechnology), or anti-FLAG Abs (2 μg, M2; Sigma-Aldrich) as another negative control. To collect the Ab/histone complex the next morning, protein A or protein G agarose beads (GE Healthcare) were incubated with the sample for 1 h at 4°C. The protein A/agarose/histone complex was washed with high salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl), high salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl), and LiCl Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl). The samples were washed twice with PBS with protease inhibitors (Roche), resuspended in Preparation Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl), and 500 mM NaCl, and LiCl Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl). The DNA amount was raised to 25 ng/ml ionomycin (Sigma-Aldrich). After 8 h, cells were assayed for luciferase activity in a Centro/Centro XS3 Microplate Luminometer LB 960 (Berthold Technologies) using the Dual-Luciferase assay system (Promega), according to the manufacturer’s instructions. T-bet promoter and HS2 reporter gene constructs

An 800-bp T-bet promoter fragment (−821 to −21) upstream from the mRNA start site was amplified from genomic DNA of CD4+ T cells. The sequences of sense and antisense primers used to amplify the fragment were as follows: 5'-TCTATGATGACCACTGTA-3’ and 5'-CCTGTCGTTATTAAGC-3’, respectively. The PCR product was sub-cloned into a TopoTA vector (Invitrogen) and was confirmed by sequencing. The T-bet promoter luciferase reporter construct pGL3-T-bet was generated by inserting the promoter fragment into the pSoul site of the pGL3-basic vector (Promega). The pGL3-prom-HS2 construct was generated by inserting a dsDNA oligo carrying three copies of the NF-AT binding site from HS2 (5’-TGATGAGATTTCGGC-3’)-12 kb) of T-bet gene: forward, 5’-TGTGGCTTTATGAAGC-3’; reverse, 5’-ATTGGAATTTGCGCCTAGCTTAG-3’.

**Reporter gene assays**

Jurkat E6.1 cells (referred to as a Jurkat) were resuspended in RPMI 1640 medium at a concentration 1 × 10⁵ cells/ml and transiently transfected by electroporation at 260 V, 950 μF with 10 μg of the reporter gene construct and 1 μg of pRL_TK encoding Renilla luciferase gene (Promega). Total DNA amount was raised to 25 μg using an empty pBluescript vector. As a positive control, we used the NF-AT-induced luciferase construct (pNFA-Tluc) described previously (20). Eighteen hours later, cells were left unstimulated or stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 250 ng/ml ionomycin (Sigma-Aldrich). After 8 h, cells were assayed for luciferase activity in a Centro/ Centro XS3 Microplate Luminometer LB 960 (Berthold Technologies) using the Dual-Luciferase assay system (Promega), according to the manufacturer’s instructions. T-bet promoter and HS2 activities were expressed as a ratio of firefly luciferase to Renilla luciferase luminescence. Results are given as the induction of luciferase activity in stimulated compared with resting cells. Each experiment was performed in triplicates.

**Results**

Early induction of T-bet requires TCR/CD28 signaling and is amplified by cytokines

T-bet is not expressed in naive CD4+ T cells, but is rapidly induced during Th1 cell development (5). To define the factors that initiate this process, we stimulated purified naive CD4+ T cells with anti-CD3 and anti-CD28 Abs alone or in the presence of the Th1-inducing cytokines IFN-γ or IL-12, or with the Th2-specific cytokine IL-4. Cells were harvested at different time points after stimulation, and gene expression levels were determined by quantitative RT-PCR (qRT-PCR; Fig. 1A). These experiments revealed that cytokine signaling alone without stimulation via the TCR did not induce T-bet transcripts (Fig. 1A, right panel). In contrast, TCR signaling alone was sufficient to induce T-bet expression as...
Early as 8 h after stimulation, indicating that TCR signaling is necessary and sufficient to induce T-bet expression in human naive CD4+ T cells. Cotreatment with IFN-γ or IL-12 amplified expression of the T-bet gene. However, these cytokines induced T-bet transcripts with different kinetics. T-bet mRNA was detectable as soon as the T-bet gene. However, these cytokines induced T-bet expression; however, T-bet mRNA (Fig. 1B) was substantially increased in the presence of IL-12 (supplementary Fig. 1).6 Addition of blocking anti-IFN-γ Abs only slightly decreased IFN-γ production.

These findings further support the notion that TCR signaling is necessary and sufficient for the early induction of T-bet in naive CD4+ T cells. IFN-γ production in response to TCR stimulation increased T-bet expression; however, T-bet mRNA (Fig. 1B) and protein (Fig. 1C) were induced in the presence of blocking anti-IFN-γ Abs, indicating the role of an IFN-γ-independent pathway in TCR-induced T-bet expression. Similarly, IL-12 treatment also increased T-bet expression at the mRNA and protein level (Fig. 1, B and C) in the presence of blocking anti-IFN-γ Abs, suggesting an IFN-γ-independent component in T-bet induction, consistent with previous results (24).

6 The online version of this article contains supplemental material.
T-bet functions are not restricted to Th1 cells, but have been shown to be involved in the generation of memory CD8+ T cells (25). Studies in mouse cells revealed that T-bet is induced in CD8+ T cells in response to IL-12 signaling and that this process is IFN-γ independent (13, 26). We found that TCR stimulation of naive human CD8+ T cells resulted in the induction of T-bet transcripts at 16 h of stimulation (Fig. 1D). T-bet expression was strongly enhanced when cells were stimulated in the presence of IL-12. In contrast to CD4+ T cells, addition of IFN-γ did not further increase T-bet expression in TCR-stimulated CD8+ T cells.

Collectively, our data demonstrate that TCR/CD28 signaling plays a critical role in the early induction of T-bet transcripts in naive CD4+ and CD8+ T cells, and that both IFN-γ and IL-12 further enhance T-bet expression during human Th1 cell development.

Identification of potential regulatory regions within the human T-bet locus

To identify potential cis elements that control T-bet expression, we mapped DNase I HS at this locus in naive CD4+ T cells and in cells developing along a Th1 or Th2 pathway. We used two complementary techniques: conventional HS assays based on Southern blot analysis (27) and QCP, a PCR-based approach (19). The first technique allowed us to analyze a contig of 16.7 kb spanning 10.7 kb upstream and 6 kb downstream of the transcription start site of the human T-bet gene. We identified two strong HS in Th1 and Th2 cells. HS2 maps 7.5 kb upstream and HS3 in the proximity of the mRNA start site, respectively (Fig. 2, A and C). HS2 was not detected in naive CD4+ T cells (Fig. 1A, upper left panel), whereas HS3 was partially sensitive to DNase I digestion in naive cells (Fig. 1A, lower left panel). HS3 overlaps with a HS recently identified in a genome-wide screen for open chromatin that revealed sensitivity to DNase I of the T-bet promoter region in unfractonated human CD4+ T cells (28).

Because we did not identify any Th1-specific HS within the 16.7 kb surrounding the mRNA start site, we screened for potential Th1-specific HS using the QCP technique, which is not limited by the presence of convenient sites for restriction enzymes. After treatment of nuclei with DNase I, sensitivity of chromatin to this enzyme was examined by qRT-PCR with primers covering the T-bet locus. The digestion profile is shown by plotting the number of remaining copies against the number of units of enzyme used in digestion. The amount of template DNA was standardized by correcting for amplification of the T-bet promoter region in Th1 and Th2 cells, the promoter region GAPDH gene as a positive control. Consistent with high expression of GAPDH in Th1 and Th2 cells, the promoter region GAPDH was highly accessible to DNase I, because the number of copies decreased rapidly with increasing DNase I concentration (Fig. 2, upper right). In contrast, the GAPDH promoter appeared to be less accessible in naive CD4+ T cells. By screening the upstream region of the T-bet gene, we identified an additional HS, HS1, located 12 kb upstream of the transcription initiation site, which was highly accessible to DNase I treatment in Th1 cells (Fig. 2B, lower left). HS1 was less accessible in Th2 cells and not detectable in naive CD4+ T cells. DNA sequences flanking HS1 were less sensitive to DNase I digestion, indicating the restricted sensitivity of this region to DNase I. The three HS identified in our experiments overlapped with conserved noncoding sequences identified in silico by alignment of human and mouse or human and dog genomic DNA sequences (Fig. 3 and supplementary Fig. 2). Together, these findings suggested an important role of HS1–3 in the regulation of T-bet gene expression.

FIGURE 2. Mapping HS to DNase I at the human T-bet locus. A, Conventional HS assay was performed on naive CD4+CD45RO– T cells and cells stimulated for 6 days in Th1 or Th2 conditions. Nuclei isolated from these cells were treated with increasing amounts of DNase I, followed by genomic DNA digestion with Acc65I (upper panel) or SpeI (lower panel) restriction enzymes. Southern blot analysis was performed using a probe localized 5 kb upstream of the mRNA start site. Shown is one representative of two experiments performed with independent donors. B, Mapping of HS to DNase I at the T-bet locus by QCP. Nuclei isolated from naive CD4+ T cells or cells stimulated overnight in Th1 or Th2 conditions were digested with increasing concentrations of DNase I, and RT-PCR was performed with primers covering the T-bet locus up to 13.5 kb upstream of the transcription start site of the T-bet gene. Digestion profiles for GAPDH promoter, RHO (upper panel), and T-bet locus at −13.5, −12, and −10.5 kb (lower panel) were generated by plotting the number of remaining copies against the number of units of enzyme used in digestion. The amount of template DNA was standardized by correcting for amplification of the DNase I-insensitive RHO. One representative of four experiments is shown. C, Positions of HS of the human T-bet gene with respect to the SpeI and Acc65I restriction sites, the localization of first exon, and the probe used for Southern blot analysis.

T cell activation and IL-12 signaling induce chromatin modifications at the human T-bet locus

Because TCR/CD28 and IL-12 signaling induced the appearance of specific HS at the T-bet locus, we analyzed the effect of these signaling pathways on histone modifications at these regulatory
elements. In particular, we were interested in histone acetylation as a mark of active genes (29). We performed ChIP assays with anti-acetyl histone H4 Abs on naive CD4⁺/H11001 T cells and cells stimulated with anti-CD3/anti-CD28 Abs alone or in the presence of either CsA or IL-12. The association of the HS1–3 regions with acetylated histones was analyzed by qRT-PCR. We found that H4 was hyperacetylated 20 h after TCR triggering at the three HS sites (Fig. 4). T cell stimulation in the presence of CsA resulted in a strong reduction of H4 acetylation at HS2 and HS3, but not at the Th1-specific HS1. Histone acetylation at HS1 was substantially increased when the cells were stimulated in the presence of IL-12. In contrast, stimulation in the presence of IL-12 did not further increase the level of histone acetylation at HS2 and HS3 of the T-bet locus compared with TCR stimulation alone, indicating that these elements were not targets for IL-12 signaling. As control, we analyzed H4 acetylation at the GAPDH promoter, which remained unchanged in the examined conditions.

These experiments indicated that TCR and cytokine signaling pathways act at distinct regulatory elements and control accessibility of the human T-bet locus in a hierarchical manner. TCR signaling was required to induce accessibility at HS2 and HS3 and appeared to be independent of IL-12 signaling, whereas accessibility at HS1 was largely controlled by IL-12. Furthermore, the finding that TCR-induced chromatin remodeling at HS2 and HS3 was sensitive to CsA indicated that NF-AT activation may be involved in the early events controlling T-bet locus remodeling following stimulation of naive CD4⁺ T cells.

TCR stimulation induces NF-AT2 binding to HS2 of the T-bet locus

Several transcriptional activators can bind directly to histone acetylases or ATP-dependent chromatin-remodeling complexes (30). This indicates that the recruitment of transcription factors to DNA and chromatin structure modifications are interdependent. Inhibition of chromatin remodeling at the T-bet locus by CsA strongly suggested the role of NF-AT proteins in this process. We therefore asked whether NF-AT was recruited to the T-bet locus. Inspection of the DNA sequence encompassing the regulatory regions of the human T-bet gene revealed a consensus binding site for NF-AT proteins located at HS2 (Fig. 5C). We stimulated naive CD4⁺ T cells via the TCR in the presence of anti-IFN-γ Abs, and in the presence or absence of CsA. Cells were harvested 16 h after stimulation, and ChIP assays with anti-NF-AT2 Abs were performed. Stimulation of naive CD4⁺ T cells via the TCR in the presence of anti-IFN-γ Abs, and in the presence or absence of CsA. Cells were harvested 16 h after stimulation, and ChIP assays with anti-NF-AT2 Abs were performed. Stimulation of naive CD4⁺ T cells via the TCR resulted in NF-AT2 binding to HS2 in the absence of CsA. NF-AT2 recruitment was strongly reduced when cells were stimulated in the presence of CsA (Fig. 5A). Consistent with the lack of a consensus binding site, NF-AT recruitment was not observed at HS3 (data not shown).

To analyze responsiveness of HS2 and HS3 to TCR activation, we have performed reporter gene assays with constructs containing the T-bet promoter or three copies of the NF-AT binding site present in HS2. We found that the HS2 element enhanced 2.6-fold the activity of the reporter gene in response to T cell stimulation (supplementary Fig. 3). As a positive control, we have used the NF-AT luc construct that contains three copies of the combined NF-AT/AP1 binding site from the human IL-2 promoter (20). We observed only a minor induction of reporter gene activity following stimulation of T cells transfected with the T-bet promoter construct. This is consistent with the absence of high-affinity NF-AT
STAT4 is recruited to HS1 in differentiating Th1 cells in vivo

To define the mechanism underlying IL-12 inducibility of the human T-bet gene, we analyzed the DNA sequence surrounding HS1, the element preferentially accessible in Th1 cells. We identified a consensus binding site for STAT factors at HS1 (Fig. 5D). Dependence of chromatin remodeling at this site on IL-12 signaling (Fig. 4) prompted us to analyze recruitment of STAT4 to this element. ChIP assays revealed that this element is bound by STAT4 in IL-12-treated CD4+ T cells in vivo (Fig. 5B). As expected, IL-12 signaling did not induce the binding of STAT4 to the GAPDH promoter (Fig. 5B). These experiments demonstrated that HS1 is an important regulatory element mediating IL-12 responsiveness via STAT4 recruitment to the human T-bet locus. To test whether the GAS element in HS1 bound STAT4 in vitro, we incubated nuclear extracts from IL-12-treated and untreated Th1 cells with biotinylated oligonucleotides corresponding to the HS1 GAS element in HS1 bound STAT4 in vitro. Th1 cells were generated as for STAT4 binding to HS1 of the T-bet locus in vitro. Th1 cells were stimulated 7 days after priming in the presence or absence of IL-12 for 45 min before harvesting. ChIP assays were performed with anti-STAT4 Abs or rabbit IgG as control. The enrichment of HS1 enhancer sequences in the precipitate was measured by RT-PCR using TaqMan probes, as in A. Shown is binding of STAT4 to HS1 of the T-bet locus (left panel) and to GAPDH promoter as control (right panel) relative to naive CD4+ T cells. The results were normalized to the amount of DNA immunoprecipitated with rabbit IgG. One representative of three experiments is shown. C. STAT4 binds to HS1 of the T-bet locus in vitro. Th1 cells were generated as for B. Cells were stimulated for 45 min with IL-12 or without IL-12 (none). Affinity capture assays were performed with double-stranded biotinylated oligos spanning the wild-type (HS1) or mutated (HS1 mut, as a control) GAS elements in HS1 of the T-bet locus. STAT4 binding was analyzed by Western blotting with anti-STAT4 Abs. Ten percent of the cell lysates were loaded as controls. One of two experiments is shown. D. Localization and sequences of the STAT and NF-AT binding sites at the human T-bet locus.

STAT4 knockdown impairs IL-12-induced T-bet gene expression

To confirm that STAT4 mediates IL-12-induced T-bet expression, we knocked down STAT4 expression using siRNAs. Naive CD4+ T cells were transfected with the indicated siRNAs and stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-12. Transfected cells were harvested 24 and 48 h after stimulation, and expression of T-bet, IL-12Rβ2, and IFN-γ transcripts was determined by qRT-PCR. STAT4 knockdown resulted in reduced expression of the three genes associated with the Th1 phenotype at both time points (Fig. 6). STAT4 knockdown had a more pronounced effect on the level of IFN-γ transcripts (more than 10-fold reduction) than on IL-12Rβ2 and T-bet mRNA levels (3- and 2-fold reduction, respectively). Consistent with the fact that STAT1 is not the major STAT molecule implicated in IL-12 signaling, knockdown of STAT1 expression had only modest or no effect on the expression of IFN-γ, IL-12Rβ2, and T-bet transcripts. These observations provide further evidence for an important role of STAT4 in the induction of T-bet during human Th1 cell differentiation.

Because IFN-γ enhanced T-bet expression, we asked whether STAT1 also plays a role in IFN-γ-induced T-bet expression in human CD4+ T cells; we have knocked down STAT1 in naive CD4+ T cells stimulated in the presence of IFN-γ. We observed that STAT1 knockdown negatively affects T-bet expression already after 8 h of stimulation (supplementary Fig. 4). These data are consistent with previous reports that have demonstrated that IFN-γ is of critical importance for the early induction of T-bet expression during mouse Th1 cell differentiation (8, 9, 11, 13).

Discussion

The transcription factor T-bet has been demonstrated to be a primary regulator of Th1 cell development (31). T-bet induces chromatin remodeling at the IFN-γ locus and IFN-γ gene transcription, as well as repression of Th2-specific cytokine genes (5, 32). Although it has been established how T-bet directs Th1 development, little is known about how expression of this gene is regulated in human cells.

To identify functional elements controlling T-bet expression, we have analyzed accessibility of chromatin at the human T-bet locus in naive CD4+ T cells and in cells differentiating along the Th1 or the Th2 pathway. By conventional Southern blot-based mapping...
and the PCR-based QCP technique, we identified three cis-regulatory elements (HS1–3) that relay information elicited at the TCR and at cytokine receptors. HS3 is proximal to the mRNA start site of the human T-bet gene and is the only HS that is detectable in naive CD4+ T cells, which revealed accessibility around the mRNA start site of the T-bet gene (28). Similar to the promoter region of the human IL12RB2 gene, this region is highly GC rich, contains several binding sites for the ubiquitously expressed transcription factor Sp1, and displays promoter activity in reporter gene assays in cell lines of hematopoietic and nonhematopoietic origin (1, 10). The region corresponding to HS3 may therefore contain the minimal promoter of the T-bet gene.

Accessibility at HS3, and also at HS2 (located 7.5 kb upstream of the mRNA start site), was strongly increased in cells stimulated via the TCR and CD28, suggesting that these elements would convey signals generated by T cell stimulation. In contrast, HS1 was only detected in Th1 cells, indicating that this element could be an enhancer directing Th1-specific T-bet expression. The positions of HS1–3 overlapped with conserved noncoding sequences identified by cross-species sequence comparisons (33, 34), supporting the importance of these elements to control T-bet expression. In contrast, we consistently failed to detect DNase I hypersensitivity 8.8 and 2.3 kb upstream of the mRNA start site in CD4+ T cells, potential regulatory elements identified in silico (Fig. 3). It is tempting to speculate that these elements may be involved in regulating T-bet expression in other tissues, such as dendritic cells and B cells (35–37).

TCR/CD28 stimulation alone was necessary and sufficient to induce T-bet expression. We found that HS2 and 3 were the major targets of TCR/CD28 signaling, and histone 4 acetylation was strongly increased at these elements following Ag receptor triggering. T-bet gene expression was sensitive to CsA, which prompted us to screen for binding sites of NF-AT transcription factors at the T-bet locus. We identified a NF-AT consensus binding site at HS2 and could demonstrate recruitment of NF-AT2 to this element following T cell activation. Importantly, H4 acetylation was strongly reduced at HS2 and 3 when cells were stimulated in the presence of CsA, suggesting that NF-AT recruitment to HS2 may initiate chromatin remodeling at this site. Work from the Rao laboratory (38) has shown that NF-AT physically interacts with the histone acetyltransferases p300/CBP. NF-AT has also been shown to interact with the ATPase subunit of the BAF chromatin remodeling complex, BRG1, to mediate nucleosome remodeling at the IFN-γ promoter in developing mouse Th1 cells (39). Together, these data suggest that NF-AT binding to HS2 following antigenic stimulation may result in the recruitment of enzymes catalyzing epigenetic modifications and chromatin remodeling at T-bet regulatory elements. CsA inhibited histone acetylation at HS3 despite the absence of a NF-AT consensus site and NF-AT binding to this element. Our data suggest that chromatin remodeling and gene transcription at the T-bet locus initiate at HS2 following NF-AT binding to this element, induced by TCR stimulation. This event would start remodeling of the entire locus and histone acetylation. Blocking NF-AT binding to HS2 would therefore abrogate initiation of this process and inhibit histone acetylation not only at HS2, but also at HS3.

T-bet expression was amplified in cells stimulated in the presence of IL-12 and IFN-γ, indicating that TCR/CD28 and cytokine signaling synergized to induce high-level T-bet expression. T cell stimulation in the presence of IL-12 strongly amplified H4 acetylation at HS1. These findings suggested that HS1 was an enhancer relaying IL-12-induced signals, a hypothesis that was validated by ChIP assays showing STAT4 binding to HS1 in IL-12-stimulated cells in vivo. This IL-12-responsive T-bet enhancer element overlaps with a STAT1/STAT4 binding site recently identified in a screen in mouse CD8+ T cells (13). Using ChIP assays, Yang et al. (13) demonstrated that STAT1 and STAT4 recognized this element in murine CD4+ and CD8+ T cells. Together, these findings suggest that HS1 relays IL-12/IFN-γ/STAT1 axes play important roles in directing Th1-specific expression of the T-bet gene, it is important to note that cytokine signaling alone cannot induce T-bet expression in naive CD4+ T cells. Ag recognition by T cells is therefore the critical “gate keeper” at the T-bet locus to avoid T-bet expression and potentially pathogenic IFN-γ secretion by bystander cytokine signaling.

We found that T-bet transcripts were expressed at much lower levels in cells stimulated in the presence of IL-4 than in cells stimulated in the absence of cytokines, suggesting the presence of active suppression of T-bet gene expression in Th2 cells. In these cells, both HS2 and HS3 were clearly detectable, although T-bet was not expressed. HS2 and HS3 accessibility in these conditions could represent a mark of recent TCR-mediated activation, but

![FIGURE 6. STAT4 knockdown impairs T-bet expression. Naive human CD4+ T cells were purified from peripheral blood by negative selection and were transfected with a control siRNA oligo (CTRL), siRNA oligos targeting STAT4 (siSTAT4), or an oligo targeting STAT1 (siSTAT1). Cells were stimulated with anti-CD3/CD28 in the presence of IL-12 and harvested after 24 or 48 h. Transcripts encoding T-bet, IL-12Rβ2, IFN-γ, STAT4, and STAT1 were measured by RT-PCR using TaqMan probes. Shown are the expression levels of T-bet (upper panel), IL-12Rβ2 (middle), and IFN-γ (lower) transcripts relative to the control at 24 h after stimulation. Knockdown efficiencies for STAT4 and STAT1 mRNAs were 60–65% and 75–85%, respectively (data not shown). Shown is one of four experiments performed with different donors.](http://www.jimmunol.org/DownloadedFrom/cite/7750)
plays a negative regulatory role through recruitment of repressors of transcription and/or chromatin remodeling. A similar observation has recently been made for conserved noncoding sequence-22, a regulatory element 22 kb upstream of the IFN-γ gene. As HS2 and 3 at the T-bet locus, conserved noncoding sequence-22 displayed increased histone acetylation and hypersensitivity to DNase I in Th1 and Th2 cells (40). Genetic deletion of this element from a reporter transgene demonstrated that this element serves as an important platform to integrate signals required for tissue-specific IFN-γ expression (6).

Recent results from our laboratory showed that TCR stimulation of naive human CD4+ T cells induced rapid recruitment of BRG1, the ATPase subunit of the BAF chromatin-remodeling complex to the IL-12Rβ2 locus, resulting in low-level IL-12Rβ2 expression (1). However, in contrast to T-bet, expression of IL-12Rβ2 was clearly less sensitive to CsA. These results underscore the importance of TCR/CD28 signaling during the early phase of Th1 cell differentiation and indicate that two pathways act in parallel to control Th1 development in human CD4+ T cells: a CsA-sensitive (impinging on T-bet) and an insensitive one (driving IL-12Rβ2).

Disclosures

The authors have no financial conflict of interest.

References

Supplementary figure 1: TCR stimulation induces IFN-γ production.
CD4⁺ T cells were stimulated as in Fig. 1C. IFN-γ level in supernatants was determined by ELISA assays.
Supplementary figure 2: Conserved Noncoding Sequences of the T-bet locus.
Extended Vista plot for T-bet locus covering 25kb upstream and 50kb downstream of the mRNA start site. CNS are indicated in pink, exons in blue, the horizontal arrow shows the position of the T-bet gene and the TBK1 binding protein 1 (TBKBP1) gene localized approximately 21kb upstream of T-bet mRNA start site.
Supplementary figure 3: T cell stimulation induces a reporter gene construct containing the NFAT site present in HS2.

Jurkat cells were transiently transfected with the following reporter gene constructs: the pGL3 vector with a SV40 promoter (pGL3prom, Promega), the pGL3prom-HS2 construct containing three copies of the NFAT binding site from HS2, the pGL3 T-bet prom construct containing an 800 bp T-bet promoter fragment (-821 to -21) upstream from the mRNA start site cloned into pGL3, or a construct containing three copies of the NFAT/AP1 binding sites from the human IL-2 promoter (pNFAT luc). The pRL_TK vector expressing Renilla luciferase was co-transfected to normalize for transfection efficiency. The next day cells were stimulated with PMA and ionomycin for 8h and the activities of both enzymes were measured in stimulated and resting cells. Results are represented as an induction of luciferase activity in stimulated to resting cells. Transfections were performed in triplicates and one of four experiments is shown.
Supplementary figure 4: STAT1 silencing results in reduced T-bet expression in IFN-g-stimulated cells.

Naïve CD4+ T cells were transfected with siRNA oligos targeting STAT1 expression as described for Fig. 6 and stimulated with anti-CD3/CD28 antibodies in the presence of IFN-g and blocking anti-IL-12 antibodies. Decrease of T-bet mRNA expression by STAT1 knock-down was detected already 8 hours after stimulation. Shown is one of three experiments.