Genome-Wide Regulatory Analysis Reveals That T-bet Controls Th17 Lineage Differentiation through Direct Suppression of IRF4

M. Refik Gökmen, Rong Dong, Aditi Kanhere, Nick Powell, Esperanza Perucha, Ian Jackson, Jane K. Howard, Maria Hernandez-Fuentes, Richard G. Jenner and Graham M. Lord

J Immunol 2013; 191:5925-5932; Prepublished online 18 November 2013;
doi: 10.4049/jimmunol.1202254
http://www.jimmunol.org/content/191/12/5925

Supplementary Material  http://www.jimmunol.org/content/suppl/2013/11/18/jimmunol.1202254.DC1

References This article cites 39 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/191/12/5925.full#ref-list-1

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

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

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The transcription factor T-bet plays an essential role in regulating both adaptive and innate immune responses. T-bet was first described in T cells as the key transcription factor that directs naïve CD4+ T cells toward the Th1 lineage, which is characterized by the production of the hallmark cytokine IFN-γ (1). T-bet directly activates the Ifng gene, and is required for CD4+ T cells to acquire the Th1 migratory program (1, 2). In addition, T-bet inhibits the differentiation of Th2 cells by a number of mechanisms (3, 4). The role of T-bet in regulating the Th17 lineage is less well characterized. Indeed, the relationship between Th1 and Th17 cells appears to be more complex than that between the Th1 and Th2 lineages. Cells expressing both IFN-γ and IL-17 are frequently isolated from humans and experimental animals with inflammatory conditions (5, 6). Recently the generation of T-bet-expressing Th17 cells in the absence of TGF-β has also been described (7), and Candida albicans–specific CD4+ T cells producing both IL-17 and IFN-γ have been shown to express both T-bet and the Th17 transcription factor RORγt (8). However, the absence of T-bet has also been shown to be associated with augmented Th17 responses in a number of settings, including cardiac allograft rejection, allergic airway inflammation, autoimmune myocarditis, and experimental autoimmune encephalomyelitis (9–14). T-bet has been shown to be a potent suppressor of Th17 development independently of STAT1, which is downstream of IFN-γ (15). The recent demonstration of the repression by T-bet of Runx1-mediated Rorc activation provides important mechanistic insight on how this phenomenon might occur (12). However, it is unclear whether a direct cell–intrinsic transcriptional mechanism exists to explain the significant effect T-bet exerts on Th17 lineage commitment. We sought to address this question by examining further the role of T-bet in directing the transcriptional program of CD4+ Th cells.

Materials and Methods

Mice

Wild-type (WT) BALB/c and Rag2−/− mice on a BALB/c background were from Harlan Laboratories, T-bet−/− mice from Taconic (Ejby, Denmark), and IFN-γ−/− mice from The Jackson Laboratory (9). All mice were

Abbreviations used in this article: ChIP-seq, chromatin immunoprecipitation sequencing; ES cell, embryonic stem cell; IFN4, IFN regulatory factor-4; qPCR, quantitative PCR; RV, retrovirus; WT, wild-type.

Received for publication August 13, 2012. Accepted for publication October 7, 2013.

This work was supported by Wellcome Trust Grant 091009 (to R.G.J. and G.M.L.). Medical Research Council Grant G0802068, British Heart Foundation, U.K., Grant PG12/36/29444, and the National Institute for Health Research Biomedical Research Centre based at Guy’s and St Thomas’ National Health Service Foundation Trust and King’s College London (to M.R.G.); and a Kidney Research U.K. clinical training fellowship (to R.G.J.). G.M.L., R.G.J., and M.R.G. designed the research; M.R.G., R.D., N.P., E.P., and G.M.L. conducted the experiments; genomic data were analyzed by A.K. and R.G.J.; M.R.G. and R.G.J. wrote the manuscript with input from the other authors.

The online version of this article contains supplemental material.

© 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00
housed in specific pathogen–free facilities either at King’s College London or at Charles River Laboratories. All experiments were performed according to King’s College London and national guidelines under the U.K. Home Office project license PPL/70/6792.

**Generation of T-bet<sup>$^{GFP}$</sup> mouse**

**Construction of targeting vector.** The genomic region of interest containing the murine Tbx21 locus was isolated by PCR from 129Sv/Pas ES cell genomic DNA. PCR fragments were subcloned into the pCR4-TOPO vector (Invitrogen). The genomic clones (containing intron 1 to exon 6) were used to construct the targeting vector. Briefly, a 5.6-kb fragment comprising Tbx21 exon 2 and 6 and a 1.6-kb fragment located downstream of the Tbx21 exon 6 STOP codon were used to flank an IRES-Flpe-ERT2 NEO cassette (ERT site-PGK-promoter Neo cDNA-FRT site-LoxP site; a distal LoxP site was introduced within Tbx21 intron 3 to have access to the conditional knockout line by deleting exons 4–6 of the Tbx21 gene (Fig. 4C).

**Screening of Tbet-targeted embryonic stem cell clones.** The Nml-linearized targeting vector was transfected into 129Sv/Pas embryonic stem (ES) cells. Positive selection was started 48 h after electroporation, by addition of 200 μg/ml G418 (150 μg/ml active component; Life Technologies). Then, 370 resistant clones were isolated, amplified, and screened by PCR and further confirmed by Southern blot. PCR and Southern blot screening conditions are available on request.

**Generation of chimeric mice and breeding scheme.** One floxed mutated Tbx21 ES cell clone was microinjected into C57BL/6 blastocysts, and gave rise to male chimeras with a significant ES cell contribution (as determined by an agouti coat color). After mating with C57BL/6 CMV-Flp–expressing females to remove the neo cassette (as determined by an agouti coat color), Southern blot was performed to remove the neo cassette. PCR and Southern blot screening conditions are available on request.

**T cell isolation and differentiation**

CD4<sup>+</sup> T cells from spleens and lymph nodes of 4- to 10-wk-old mice were purified by CD4 positive selection (Miltenyi Biotec) followed by sorting of naive CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>−</sup>CD4<sup>+</sup> cells using a FACSaria II BD Biosciences. Cells were activated by plate-bound anti-CD3 and anti-CD28 (both 10 μg/ml; clones 145-2C11 and 37.51, respectively; Bio X Cell). T1 conditions comprised recombiant human IL-2 (20 ng/ml; R&D Systems) and mouse IL-12 (20 ng/ml; eBioscience, San Diego, CA), and anti–IFN-γ (20 ng/ml; Bio X Cell). Th1 conditions comprised recombiant human IL-2 (20 ng/ml; R&D Systems) and mouse IL-12 (20 ng/ml; eBioscience, San Diego, CA), and anti–IFN-γ (20 ng/ml; Bio X Cell). Th17 conditions comprised plate-bound anti-CD3 only, with soluble anti–CD28 (5 μg/ml) added to culture medium, recombinant human TGF-β (2 ng/ml; R&D Systems), IL-6 (20 ng/ml), IL-1β (10 ng/ml; both eBioscience), anti–IFN-γ (20 μg/ml), and anti–IL-10 (10 μg/ml) were also added.

**Flow cytometry**

In all experiments, freshly isolated or in vitro cultured cells were restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 4 h, with the addition of monomycin (2 μM; Sigma-Aldrich) for the final 2 h. A LIVE/DEAD fixable dead cell stain (Invitrogen) was used according to the manufacturer’s instructions to discriminate live cells. Cells were fixed with freshly prepared 4% formaldehyde, and intracellular and intranuclear Ags were stained using eBioscience nuclear Permeabilization Buffer.

**T cell transfer colitis model**

Rag2<sup>−/−</sup> mice received an i.p. injection of 5 × 10<sup>7</sup> naive CD4<sup>+</sup> T cells, isolated as described above. Mice were assessed regularly for signs of colitis, including diarrhea, rectal bleeding, and weight loss, and for signs of peritonism. Mice were culled at 1–6 wk after adoptive transfer of naive CD4<sup>+</sup> T cells, to assess in vivo T cell differentiation and induction of colitis.

**Chromatin immunoprecipitation sequencing**

Following 7 d of culture, polarized Th1 cells from WT and T-bet<sup>−/−</sup> mice were activated for 4 h with PMA (50 ng/ml) and ionomycin (1 μg/ml), crosslinked with 1% formaldehyde, lysed, and sonicated at 24 W for 10 × 30-s pulses using a Misonix Sonicator 3000. The resulting whole-cell extract was incubated overnight at 4°C with Dynal Protein G Beads preincubated with 10 μl purified rabbit anti–T-bet polyclonal antiserum (9856) (1). Beads were washed, bound complexes were eluted, and crosslinks were reversed by heating at 65°C. Immunoprecipitants and input DNA were then purified by treatment with RNase A, protease K, and phenol:chloroform extraction. Libraries were constructed from immunoprecipitants and input DNA by standard Illumina protocols, except that DNA in the range 150–350 bp was gel purified after PCR amplification. The library was quantified using an Agilent bioanalyzer and subjected to 35–bp single-end read sequencing with an Illumina Genome Analyzer II.

**Data analysis**

Initial processing was performed with the CASAVA pipeline. Reads were aligned to the Mouse NCBI37/mm9 reference genome with ELAND, background corrected using whole-cell extract data, and converted to tags per million total reads. Significant peaks were identified with MACS (16), using a q-value threshold of 10<sup>−4</sup>.

Chromatin immunoprecipitation sequencing (ChiP-seq) experiments using human cells are described elsewhere (17). Gene-specific ChiP for the Ilf4 and Irf4 promoters was performed on the whole-cell extracts, as described above using monoclonal anti–T-bet IgG1 (4B10, eBioscience) and mouse IgG1 isotype (eBioscience). All raw and processed ChIP-seq data are available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession numbers GSE40623 (mouse) and GSE31320 (human).

**Real-time PCR and gene expression microarrays**

All in vitro–cultured cells derived from naive precursors were cultured for 7 d; both freshly isolated and in vitro–cultured cells for real-time PCR and gene expression microarrays were restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 4 h and then lysed in TRIzol (Invitrogen). For real-time PCR experiments, extracted RNA was reverse transcribed to cDNA, using an iScript Select cDNA Synthesis Kit (Bio-Rad laboratories) according to the manufacturer’s instructions. All PCR experiments were performed using TaqMan gene expression assays (Applied Biosystems), using the mouse β-actin gene (Actb) as an internal control in each well. TaqMan primers were as follows: Ilf4 Mn01168134_m1; Ilf4 Mn099999154_m1; Irf4 Mn00439619_m1; Tbx21 Mn00450960_m1; Gata3 Mn01337569_m1; Rora Mn00443103_m1; Rora Mn01261021_g1; Ifng Mn0516431_m1; Actb Mn00607939_s1.

Gene expression microarray analysis was performed using Affymetrix GeneChip Mouse Gene 1.0 ST arrays according to the manufacturer’s instructions. Array images were analyzed using Microarray Analysis Software (MASS) with the default settings, and normalization was performed by robust multiarray average. For comparison with T-bet binding results, data for multiple transcripts were averaged for each gene.

Mouse array data can be accessed at ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-TABM-1187.

**Liferucase assay**

A 1489-bp promoter region upstream of Irf4 exon 1 was amplified with BALB/c DNA, using forward primer 5′-CTCGAGGATCCCTGCTCCTGCTTCTTATGAGCAAGAAGGAGG-3′ and reverse primer 5′-AAAGCTTATGAAATACATTAGAAGCCGGAG-3′. TG to CA mutations were introduced into T-bet consensus sequences within this region, using primer 1 5′-GGAGG—GAAATGGATGGAGCCACCATTTGTG—GATTAAAAGAAAAAC-3′ and primer 2 5′-GATAAAGAAAAACCAAGAAGACATG—TGAAATG—3′ for the 435–436 position within the promoter. All PCR products were verified by sequencing. These WT and mutant Irf4 promoter sequences were digested using XhoI and HindIII and cloned into a pGL4 vector (Promega, Madison, WI). HEK293 cells were transfected by calcium chloride–mediated gene transfer: 4 × 10<sup>5</sup> HEK293 cells were cotransfected with either 0.5 μg retrovirus (RV)—T-bet–GFP or 0.5 μg RV-GFP, together with 5 ng WT or mutant Irf4 promoter-pGL4. Firefly luciferase activity was assayed 24 h after transfection, using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Results are presented as Renilla luciferase activity normalized to firefly luciferase activity.

**Retroviral gene transduction**

A plasmid encoding mouse IFN regulatory factor-4 (IRF4), pCDNA FlgMIRF4, was kindly donated by Dr. Kiki Honma and Professor Katsuyuki Yui of Nagasaki University, Japan. The IRF4-encoding sequence was cloned into the MSCV-IRES-eGFP vector. MSCV—T-bet–GFP and RV-GFP vectors were produced as previously described (1). HEK293/FT cells were transfected with the vector plasmids, along with gag-pol and eco-env, using calcium phosphate precipitation. Viral supernatants were collected 48 h later. Naive mouse
T cells were sorted and maintained as before. After activation for 24 h, cells were activated for an additional 48 h and then cultured for 3 d with cytokines, as described. Cells were stained for Thy1.1 expression and sorted using a FACSaria II. Total RNA isolation and real-time PCR analysis were performed as described above.

Results

T-bet directly represses the expression of Irf4

To determine novel mechanisms by which T-bet regulates Th cell differentiation, we sought to identify T-bet target genes in primary mouse T cells using ChIP coupled with massively parallel sequencing (ChIP-seq). We purified naive CD4+ T cells from WT mice, polarized these in vitro into Th1 cells, and performed ChIP for T-bet. Efficacy of Th1 polarization was confirmed by flow cytometry, quantitative PCR (qPCR), and ELISA (data not shown). As a control to verify the specificity of the technique, we performed ChIP for T-bet in cells from T-bet−/− mice. Th1 cells were used for this experiment, as they are the only subset with T-bet expression. With this technique, 14,880 significant T-bet binding sites were identified in WT cells, with a false-positive rate of 0.7%. Assignment of T-bet binding sites to genes with transcription start sites located within 2 kb identified 3982 genes potentially regulated by T-bet. These T-bet targets included the key Th1 genes Ifng, Tnf, Cxcr3, and Gzmb, providing confidence in the dataset (Supplemental Fig. 1A). We also found that in the mouse, T-bet targets a number of genes involved in T cell trafficking, including Itgal, Ccr5, and Icam1 (not shown), as previously demonstrated in human T cells using ChIP-Chip (18).

We next sought to identify genes both bound and regulated by T-bet. To identify genes regulated by T-bet, we profiled gene expression in Th1 cells generated in vitro from naive precursors from WT and T-bet−/− mice. As for the ChIP-seq experiment described above, efficacy of skewing was confirmed using intracellular staining and FACS analysis, qPCR, and ELISA (not shown).

Overlap of the binding and expression data yielded a subset of genes that were bound by T-bet within 2 kb of the transcription start site and also maximally differentially expressed in the absence of T-bet (Fig. 1A, Supplemental Table 1A). To focus on the role of T-bet in Th17 differentiation, this list was examined for the presence of genes with well-described roles in Th17 differentiation. Crucially, although the Th17-related genes Rorc, Rora, Batf, and Il23r were found to be bound by T-bet, the expression of these genes was not significantly affected by loss of this transcription factor in the in vitro system, and the Il17a gene itself was not a target of T-bet (Supplemental Fig. 1B). This approach instead revealed that T-bet represses the gene encoding the transcription factor IRF4, which has previously been shown to be required for optimal Th17 development (19, 20). ChIP-seq shows that the If4l/IRF4 promoter is bound by T-bet, both in mouse and in human Th1 cells (Fig. 1B). In the mouse, T-bet binding colocalized with the transcription initiation marker H3K4me3 (21). Detailed comparison of binding sites in mouse and human showed that three of the binding peaks are conserved between mouse and human, whereas one binding peak unique to each species is also observed (Supplemental Fig. 2); such evolutionary divergence of transcription factor binding sites, despite conserved functional targets, has been described by other groups (22). T-bet binding to the If4l promoter was confirmed using gene-specific ChIP, with binding to the Ifng promoter used as a positive control (Fig. 1C).

The finding that Irf4 expression was increased in the absence of T-bet on microarray was confirmed by qPCR (Fig. 2A). This increased expression of Irf4 in vitro-generated Th cell subsets in the absence of T-bet was confirmed by immunoblotting (Fig. 2B). These data suggest that transcriptional repression of Irf4 may be a mechanism by which T-bet regulates Th17 development.

Given that one of the key effects of T-bet expression on Th cell differentiation is the induction of IFN-γ expression, we next sought to determine whether T-bet directly suppressed Irf4 expression

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** T-bet binds the promoter of Irf4/IRF4. (A) Scatter plot of genes up- and downregulated in T-bet−/− compared with WT Th1 cells, showing those genes determined by ChIP-seq to be bound by T-bet within 2 kb of the transcription start site (red). The full list of bound genes with fold change > 2.0 or < 0.5 is provided in Supplemental Table 1A. (B) T-bet binding at the Irf4 locus in Th1 cells from WT and T-bet−/− mice, and the IRF4 locus in human Th1 cells. The number of sequencing reads from T-bet ChIP-enriched DNA are plotted per million background-subtracted total reads and aligned with the mouse genome. Gene structures are marked at the bottom of the figure. The sites of H3K4me3 occupancy in mouse Th1 cells are also shown as reads per million. (C) T-bet binding of the Ifng and Irf4 promoters demonstrated by gene-specific ChIP. ChIP was performed on chromatin derived from WT and T-bet−/− Th1 cells after 1 wk of in vitro culture and restimulation with PMA and ionomycin. **p < 0.005, paired Student t test.
The absence of T-bet is associated with augmented Th17 responses

Having determined that T-bet can act as a transcriptional repressor of the Ifr4 gene, we next sought to investigate the role of T-bet in Th17 differentiation in vivo, using the T cell transfer model of colitis (24, 25). Naive CD4+ T cells were purified from WT and T-bet−/− mice and adoptively transferred into sex- and strain-matched Rag2−/− recipients. Interestingly, adoptive transfer of T-bet−/− naive CD4+ T cells resulted in more severe disease, as evidenced by the more profound weight loss and increased colon weight seen in the recipients of these cells (Fig. 3A, Fig. 3B). The adoptive transfer of WT naive CD4+ T cells was characterized by predominant differentiation toward a Th1 phenotype, with the production of large amounts of IFN-γ by CD4+ T cells recovered from the mesenteric lymph nodes of colitic mice. Importantly, however, T-bet−/− naive CD4+ T cells were seen to differentiate predominantly into IL-17–secreting effectors (Fig. 3C, Supplemental Fig. 3A). The same phenomenon was evident in splenic CD4+ T cells from colitic mice assessed by qPCR (Fig. 3D) and in the supernatants of cultured colonic mucosa from mice that had received T-bet−/− naive CD4+ T cells (Supplemental Fig. 3B). These data together suggest that augmented Th17 differentiation seen in the absence of T-bet also occurs in vivo, and that T-bet–deficient Th17 responses are sufficient to cause disease. Crucially, the increased Ifr4 transcript levels seen in the absence of T-bet in vitro were also observed in vivo in the T cell transfer colitis model (Fig. 3E).

T-bet inhibits Th17 development independently of IFN-γ

Having shown that Th17 differentiation is augmented in the absence of T-bet, we next sought to determine whether this suppressive effect of T-bet on Th17 differentiation is dependent on
IFN-γ, because IFN-γ itself has been proposed to be a direct negative regulator of Th17 differentiation (26, 27). To this end, the effects of ectopic expression of T-bet in CD4+ T cells from mice lacking both T-bet and IFN-γ were assessed. Naive CD4+ T cells were extracted from T-bet+/− mice, because IFN-γ−/− mice are not simply due to reduced IFN-γ production (Fig. 4D), suggesting that continued T-bet expression is required for maintenance of the Th1 phenotype. However, inhibition of T-bet expression at 24 h in developing Th17 cells was not associated with de-repression of IL-17A expression. This finding suggests that the effect of T-bet on the Th1/Th17 lineage choice is separable from IFN-γ transactivation. These data demonstrate that repression of Th17 commitment possibly occurs early post activation, whereas continuous T-bet expression is required to maintain Th1 polarity.

Expression of IRF4 rescues T-bet-mediated suppression of Th17 differentiation

Having established that T-bet binds the Irf4 gene, and that T-bet binding represses Irf4 transcription, we next sought to determine whether ectopic expression of IRF4 reverses the T-bet-mediated suppression of Th17 differentiation. As shown in Fig. 5, ectopic expression of T-bet alone was associated with reduced Il17a mRNA levels, whereas ectopic expression of Irf4 alone led to increased Il17a transcript levels. Crucially, when Irf4 and T-bet were coexpressed, there was an abrogation of the T-bet-mediated suppression of IL-17A expression, with Il17a transcript levels in the double-transduced cells being comparable with those in untransduced cells. These data suggest that optimal Th17 development is maintained in the face of T-bet expression when cells can express Irf4 in the absence of T-bet-mediated transcriptional repression.

Discussion

The mechanisms controlling Th cell lineage commitment have been the focus of much study over recent years. However, a paradox...
currently exists, whereby T-bet has been associated with both inhibition and promotion of Th17 cells. To resolve this at a mechanistic level, we have used multiple gene–targeted mice to demonstrate that T-bet represses Th17 lineage commitment in a temporally defined manner that is genetically independent of IFN-γ. Using an unbiased approach, we have combined ChIP-seq and expression data to identify a key cell-intrinsic transcriptional mechanism by which T-bet represses Th17 lineage choice via direct trans-repression of IRF4 (Figs. 1, 2).

Our findings from the T cell transfer colitis model showing augmented Th17 responses in the absence of T-bet (Fig. 3, Supplemental Fig. 2) appear to conflict with previous reports describing a lack of colitis when T-bet^−/−^CD4^+^CD62L^+^ T cells were transferred into immunodeficient hosts (24). This discrepancy is most likely explained by different patterns of colonization by commensal gut organisms in different animal facilities. Gut-residing segmented filamentous bacteria have been shown to be essential for the development of systemic Th17 responses (29); the presence of these bacteria in the mouse colonies used in the experiments described in this article has been confirmed by microbial sequencing analysis (not shown).

translate. As the interaction between T-bet and Runx1 would seem to affect the ability of T-bet to bind target DNA sequences (12), it is likely that T-bet–mediated suppression of If4 expression occurs in different set-

FIGURE 4. T-bet suppresses IL-17 production independently of IFN-γ, within the first 24 h following stimulation. CD4^+^ T cells from T-bet^−/−^ × IFN-γ^−/−^ mice were cultured in Th0 conditions (IL-2 only) and transduced with either RV encoding both T-bet and GFP (RV–T-bet) or control RV encoding GFP only (RV-GFP). (A) Expression of cytokines and the transcription factor Gata3 relative to Actb in sorted GFP^+^ cells. **p < 0.005, paired Student t test. (B) ELISA for IL-17A protein performed on the supernatants from 1 × 10^6^ GFP^+^ cells restimulated for 24 h with plate-bound anti-CD3 and anti-CD28 Abs. Data are representative of three independent experiments. *p < 0.005, paired Student t test. (C) Strategy for generation of T-bet^fl/fl^ mouse line, as described in Materials and Methods. (D) Naive CD4^+^ T cells from T-bet^fl/fl^ mice were cultured in either Th1 or Th17 conditions and transduced at 24 h with either RV encoding Cre recombinase (pMY-cre, top) or control RV (pMY-GFP, bottom). Cells were restimulated with PMA and ionomycin at day 4 prior to intracellular staining.

FIGURE 5. Ectopic expression of IRF4 rescues T-bet–mediated suppression of Th17 differentiation. Naive CD4^+^ T cells from T-bet^−/−^ mice were transduced with either both MSCV–T-bet–Thy1.1 and MSCV-IRF4-eGFP or both MSCV-Thy1.1 and MSCV-eGFP at 36 h, and cultured in Th17 conditions. (A) The cell-sorting strategy on the basis of eGFP and Thy1.1 expression at day 5. (B) qPCR for Il17a mRNA was performed on sorted populations. Representative of three experiments.

5930 T-BET SUPPRESSES Th17 VIA IRF4

by guest on April 18, 2017 http://www.jimmunol.org/ Downloaded from
tions, or where T-bet is in excess. Further work is required to determine the relative contributions of these complementary mechanisms. It is likely that lineage-specifying transcription factors such as T-bet employ a range of mechanisms to suppress the gene programs of alternative lineages (30).

We show that T-bet acts as a direct transcriptional repressor at the Irf4 promoter through sequence-specific DNA binding. The precise mechanism of this repression remains to be determined. It has been shown that T-bet can repress the genes Socs1, Socs3, and Tcf7 in fully committed Th1 cells through recruiting the transcriptional repressor Bcl-6 to their promoters (31). It is possible that T-bet-mediated repression of Irf4 expression is similarly dependent on a co-repressor. Blocking of the DNA binding of an activator of Irf4 expression would seem unlikely, given that the phenomenon was observed in HEK293 cells. Our ChIP-seq and gene expression data suggest that T-bet represses the expression of more of its genomic targets than it activates (data not shown); the mechanisms of T-bet-mediated repression at these loci are yet to be fully described.

The relationship between T-bet and IRF4 represents a new axis of regulation of Th cell fate. The importance of IRF4 in Th17 differentiation has been underlined by a study using an iterative systems approach to delineate the Th17 transcriptional regulatory network (32), in which IRF4 was shown to bind with BATF and thereby contribute to initial chromatin accessibility and the recruitment of ROR-γt.

Mucosal IRF4 levels are now known to correlate with proinflammatory cytokine production in patients with inflammatory bowel disease, whereas Irf4−/− mice are protected from T cell transfer colitis (33). The demonstration that IRF4 is itself regulated by T-bet is further evidence of the role of T-bet in regulating mucosal immune responses (34). In addition, IRF4 has an important role in the differentiation and function of a number of Th cell subsets besides the Th17 lineage, including a function in promoting Th2 differentiation and preventing Th1 development (35, 36), and in the generation of IL-9–secreting Th9 cells (37). T-bet has been shown to repress IL-9 production in Th9 conditions, suggesting that T-bet might be acting via suppression of IRF4 in this cell lineage also (38). Although the expression of IRF4 in Th2 cells has been shown to be essential for the suppression of Th2-mediated inflammation (39), any functional interaction between T-bet and IRF4 in Th2 cells has yet to be determined. The transcriptional control of IRF4 by T-bet represents a further mechanism by which T-bet regulates inflammation that is likely to be relevant for understanding the pathogenesis of a variety of autoimmune conditions.

Acknowledgments
We thank S. Heck, P.J. Chana, and H. Graves of the Guy’s and St Thomas’/King’s College London Comprehensive Biomedical Research Centre Flow Cytometry Core for support; M. Arno and A. Aldecoa-Otalora Astarloa of the King’s College London Genomics Centre for help with expression arrays; Justyna Osińska of University College London Genomics for performing the sequencing runs; and A. Hayday, K. Homma, K. Yui, A. Villarino, and A. Abbas for the generous supply of expression plasmids.

Disclosures
The authors have no financial conflicts of interest.

References


S1A

Gökmen et al. Supplementary figures
Cacac motifs
Mm

Mm Th1 T-bet

Mm KO T-bet

Hu Th1 T-bet

Conservation

Ir4
A

% of MLN CD4+ cells producing IL-17A

% of MLN CD4+ cells producing IFN-γ

% of MLN CD4+ cells producing IL-4

B

IL-17

IL-22

TNFα

IFNγ

*p < 0.001
## Supplementary table

### 1A

**Genes bound by T-bet at promoter and upregulated in T-bet−/− vs WT Th1 cells (fold change > 2.0)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>Gene</th>
<th>Fold change</th>
<th>Gene</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnfrsf8</td>
<td>5.42</td>
<td>Prrg4</td>
<td>2.71</td>
<td>Irf4</td>
<td>2.30</td>
</tr>
<tr>
<td>Il21</td>
<td>4.96</td>
<td>Tnfsf9</td>
<td>2.67</td>
<td>Lrc8d</td>
<td>2.27</td>
</tr>
<tr>
<td>Gad2</td>
<td>4.95</td>
<td>Trib3</td>
<td>2.66</td>
<td>Il1r2</td>
<td>2.27</td>
</tr>
<tr>
<td>Ecm1</td>
<td>4.81</td>
<td>Rnf19b</td>
<td>2.66</td>
<td>Spred2</td>
<td>2.25</td>
</tr>
<tr>
<td>Spef2</td>
<td>4.46</td>
<td>Ifngr2</td>
<td>2.66</td>
<td>Socs2</td>
<td>2.25</td>
</tr>
<tr>
<td>Il24</td>
<td>4.37</td>
<td>Gp49a</td>
<td>2.64</td>
<td>Acvr1c</td>
<td>2.24</td>
</tr>
<tr>
<td>Lift</td>
<td>4.36</td>
<td>Prdm1</td>
<td>2.63</td>
<td>F2rl2</td>
<td>2.19</td>
</tr>
<tr>
<td>Lta</td>
<td>4.07</td>
<td>Mlap6</td>
<td>2.61</td>
<td>Gadd45b</td>
<td>2.19</td>
</tr>
<tr>
<td>Cish</td>
<td>4.06</td>
<td>Pbpj</td>
<td>2.57</td>
<td>Nucb2</td>
<td>2.16</td>
</tr>
<tr>
<td>Ccr4</td>
<td>3.76</td>
<td>Lirb4</td>
<td>2.58</td>
<td>Oas3</td>
<td>2.15</td>
</tr>
<tr>
<td>Gzmb</td>
<td>3.53</td>
<td>Dusp6</td>
<td>2.53</td>
<td>Irf8</td>
<td>2.11</td>
</tr>
<tr>
<td>Serpinb5</td>
<td>3.27</td>
<td>Traf1</td>
<td>2.51</td>
<td>Bcat1</td>
<td>2.09</td>
</tr>
<tr>
<td>Cyslr1</td>
<td>3.17</td>
<td>Sap30</td>
<td>2.49</td>
<td>Plscr1</td>
<td>2.09</td>
</tr>
<tr>
<td>Pcx</td>
<td>3.08</td>
<td>Batf3</td>
<td>2.47</td>
<td>Sgk3</td>
<td>2.08</td>
</tr>
<tr>
<td>2010109K11Rik</td>
<td>2.97</td>
<td>Mgc</td>
<td>2.46</td>
<td>Mgl1</td>
<td>2.07</td>
</tr>
<tr>
<td>Il4</td>
<td>2.92</td>
<td>Gem</td>
<td>2.42</td>
<td>Pla2g12a</td>
<td>2.07</td>
</tr>
<tr>
<td>Gpr65</td>
<td>2.88</td>
<td>Osbp3</td>
<td>2.39</td>
<td>Acat2</td>
<td>2.05</td>
</tr>
<tr>
<td>Mxi1</td>
<td>2.78</td>
<td>Sypl</td>
<td>2.36</td>
<td>Fkbp1a</td>
<td>2.03</td>
</tr>
<tr>
<td>Cpd</td>
<td>2.76</td>
<td>Isg20</td>
<td>2.35</td>
<td>Sc4mol</td>
<td>2.01</td>
</tr>
<tr>
<td>Bmp2k</td>
<td>2.75</td>
<td>Egln3</td>
<td>2.33</td>
<td>Slc33a1</td>
<td>2.00</td>
</tr>
<tr>
<td>Cdk6</td>
<td>2.75</td>
<td>Dusp4</td>
<td>2.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Genes bound by T-bet at promoter and downregulated in T-bet−/− vs WT Th1 cells (fold change < 0.5)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>Gene</th>
<th>Fold change</th>
<th>Gene</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Il18r1</td>
<td>0.90</td>
<td>Msh5</td>
<td>0.44</td>
<td>Cxcr5</td>
<td>0.36</td>
</tr>
<tr>
<td>Zhx2</td>
<td>0.49</td>
<td>Ccl3</td>
<td>0.43</td>
<td>Csf1</td>
<td>0.34</td>
</tr>
<tr>
<td>Pdcd4</td>
<td>0.49</td>
<td>Sntb2</td>
<td>0.43</td>
<td>Tnfsf4</td>
<td>0.30</td>
</tr>
<tr>
<td>Cmah</td>
<td>0.49</td>
<td>Serpine2</td>
<td>0.42</td>
<td>Ptlg1</td>
<td>0.27</td>
</tr>
<tr>
<td>Hopx</td>
<td>0.49</td>
<td>Lrig1</td>
<td>0.43</td>
<td>Nkg7</td>
<td>0.21</td>
</tr>
<tr>
<td>Dapk2</td>
<td>0.48</td>
<td>Cd7</td>
<td>0.40</td>
<td>Art2b</td>
<td>0.18</td>
</tr>
<tr>
<td>Arsb</td>
<td>0.47</td>
<td>Pde5a</td>
<td>0.39</td>
<td>Ctsw</td>
<td>0.13</td>
</tr>
<tr>
<td>Cd27</td>
<td>0.46</td>
<td>Samd3</td>
<td>0.37</td>
<td>Irl1</td>
<td>0.12</td>
</tr>
<tr>
<td>Pogk</td>
<td>0.46</td>
<td>Lax1</td>
<td>0.37</td>
<td>Cxcr3</td>
<td>0.09</td>
</tr>
</tbody>
</table>

### 1B

T-bet consensus sequences in Irf4 promoter (chr13:30839613-30841112)

<table>
<thead>
<tr>
<th>Start</th>
<th>Stop</th>
<th>Strand</th>
<th>Score</th>
<th>p-value</th>
<th>q-value</th>
<th>Matched sequence</th>
<th>Mutated sequence</th>
<th>Construct name</th>
</tr>
</thead>
<tbody>
<tr>
<td>216</td>
<td>225</td>
<td>+</td>
<td>7.49645</td>
<td>0.000096</td>
<td>0.399</td>
<td>GGGTTGACCC</td>
<td>GGGGATGACC</td>
<td>Mut 1</td>
</tr>
<tr>
<td>449</td>
<td>458</td>
<td>+</td>
<td>10.1624</td>
<td>0.000138</td>
<td>0.187</td>
<td>AACAGAGAAA</td>
<td>AACCAAGAA</td>
<td>Mut 2</td>
</tr>
</tbody>
</table>
Supplementary Figures & Table

Supplementary Figure 1. T-bet binding at selected genes in mouse Th1 cells. Gene structures are marked at the bottom of the figure; the vertical axes depict reads per million background-subtracted total sequences.

A: T-bet binding at selected Th1-related genes: *Ifng, Tnf, Gzmb, Cxcr3*

B: T-bet binding at selected Th17-related genes: *Rorc, Rora, Il17a, Il23r, Batf, Ahr*

Supplementary Figure 2. Comparison of T-bet binding at *Irf4/IRF4* in mouse and human.

The number of sequencing reads from T-bet ChIP-enriched DNA are plotted per million background-subtracted total reads and aligned with the mouse (mm9, chr13:30,838,900-30,841,40) or human (hg18, chr6:334,200-337,000) genomes. PhyloP basewise sequence conservation in mammals is shown below (data from UCSC genome browser). Gene structures are marked at the bottom of the figure, with the transcription start site denoted with an arrow. T-bet binding sites are delineated by dashed lines and labeled A-F. Sites A, B and F are conserved in mouse and human. Site C is weak in both species. Site D is specific to mouse and site E to human.

Supplementary Figure 3. Augmented Th17 responses in the absence of T-bet in the T-cell transfer colitis model.

A: Summary of flow data from CD4$^+$ T cells extracted from MLN of four mice in each experimental group, showing increased IL-17 and IL-4 and decreased IFN-$\gamma$ production in the absence of T-bet (Mann Whitney U test).
B: Cytokine production as determined by *ex vivo* colon organ culture. Equal volumes of colonic mucosa were obtained *ex vivo* from mice four weeks after adoptive transfer, using a dermatological punch biopsy instrument. These mucosal samples were incubated in culture medium for 24h, following which ELISA was performed on the culture supernatant.

**Supplementary Table**

1A. Genes bound by T-bet at promoter and upregulated in T-bet<sup>−/−</sup> vs. WT Th1 cells (fold change > 2.0); genes bound by T-bet at promoter and downregulated in T-bet<sup>−/−</sup> vs. WT Th1 cells (fold change < 0.5).

1B. T-bet consensus sequences in the *Irf4* promoter and strategy for site-directed mutagenesis.