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Temporal Dissection of T-bet Functions

Jennifer L. Matsuda,* Thaddeus C. George,† James Hagman,* and Laurent Gapin2*

T-bet is a transcription factor of the T-box family that regulates the expression of numerous immune system-associated genes. T-bet directs the acquisition of the Th1-associated genetic program in differentiating CD4+ T cells, partly as a result of its transactivation of the hallmark Th1-type cytokine IFN-γ (4, 5). These results were confirmed by the generation of T-bet-deficient mice, which lack Th1 cells and instead exhibit an enlarged Th2 compartment (6). In addition to Th1 cells, T-bet is detected in several other immune cells, including CD8+ T cells (3, 5, 6), dendritic cells (7), B cells (8), NK cells and NKT cells (9–11). T-bet regulates expression of numerous immune system-associated genes, including cytokines (4), cytokine receptors (9–12), chemokines (7, 11), chemokine receptors (4, 11, 13) and cytotoxicity (9, 11). Altogether these results have established T-bet as an essential transcription factor in the generation and regulation of proper immune responses.

Various T-bet mutant constructs as well as T-bet−/− mice have proven invaluable tools in the study of T-bet function. However, one limitation of these approaches is that they do not allow for subsequent reversal of T-bet expression, a prerequisite for understanding the temporal relationship between T-bet activity and its role in the immune system. Exactly how and when T-bet is required for the regulation of its putative target genes is unknown. In previous studies, T-bet transactivated IFN-γ reporter constructs in vitro (4, 14, 15), suggesting a possible direct and active role of T-bet in IFN-γ transcription. In addition, T-bet was important for maintaining histone hyperacetylation at the IFN-γ promoter (16, 17). By maintaining the chromatin structure in an “open” configuration, T-bet facilitated the binding of relevant transcription factors, such as NFAT, to the IFN-γ promoter for active transcription after activation (16).

In the present studies, we have modified T-bet by fusing its C terminus with a mutated ligand binding domain of human estrogen receptor α (ER). This fusion protein resulted in a tamoxifen-regulated T-bet, T-bet-ER, which could rapidly, reversibly and repeatedly be switched on or off by the addition or removal of 4-hydroxytamoxifen (4-HT). Ectopic expression of T-bet-ER in Th2 cells or in T-bet−/− Th1 cells in vitro allowed direct examination of the temporal requirements for T-bet activity in regulating three of its target genes, IFN-γ, CD122, and CxCR3.

Materials and Methods

Mice

C57BL/6 mice were originally purchased from The Jackson Laboratory. T-bet−/− mice on the C57BL/6 background have been described previously (6). Mice were housed at the University of Colorado Health Science Center under specific pathogen-free conditions. Handling of mice and experimental procedures were in accordance with institutional requirements for animal care and use.

Retroviral transduction and constructs

Bicistronic retrovirus (RV) expressing T-bet upstream of an internal ribosomal entry sequence (IRES) and enhanced GFP (eGFP) has been described previously (3, 4, 18). The T-bet-ER construct was generated by fusing the nucleotide sequence encoding a modified hormone-binding domain (aa 282–595) of human estrogen receptor (19) to the 3′ end of the T-bet coding via the linker sequence His-Ala-Gly-Ala-Ile. The whole construct was then ligated upstream of an internal ribosomal entry sequence (IRES) and eGFP. Bicistronic retrovirus (RV) expressing T-bet upstream of an internal ribosomal entry sequence (IRES) and enhanced GFP (eGFP) has been described previously (3, 4, 18). The T-bet-ER construct was generated by fusing the nucleotide sequence encoding a modified hormone-binding domain (aa 282–595) of human estrogen receptor (19) to the 3′ end of the T-bet coding via the linker sequence His-Ala-Gly-Ala-Leu-Thr-Gly-Ala-Leu-Thr-Glu-Ala-Ile. The whole construct was then ligated upstream of an IRES and eGFP in an MSCR-based retroviral construct (MSCV-IRES-eGFP). Retroviral constructs were cotransfected into 293T cells together

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with the retroviral packaging vector pCL-Eco using LipofectAMINE 2000 (Invitrogen Life Technologies) following the manufacturer’s instructions. The medium was replaced on the following day and left overnight before collection and filtration with a 0.45-μm filter (Milllex HV; Millipore).

**Cell culture and stimulation**

CD4+ T cells were purified from the spleen and lymph nodes of 6- to 8-wk-old C57BL/6 or T-bet−/− mice with the use of anti-CD4 coated magnetic beads (Miltenyi Biotec). For Th differentiation, cells (2 × 10^6/ml) were stimulated with 1 μg/ml soluble anti-CD3ε and 1 μg/ml anti-CD28 (145.2C11 and 37.51, respectively; eBioscience) in RPMI 1640 medium (without phenol red) but supplemented with 10% FCS, l-glutamine, penicillin-streptomycin, nonessential amino acids, essential amino acids, sodium pyruvate, and 2-ME. Neutral conditions are defined as stimulation in the presence of anti-CD3ε and anti-CD28, followed by expansion with IL-2. For Th1 differentiation, cells were stimulated in the presence of 5 ng/ml rIL-12 p70 (eBioscience) and 10 μg/ml anti-IL-4 (11B11; eBioscience). For Th2 differentiation, cells were stimulated in the presence of 20 ng/ml recombinant mouse IL-4 (eBioscience) and 5 μg/ml anti-IFN-γ (XM13; eBioscience). Ab After 24 h after stimulation, T cells were resuspended in retroviral supernatant containing polybrene (8 μg/ml; Sigma-Aldrich) and were centrifuged at 6000 × g for 90 min at 25°C. After spin infection, cells were cultured in fresh medium containing the same polarizing milieu used in the initial stimulation IL-2 (40 U/ml) for 2 additional days. After 3 days of differentiation, cells were expanded in the absence of cytokine polarizing conditions but in the presence of IL-2 (40 U/ml). 4-HT (Calbiochem) was added on a daily basis as indicated. Alternatively, to remove the 4-HT, the cells were washed twice in complete medium and replaced in culture under identical conditions, except for the absence of 4-HT. At the end of the experiment, the cells were left unstimulated or were stimulated with PMA (20 nM) and ionomycin (1 μM) for 6 h in the presence of GolgiStop (BD Pharmingen) for the last 5 h. Alternatively, the cells were transferred to anti-CD3ε-coated wells (1 μg/ml) for 6 h stimulation, the last 5 h in the presence of GolgiStop (BD Pharmingen). Protein synthesis was inhibited with 10 μg/ml cycloheximide (CHX) (Sigma-Aldrich), which was added for 30 min before the addition of 4-HT.

**Abs and flow cytometry**

mAbs used in this study for flow cytometry included PE-labeled anti-CD122 clone 5H4, PE-Cy7-labeled anti-IFN-γ clone XMG1.2, PE-labeled anti-CxCR3, PE-Cy7-labeled Rat IgG1 isotype control, purified anti-T-bet clone 4B10, and Cy3-labeled goat anti-mouse IgG1. Abs were purchased from eBioscience, except the anti-CxCR3 mAb (R&D Systems), anti-T-bet clone 4B10 (Santa Cruz Biotechnology), and Cy3-labeled goat anti-mouse IgG1 (Invitrogen Life Technologies). For intracellular cytokine staining, cells were permeabilized using Cytofix/Cytoperm (BD Pharmingen) and stained according to the manufacturer’s protocol. Cells were analyzed using a FACScalibur (BD Pharmingen). For intracellular T-bet staining, cells were prepared by methanol permeabilization. Cells were suspended in 2% paraformaldehyde fixation buffer, incubated for 10 min at 37°C, pelleted, and resuspended in ice-cold 90% methanol for 30 min at 4°C. Cells were then stained with purified T-bet Ab for 1 h at room temperature, washed, and subsequently stained with Cy3-labeled goat anti-mouse IgG1 for 30 min at room temperature. Colocalization studies were analyzed on the ImageStream (Amnis) (20). Where indicated, cells were sorted with a MoFlo cytometer (DakoCytomation).

**Western blot analysis**

CD4 T cells were enriched from either C57BL/6 or T-bet−/− mice and cultured under neutral or Th1 conditions and transduced with indicated RV. Unless otherwise indicated, tamoxifen was added daily starting at day 3 (when cells were expanded in IL-2) until the end of the culture period. Following the expansion period, cells were sorted for GFP expression before preparation of protein extracts. Cells were pelleted and lysed in 1% Nonidet P-40 lysis buffer (150 mM NaCl, 10 mM Tris pH 7.5, 10 mM sodium pyrophosphate, 2 mM sodium orthovandate, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.4 mM EDTA, 1 mM aprotinin, 1 mM l-antitrypsin, and 1 mM leupeptin). Lysates were kept on ice for 15 min before centrifugation at 14,000 rpm for 10 min at 4°C. Lysate was combined with NuPage LDS sample buffer and reducing agent and denatured for 10 min at 70°C. Proteins were separated using the NuPage system (Invitrogen Life Technologies), transferred to polyvinylidene difluoride membranes and visualized with specific Abs followed by ECL. Abs to Erk2 and T-bet were obtained from Cell Signaling Technologies and Santa Cruz Biotechnology, respectively. Quantification and imaging was performed on the Odyssey Imaging System (LiCor Biosciences).

**Quantitative PCR**

Total mRNA was extracted from sorted cells using TRIzol solution (Invitrogen Life Technologies). RNA was treated with the DNA-free kit (Ambion) to remove contaminating DNA from RNA preparations. Reverse transcription was conducted by using the SuperScript III Kit (Invitrogen Life Technologies) following the manufacturer’s instructions. The amount of amplicon generated during the PCR was monitored with a DNA engine Opticon 2 apparatus (MJ Research) by using gene-specific primers and probes and the Platinum Quantitative PCR SuperMix UDG (Invitrogen Life Technologies). The sequences of the primers and probes have been published previously (10, 21).

**Results**

**Validation of T-bet-ER function**

We generated T-bet-ER by inserting the hormone-binding domain of the modified estrogen receptor, ERtam (19, 22), in frame at the C′ end of the coding sequence of T-bet. This construct was then cloned into a bicistronic MSCV-based RV expressing T-bet-ER upstream of an IRES and enhanced GFP (Fig. 1A). Ectopic expression of T-bet is sufficient to induce production of IFN-γ by otherwise nonpermissive Th2 cells (3, 4, 23). Therefore, we first examined whether T-bet-ER functions as expected by analyzing transduced Th2 cells. Increasing concentrations of 4-HT were maintained in the cultures for a total of 7 days. The cells were restimulated using PMA/ionomycin, and IFN-γ production was assessed by intracellular cytokine staining (Fig. 1B). Production of IFN-γ by Th2 cells transduced with the control RV was negligible regardless of the concentration of 4-HT present in the culture. Th2 cells transduced with T-bet-ER demonstrated a slight increase in IFN-γ production when cultivated in medium alone, but this result was variable between experiments and remained minimal. Addition of 10 nM 4-HT to the cultures was sufficient to induce T-bet-ER-expressing Th2 cells (GFP+) to produce IFN-γ (Fig. 1B). Higher concentrations of 4-HT appeared toxic and reduced the percentage of GFP+ cells in the cultures. Regardless, the ability to induce IFN-γ only in the presence of 4-HT established that T-bet-ER behaves as expected having the necessary properties of low background and efficient inducibility by the synthetic drug.
To determine the levels of T-bet induced during the forced constitutive expression of T-bet by T-bet RV or the modulated form of T-bet in T-bet-ER-RV transduced cells, we performed Western blot analysis. CD4 T cells from T-bet−/− mice were transduced under neutral conditions with the empty GFP-RV, T-bet-RV, or T-bet-ER-RV and cultured, or not, in the presence of 4-HT. In parallel, wild-type (C57BL/6) Th1 cells were differentiated and transduced with empty GFP-RV as a control for endogenous T-bet levels. All cells were sorted for GFP expression. Protein lysates were extracted and analyzed by Western blotting. Quantification by chemiluminescence of T-bet bands as compared with Erk2 loading controls revealed T-bet-ER protein levels to be more comparable to endogenous T-bet levels (~1.0 times endogenous without tamoxifen, ~1.7 times endogenous levels with tamoxifen) than the high T-bet levels observed when cells expressed T-bet-RV (~28 times endogenous levels without tamoxifen, ~29 times endogenous levels with tamoxifen) (Fig. 2A). Consistent with this, the cells for intracellular T-bet by flow cytometry revealed the levels of T-bet detected in the cells transduced with T-bet-ER-RV to be slightly higher than endogenous T-bet levels of Th1 cells and lower than the level observed when cells were transduced with T-bet-RV (Fig. 2B).

To measure the subcellular localization of T-bet-ER before and after exposure to 4-HT, we analyzed cells stained for T-bet expression and counterstained with the nuclear dye DRAQ5 using the ImageStream multispectral imaging flow cytometer (Amnis). This instrument captures spatially registered images of multiple markers within individual cells simultaneously. Nuclear localization of T-bet was quantified by measuring the similarity of the T-bet (Cy3) and the nuclear (DRAQ5) image pair on a per-cell basis (24). Visualization of nuclear translocation is shown as images of six individual cells in Fig. 2C. For this analysis, T-bet−/− CD4 T cells were transduced with T-bet-ER-RV and cultured in the absence (Fig. 2C, left panel) or presence (Fig. 2C, right panel) of 4-HT. All analyses were prepped on GFP+ cells. Four images are shown for each individual cell, a brightfield image, a T-bet Cy3 image, a DRAQ5 (nuclear stain) image, and a composite image (overlay of T-bet and DRAQ5). In the absence of 4-HT (Fig. 2C, left panel), T-bet clearly does not colocalize with DRAQ5, with the composite images retaining distinct fluorescence for both T-bet Cy3 and DRAQ5. By contrast, when cells are cultured with 4-HT (Fig. 2C, right panel), nuclear translocation of T-bet is readily observed.

However, not only is visualization of individual cells feasible with ImageStream analysis, entire populations may be analyzed and assessed for the extent of nuclear localization of T-bet under various conditions. To better understand the kinetics of the nuclear translocation of T-bet, we performed a time course experiment by adding or removing 4-HT at different times before analysis (Fig. 2D). The cells were analyzed on the ImageStream, and the similarity of subcellular localization for T-bet and DRAQ5 was assessed on thousands of individual cells for each condition. When T-bet is localized in the nucleus, T-bet and DRAQ5 images will appear similar and have a large positive value. The percentage of a given population with nuclear-localized T-bet can be obtained by gating on events with large similarity values. As shown in Fig. 2D, T-bet−/− CD4 T cells transduced with empty GFP-RV and stained with anti-T-bet and DRAQ5 exhibit only 4% similarity between T-bet and DRAQ5, defining the level of background in the experiment. As a control for the specificity of 4-HT, we compared the extent of nuclear translocation of T-bet in T-bet−/− CD4 T cells transduced with T-bet RV and cultured in either the presence or absence of 4-HT (Fig. 2D, top row). The similarity of the DRAQ5 nuclear staining and the T-bet staining was comparable between these two conditions, ~70% in each case, indicating extensive nuclear localization of T-bet in these cells. For T-bet−/− CD4 T cells transduced with T-bet-ER RV, we observed 17% similarity of T-bet/DRAQ5 in the absence of tamoxifen, indicating some translocation of T-bet-ER into the nuclei. This modest degree of similarity of T-bet-ER and DRAQ5, although a bit higher than background (4% with empty RV), is consistent with a previous report demonstrating that ER-fused proteins can accumulate in punctate foci within the nucleus in the absence of ligand binding (25). Despite some nuclear localization of T-bet-ER in the absence of 4-HT, no significant or consistent effects of T-bet-ER on downstream target genes were observed in the absence of 4-HT in the course of our studies.

When T-bet-ER transduced T-bet−/− CD4 T cells were incubated with 4-HT, we found that significant nuclear translocation was observed as early as 2 h after adding 4-HT (Fig. 2D, middle row). Consistent with this, we observed nuclear translocation of T-bet following the addition of tamoxifen by Western blot analysis on nuclear and cytoplasmic extracts of T-bet-ER-GFP+ T-bet−/− sorted cells (data not shown). Conversely, when we removed 4-HT at different times before analysis, we found that T-bet remained mostly in the nucleus, even 48 h after the removal of 4-HT (Fig. 2D, bottom row).

Delayed induction of T-bet activity in developing Th2 cells is sufficient to induce IFN-γ production and CD122high,CxCR3high phenotype

Next, differentiating Th2 cells were transduced with empty GFP, native T-bet or T-bet-ER RVs and 4-HT (10 nM) was added to the cultures at different time points (Fig. 3A). We reported previously that ectopic expression of T-bet in T-bet−/− NKT cells induces expression of IL-2/IL-15Rβ (CD122) mRNA and cell surface expression of CD122 (10, 11). Furthermore, recent data have emphasized the critical role of T-bet and its homolog Eomes in enhancing CD122 expression and consequently in controlling the survival of IL-15-dependent cells, including NKT, NK, and CD8 memory cells (9). In addition, T-bet has been involved in the control of Th1 cell migration to inflammatory sites through its regulation of expression of the chemokine receptor CxCR3 (11, 13). Altogether these results emphasize the importance of T-bet in regulating CD122 and CxCR3 expression in addition to IFN-γ. For this reason, we focused our studies on these three putative T-bet target genes. In each experiment, cells were stained directly to assess expression levels of CD122 and CxCR3 on their surfaces (Fig. 3B). Alternatively, cells were restimulated either with PMA/ionomycin or with anti-CD3ε-coated plates, and the production of IFN-γ was assessed by intracellular staining (Fig. 3C). The percentages in the top right quadrant of each dot plot in Fig. 3 indicate the proportion of GFP+ cells that stained positively for IFN-γ, CD122, or CxCR3. Although cells transduced with the empty GFP retrovirus failed to express IFN-γ, CD122, or CxCR3 at high levels, cells expressing native T-bet produced IFN-γ and were CD122high and CxCR3high, regardless of the duration of incubation with 4-HT (Fig. 3, C and D). For the T-bet-ER expressing cells, addition of 4-HT from the start of the cultures induced IFN-γ production and expression of high levels of CD122 and CxCR3 (Fig. 3, B–D). Interestingly, the IFN-γ CD122high,CxCR3high phenotype of the T-bet-ER expressing Th2 cells was critically dependent upon the day of 4-HT addition in the cultures (Fig. 3, C and D). CD4+ T cells that had been differentiated under Th2 conditions for 3 days, a time frame sufficient to establish the Th2 phenotype (data not shown), and further expanded in IL-2 for a total period of 7 days remained responsive to the activity of T-bet mediated through the addition of 4-HT. Addition of 4-HT for the last 12–24
FIGURE 2. Characterization of T-bet in T-bet-ER-expressing CD4 T cells. A, Western blot analysis comparing endogenous T-bet levels in wild-type (B6) Th1 cells transduced with empty GFP-RV vs T-bet levels in T-bet−/− CD4 T cells transduced with T-bet-RV or T-bet-ER-RV in the presence or absence of tamoxifen (4-HT). B, Intracellular staining of T-bet in wild-type Th1 cells transduced with empty GFP-RV as compared with T-bet−/− CD4 T cells transduced with either T-bet-RV or T-bet-ER-RV. C, ImageStream fluorescence imaging of subcellular localization of T-bet-ER in T-bet−/− CD4 T cells. Brightfield, T-bet Cy3 (green), nuclear stain DRAQ5 (red), and composite images for individual T-bet−/− CD4 T cells transduced with T-bet-ER RV. All images were pregated on GFP− cells only. The left panel shows T-bet-ER transduced T-bet−/− CD4 T cells in the absence of tamoxifen, where T-bet is localized mainly in the cytoplasm. In the right panel, T-bet-ER T-bet−/− cells were cultured in the presence of tamoxifen for 96 h. Significant colocalization of T-bet and the nuclear stain DRAQ5 indicates that T-bet has translocated into the nucleus. D, Determination of the extent of nuclear translocation of T-bet relative to the time of addition or removal of 4-HT. The extent of nuclear translocation of T-bet was determined by assessing the similarity of T-bet staining and DRAQ5 staining of individual cells. Similarity data were compiled for entire populations in histograms. Percentages in plots indicate the extent of colocalization of T-bet and DRAQ5 for each population of cells analyzed. All analyses are pregated on GFP− (transduced) cells. Populations analyzed include T-bet−/− CD4 T cells transduced with empty GFP-RV, T-bet-RV, or T-bet-ER-RV ± 4-HT. In the top row of histograms, control samples were analyzed to determine the level of background staining observed and to ensure that 4-HT did not have a nonspecific effect on T-bet RV, which does not express the ER. In the middle row of histograms, 4-HT was not added (medium) or was added 2, 12, 24, or 96 h before intracellular staining of the cells. By contrast, the bottom row shows similarity plots for T-bet-ER transduced cells cultured in the presence of 4-HT for 96 h before staining or cultured with 4-HT 96 h before staining and 24 or 48 h after removal of 4-HT (cells were in the presence of 4-HT for a total of 96, 72, or 48 h, respectively). Data are representative of three independent experiments.
were transduced with either a GFP-expressing retrovirus (addition to the culture medium is shown. Cells were analyzed as in the top right quadrant of each dot plot. C, Percentage of GFP⁺ cells that are producing IFN-γ as a function of the day of 4-HT addition to the culture medium. Cells were analyzed as in B after restimulation with PMA/ionomycin or anti-CD3 mAb. The cells were transduced with either a GFP-expressing retrovirus (○), a T-bet-expressing retrovirus (△), or T-bet-ER-expressing retrovirus (●). D, Percentage of CD122⁺ (●) or CxCR3⁺ (●) cells as a function of 4-HT addition to the culture medium for cells expressing GFP (□), T-bet (△), or T-bet-ER (●). The presented data are representative of four independent experiments with similar results.

FIGURE 3. Induction of T-bet activity as a function of time. A, CD4⁺ T cells were stimulated with anti-CD3/anti-CD28 mAbs under Th2 conditions. After 24 h, the cells were transduced using GFP-RV, T-bet-RV, or T-bet-ER-RV and returned to Th2 culture conditions for 2 more days. The cells were then expanded with IL-2 and analyzed on day 7. The diagram depicts the days of 4-HT addition (10 nM) during the course of the experiment. B, On day 7, the cells were restimulated by PMA/ionomycin and subjected to intracellular cytokine staining for IFN-γ. Alternatively, the cells were directly stained for CD122 or CxCR3 expression. The percentage of GFP⁺ cells that express IFN-γ, CD122, or CxCR3 is indicated in the top right quadrant of each dot plot. C, Percentage of GFP⁺ cells that are producing IFN-γ as a function of the day of 4-HT addition to the culture medium is shown. Cells were analyzed as in B after restimulation with PMA/ionomycin or anti-CD3 mAb. The cells were transduced with either a GFP-expressing retrovirus (○), a T-bet-expressing retrovirus (△), or T-bet-ER-expressing retrovirus (●). D, Percentage of CD122⁺ (●) or CxCR3⁺ (●) cells as a function of 4-HT addition to the culture medium for cells expressing GFP (□), T-bet (△), or T-bet-ER (●). The presented data are representative of four independent experiments with similar results.

Role of T-bet on expression of CD122 and CxCR3 in Th cells

Our results provide evidence that T-bet has a direct role in promoting the expression of both CD122 and CxCR3 in addition to IFN-γ in Th1 cells. We reasoned, therefore, that CD122 and CxCR3 levels should be reduced in situations where T-bet is normally absent, such as in wild-type Th2 cells or in Tbet⁻/⁻ T cells. To assess this, we differentiated both wild-type and T-bet⁻/⁻ CD4 h of the culture was sufficient to induce the production of some IFN-γ, although significantly increased CD122 and CxCR3 expression required longer incubation with the 4-HT (Fig. 3, C and D). These results confirmed that T-bet activity is necessary for the regulation of these three genes and defined the required incubation period necessary for T-bet-mediated activation.

Persistent T-bet activity is necessary for IFN-γ production and the CD122⁺, CxCR3⁺ phenotype

Although the previous experiment established that T-bet is a crucial regulator of IFN-γ production and CD122 and CxCR3 expression, it is not clear whether the effects of T-bet or the T-bet-activated signals it might trigger on T cells are transient or permanent. Therefore, we performed an experiment in which 4-HT was provided from the start of the experiment, followed by its removal as a function of time to deactivate T-bet activity (see diagram of Fig. 4A). The results shown in Fig. 4B established that Th2 cell cultures in which 4-HT was added for up to 4 days during their differentiation, a time frame sufficient to induce T-bet activity efficiently (see Fig. 3), but then washed off afterwards, did not differentiate into IFN-γ-producing cells and did not express high levels of surface CD122 or CxCR3 (Fig. 4, B–D). T-bet activity had to be maintained for at least 5–6 days to visualize its effects at the time of analysis at day 7. These results suggest that T-bet activity is short-lived and that T-bet is not able to permanently imprint its activity on Th2 cells, at least during this time period.

Acute activity of T-bet at the time of TCR stimulation is important for the production of IFN-γ

T-bet has been implicated in the chromatin remodeling of the IFN-γ locus during Th1 differentiation (12, 16, 26) and transactivated IFN-γ reporter constructs in vitro (4, 14, 15). Our results suggest that T-bet activity is required acutely in order for IFN-γ to be produced and for CD122 and CxCR3 to be expressed at high levels. We wanted to assess whether prior activation of T-bet activity enhanced responsiveness to its acute expression, as evidenced by changes in outcomes of Th2 differentiation. To do so, we provided 4-HT to Th2 developing cultures for 3 or 4 days before washing it out. 4-HT was then subsequently added or not for the last 12 h before restimulation and analysis of IFN-γ production (Fig. 5A). We chose to focus on IFN-γ, as the addition of 4-HT for 12 h is not sufficient to induce measurable staining of CD122 or CxCR3 (Fig. 3). In agreement with our previous results (Fig. 4), removal of 4-HT following its induction for the first 3 or 4 days of the differentiation culture, minimally affected the differentiation of Th2 cells and IFN-γ production by these cells remained low (Fig. 5B). By contrast, induction of T-bet activity by the addition of 4-HT 12 h before the analysis induced the production of IFN-γ by the transduced Th2 cells (Fig. 5, B and C). No significant difference was observed whether or not T-bet activity had been previously induced in the cells during their differentiation. These results emphasize the crucial role of T-bet activity at the time of stimulation and suggest that expression of T-bet during Th2 differentiation is not sufficient to reprogram the cells toward an IFN-γ-secreting Th1 program if T-bet activity is not maintained.

Role of T-bet on expression of CD122 and CxCR3 in Th cells

Our results provide evidence that T-bet has a direct role in promoting the expression of both CD122 and CxCR3 in addition to IFN-γ in Th1 cells. We reasoned, therefore, that CD122 and CxCR3 levels should be reduced in situations where T-bet is normally absent, such as in wild-type Th2 cells or in Tbet⁻/⁻ T cells. To assess this, we differentiated both wild-type and T-bet⁻/⁻ CD4
T cells under Th1 and Th2 conditions and determined their phenotype (Fig. 6A). As expected, only the wild-type Th1 cells, which express T-bet, expressed high levels of CD122 and CxCR3. The phenotype of the T-bet\textsuperscript{-/-} Th1-differentiated cells was comparable to that of wild-type Th2 cells.

Effect of temporal activity of T-bet in T-bet\textsuperscript{-/-} Th1 cells
Our data in Th2 cells strongly suggest that expression of T-bet during the Th differentiation period is not sufficient to program the cells to express CD122, CxCR3, and IFN-\gamma. Instead, T-bet appears necessary for the maintenance of this phenotype. However, the requirement for continuous expression of T-bet may be due to our use of Th2 cells. Perhaps some factor(s) critical to amplifying or driving Th1 differentiation following T-bet expression are inherently lacking in these cells. For example, expression of IL-12R and exposure to IL-12 during cellular differentiation is critical to the polarization of naive CD4 T cells into Th1 cells. Therefore, to address this possibility we repeated our experiments using...

**FIGURE 4.** Loss of T-bet activity as a function of time. A, CD4\textsuperscript{+} T cells were stimulated with anti-CD3/anti-CD28 mAbs under Th2 conditions. After 24 h, the cells were transduced using GFP-RV, T-bet-RV, or T-bet-ER-RV and cultured under Th2 conditions for 2 more days. The cells were then expanded with IL-2 and analyzed on day 7. The diagram depicts the days of presence of 4-HT in the culture medium (10 nM) during the course of the experiment. All cells, except the medium only control, were incubated with 4-HT following retroviral transduction on day 1. The following day, 4-HT was removed from one well for each RV (these samples are defined as having 1 day in the presence of 4-HT). The next day, 4-HT was removed from one more well for each RV (2 days in presence of 4-HT), and so on. B, On day 7, the cells were restimulated by PMA/ionomycin and subjected to intracellular cytokine staining for IFN-\gamma. Alternatively, the cells were directly stained for CD122 or CxCR3 expression. The percentage of GFP\textsuperscript{+} cells that express IFN-\gamma, CD122, or CxCR3 is indicated in the top right quadrant of each dot plot. C, Percentage of IFN-\gamma producing cells as a function of the number days in presence of 4-HT is shown. Cells were analyzed as in B after restimulation with PMA/ionomycin or anti-CD3 mAb. The cells were transduced with either a GFP-expressing retrovirus (■), a T-bet-expressing retrovirus (○), or a T-bet-ER-expressing retrovirus (●). D, Percentage of CD122\textsuperscript{high} or CxCR3\textsuperscript{high} cells as a function of 4-hydroxytamoxifen addition to the culture medium for cells expressing GFP (■), T-bet (○), or T-bet-ER (●). The presented data are representative of four independent experiments with similar results.

**FIGURE 5.** Acute activity of T-bet is required for the production of IFN-\gamma by Th2 cells. CD4\textsuperscript{+} T cells were stimulated with anti-CD3/anti-CD28 mAbs under Th2 conditions. After 24 h, the cells were transduced using GFP-RV, T-bet-RV, or T-bet-ER-RV and replaced under Th2 conditions for 2 more days. The cells were then expanded with IL-2 and analyzed on day 7. A, The diagram depicts the days of presence of 4-HT in the culture medium (10 nM) during the course of the experiment. B, On day 7, the cells were restimulated by PMA/ionomycin and subjected to intracellular cytokine staining for IFN-\gamma. The results of T-bet-ER-expressing Th2 cells are depicted. Experimental conditions (A–F) are indicated. The percentage of GFP\textsuperscript{+} cells that produce IFN-\gamma is indicated in the top right quadrant. C, The percentage of IFN-\gamma-expressing cells for the different experimental conditions was compiled for Th2 cells transduced with GFP-RV, T-bet-RV, or T-bet-ER-RV. The presented data are representative of two independent experiments with similar results.
T-bet<sup>−/−</sup> cells cultured under Th1-polarizing conditions, when 4-HT was added or removed over time (Fig. 6B). The results obtained were comparable to those observed for Th2 cells. This suggests that other factors potentially present in Th1 cells are not likely to alter the dependence upon T-bet for the CD122<sup>high</sup> and CxCR3<sup>high</sup>IFN-γ<sup>+</sup> phenotype.

IFN-γ, CD122, and CxCR3 are potential direct gene targets of T-bet

We took advantage of our unique system for the induction of T-bet activity to address the question of whether IFN-γ, CD122 and CxCR3 are direct or indirect gene targets of T-bet. T-bet can trans-activate the IFN-γ promoter in vitro (4, 14) and chromatin immunoprecipitation experiments have detected T-bet bound to the CD122 and IFN-γ promoters (10, 27). However, recent data have suggested that the functions of T-bet cannot be determined from binding studies to individual target promoters alone (27). Therefore, it remains possible that T-bet acts indirectly by inducing another activator. This would require new protein synthesis for translation of the intermediate gene product. To test for this possibility, CD4<sup>+</sup> T cells were differentiated under Th2 conditions and transduced with the T-bet-ER retrovirus. After 7 days of culture, GFP<sup>+</sup> cells were sorted and placed in culture. New protein synthesis was blocked or not by the addition of CHX before the induction of T-bet activity for 6 h (Fig. 7). Under these conditions, the induction of CD122, IFN-γ, and CxCR3 mRNA was measured by quantitative PCR. Results are shown as mean ± SD. Data are representative of four independent experiments.

Discussion

In this report, we have generated and characterized an inducible form of T-bet in a retroviral delivery system. We determined that the levels of T-bet in cells expressing T-bet-ER are much more comparable to the levels of endogenous T-bet in Th1 cells than when T-bet is forcibly expressed using T-bet RV. Using a novel imaging technology, we were able to observe the subcellular localization of T-bet under different conditions. We found that T-bet-ER translocates to the nucleus in the presence of 4-HT. Significant nuclear translocation of T-bet occurred within 2 h after 4-HT addition. Despite this rapid nuclear translocation of T-bet upon 4-HT addition, demonstrable effects on IFNγ, CxCR3, and CD122 expression levels were delayed, requiring between 12 and 48 h. The lag in the downstream effects of T-bet might be due to a dependence on other Th1-specific factors and/or a requirement for progressing through the cell cycle (Ref. 30 and data not shown). In an attempt to determine how long T-bet-ER remains in the nucleus after the removal of 4-HT, we looked for changes in percentage of T-bet/DRAQ5 similarity when 4-HT was removed at different time points before analysis. Even when 4-HT had been removed over time (comparable to Fig. 4 for wild-type Th2 cells), the column on the right shows data derived from cells where tamoxifen was removed over time (comparable to Fig. 4 for wild-type Th2 cells). Data are representative of two independent experiments.
tion domain. In contrast, the transcriptional activity of the IFN-γ promoter is crucially dependent on T-bet. In this context, removal of T-bet would lead to the complete loss of factor binding and of transcriptional activity of the IFN-γ promoter. Future experiments aimed at identifying other transcription factors implicated in the control of expression of these genes and assessing their activity and binding in the presence and absence of T-bet are warranted.

Recent data have suggested a different scenario for the development of Th1 and Th2 cells (32). It has been argued that the main role of T-bet is to negatively regulate GATA-3 function, rather than to positively regulate the IFN-γ gene (32). Such inhibition was proposed to release the inhibition of STAT4 imposed by GATA-3 in Th2 cells (33). STAT4 signaling induced by IL-12 would then induce the production of IFN-γ. Our results do not support this model. In our experiments, fully differentiated Th2 cells were induced to produce IFN-γ through the induction of T-bet activity associated with IL-2 signaling, but in the absence of exogenous IL-12. These results are consistent with a central role for T-bet in Th1 development. We conclude that T-bet directly regulates the expression of IFN-γ as well as several other important genes, including cytokine receptors, which impart the cells with reinforcing signals necessary to their commitment.

Martins et al. (34) recently suggested that while T-bet is essential for the establishment of the IFN-γ gene activity, it might not be required for its maintenance in Th1 cells. These results are in apparent contradiction with our current data showing that T-bet activity is absolutely required at the time of the secondary activation to induce IFN-γ production. In their experiments T cells were activated and restimulated weekly under Th1 conditions and retrovirally transduced with a mutant form of T-bet, in which the transactivation domain was replaced by the Drosophila active repressor engrailed. In these conditions, IFN-γ expression by Th1 cells became resistant to the action of the dominant negative construct as Th1 maturation progressed in time. Differences between the two sets of experiments may be related to their time frame (1 vs 3 wk) as well as to the extensive restimulation under Th1 conditions that might favor a T-bet-independent maintenance of the cytokine gene activity (34). Our control of T-bet activity by the addition or removal of 4-HT from the cell cultures clearly established the need for constant T-bet activity to induce IFN-γ by Th2 cells or by T-bet−/− Th1 cells. Attempts at inducing T-bet activity at latter time points after several rounds of Th2 differentiation were unsuccessful due to activation induced cell death following re-stimulation of the cells with anti-CD3ε (data not shown). However, two other T-bet targets, CD122 and CxCR3, were also acutely dependent upon T-bet activity to be expressed by Th2 cells and T-bet−/− Th1 cells. These results reinforce the idea that, at least in early time points following differentiation of T cells, T-bet activity has to be maintained and is not dispensable to observe its effects.

The concomitant and acute regulation of IFN-γ, CD122, and CxCR3 by T-bet is interesting with regards to its potential role in immunity. Indeed, T-bet was recently found to be expressed in both Ag-specific effector and memory T cells from mice infected with lymphocytic choriomeningitis virus and vaccinia virus (9). T-bet is also expressed in NK and NKT cells (10). The expression of T-bet activity in these cytolytic effector cells suggests potential conserved gene regulation with a central role of T-bet in controlling the development/survival (CD122 expression), function (IFN-γ secretion), and trafficking (CxCR3 expression) of these cells (11).

The control of CxCR3 expression by T-bet is of particular interest. Recent reports (35, 36) suggested that CxCR3, in addition to its critical role in recruiting T cell during allograft rejection, is also important in regulating NK cell mobilization under normal and pathogenic conditions (37). CxCR3 is also essential for NK-dependent antitumor responses in vivo (38). Furthermore, CxCR3-mediated trafficking of NK cells to draining lymph nodes is critical for induction of Th1 responses by providing an early source of IFN-γ (39). Thus, T-bet and its homolog Eomes (3) are likely to play a central role in orchestrating inflammatory immune responses. Future studies aimed at regulating T-bet activity temporally in vivo will be essential to our understanding of the role of this transcription factor in regulating immune responses. Spatial and temporal regulation of T-bet activity through the generation of cell type-specific T-bet-ER transgenic mice will aid in deciphering the diverse roles of T-bet in the immune response, from its regulation of IFN-γ production (6), to its regulation of NK and NKT cell development and trafficking (10) and ultimately to its regulation of autoimmune diseases (40).

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Disclosures
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References