

T-bet is a STAT1-induced regulator of IL-12R expression in naïve CD4⁺ T cells

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T helper type 1 (T_H1) cell development involves interferon- γ (IFN- γ) signaling through signal transducer and activator of transcription 1 (STAT1) and interleukin-12 (IL-12) signaling through STAT4 activation. We examined here T-bet regulation and evaluated the actions of T-bet in STAT1- and STAT4-dependent T_H1 development processes. We found that T-bet expression during T cell activation was strongly dependent on IFN- γ signaling and STAT1 activation, but was independent of STAT4. Ectopic T-bet expression strongly increased IFN- γ production in T_H2 cells activated by PMA-ionomycin, but weakly increased IFN- γ production in T_H2 cells stimulated by IL-12-IL-18 or OVA peptide-antigen-presenting cell stimulation. In contrast, IL-12-IL-18-induced IFN- γ production remained STAT4-dependent despite ectopic T-bet expression. Ectopic T-bet expression selectively induced expression of IL-12R β 2, but not IL-18R α , in wild-type and STAT1^{-/-} T_H2 cells, but did not extinguish expression of GATA-3 and T_H2 cytokines. Finally, ectopic T-bet did not directly induce expression of endogenous T-bet independently of IFN- γ or STAT1. Thus, T-bet is induced by IFN- γ and STAT1 signaling during T cell activation. In addition, T-bet mediates STAT1-dependent processes of T_H1 development, including the induction of IL-12R β 2.

T helper type 1 (T_H1) cell development involves the actions of both interferon- γ (IFN- γ) and interleukin-12 (IL-12)^{1,2}. Naïve CD4⁺ T cells express functional IFN- γ receptors, but induce and maintain expression of the IL-12 receptor (IL-12R) signaling subunit IL-12R β 2 only after activation in appropriate cytokine conditions^{3,4}. IFN- γ promotes IL-12R β 2 expression, whereas IL-4 opposes its expression³. IL-12 and signal transducer and activator of transcription 4 (STAT4) are not absolutely required for IFN- γ production, as some cell types such as CD8⁺ T cells can produce abundant IFN- γ independently of IL-12 and STAT4⁵. In addition, STAT4-independent IFN- γ production by CD4⁺ T cells has been reported under some conditions^{6,7}. However, IL-12 strongly augments T_H1 development and IFN- γ production by CD4⁺ T cells⁸ and STAT4^{-/-} CD4⁺ T cells showed markedly reduced T_H1 development and IFN- γ production^{5,9,10}. Thus both STAT1 and STAT4 contribute to T_H1 development.

T-bet is a T_H1-specific transcription factor that increases IFN- γ production in T_H2 cells, represses T_H2 cytokine expression and directly activates IFN- γ reporter activity¹¹. T-bet was initially proposed to be induced by IL-12 and STAT4 activation^{11,12}, to inhibit GATA-3 and to act as a master switch for T_H1 development^{12,13}. However, another study placed T-bet expression before IL-12 and STAT4 in T_H1 development¹⁴ and concluded T-bet could prime STAT4^{-/-} CD4⁺ T cells for IFN- γ production. In addition, ectopic T-bet expression induced expression of IL-12R β 2 and of the endogenous gene encoding T-bet,

which suggested that T-bet was STAT4-independent, stochastic and stabilized by autoactivation. This study also presented evidence that T-bet remodeled the IFN- γ locus¹⁴.

These studies left several issues unresolved. First, it was unclear whether T-bet was induced stochastically or regulated by unrecognized factors. Indeed, it has been suggested that T-bet is regulated by IFN- γ signaling through STAT1¹⁵. This raises the possibility that the induction of IL-12R β 2 and endogenous T-bet by ectopic T-bet¹⁴ is not due to T-bet itself, but is actually caused indirectly by IFN- γ . Second, the requirement for STAT4 in IFN- γ production is unresolved. There are at least two distinct physiologic pathways that can independently induce IFN- γ , including T cell receptor (TCR) signaling and IL-12-IL-18 signaling¹⁶⁻¹⁸. Studies that used antigen-pulsed-antigen-presenting cells (antigen-APCs) or cytokine stimulation show STAT4-dependent IFN- γ production^{5,9,10,16}, whereas studies that used phorbol 12-myristate 13-acetate + ionomycin (PMA-ionomycin) to induce IFN- γ production by CD4⁺ T cells report STAT4-independent IFN- γ production¹⁴. Third, because only PMA-ionomycin stimulation has been used to analyze the effects of ectopic T-bet expression^{11,14}, the role played by T-bet in either TCR- or cytokine-induced pathways of IFN- γ production has not been tested.

We have addressed several of these issues here by examining the expression and effects of T-bet in wild-type, STAT1^{-/-} and STAT4^{-/-} T cells. Our results show that T-bet expression during T cell activation was strongly dependent on IFN- γ and STAT1, but was independent of

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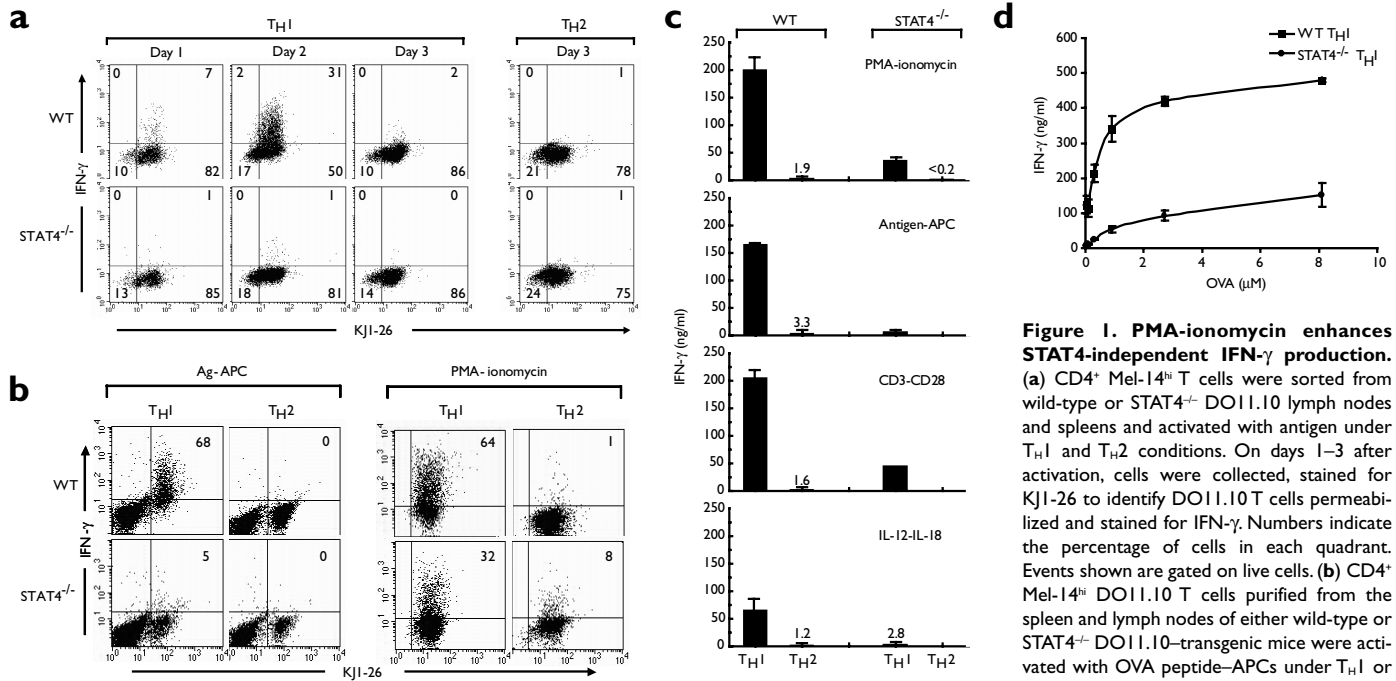


Figure 1. PMA-ionomycin enhances STAT4-independent IFN- γ production.

(a) CD4⁺ Mel-14^{hi} T cells were sorted from wild-type or STAT4^{-/-} DO11.10 lymph nodes and spleens and activated with antigen under T_H1 and T_H2 conditions. On days 1–3 after activation, cells were collected, stained for KJ1-26 to identify DO11.10 T cells permeabilized and stained for IFN- γ . Numbers indicate the percentage of cells in each quadrant. Events shown are gated on live cells. (b) CD4⁺ Mel-14^{hi} DO11.10 T cells purified from the spleen and lymph nodes of either wild-type or STAT4^{-/-} DO11.10-transgenic mice were activated with OVA peptide-APCs under T_H1 or T_H2 conditions, collected on day 7 and restimulated with either PMA-ionomycin or

OVA peptide-APCs and anti-IL-12 for 24 h. Brefeldin A treatment was included for the final 2.5 h. Cells were stained for KJ1-26 and CD4, permeabilized, fixed and stained for intracellular IFN- γ . Events shown are gated on live cells. Numbers represent the percentages of KJ1-26⁺CD4⁺ cells that stained positive for IFN- γ relative to the isotype control. (c) Wild-type and STAT4^{-/-} T_H1 cells were collected on day 7 after primary stimulation and restimulated with PMA-ionomycin, plate-bound anti-CD3 + anti-CD28, antigen-APC + anti-IL-12 or IL-12-IL-18. After 48 h, culture supernatants were collected and IFN- γ was measured by ELISA in triplicate. (d) Wild-type or STAT4^{-/-} T_H1 cells were collected on day 7 after primary stimulation and restimulated with OVA peptide-APCs and anti-IL-12. Culture supernatants were collected after 48 h and IFN- γ was measured by ELISA.

STAT4. Ectopic T-bet expression strongly increased IFN- γ production in T_H2 cells activated by PMA-ionomycin, but only weakly increased IFN- γ production activated by antigen-APC or IL-12-IL-18. This indicated that expression of ectopic T-bet into T_H2 cells has not generated completely normal T_H1 cell development. IFN- γ production induced by IL-12-IL-18 stimulation remained strongly STAT4-dependent, despite retroviral T-bet expression; this indicated that STAT4 was also important for the IFN- γ production induced by these cytokines. Notably, ectopic T-bet expression selectively induced IL-12R β 2 expression, but did not induce IL-18R α expression in wild-type or STAT1^{-/-} T_H2 cells. In addition, T-bet did not extinguish expression of GATA-3 or T_H2 cytokines or directly induce endogenous T-bet. Our results suggest that T-bet is induced by IFN- γ -STAT1 signaling during T cell activation and that T-bet mediates STAT1- but not STAT4-dependent processes of T_H1 development that include the induction of IL-12R in CD4⁺ T cells.

Results

T_H1 development conditionally requires STAT1

IFN- γ alone does not fully induce T_H1 development^{3,19,20}, but it has crucial actions early in this process. One key role played by IFN- γ is the regulation of IL-12R β 2 expression³, which facilitates subsequent IL-12 signaling^{4,9,10,21,22}. To test the roles played by STAT1 and STAT4 in T_H1 development, we compared naïve wild-type, STAT1^{-/-} and STAT4^{-/-} DO11.10 T cells primed *in vitro* under various conditions (Web Fig. 1 online). Under T_H2 conditions (IL-4 + IL-12 neutralization), STAT1^{-/-} and STAT4^{-/-} cells both developed T_H2 phenotypes that were similar to wild-type T cells, as expected. Under T_H1 conditions (IL-12 + IL-4 neutralization), STAT1^{-/-} T cells produced ~50% as much IFN- γ as wild-type controls, but STAT4^{-/-} T cells showed much more reduced IFN- γ

production (Web Fig. 1 online). However, in suboptimal T_H1-inducing conditions—in which endogenous IL-4 was not neutralized—STAT1^{-/-} T cells showed a more impaired production of IFN- γ compared to wild-type T_H1 cells (Web Fig. 1 online). This result indicates a conditional requirement for STAT1 in T_H1 development *in vitro* that is best revealed under suboptimal T_H1-inducing conditions.

We next examined IL-12R β 2 expression in the T cells described above on day 7 by RNA blot (northern) analysis (Web Fig. 2 online). In T_H1 conditions that neutralized IL-4, IL-12R β 2 expression was seen in wild-type and STAT1^{-/-} T cells. However, when IL-4 was not neutralized, IL-12R β 2 was not expressed by STAT1^{-/-} T cells, which was consistent with impaired T_H1 development (Web Fig. 1 online). To determine whether this defective IL-12R β 2 expression in STAT1^{-/-} T cells was absolute or a result of defective maintenance after transient induction, we examined IL-12R β 2 surface expression after primary activation (Web Fig. 3 online). We found that IL-12-treated wild-type T cells induced IL-12R β 2 expression by day 2 and maintained expression on day 7, with or without neutralization of IL-4. In contrast, STAT1^{-/-} T cells transiently expressed IL-12R β 2 in the presence of IL-12 either with or without neutralization of IL-4, but expression was maintained at day 7 only with neutralization of IL-4. Thus, STAT1 is important in maintaining IL-12R β 2 expression in the presence of endogenous amounts of IL-4.

STAT4-dependent IFN- γ production

STAT4^{-/-} T cells produced much less IFN- γ than wild-type T cells when cultured in T_H1-inducing conditions for 7 days (Web Fig. 1 online). To determine whether IFN- γ production by CD4⁺ T cells was STAT4-dependent at earlier time points, we compared wild-type and STAT4^{-/-}

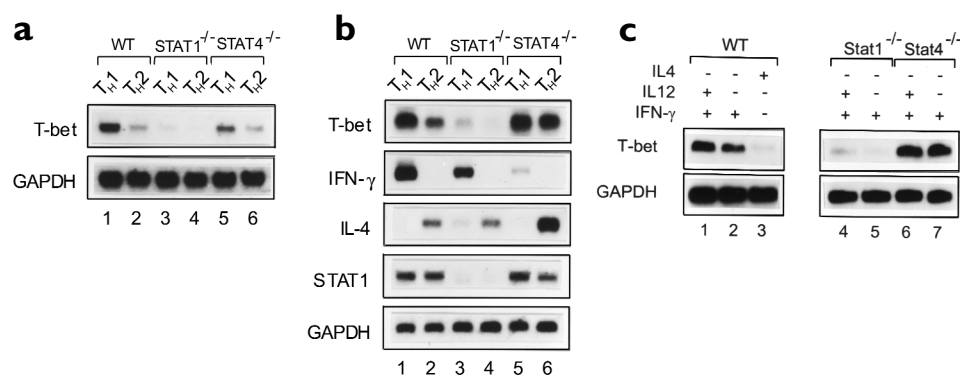


Figure 2. Expression of the gene encoding T-bet is STAT1-dependent. (a,b) DO11.10 T cells were polarized in two cycles to T_{H1} or T_{H2}. T cells were then collected and restimulated either with (a) plate-bound anti-CD3 for 24 h or (b) PMA-ionomycin for 3 h. Total RNA was prepared and RNA blot analysis done for the indicated transcripts as described³. (c) DO11.10 primary T cells from wild-type, STAT1^{-/-} or STAT4^{-/-} mice were activated in the presence of the indicated cytokines (+) and neutralizing cytokine antibodies (-). After 48 h of stimulation, cells were collected and RNA was extracted. T-bet and GAPDH mRNA levels were determined by RNA blot analysis with full-length cDNA as probe.

T cells for IFN-γ production by intracellular cytokine staining on days 1, 2 and 3 after primary activation (Fig. 1a). IFN-γ production by antigen-APC-activated wild-type T cells peaked on day 2, at which point 31% of cells were IFN-γ-positive. In contrast, IFN-γ production by antigen-APC-activated STAT4^{-/-} T cells was 1% or less at all times tested and was similar to the amounts detected in control T_{H2} cultures.

These results agree with previous reports that claim antigen-induced IFN-γ production by CD4⁺ T cell is STAT4-dependent^{9,10,23,24}. However, there are other reports that claim IFN-γ production by CD4⁺ T cells is STAT4-independent^{5,25,26}. Because these studies have used a variety of activation stimuli, we compared the degree of STAT4-dependence of IFN-γ production in T cells stimulated by different stimuli (Fig. 1b,c). Wild-type and STAT4^{-/-} DO11.10 CD4⁺ T cells were primed for 7 days with antigen-APCs under T_{H1}- or T_{H2}-inducing conditions, restimulated with either antigen-APCs or PMA-ionomycin, and then cytokine production was measured first by intracellular cytokine staining (Fig. 1b). IL-12 was neutralized when antigen-APC stimulation was used in order to prevent TCR-dependent induction of IFN-γ *via* STAT4 activation. Antigen-APC stimulation induced IFN-γ production in 68% of wild-type T cells, but only 5% of STAT4^{-/-} T cells; this represented a >90% reduction in the absence of STAT4. In contrast, PMA-ionomycin induced IFN-γ production in 64% of wild-type T cells and 32% of STAT4^{-/-} T cells, which represented only a 50% reduction in the absence of STAT4.

Because intracellular cytokine staining reflects a short time-frame of cytokine production, we used enzyme-linked immunosorbent assay (ELISA) to quantify cytokine production over a longer time period (Fig. 1c). By ELISA, antigen-APC stimulation induced 165 ng/ml of IFN-γ from wild-type T cells compared to 6 ng/ml from STAT4^{-/-} T cells (Fig. 1c); this represented a 97% reduction in IFN-γ production in the absence of STAT4. PMA-ionomycin induced 200 ng/ml of IFN-γ from wild-type T cells compared to 35 ng/ml from STAT4^{-/-} T cells, or an 80% reduction in IFN-γ without STAT4. IL-12 and IL-18 induced 65 ng/ml of IFN-γ from wild-type T cells compared to 3 ng/ml from STAT4^{-/-} T cells (Fig. 1c), or a 95% reduction without STAT4. Anti-CD3 + anti-CD28 induced 205 ng/ml of IFN-γ from wild-type T cells compared to 45 ng/ml from STAT4^{-/-} T cells (Fig. 1c), which represented an 80% reduction without STAT4. We also compared IFN-γ production by wild-type and STAT4^{-/-} T_{H1} cells that were restimulated on day 7 with various doses of ovalbumin (OVA) peptide (Fig. 1d). With increasing peptide concentration, IFN-γ production in both wild-type and STAT4^{-/-} T cells increased, but STAT4^{-/-} T_{H1} cells produced comparatively less IFN-γ compared to wild-type T cells at all doses.

These direct comparisons show that the dependence of IFN-γ production on STAT4 varies greatly depending on the method of activation and cytokine measurement used. Thus, we evaluated the role played by

T-bet in IFN-γ production with the use of a combination of activation and assay conditions to better define its precise locus of action.

T-bet expression is STAT1-dependent

Initially T-bet was reported to be IL-12 and STAT4-dependent^{11,12}, but later was reported to be STAT4-independent¹⁴. We examined T-bet regulation in wild-type, STAT1^{-/-} and STAT4^{-/-} DO11.10 T cells differentiated in T_{H1} and T_{H2} conditions for two cycles (Fig. 2a,b). Resting T cells were collected on day 14 after initial activation and were restimulated with anti-CD3 (Fig. 2a) or PMA-ionomycin treatment (Fig. 2b). Expression of the gene encoding T-bet after restimulation was T_{H1}-specific and expressed in wild-type and STAT4^{-/-} T cells, but was greatly reduced in STAT1^{-/-} T_{H1} cells (Fig. 2a,b). As controls, we confirmed IFN-γ and IL-4 mRNA expression. Both wild-type and STAT1^{-/-} T_{H1} cells produced abundant IFN-γ mRNA, whereas STAT4^{-/-} T_{H1} cultures showed a large reduction in IFN-γ mRNA levels (Fig. 2b).

We also examined T-bet expression at earlier time points after activation and assessed the requirement for IFN-γ separately from that for IL-12. Naïve wild-type, STAT1^{-/-} and STAT4^{-/-} DO11.10 T cells were activated with antigen-APCs in the presence of IL-4, IFN-γ or IFN-γ + IL-12 (Fig. 2c). Two days after activation, T-bet was expressed by wild-type T cells in the presence of IFN-γ alone or with IFN-γ + IL-12, but not with IL-4 (Fig. 2c). STAT4^{-/-} T cells, but not STAT1^{-/-} T cells, expressed T-bet in the presence of IFN-γ or IFN-γ + IL-12. Thus, during T cell activation, T-bet expression is strongly dependent on IFN-γ and STAT1, but not STAT4.

IFN-γ dependence on T-bet varies with stimuli

In addition to induction by PMA-ionomycin, IFN-γ production can be induced by two distinct physiologic pathways: antigen or IL-12-IL-18 stimulation. We tested T-bet's role in each of these modes of IFN-γ induction. First, wild-type or STAT4^{-/-} CD4⁺ DO11.10 T cells—activated in T_{H1} or T_{H2} conditions—were infected with T-bet-expressing or control retrovirus, purified and expanded. These cells were then restimulated with PMA-ionomycin, antigen-APCs or IL-12-IL-18, and IFN-γ production was measured by ELISA (Fig. 3a). With PMA-ionomycin stimulation, T-bet strongly increased IFN-γ expression, inducing the production of 1180 ng/ml of IFN-γ in wild-type T_{H2} cells and 1800 ng/ml of IFN-γ in STAT4^{-/-} T_{H1} cells compared to 1380 ng/ml in wild-type T_{H1} controls. When these same cells were stimulated with antigen-APCs, the effect of T-bet was quantitatively smaller, inducing only 70 ng/ml of IFN-γ in wild-type T_{H2} cells or 290 ng in STAT4^{-/-} T_{H1} cells compared to 2002 ng/ml in wild-type T_{H1} controls. When stimulated with IL-12-IL-18, the effects of T-bet were also quantitatively smaller than those induced by PMA-ionomycin, inducing 250 ng/ml of IFN-γ in wild-type T_{H2} cells or 10 ng of IFN-γ in STAT4^{-/-} T_{H1} cells, compared

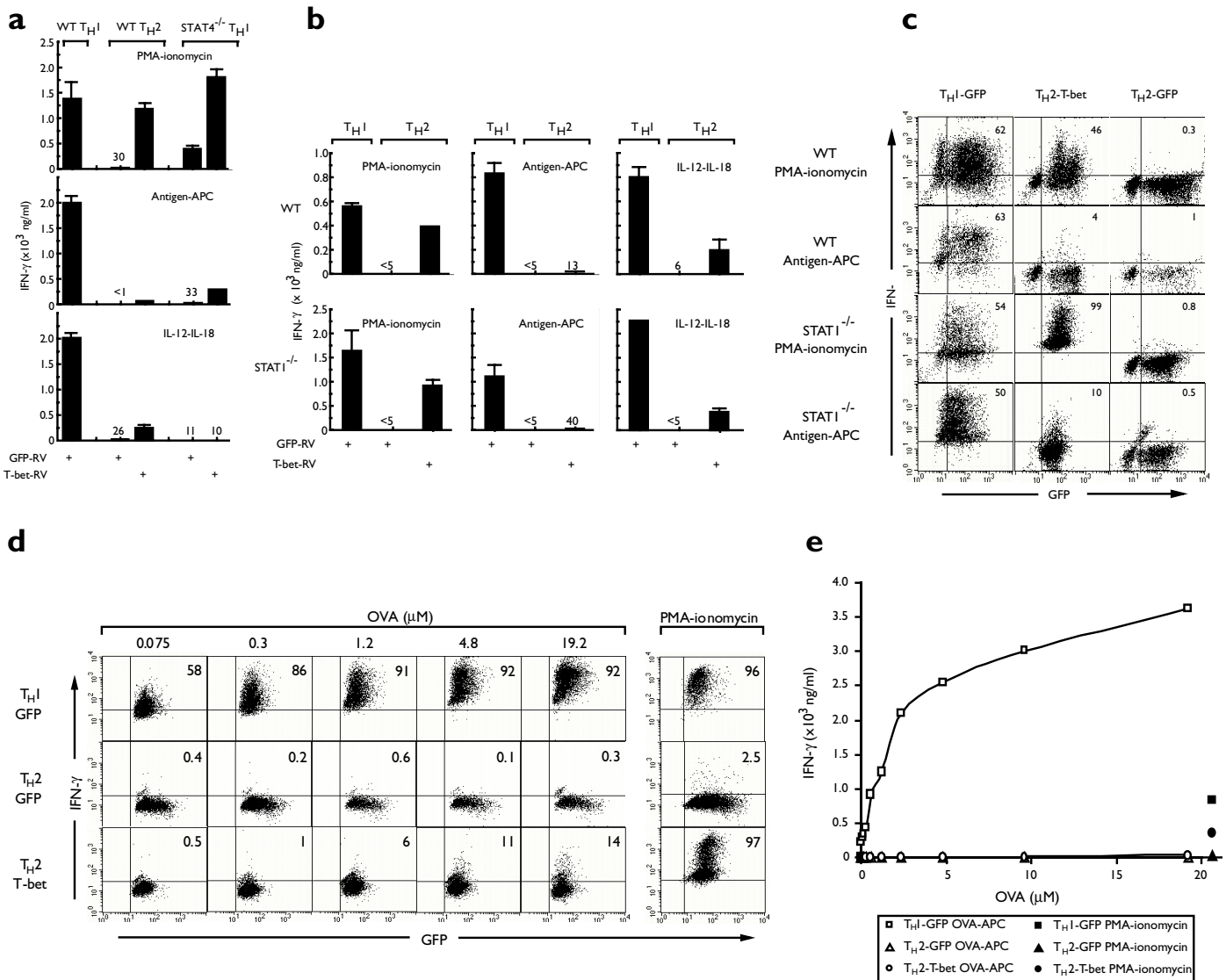


Figure 3. The effect of T-bet on cytokine production in STAT4^{-/-} and STAT1^{-/-} T cells. (a) Naïve wild-type or STAT4^{-/-} DO11.10 T cells activated with OVA peptide-APCs in T_{H1} or T_{H2} conditions were infected on day 2 with either T-bet-expressing (T-bet-RV) or control retrovirus (GFP-RV). On day 7, CD4⁺ and GFP⁺ T cells were purified by two-color sorting, expanded for another 7 days, collected at day 14 and restimulated with PMA-ionomycin, OVA peptide-APCs or IL-12-IL-18. Supernatants were analyzed for IFN- γ by ELISA. (b) Naïve wild-type or STAT1^{-/-} DO11.10 T cells were activated with OVA peptide-APCs in T_{H1} or T_{H2} conditions, infected on day 2 with either T-bet or control retrovirus, then collected, restimulated and analyzed as in a. (c) Cells in b were restimulated for 24 h as indicated. Brefeldin A was added for the final 2.5 h, then cells were stained for KJ1-26 expression, permeabilized, fixed and stained for intracellular IFN- γ . Data are gated on KJ1-26⁺ cells. Numbers indicate the percentage of GFP⁺ cells that stained positive for IFN- γ relative to the isotype control. (d) Wild-type DO11.10 cells in b were restimulated for 24 h with OVA peptide + APCs or with PMA-ionomycin. Brefeldin A was added and cells were treated as in c. Data are gated on GFP⁺ KJ1-26⁺ cells. Numbers indicate the percentage of GFP⁺ cells that stained positive for IFN- γ relative to the isotype control. (e) Cells in b were restimulated with OVA peptide and APCs or PMA-ionomycin. After 48 h, supernatants were analyzed for IFN- γ by ELISA.

to 2111 ng/ml in wild-type T_{H1} controls. Thus, T-bet strongly increased IFN- γ production in T_{H2} cells or STAT4^{-/-} T cells when the cells were stimulated with PMA-ionomycin, but did not strongly increase IFN- γ production when stimulated with antigen-APC or IL-12-IL-18. In addition, IL-12-IL-18-induced IFN- γ remained dependent on STAT4 in T_{H1} cells, despite ectopic T-bet expression.

Next, wild-type or STAT1^{-/-} CD4⁺ DO11.10 T cells were activated under T_{H1} or T_{H2} conditions, infected with T-bet-expressing or control retrovirus, purified, expanded and restimulated with PMA-ionomycin, antigen-APCs or IL-12-IL-18, and IFN- γ production was measured by ELISA (Fig. 3b). STAT1^{-/-} T_{H1} control cells showed

strong IFN- γ production in response to all modes of restimulation, which was consistent with previous data (Web Fig. 1a online). With PMA-ionomycin stimulation, T-bet strongly increased IFN- γ production in both wild-type and STAT1^{-/-} T_{H2} cells (Fig. 3b). However, with antigen-APC stimulation, the effects of T-bet were quantitatively smaller than those observed with PMA-ionomycin. With antigen-APC stimulation, T-bet induced only 13 ng/ml of IFN- γ in wild-type T_{H2} cells compared to 860 ng/ml in wild-type T_{H1} cells, and only 40 ng/ml of IFN- γ in STAT1^{-/-} T_{H2} cells compared to 1120 ng/ml in STAT1^{-/-} T_{H1} controls (Fig. 3b). With IL-12-IL-18 stimulation, T-bet partially restored IFN- γ production, inducing 210 ng/ml IFN- γ

production in wild-type T_H2 cells compared to 820 ng/ml in wild-type T_H1 cells, and 400 ng/ml IFN- γ in $STAT1^{-/-}$ T_H2 cells compared to 2300 ng/ml in $STAT1^{-/-}$ T_H1 controls (**Fig. 3b**).

We also used intracellular cytokine staining to compare PMA-ionomycin stimulation to antigen-APC stimulation in these cells (**Fig. 3c**). As measured by intracellular cytokine staining, PMA-ionomycin stimulation allowed T-bet to strongly augment IFN- γ production in T_H2 cells, inducing IFN- γ expression to 46%, compared to 62% in T_H1 cells and <1% in control T_H2 cells that lacked ectopic T-bet expression. Similarly, in $STAT1^{-/-}$ T cells, PMA-ionomycin stimulation enabled T-bet to induce IFN- γ expression in 99% of T_H2 cells, compared to 54% in T_H1 cells and <1% in control T_H2 cells lacking ectopic T-bet expression (**Fig. 3c**). However, when these same cells were restimulated with antigen-APCs, T-bet had a much weaker effect. With antigen-APC stimulation, T-bet drove only 4% of IFN- γ expression in T_H2 cells, compared to 63% in T_H1 cells and 1% in control T_H2 cells that lacked ectopic T-bet expression. Similarly in $STAT1^{-/-}$ T cells stimulated with antigen-APC stimulation, T-bet drove only 10% of IFN- γ expression in T_H2 cells compared to 50% of expression in T_H1 cells and <1% in control T_H2 cells that lacked ectopic T-bet expression.

As a control, we confirmed that the peptide dose used for antigen-APC stimulation in these experiments was sufficient to induce activation relative to PMA-ionomycin (**Fig. 3d,e**). We found that in control T_H1 cells, 0.3 μ M OVA peptide induced nearly maximal IFN- γ production by intracellular cytokine staining, inducing 86% IFN- γ expression compared to the maximum of 92% with 20 μ M OVA peptide or 96% with PMA-ionomycin treatment. In contrast, in T-bet-expressing T_H2 cells, 0.3 μ M OVA peptide induced only 1% IFN- γ expression, which increased to only 14% at 20 μ M OVA peptide compared to 97% induced by PMA-ionomycin in these same cells. Similarly, when ELISA was used to measure IFN- γ (**Fig. 3e**), 0.3 μ M OVA peptide and PMA-ionomycin induced similar amounts of IFN- γ in control T_H1 cells. Thus, compared to PMA-ionomycin, 0.3 μ M OVA peptide induces similar amounts of IFN- γ , as measured by both ELISA and intracellular cytokine staining. However, even at the highest antigen dose (19 μ M), T-bet-expressing T_H2 cells only produced 11 ng/ml of IFN- γ compared to 3600 ng/ml in control T_H1 cells. Thus, T-bet strongly increased IFN- γ production in T_H2 cells when stimulated by PMA-ionomycin, but not when stimulated by antigen-APCs. T-bet could also partially restore IFN- γ induced by IL-12-IL-18 in wild-type and $STAT1^{-/-}$ T_H2 cells, but not in $STAT4^{-/-}$ T_H1 cells.

T-bet expression during T_H2 development

It has been reported that T-bet inhibits T_H2 cytokines to various degrees. For example, T-bet induces a stronger reduction in IL-5 production than IL-4¹¹. However, in that study¹¹, T_H2 cytokine secretion

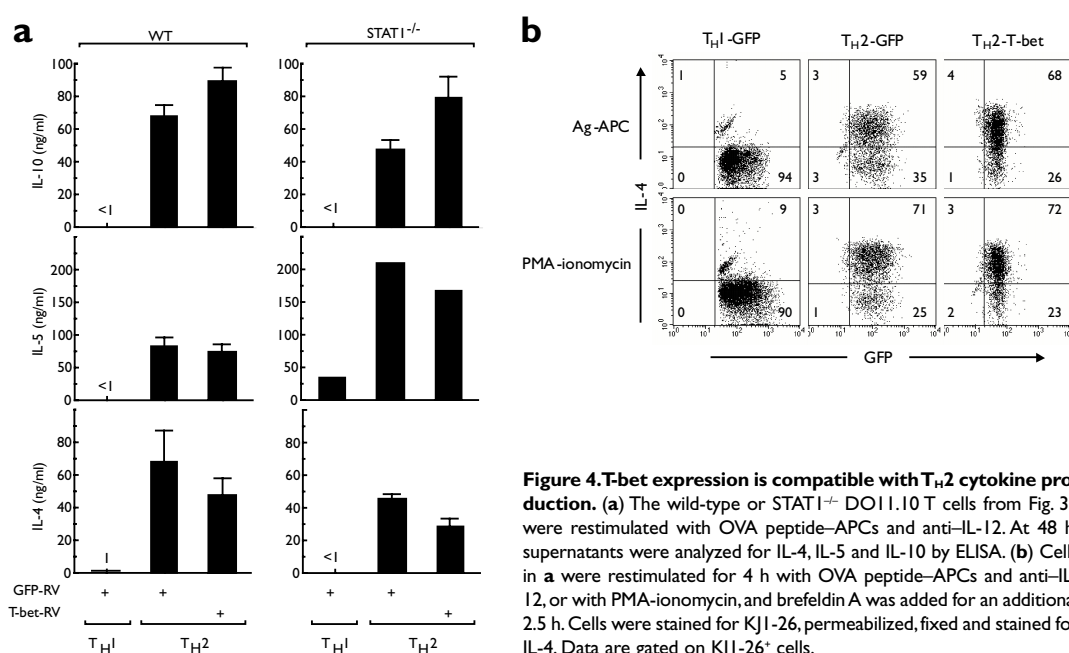


Figure 4. T-bet expression is compatible with T_H2 cytokine production. (a) The wild-type or $STAT1^{-/-}$ DO11.10 T cells from Fig. 3b were restimulated with OVA peptide-APCs and anti-IL-12. At 48 h, supernatants were analyzed for IL-4, IL-5 and IL-10 by ELISA. (b) Cells in a were restimulated for 4 h with OVA peptide-APCs and anti-IL-12, or with PMA-ionomycin, and brefeldin A was added for an additional 2.5 h. Cells were stained for KJ1-26, permeabilized, fixed and stained for IL-4. Data are gated on KJ1-26⁺ cells.

had not been quantified by ELISA. Thus, with the use of ELISA, we analyzed T_H2 cytokine production in response to antigen-APC in wild-type and $STAT1^{-/-}$ $CD4^+$ DO11.10 T cells activated under T_H1 or T_H2 conditions and infected either with T-bet-expressing or control retrovirus (**Fig. 4a**). Control, green fluorescent protein-retrovirus (GFP-RV), T_H2 but not T_H1 cells produced IL-4, IL-5 and IL-10 (**Fig. 4a**). T-bet-RV-expressing T_H2 cells also produced IL-4, IL-5 and IL-10 at amounts similar to GFP-RV-infected T_H2 control cells when stimulated by antigen-APCs (**Fig. 4a**) or with PMA-ionomycin (data not shown). We confirmed these findings with the use of intracellular cytokine staining for IL-4 production (**Fig. 4b**). With antigen-APC stimulation, 68% of T-bet-expressing T_H2 cells were IL-4-positive, compared to 59% of control (GFP-RV) T_H2 cells. With PMA-ionomycin stimulation, 72% of T-bet-expressing T_H2 cells were IL-4-positive, compared to 71% of control T_H2 cells. Thus, both by ELISA and intracellular cytokine staining, T-bet-expressing T_H2 cells produced T_H2 cytokines similar to T_H2 controls, despite T-bet's increased PMA-ionomycin-induced IFN- γ production by these cells (**Fig. 3b**).

As a control, we confirmed T-bet expression in these cells (**Fig. 5**) by both immunoblot and RNA blot analysis. Immunoblotting showed that T-bet was expressed in T-bet-RV-expressing T_H2 cells; it was not detected in control T_H2 cells (both wild-type and $STAT1^{-/-}$ T cells) (**Fig. 5a**). For wild-type cells, T-bet protein in T-bet-RV-infected T_H2 cells was expressed at similar amounts to control T_H1 cells (**Fig. 5a**, lanes 1 and 3). T-bet-RV-infected $STAT1^{-/-}$ T_H2 cells expressed more T-bet protein than $STAT1^{-/-}$ T_H1 controls (**Fig. 5a**, lanes 4 and 6), which was consistent with the $STAT1$ -dependence of endogenous T-bet expression. Because T-bet-expressing T_H2 cells unexpectedly produced T_H2 cytokines, we examined GATA-3 mRNA and protein expression in these same cells (**Fig. 5a,b**). Control T_H1 cells lacked GATA-3 protein and mRNA, but T-bet-expressing T_H2 cells showed GATA-3 expression in amounts that were similar to control T_H2 cells (**Fig. 5a,b**). Thus, retroviral T-bet expression did not inhibit GATA-3 induction in T_H2 conditions (**Fig. 5**), despite T-bet's augmentation of PMA-ionomycin-induced IFN- γ in these cells (**Fig. 3**).

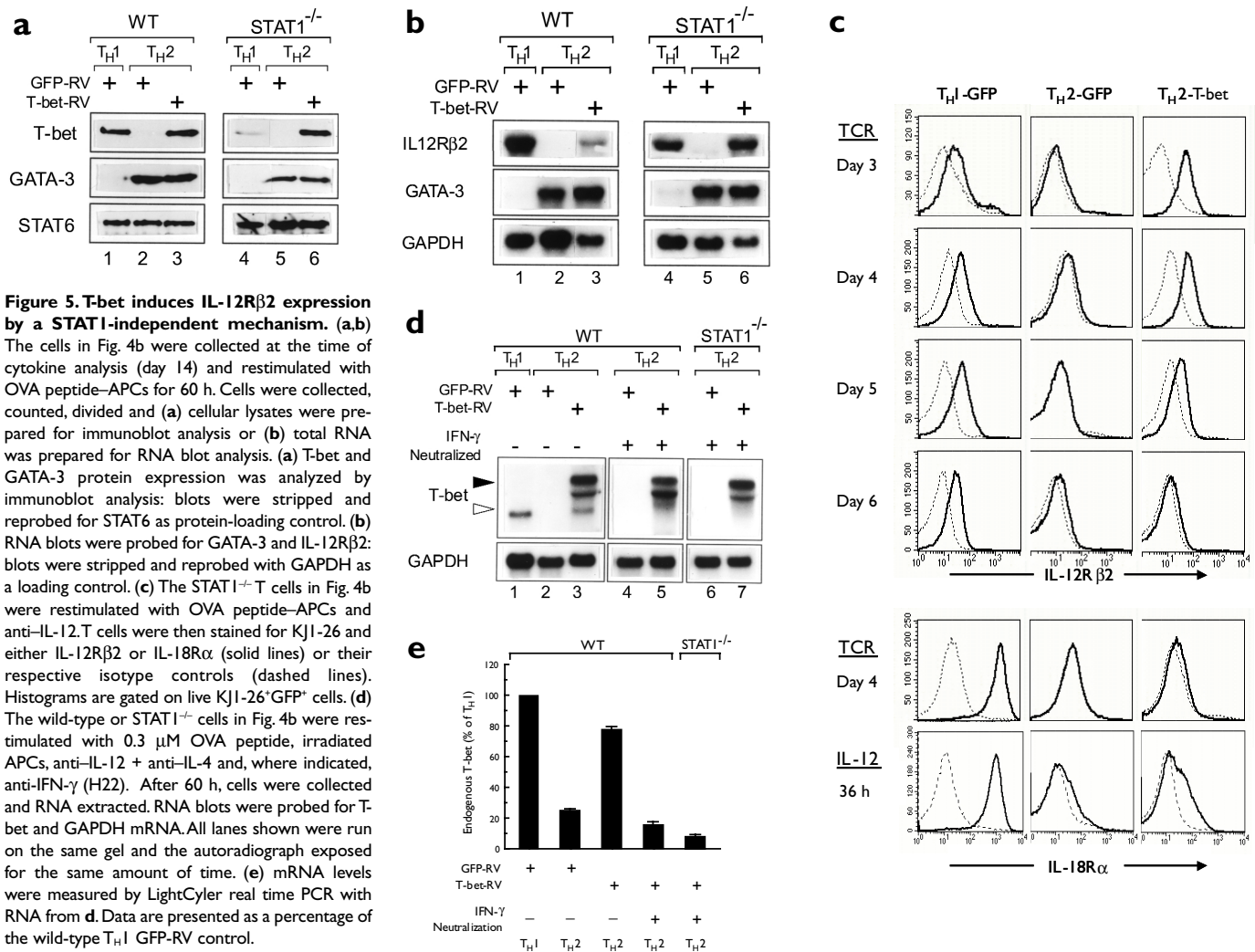


Figure 5. T-bet induces IL-12Rβ2 expression by a STAT1-independent mechanism. (a,b) The cells in Fig. 4b were collected at the time of cytokine analysis (day 14) and restimulated with OVA peptide-APCs for 60 h. Cells were collected, counted, divided and (a) cellular lysates were prepared for immunoblot analysis or (b) total RNA was prepared for RNA blot analysis. (a) T-bet and GATA-3 protein expression was analyzed by immunoblot analysis: blots were stripped and reprobed for STAT6 as protein-loading control. (b) RNA blots were probed for GATA-3 and IL-12Rβ2: blots were stripped and reprobed with GAPDH as a loading control. (c) The STAT1^{-/-} T cells in Fig. 4b were restimulated with OVA peptide-APCs and anti-IL-12. T cells were then stained for KJ1-26 and either IL-12Rβ2 or IL-18Rα (solid lines) or their respective isotype controls (dashed lines). Histograms are gated on live KJ1-26⁺GFP⁺ cells. (d) The wild-type or STAT1^{-/-} cells in Fig. 4b were restimulated with 0.3 μM OVA peptide, irradiated APCs, anti-IL-12 + anti-IL-4 and, where indicated, anti-IFN-γ (H22). After 60 h, cells were collected and RNA extracted. RNA blots were probed for T-bet and GAPDH mRNA. All lanes shown were run on the same gel and the autoradiograph exposed for the same amount of time. (e) mRNA levels were measured by LightCycler real time PCR with RNA from d. Data are presented as a percentage of the wild-type T_{H1} GFP-RV control.

STAT1-independent IL-12Rβ2 expression by T-bet

Because T-bet may induce IL-12Rβ2 expression¹⁴ indirectly *via* IFN-γ³, we examined IL-12Rβ2 expression during ectopic T-bet expression in wild-type and STAT1^{-/-} T cells to block IFN-γ signaling (Fig. 5b,c). Using RNA blot analysis, we found that IL-12Rβ2 mRNA was expressed in control T_{H1} but not control T_{H2} cells (Fig. 5b, lanes 1 and 2), as expected. However, IL-12Rβ2 mRNA expression was detected in T-bet-expressing wild-type T_{H2} cells (Fig. 5b, lane 3) and in STAT1^{-/-} T_{H2} cells (Fig. 5b, lane 6), which suggested that T-bet may mediate IFN-γ- and STAT1-induced IL-12Rβ2 expression. Using fluorescence-activated cell sorting (FACS) analysis, we examined IL-12Rβ2 surface expression at several time points after antigen activation in STAT1^{-/-} T cells (Fig. 5c). On day 3 after antigen activation, control STAT1^{-/-} T_{H2} cells lacked IL-12Rβ2 as expected, but T-bet-expressing STAT1^{-/-} T_{H2} cells expressed IL-12Rβ2 more highly than T_{H1} controls (Fig. 5c). On day 4, T-bet-infected STAT1^{-/-} T_{H2} cells expressed IL-12Rβ2 at amounts similar to control T_{H1} cells. T-bet-induced IL-12Rβ2 expression gradually declined on days 5 and 6, which may be consistent with decreased retroviral long terminal repeat (LTR)-driven T-bet expression, as T cells become quiescent at these times after activation by antigen-APC.

As a control, we evaluated expression of another T_{H1}-specific receptor, IL-18Rα^{27,28} (Fig. 5c). On day 4 after antigen-APC-activation, control

T_{H1} but not T_{H2} cells expressed IL-18Rα, as expected. However, T-bet-infected T_{H2} cells did not express IL-18Rα. Notably, IL-12 treatment of T-bet-expressing T_{H2} cells induced IL-18Rα, which was consistent with published data showing that IL-12 signaling induces IL-18Rα expression^{27,29,30}. Thus, T-bet can induce STAT1-independent IL-12Rβ2 expression in T_{H2} cells, enabling IL-12 to induce IL-18Rα expression; this explains why IL-12-IL-18 treatment partially restored IFN-γ production in T-bet-infected T_{H2} cells (Fig. 3b).

Lack of STAT1-independent T-bet autoactivation

An earlier study concluded that T-bet induced its own expression by autoactivation¹⁴, but this study predated the recognition of IFN-γ and STAT1-mediated induction of T-bet. The conditions used in this study¹⁴ did not neutralize PMA-ionomycin-induced IFN-γ, which therefore allowed IFN-γ, rather than ectopic T-bet, to induce endogenous T-bet expression. To distinguish between these two possibilities, we repeated this analysis, but in addition we neutralized IFN-γ or blocked IFN-γ signaling by using STAT1^{-/-} T cells. We analyzed retroviral and endogenous T-bet expression in two ways. First, we used RNA blot analysis to distinguish between the endogenous and retroviral T-bet transcripts based on differences in size (Fig. 5d). T_{H1} or T_{H2} cells infected with control or T-bet retroviruses were activated and RNA was prepared (see Methods). In these experiments, electrophoresis of RNA was prolonged to increase

the separation between transcripts. T_H1 cells that express only endogenous T-bet, and not retroviral T-bet, show a single band of hybridization with the T-bet probe, which thus defined the endogenous T-bet transcript (Fig. 5d, lane 1, open arrow). These RNA blots resolved two specific retroviral T-bet transcripts, which—in RNA blots of lower resolution—appeared as a single broad high molecular weight band; this may have been due to splice and polyadenylation variations in the T-bet virus (Fig. 5d, lane 3, filled arrows). The endogenous T-bet band and the retroviral T-bet bands were both specific, as they were both absent in control T_H2 cells infected only with the control retrovirus (GFP-RV) (Fig. 5d, lane 2), which were shown to lack T-bet protein by immunoblot analysis (Fig. 5a). When IFN- γ was not neutralized, T-bet-RV-infected T_H2 cells activated by antigen-APC expressed both the retroviral and endogenous T-bet transcripts (Fig. 5d, lane 3), which was consistent with autoactivation¹⁴. However, when these T cells were activated under IFN- γ neutralizing conditions, the endogenous T-bet transcript was reduced in appearance and no band was distinguishable above the lane background (Fig. 5d, lane 5). In addition, T-bet-expressing STAT1^{-/-} T_H2 cells activated by antigen-APCs showed only retroviral T-bet transcripts, and did not show a distinguishable endogenous T-bet transcript (Fig. 5d, lane 7). We noted that the retroviral T-bet transcripts were expressed at higher levels than the endogenous T-bet transcripts, whereas—with GATA-3 autoactivation^{31,32}—endogenous GATA-3 transcripts were expressed at much higher levels than retroviral GATA-3 transcripts.

We also independently verified these results by specifically quantifying the endogenous T-bet transcripts with quantitative reverse-transcribed-polymerase chain reaction (RT-PCR) in these cells (Fig. 5e), as has been described¹⁵. The range of T-bet expression seen in T_H1 and T_H2 cells (Fig. 5e) was similar to that observed in published findings¹⁵. T-bet-expressing T_H2 cells expressed endogenous T-bet mRNA at levels that were similar to T_H1 control cells when IFN- γ was not neutralized; this was again consistent with autoactivation¹⁴. However, when IFN- γ was neutralized or when STAT1^{-/-} T_H2 cells were used, the levels of endogenous T-bet mRNA were reduced to those observed in T_H2 controls (Fig. 5e). Thus, in the absence of IFN- γ signaling or STAT1, ectopic T-bet expression does not appear to induce the endogenous T-bet transcript, as measured by RNA blot analysis and quantitative RT-PCR analysis.

Discussion

T-bet was proposed to be the master switch for T_H1 development^{11–14} based on its induction of IFN- γ , repression of T_H2 cytokines and its apparent induction by IL-12 and STAT4¹¹. A previous study showed that T-bet was STAT4-independent and acted before IL-12 in T_H1 development¹⁴. In addition, this study suggested that T-bet expression was induced stochastically, but was stabilized by autoactivation¹⁴, similar to the autoactivation reported for GATA-3^{31–34}. More recently, T-bet expression has been described as being IFN- γ and STAT1-dependent¹⁵.

Here, we have further distinguished the STAT1 and STAT4-dependent role played by T-bet in T_H1 development. We found differing capacities for T-bet in increasing IFN- γ production induced by PMA-ionomycin, antigen-APCs or cytokines. T-bet also selectively induced IL-12R β 2 but not IL-18R α expression in the absence of STAT1, and T-bet expression was compatible with T_H2 development and GATA-3 expression. In addition, T-bet was not induced directly by cell-intrinsic autoactivation, but was induced by an autocrine pathway involving IFN- γ and STAT1.

Our analysis makes two key points about the roles played by STAT1 and STAT4 in T_H1 development. First, STAT1 is only conditionally required for T_H1 development: it is required for maintaining IL-12R β 2

expression only in the presence of IL-4, but not for IL-12-induced T_H1 development in the absence of IL-4. Second, the STAT4-dependence of IFN- γ production varies according to the method of T cell activation and cytokine measurement. It is greatest in cases of induction by antigen-APC or IL-12–IL-18, which is consistent with published data^{5,9,10,21,22,35}, and least in cases of induction by PMA-ionomycin, which is also consistent with published data¹⁴. Thus, discrepancies regarding the role played by STAT4 in IFN- γ production may be due to the different methods of activation and cytokine measurement used. To better ascertain the role played by T-bet in both STAT4-independent and -dependent processes, we therefore compared the effects of ectopic T-bet expression using all modes of T cell activation and cytokine assays.

We found that T-bet was active in inducing IFN- γ production by T_H2 cells stimulated with PMA-ionomycin, but not with antigen-APCs or cytokines. T-bet also strongly induced IFN- γ production in STAT4^{-/-} T_H1 cells stimulated with PMA-ionomycin, but not antigen-APCs or cytokines. Previously, T-bet has been reported to induce IFN- γ production in STAT4^{-/-} T_H1 cells with PMA-ionomycin¹⁴, which is consistent with our results. However, these previous studies did not use additional modes of T cell activation in analyzing T-bet. It is not clear why T-bet synergizes more strongly with PMA-ionomycin compared to antigen-APC or IL-12–IL-18 for IFN- γ production. This effect may be related to the quantitatively smaller STAT4-dependence of IFN- γ induced by PMA-ionomycin compared to other stimuli. But whatever the mechanism for this marked synergy of T-bet and PMA-ionomycin, this result makes two further key points. First, because T-bet strongly increases IFN- γ under these conditions, T-bet must play an essential role in IFN- γ regulation. Second, the difference in the responses of control T_H1 cells and T-bet-expressing T_H2 cells to antigen-APCs or cytokines emphasizes that ectopic T-bet expression in developing T_H2 cells did not produce a phenotype that was identical to T_H1 . Rather, compared to T_H1 cells, T-bet-expressing T_H2 cells retained some differences.

To further characterize the differences between T_H1 cells and T-bet-expressing T_H2 cells, we measured IL-4, IL-5 and IL-10 production. We found that T-bet-expressing T_H2 cells differed from T_H1 cells in that they produced IL-4, IL-5 and IL-10 at concentrations that were similar to T_H2 cells. This cytokine profile was similar to the profile obtained from T cells differentiated under T_H2 conditions but with the addition of IFN- γ ³. Such cells produced T_H2 cytokines, but also maintained IL-12R β 2 expression. In addition, when restimulated with antigen-APCs, these cells did not produce IFN- γ at concentrations similar to T_H1 cells but they could produce IFN- γ if IL-12 was added³. Thus, neither IFN- γ treatment nor ectopic T-bet expression prevented T_H2 differentiation, but both allowed for later IL-12-induced IFN- γ production.

T-bet maintained IL-12R β 2 expression even when expressed in STAT1^{-/-} T cells, which indicated that T-bet is a sufficient mediator for IFN- γ -induced IL-12R β 2 expression. T-bet did not induce IL-18R α , and yet T-bet-expressing T_H2 cells were partially restored in IL-12–IL-18-induced IFN- γ production. In addition, IL-12 signaling induces IL-18R α expression²⁷. We showed here that IL-12 stimulation of T-bet-expressing T_H2 cells could induce IL-18R α . Thus, T-bet expression appears to restore cytokine-induced IFN- γ production in T_H2 cells at least by maintaining IL-12R β 2 expression. However, this finding does not exclude the possibility that T-bet might also be required at other steps in the process, such as direct interactions with the gene encoding IFN- γ , which may be necessary for IFN- γ production.

While they confirm published reports that T-bet expression is STAT4-independent¹⁴ and STAT1-dependent¹⁵, our results argue against

a possible T-bet autoactivation pathway¹⁴. Our data reproduced the induction of endogenous T-bet by ectopic T-bet when autocrine IFN- γ signaling occurred, but not when IFN- γ was neutralized or in STAT1^{-/-} T cells. T-bet autoactivation was invoked as a mechanism to stabilize T_H1 commitment¹⁴. Because an exocrine pathway may be less stable than a cell intrinsic pathway, it is important to distinguish between these two types of mechanisms.

Finally, T-bet^{-/-} mice have diminished *in vitro* IL-12-induced T_H1 differentiation³⁶. Our results suggest that at least one mechanism for this effect could be reduced IL-12R β 2 expression, although IL-12R expression and signaling were not evaluated in T-bet^{-/-} T cells³⁶. CD8⁺ T cells from these T-bet^{-/-} mice produced normal amounts of IFN- γ ³⁶. Notably, CD8⁺ T cells may not require IL-12 signaling for antigen-induced IFN- γ production⁵. Thus, IL-12 signaling may be a CD4-specific requirement for IFN- γ production that is provided by T-bet. Conceivably, other CD4-specific actions of T-bet could involve direct actions on the gene encoding IFN- γ , which would not be required in CD8⁺ T cells.

Methods

Reagents. DO11.10 TCR-transgenic mice on wild-type, STAT1-deficient or STAT4-deficient backgrounds were as described³⁷. The antibody specific to murine IL-12R β 2 (PDL-HAM10B9)³⁸ was a gift of Protein Design Labs (Fremont, CA). An antibody to GST hamster (PIP), which was used as an isotype control, was a gift of R. D. Schreiber (St. Louis, MO). Biotinylated goat anti-murine IL-18R α was from R&D Systems (Minneapolis, MN). Biotinylated goat anti-hamster, used as an isotype control, was from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-T-bet (4B10) was a gift of L. Glimcher (Boston, MA). Other cytokines and antibodies were as described³¹. All animal experiments were approved by the Washington University Animal Studies Committee.

Constructs. The T-box proteins Tb-lym³⁹ and T-bet¹¹ have been cloned and described, but represent the same gene. Sequence comparisons between Tb-lym and T-bet showed two differences: a silent C \rightarrow T change at T-bet position 706 and a nonconservative C \rightarrow T change at T-bet position 1628, which changed the coding sequence from Pro⁴⁸² in T-bet to Ser⁴⁸² in Tb-lym. To determine which form is expressed in the BALB/c strain, RT-PCR was used to amplify the region between base pairs 1147 to 1696 of the T-bet sequence from RNA obtained from BALB/c background DO11.10 T_H1 cells with the primers TBF3 (sense) GGATTCGGGAGAAGCTTTGAGTC and TBR1 (antisense) CCACTGGAAGGATAGGG GGATA. The amplified region was sequenced and was identical to that reported for T-bet. Tb-lym was amplified from the cDNA template³⁹ with the primers Tglym-bgl2-5' GGAA-GATCTAAGGACCTCGGGTCTC and Tglym3'-Xho GCCTCGAGCACCTTCCAATT CAGCGGCA. The product was digested with *Bgl*III and *Xho*I, and cloned into *Bgl*III-*Xho*I-digested GFP-RV⁴⁰, which generated Tb-lym-RV. T-bet-RV was then generated from Tb-lym-RV with Stratagene (La Jolla, CA) Quickchange Site-directed mutagenesis as described⁴¹. The primers 5'Tbmut1 CCCTCCAGCCGGAGCCAGCGACTCAGG and 3'Tbmut1 CCTGAGTCTGGGCTCCGGCTCGAGGG were used. Tb-lym-RV and T-bet-RV were verified by sequencing. Retroviruses that contained T-bet cDNA were generated in the GFP-RV vector⁴⁰ and the functional analyses presented were done with T-bet-RV.

T cell activation and retroviral infections. Naïve (MEL-14⁴⁰) CD4⁺ DO11.10 T cells were sequentially purified, first by isolation of CD4⁺ cells with Dynal CD4 beads and DETACHaBEAD (DynaL Biotech, Oslo, Norway), then by isolation of MEL-14⁴⁰ CD4⁺ cells with mouse CD62L microbeads (Miltenyi Biotech, Auburn, CA). These cells were stimulated in 7-day cycles, starting with activation on day 0; this was followed by expansion on day 3 and the collection of resting cells on day 7. For primary stimulation, purified naïve T cells were activated with 0.3 μ M OVA peptide (amino acids 323–339)⁸ and irradiated BALB/c splenic APCs (2,000 rad) (OVA peptide-APCs), in the presence of 10 U/ml of IL-12 and 10 μ g/ml of anti-IL-4 (11B11) for T_H1 differentiation and 100 U/ml of IL-4, 3 μ g/ml of anti-IL-12 (TOSH) and 10 μ g/ml of anti-IFN- γ (H22) for T_H2 differentiation. For retroviral infections, cells were infected 36 h after activation by the addition of retroviral supernatant and 2 μ g/ml of polybrene, as described^{37,40}. On day 3, cells were expanded into fresh media plus 40 U/ml of IL-2 and resting cells were collected on day 7 for further use, typically for purification by cell sorting as described^{37,40}. Sorted cells were restimulated with 0.3 μ M OVA peptide and irradiated BALB/c spleen APCs in the presence of 10 μ g/ml of anti-IL-4 for T_H1 and 3 μ g/ml of anti-IL-12 and 10 μ g/ml of anti-IFN- γ for T_H2 cells and collected after another 7 days.

RNA blot and immunoblot analysis. For Web Figs. 1–3, naïve wild-type or STAT1^{-/-} CD4⁺ DO11.10 T cells were activated for 7 days as described above and collected on day 7 for RNA preparation. In Fig. 2, naïve wild-type, STAT4^{-/-} or STAT1^{-/-} CD4⁺ DO11.10 T cells were activated under T_H1 or T_H2 conditions for two 7-day cycles as described above, collected and restimulated with plate-bound anti-CD3 for 24 h (Fig. 2a) or PMA (50 ng/ml) and

ionomycin (1 μ M) for 3 h (Fig. 2b). In Fig. 5, naïve CD4⁺ DO11.10 T cells were activated, infected, sorted and restimulated as described above. On day 14 after primary stimulation, resting cells were collected and restimulated with 0.3 μ M OVA peptide and irradiated BALB/c spleen APCs in presence of 10 μ g/ml of anti-IL-4 (11B11) and 3 μ g/ml of anti-IL-12 (TOSH) for T_H1 cells and the same plus 10 μ g/ml of anti-IFN- γ (H22) for T_H2 cells, unless otherwise indicated. The cells were collected 60 h after restimulation and live cells were purified on a density gradient (Histopaque-1119; Sigma, St. Louis, MO). For RNA blot analysis, RNA was extracted with RNeasy kit (Qiagen, Valencia, CA), 10 μ g/lane was separated by gel electrophoresis and transferred to Zeta Probe membrane (BioRad, Richmond, CA). Full-length mouse IFN- γ , IL-4, STAT1, GATA-3, IL-12R β 2, T-bet and GAPDH cDNA were labeled and used as probe at 10⁶–10⁷ cpm/ml. For immunoblot analysis, total cellular lysates were prepared and analyzed as described³⁷.

Intracellular cytokine staining, FACS analysis and ELISA. Intracellular cytokine staining for IFN- γ was done as described^{5,42} with the modification that T cells were activated either with PMA-ionomycin as described^{10,14,42} or with antigen-APC or IL-12-IL-18 treatment. For antigenic stimulation, we used 0.3 μ M OVA peptide (or other concentrations, as indicated), irradiated BALB/c spleen APCs as described²³ and 3 μ g/ml anti-IL-12 (TOSH). For IL-12-IL-18 stimulation, we used 10 U/ml of murine IL-12 and 50 ng/ml of murine IL-18. For PMA-ionomycin stimulation, we used 50 ng/ml of PMA and 1 μ M ionomycin. Peaks of production for different cytokines were measured by intracellular cytokine staining and varied. IL-4 production was measured after 6 h of stimulation and IFN- γ after 24 h. Brefeldin A (1 μ g/ml, Epicenter Technology, Epicenter Technologies, Madison, WI) was added for the final 2.5 h of each stimulation. After stimulation, the cells were collected and stained for surface markers, fixed, permeabilized, stained with phycoerythrin (PE)-conjugated cytokine antibody or isotype control antibody (Pharmingen, San Diego, CA), and analyzed on a FACSCaliber (Becton Dickinson, San Jose, CA). For ELISA, the cells were stimulated as described above for intracellular cytokine staining. After 48 h of stimulation, culture supernatants were collected and cytokine concentrations were measured as described³². For FACS analysis of IL-12R β 2 and IL-18R α expression, cells were stimulated as described in the figure legends. For IL-12R β 2 staining, cells were collected at various times and stained with the primary antibody PDL-HAM10B9 or an isotype control; this was followed by staining with biotinylated goat anti-Armenian hamster (Jackson Immunoresearch Laboratories) and PE-streptavidin (Pharmingen) as described³⁸. For IL-18R α staining, cells were stained with biotinylated goat anti-murine IL-18R α followed by PE-streptavidin.

Quantitative RT-PCR analysis of T-bet expression. For quantitative RT-PCR experiments, we used a Real-time LightCycler PCR^{43,44} as described^{18,43}. cDNA was reverse transcribed from 4 μ g of total RNA isolated from the cells in Fig. 5d with 0.5 μ g oligo(dT)₁₂₋₁₈ and Superscript RT (Gibco-BRL, Gaithersburg, MD). A total reaction volume of 20 μ l contained 1 μ l of cDNA, 2 mM MgCl₂, 0.2 mM dNTPs, 1 μ l of 1 mg/ml of bovine serum albumin, 2 μ l of 10 \times PCR buffer, 1 μ l of Taq polymerase (Promega, Madison, WI) and 50 ng of each primer. Samples were normalized to HPRT for RT-PCR. Primers for endogenous T-bet expression were sense T-bet 5'-TGCCTGCAGTGCTTCTAACA-3'; antisense T-bet 5'-TGCCCGCTTCTCTCCAACCA-3'. Primers for murine HPRT were sense HPRT 5'-GCGACCCGACCTCCAGC-3'; antisense HPRT 5'-TTAGGCTTTGATTTG-GCTTTT-3'. LightCycler PCR comprised a 2-min denaturation at 95°C, followed by cycles of 95°C for 1 s, 60°C for 10 s, 72°C for 30 s and 84°C for 0 s at which point fluorescence was measured. Specific product was confirmed by melting-curve analysis that consisted of 65°C for 15 s annealing, followed by a slow ramp to 95°C at 0.2 °C/s with continuous fluorescence measurement.

Note: Supplementary information is available on the Nature Immunology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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