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Transcription factor T-bet represses T_H17 differentiation by preventing Runx1-mediated activation of the RORγt gene

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Abstract

Overactive T_H17 responses are tightly linked to the development of autoimmunity, yet the factors that negatively regulate differentiation of this lineage remain unknown. Here, we report that T-bet suppresses the development of the T_H17 cell lineage by inhibiting the transcription of *Rorc*. T-bet interacts with the transcription factor Runx1 and this interaction blocks Runx1-mediated transactivation of *Rorc*. T-bet residue Tyr^{304} is required for T-bet-Runx1 complex formation, for blocking Runx1 activity and for inhibiting the T_H17 differentiation program. These data reinforce the concept of master regulators that shape immune responses by simultaneously activating one genetic program while silencing the activity of competing regulators in a common progenitor cell.

The signals received during an infection trigger a strong adaptive immune response tailored to combat a particular class of pathogen. In the presence of cytokines produced by cells of innate immunity, naive $CD4^+$ T cells differentiate into a T_H cell subset with distinct

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V.L. designed and executed experiments and prepared the manuscript. X.C. performed ChIP assays and J.H.S. performed DNA pull-down and co-immunoprecipitation assays. E.S.H. created T-bet mutant retroviral constructs, E.J. performed doxycycline transgenic T cell experiment and M.O. generated IL-23R.GFP mice. V.K.K. contributed to discussions and manuscript preparation. A.N.B. provided technical assistance. L.H.G. supervised the research, designed experiments and participated in the manuscript preparation.

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functions and cytokine profile. Until recently two main T_H subsets were described: T_H1 and $T_H2$1. T_H1 cells, which predominantly secrete interferon-γ (IFN-γ), are essential for immunity against intracellular microorganisms, while T_H2 cells, which secrete IL-4, IL-5 and IL-13, are important for protection against parasites and extracellular pathogens. More recently, a third subset of T_H cells, called T_H17, has been described2–7. T_H17 cells produce IL-17A, IL-17F, IL-21 and IL-22, which protect the host against bacterial and fungal infections encountered at mucosal surfaces8. In mice, T_H17 differentiation is initiated by the combination of transforming growth factor-β (TGF-β) and IL-6 or IL-21, which induces the expression of retinoic acid receptor-related orphan nuclear receptor, RORγt, and IL-23R7, 9–12. This acquisition of responsiveness to IL-23 is necessary for the terminal differentiation of the T_H17 cell lineage and for the maintenance of T_H17 functions *in vivo*13.

Generally, lineage-specific transcription factors and cytokines can inhibit the differentiation of other T_H subsets. T-bet suppresses the generation of T_H2 cells by blocking the expression of the T_H2 polarizing cytokine IL-4, and by interfering with the activity of the T_H2-cell–specific transcription factor, GATA314, 15. Results from several studies have indicated that T_H17 responses are stronger in T-bet-deficient animals, although the mechanism underlying this phenomenon was not described16–19. This raises the question of whether, analogous to its role in inhibiting the T_H2 pathway, T-bet also actively suppresses T_H17 differentiation. Furthermore, several studies have reported that the re-programming of committed T_H17 and T_H2 cells into effector cells with T_H17-T_H1 and T_H2-T_H1 phenotypes is driven by T-bet in response to inflammatory cytokines such as IL-12 and interferons20, 21.

In this study we sought to determine whether T-bet plays a regulatory role in the development of the T_H17 lineage. We investigated T_H17 differentiationin *Tbx21*$^{-/-}$ and WT $CD4^+$ T cells *in vitro* and *in vivo* during experimental autoimmune encephalomyelitis (EAE). Here we report that T-bet has a negative effect on the expression of the T_H17-cell-specific transcription factor RORγt and T_H17 cytokine genes. Ectopic T-bet expression in naïve T_H precursor cells or in committed T_H17 cells was sufficient to repress the expression of RORγt and T_H17 signature cytokine genes under T_H17 polarizing conditions. Mechanistic studies revealed that interaction of T-bet with Runx1 via the T-bet residue Tyr^{304} is critical for blocking Runx1-mediated transactivation of the *Rorc* promoter and for inhibiting T_H17 lineage commitment.

RESULTS

T-bet deficiency promotes IL-17A production *in vitro*

T-bet, encoded by the *Tbx21* gene, is a transcriptional activator of IFN-γ and the key regulator of the T_H1 differentiation program22. In addition to promoting differentiation of naive $CD4^+$ T cells into the T_H1 subset, T-bet actively suppresses the development of the T_H2 lineage 14, 15. To investigate whether T-bet expression has a similar antagonistic effect on the development of IL-17A producing T_H cells, we cultured *Tbx21*$^{-/-}$ and wild-type (WT) $CD4^+$ T cells under non-skewing conditions or differentiated them into T_H1 cells or T_H17 cells which were grown in the absence or presence of IL-23 (T_H17 and T_H17+IL-23 conditions). Since IFN-γ has a negative effect on the polarization of T_H17 cells and *Tbx21*$^{-/-}$ T cells produce significantly less IFN-γ than WT $CD4^+$ T cells, *Ifng*$^{-/-}$ T_H cells

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were also tested to delineate T-bet- versus IFN-γ-mediated effects on T_H17 development. After five days of *in vitro* differentiation, *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ and WT T_H0, T_H1, T_H17 and T_H17+IL-23 cells were briefly stimulated with phorbol myristate acetate and ionomycin (PMA+I). We observed a higher percentage of IL-17A producing cells in T-bet-deficient T_H0 and T_H1 cultures when compared to *Ifng*$^{-/-}$ and WT cultures (Fig. 1a). Although a similar percentage of IL-17A producing cells was detected under T_H17 polarizing conditions, the amount of IL-17A secreted by *Tbx21*$^{-/-}$ T_H cells was higher than that secreted by *Ifng*$^{-/-}$ and WT T_H cells under all differentiating conditions (Fig. 1b).We did not observe substantial differences in the amount of *Rorc* mRNA expression amongst different T_H subsets at 24 hours after activation (data not shown). However, the enhanced IL-17A production by *Tbx21*$^{-/-}$ T_H0 and T_H1 cultures correlated with a 2-fold increase in the expression of *Rorc* mRNA after 5 days of culture. In contrast, *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ and WT T_H17 cells expressed similar levels of *Rorc* mRNA (Fig. 1c). These results show that T-bet deficiency promotes development of IL-17A producing cells under all polarizing conditions independently of IFN-γ and suggest that T-bet-mediated effects on the generation of IL-17A producing cells *in vitro* may be through the transcriptional regulation of *Rorc* and/or *Il17a* genes in T_H0-T_H1 and T_H17 cells, respectively.

T_H17 responses in *Tbx21*$^{-/-}$ and WT mice during EAE

Tbx21$^{-/-}$ mice are protected from developing EAE23. At the time when the results of this study were reported, T_H17 cells were yet to be discovered, and the resistance of *Tbx21*$^{-/-}$ mice to central nervous system (CNS)-specific autoimmune attack was ascribed to the polarization shift of $CD4^+$ T cells from a pathogenic T_H1 to a protective T_H2 response23. Considering the propensity of T-bet-deficient $CD4^+$ T cells to develop into IL-17A-producing cells *in vitro*, we investigated whether *Tbx21*$^{-/-}$ mice generated T_H17 responses during EAE, the pathology of which is widely accepted to be dependent on T_H17 cells. To determine the types of cytokines produced by CNS-infiltrating $CD4^+$ T cells, we performed intracellular cytokine staining on mononuclear cells isolated from the CNS of *Tbx21*$^{-/-}$ and WT mice during the peak of disease (day 17 post-immunization). In WT mice, three different cytokine producing populations entered the CNS: those that produced IFN-γ alone (the majority of $CD4^+$ T cells), those that produced only IL-17A, and those that produced both cytokines (Fig. 2a). In contrast, in the CNS of *Tbx21*$^{-/-}$ mice, IL-17A producing $CD4^+$ T cells represented the majority of cytokine producing cells at day 17 post-immunization (Fig. 2a). Consistent with the role of T-bet in controlling expression of the IFN-γ gene, there was a deficiency in IFN-γ-producing $CD4^+$ T cells in the CNS of *Tbx21*$^{-/-}$ mice (Fig. 2a). Collectively, there was a shift in the T_H1-T_H17 balance in the CNS of *Tbx21*$^{-/-}$ mice during EAE characterized by the preferential recruitment of T_H17 cells and significant reduction in the frequency and absolute numbers of IFN-γ-producing $CD4^+$ T cells (Fig. 2b). Moreover, $CD4^+$ T cells isolated from the CNS of *Tbx21*$^{-/-}$ mice secreted significantly higher levels of IL-17A than WT $CD4^+$ T cells at day 17 post-immunization (Fig. 2c). Thus, there is a strong recruitment of IL-17A-secreting T_H17 cells into the CNS of *Tbx21*$^{-/-}$ mice.

IL-23R signaling drives the pathogenic potential of CNS-infiltrating T_H17 cells by promoting the expression of proinflammatory chemokines and by suppressing the expression of IL-10[4], 24. In addition, IL-23R signaling is essential for the terminal

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differentiation of T_H17 cells and their long term survival13, 25. To address the role of T-bet in controlling IL-23R expression in T_H17 cells, we crossed IL-23R.GFP reporter mice onto a T-bet-deficient background. On day 17 after $MOG_{35–55}$ plus CFA immunization, mononuclear cells were isolated from the draining lymph nodes, spleen and CNS of *Tbx21*$^{-/-}$-IL-23R.GFP and WT-IL23R.GFP reporter mice and the percentage of IL-23R positive cells within the $CD4^+$ population was determined by flow cytometry. As shown in Fig. 2d, *Tbx21*$^{-/-}$ mice displayed a higher percentage of IL-23R$^+$ $CD4^+$ cells in the lymph nodes and the CNS versus controls, while in spleen the frequency of IL-23$^+$ $CD4^+$ T cells was similar. In addition, there was a significantly higher percentage of $CD4^+$ cells expressing the T_H17-specific chemokine receptor, CCR6 in the lymph nodes and the CNS of *Tbx21*$^{-/-}$ mice (Fig. 2d). We looked at the expression of other T_H17 signature genes in purified $CD4^+$ T cells isolated from the CNS of *Tbx21*$^{-/-}$ and WT mice during the disease peak and observed higher levels of *Rorc*, *Il23r*, *Il17a* and *Il17f* transcripts in *Tbx21*$^{-/-}$ $CD4^+$ T cells, while the levels of *Il21* and *Il22* were more variable between *Tbx21*$^{-/-}$ and WT mice (Fig. 2e). The enhanced Type 17 response in *Tbx21*$^{-/-}$ mice could be intrinsic to the T cell or reflect differences in cytokine production by innate immune cells which could create a milieu more conducive for the polarization of T_H17 cells *in vivo*. To differentiate between these two possibilities we performed functional analysis of $CD4^+$ T cells in the CNS of *Tbx21*$^{F/F}$ and *Tbx21*$^{F/F}$ *Cd4*^{cre} mice 14 days after EAE induction (Supplementary Fig. 1). Our data demonstrate that T cell-specific deletion of T-bet results in an augmented T_H17 response in the CNS during EAE, suggesting that this is a T cell-intrinsic phenomenon. Thus, a dominant T_H17 response is generated in *Tbx21*$^{-/-}$ mice following $MOG_{35–55}$ plus CFA immunization, suggesting that T-bet expression limits the magnitude of Type 17 responses in the CNS during EAE.

T-bet expression blocks T_H17 differentiation

The experiments described above suggest strongly that T-bet negatively regulates T_H17 cell lineage commitment. To directly assess whether T-bet plays a negative role in T_H17 differentiation, naïve $CD4^+$ T cells ($CD62L^{hi}CD25^{lo}$) were activated under T_H17 polarizing conditions. After 24 h, activated $CD4^+$ cells were transduced with empty retrovirus (EV-RV) or T-bet-RV containing T_H17 skewing cytokines + neutralizing IL-4 and IFN-γ antibodies. After 5 days of culture under T_H17 polarizing conditions, the cytokine production by sorted GFP$^+$ cells was determined by intracellular cytokine staining (ICS). Transduction of both *Tbx21*$^{-/-}$ and WT naïve $CD4^+$ T cells with T-bet resulted in a substantial decrease in the percentage of IL-17A producing cells and a marked increase in the frequency of IFN-γ-IL-17A double producers and IFN-γ single producers (Fig. 3a). T-bet expression resulted in a marked reduction of *Rorc* and *Rorc*-target genes (*Il17a* and *Il17f*), *Il21* and *Il23r* (Fig. 3b). Although we observed no significant difference in the amount of *Il22* transcripts between *Tbx21*$^{-/-}$ and WT T_H17 cells, T-bet over-expression augmented *Il22* mRNA expression in both *Tbx21*$^{-/-}$ and WT T_H17 cells (Fig. 3b). Ectopic expression of T-bet therefore prevents differentiation of T_H precursors into T_H17 cells under T_H17 polarizing conditions by blocking the expression of the T_H17 cell lineage-specific transcription factor, RORγt and consequently, RORγt-target genes.

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In a separate series of experiments, we utilized the transgenic inducible T-bet expression system, where T-bet is induced in naïve T_H precursors in response to doxycycline treatment. Naïve $CD4^+$ T cells were activated under T_H17 polarizing conditions for 24 h. On the second day, T-bet expression was induced by the administration of 0.5 μg/ml of doxycycline in the absence or presence of neutralizing IFN-γ antibody. The amount of RORγt and T-bet mRNA and protein was determined by real-time PCR and immunoblotting, respectively. Induction of T-bet expression in transgenic T_H precursors by doxycycline treatment resulted in marked reduction of both RORγt transcripts and protein under T_H17 polarizing conditions (Fig. 3c–d). We observed suppression of RORγt by T-bet even in the presence of neutralizing IFN-γ antibody demonstrating that this mode of RORγt suppression is independent of IFN-γ.

T-bet can redirect fully differentiated T_H2 cells into the T_H1 pathway22. To determine whether T-bet could similarly reprogram committed T_H17 cells, we differentiated naïve $CD4^+$ T cells ($CD62L^{hi}CD25^{lo}$) under T_H17 polarizing conditions for 6 days after which *Tbx21*$^{-/-}$ and WT T_H17 cells were re-activated for 24 h and transduced with either EV-RV or T-bet-RV under T_H17 polarizing conditions. Forty-eight hours after retroviral transduction, GFP^+ cells were sorted and intracellular cytokines examined. There was a 50% reduction of IL-17A producing cells and an increase in the frequency of IFN-γ-producing cells after transduction with T-bet-RV (Fig. 3e). Similar to the experiments above, enforced T-bet expression in fully differentiated T_H17 cells resulted in reduced levels of *Rorc*, *Il17a*, *Il17f*, *Il21* and *IL23r* transcripts and increased expression of *Il22* (Fig. 3f). These results indicate that ectopic expression of T-bet is sufficient to suppress expression of the Type 17 signature genes in committed T_H17 cells even in the presence of T_H17 polarizing cytokines.

T-bet inhibits Runx1 activity

Our results support the idea that the negative effect of T-bet on T_H17 differentiation could be mediated by inhibition of the T_H17-cell-specific transcription factor RORγt. To determine whether T-bet binds to the *Rorc* promoter, we performed chromatin immunoprecipitation (ChIP) assays on T-bet-bound chromatin from nuclear lysates of non-polarized T_H0 cells and differentiated T_H1 and T_H17 cells. We detected modest, but reproducible, binding of T-bet to a site located approximately 2kb upstream of the first exon of *Rorc* (the thymus-specific isoform) in non-skewed T_H0 cells, and we found marked enrichment of T-bet bound to the same -2kb site in T_H1 cells (Fig. 4a–b). We did not detect binding of T-bet to the *Rorc* or *Ifng* promoter in T_H17 cells (data not shown). To test whether T-bet directly inhibits *Rorc* gene expression, we performed reporter luciferase assays with the –2kb *Rorc*-luc promoter in HEK293 cells. Transfection of T-bet had no effect on *Rorc*-luc activity indicating that T-bet might not directly suppress *Rorc* transcription (Fig. 4c). T-bet generally does not act as a direct transcriptional repressor 15, 26, 27. Instead, T-bet exerts its negative effect on gene expression by binding to and sequestering transcriptional activators away from regulatory regions15, 26–29. Runx1 induces RORγt expression30. There are two Runx1 binding sites immediately upstream of the T-bet binding site (2kb upstream of the first exon of the *Rorc(T)* gene). This finding prompted us to investigate whether T-bet could inhibit RORγt expression by blocking Runx1 transcriptional activity. To analyze the regulation of the *Rorc* gene, HEK293 cells

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were transfected with a −2kb *Rorc*-luc reporter construct in the presence of increasing concentrations of Runx1 plasmid with or without T-bet expression vector. Runx1 expression increased luciferase activity in a dose-dependent manner, which was blocked by co-expression of T-bet (Fig. 4c). Furthermore, T-bet blocked Runx1-mediated transactivation of the *Rorc* promoter in a dose-dependent manner (Fig. 4d). Several other transcription factors, namely Irf4, Batf and STAT3, control T_H17 differentiation by positively regulating *Rorc* expression31–33. Relative expression of *Irf4* and *Batf* was similar in *Tbx21*$^{-/-}$ and WT T_H cells as determined by RT-PCR (Supplementary Fig. 2). It is currently unknown whether regulation of *Rorc* expression by Irf4 and Batf is mediated by direct binding of these transcription factors to the *Rorc* locus, but the binding sites for STAT3 in the *Rorc* and *Il17a* loci have been clearly defined33. To determine whether there was a difference in the binding of STAT3 to its target sequences in the *Rorc* and *Il17a* loci, we performed ChIP on STAT3-bound chromatin from nuclear lysates of non-polarized T_H0 and T_H17 cells. The binding of STAT3 to its target sites in the intergenic or intragenic regions of the *Rorc* locusor *Il17a* locus was similar in *Tbx21*$^{-/-}$ and WT T_H0 and T_H17 cells (Supplementary Fig. 3). These data suggest that interference with Runx1, but not STAT3, transcriptional activity is the likely mechanism by which T-bet blocks the expression of RORγt.

T-bet interacts with Runx1

To examine further the mechanism of T-bet-mediated repression of *Rorc* expression, we investigated whether T-bet could interact with Runx1. First, we over-expressed T-bet with Myc-tagged Runx1, Runx2 and Runx3 and performed co-immunoprecipitation experiments in HEK293 cells. T-bet interacted with both Runx1 and Runx3, but not Runx2 in HEK293 cells (Fig. 5a). To determine in which T_H subset T-bet and Runx1 interact, we performed immunoblot analysis of Runx1 and T-bet protein expression in unskewed T_H0 and differentiated T_H1 and T_H17 cells. We detected T-bet and Runx1 expression in non-polarized T_H0 cells (Fig. 5b). There was a marked reduction in the expression of Runx1 protein in *in vitro* differentiated T_H1 cells and conversely, a marked decrease in levels of T-bet protein in *in vitro* differentiated T_H17 cells (Fig. 5b). These data suggest that interaction between T-bet and Runx1 could occur in non-polarized T_H0 cells in which both proteins are co-expressed. Indeed, Runx1 immunoprecipitation confirmed the presence of an endogenous T-bet-Runx1 interaction in non-polarized WT T_H0 cells, but not in differentiated T_H1 and T_H17 cells (Fig. 5c and data not shown). T-bet-Runx1 interaction in uncommitted T_H0 cells was also confirmed in the reverse co-immunoprecipitation assay (Fig. 5c). These data suggest that a functionally important interaction between T-bet and Runx1 most likely occurs in uncommitted T_H cells, but not in fully differentiated T_H1 and T_H17 cells because of restrictive expression of Runx1 and T-bet, respectively, in these T_H cells.

Based on the luciferase data and co-immunoprecipitation experiments, one could hypothesize that T-bet interaction with Runx1 could block Runx1 binding to its consensus sites located -2kb in the *Rorc(T)* promoter . We detected Runx1 binding to the oligo containing the WT Runx1 target sequence, but not to the oligo in which the Runx1 target sequence was mutated by a T to A substitution (Fig. 5d). T-bet bound to the WT oligo containing a T-bet specific half-site, but not to the oligo in which the T-bet half-site was mutated (Fig. 5d). After confirming that Runx1 and T-bet binding to the -2kb *Rorc(T)* site

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was sequence-specific, we performed a DNA pull-down assay with the WT oligo and Runx1 in the absence or presence of increasing concentrations of T-bet. In the absence of T-bet, Runx1 bound strongly to the DNA oligo containing the WT Runx1 binding site (Fig. 5e). Increasing concentrations of T-bet ablated the ability of Runx1 to bind to its target sequence in a dose-dependent manner showing that T-bet interaction with Runx1 interferes with Runx1 binding to its -2kb *Rorc(T)* site (Fig. 5e).

Runx1 reversed T-bet effects on T_H17 polarization

Next we investigated whether Runx1 over-expression could block the inhibitory effects of T-bet on T_H17 differentiation. Purified $CD4^+$ T cells were transduced with various combinations of retroviruses expressing GFP alone (EV-GFP), Thy1.1 alone (EV-Thy1.1), Runx1-GFP and/or T-bet-Thy1.1. The transduced cells were grown under T_H17 conditions for 5 days after which GFP-Thy1.1 double-positive cells were sorted and examined for T_H17 commitment. Retroviral transduction of T_H cells with T-bet had a negative effect on T_H17 commitment under T_H17 polarizing conditions, while transduction of T_H cells with Runx1 augmented the differentiation of T_H17 cells (Fig. 6a–b). Over-expression of Runx1 reversed the inhibitory effect of T-bet and fully restored T_H17 polarization in cells co-expressing Runx1 and T-bet (EV vs. T-bet-Runx1). However, *Rorc* expression was only partially up-regulated, suggesting that there may be additional mechanism(s) by which T-bet inhibits transcription of the *Rorc* gene in T_H17 cells (Fig. 6b). Conversely, transduction of purified *Tbx21*$^{-/-}$ $CD4^+$ T cells with Runx1 dominant negative (DN) retrovirus during T_H17 differentiation reversed the effects of T-bet deficiency on IL-17A production by T_H17 cells (Supplementary Fig. 4).

In addition to directly promoting RORγt expression, Runx1 also acts as a co-activator and together with RORγt induces the expression of the *Il17a* and *Il17f* genes30. T-bet suppresses T_H17 differentiation by inhibiting the expression of the *Rorc* gene (Fig. 3). However, it is possible that T-bet's interaction with Runx1 serves to sequester this transcriptional co-activator and blocks the expression of T_H17 signature genes in this manner. To investigate this possibility, we asked whether RORγt was able to restore a T_H17 developmental program in T_H cells co-expressing T-bet and RORγt under T_H17 polarizing conditions. Expression of RORγt independently of the T-bet transcriptional block (i.e. from the retroviral LTR control elements) in developing T_H17 cells was unable to fully reverse the T-bet mediated inhibition of T_H17 differentiation (Fig. 6c–d). We were unable to co-immunoprecipitate T-bet and RORγt in 293HEK cells, suggesting that the sequestration of RORγt from its target genes by T-bet is unlikely. These data demonstrate that in addition to inhibiting the transcription of the *Rorc* gene, T-bet interaction with Runx1 depletes the pool of free Runx1 which is available for the formation of transcriptionally active Runx1-RORγt complexes in T_H17 cells.

T-bet Tyr^{304} is crucial for suppression of T_H17 cells

To investigate which amino-acid residue is important for T-bet-Runx1 complex formation, we tested the ability of a series of T-bet mutants to interact with Runx1 in HEK293 cells. Two of these point mutants, T-bet$_{S508A}$ and T-bet$_{Y525F}$, were shown previously to be functionally important in IL-2 and in T_H2 lineage suppression, respectively15, 27.

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Coimmunoprecipitation studies revealed that Runx1 interacted with the T-bet$_{Y265F}$, T-bet$_{S508A}$ and T-bet$_{Y525F}$ mutants, but not with the T-bet$_{Y304F}$ mutant (Fig. 7a). Furthermore, the T-bet$_{Y304F}$ mutant was unable to suppress Runx1 transcriptional activity in the -2kb *Rorc*-luciferase assay (Fig. 7b) suggesting that this T-bet Tyr^{304} residue may be important for the suppression of T$_H$17lineage commitment. To answer this question, sorted naïve T$_H$ cells were activated under T$_H$17 polarizing conditions for 24 h and transduced with control, WT T-bet, T-bet$_{Y304F}$ and T-bet$_{Y525F}$ mutant retroviruses. The transduced cells were cultured under T$_H$17 polarizing conditions for an additional 5 days and the percentage of IFN-γ and IL-17 producing cells was determined by ICS. WT T-bet and the T-bet$_{Y525F}$ control mutant suppressed T$_H$17differentiation under T$_H$17 polarizing conditions. In contrast, mutation of T-bet residue Tyr^{304} abrogated the ability of T-bet to repress T$_H$17 lineage commitment (Fig. 7c). When compared with WT T-bet and the T-bet$_{Y525F}$ mutant, the T-bet$_{Y304F}$ mutant was unable to suppress expression of *Rorc*, *Il17a*, *Il17f* and *Il23r* in developing T$_H$17 cells (Fig. 7d). These data indicate that T-bet residue Tyr^{304} is important for T-bet-Runx1 complex formation, for inhibition of Runx1 transcriptional activity and for suppression of T$_H$17 lineage development.

In DNA pull-down assays, the T-bet$_{Y304F}$ mutant failed to bind to the T-bet binding site in the -2kb *Rorc(T)* promoter region suggesting that this residue is also important for T-bet binding to DNA (Fig. 7e). To delineate whether T-bet-mediated inhibition of Runx1 activity was dependent on DNA binding or T-bet-Runx1 protein-protein interaction, we investigated whether WT T-bet could inhibit Runx1 binding to its target site using oligos in which the T-bet binding site was mutated. T-bet blocked Runx1 binding to the Runx1-specific sequence independently of T-bet's ability to bind to DNA (lane 3, Fig. 7f). Collectively, these data suggest that the T-bet-Runx1 protein-protein interaction is mainly responsible for inhibition of Runx1 activity with a T-bet protein-DNA interaction playing a minor if any role.

DISCUSSION

Lineage-specific transcription factors can both activate and repress differentiation programs. T-bet simultaneously promotes T$_H$1 differentiation and represses T$_H$2 differentiation 3, 6. While several studies have reported an enhanced Type 17 response in *Tbx21*$^{-/-}$ animals in different disease models16–19, no mechanistic explanation for this increase in T$_H$17 responses was provided. Here, we demonstrate that T-bet suppresses T$_H$17 cell lineage commitment by inhibiting the transcription of the T$_H$17-cell-specific transcription factor RORγt and its target genes. T-bet did not directly repress the *Rorc* promoter. Instead, T-bet interacted with the Runx1 transcription factor and blocked Runx1-mediated transactivation of *Rorc*. Over-expression of Runx1 was sufficient to reverse the inhibitory effects of T-bet on IL-17A production by T$_H$17 cells. Furthermore, we demonstrate that T-bet residue Tyr^{304} is crucial not only for T-bet-Runx1 complex formation, but also for blocking Runx1 activity and for inhibiting the T$_H$17 differentiation program. Thus, our data uncover a molecular mechanism to explain the exaggerated T$_H$17 responses observed in T-bet deficient hosts.

In addition to activating a set of genes that promote T$_H$ cell differentiation towards a specific lineage, a master regulator can also suppress the developmental program of the opposing T cell lineages34. Among the T$_H$ cell-specific transcription factors, T-bet seems to be

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particularly active in this regard. The ability of T-bet to negatively regulate the differentiation of T_H2 cells15, 22, IL-2 production from T_H1 cells27 and tumor necrosis factor (TNF) production in dendritic cells26 prompted us to investigate the contribution of T-bet to the regulation of the T_H17 response. Our data show that T-bet deficiency results in an augmented Type 17 response *in vitro* and *in vivo* during CNS inflammation in the EAE model of multiple sclerosis. Our results differ from a previous publication which reported that mice injected with T-bet-specific siRNA had lower expression of IL-23R and lacked T_H17 cells after immunization with myelin basic protein (MBP) $Ac_{1–11}$ plus CFA or $MOG_{35–55}$ plus CFA35, 36. We demonstrate instead that T_H17 cells are present in the CNS of *Tbx21*$^{−/−}$ mice in greater numbers and with strong expression of T_H17 signature genes after $MOG_{35–55}$ plus CFA induced EAE. The disparity between the two studies may arise from differences in experimental conditions, such as the use of T-bet-specific siRNA rather than complete genetic deletion *in vivo*. In support of our results, other studies also detected a higher frequency of myelin-specific T_H17 cells in $MOG_{35–55}$ plus CFA immunized *Tbx21*$^{−/−}$ mice than WT mice36. Interestingly, despite a strong T_H17 response, *Tbx21*$^{−/−}$ mice are largely protected from the development of EAE. This observation might be explained by postulating that in the inflammatory milieu of the CNS, T-bet controls expression of a novel set of genes that is important for the pathogenicity, but not for the development of T_H17 cells. In support of this hypothesis, a recent study reported that T-bet is expressed in IL-23-treated T_H17 cells and these T-bet-expressing T_H17 cells were pathogenic during CNS inflammation pointing to the important functional role of T-bet in a subset of T_H17 cells37. Alternatively, the presence of both T_H1 and T_H17 cells might be required for CNS pathology. Finally, T-bet expression in other cell types might be important for driving disease development.

Here, we have focused on T-bet's function in T_H17 lineage commitment of $CD4^+$ T cells. In this study, we have demonstrated that T-bet over-expression in naïve T_H cell precursors or committed T_H17 cells had a negative effect on *Rorc* transcription and consequently on the expression of *Rorc*-target genes. In addition, we observed downregulation of *Il21* gene expression. T-bet suppressed IL-21 in T_H1 cells by interacting with NFATc2, thus preventing NFATc2 from binding to the *Il21* promoter29. Since IL-21 promotes IL-23R expression in T_H17 cells10, T-bet-mediated suppression of *Il21* could also contribute to reduced levels of *Il23r* in T_H17 cells after transduction with a T-bet expressing retrovirus. In contrast to *Rorc*, *Il17a*, *Il17f*, *Il23r*, which were suppressed by T-bet, the expression of *Il22* was augmented by ectopic expression of T-bet. The aryl hydrocarbon receptor (AHR) controls IL-22 production by T_H17 cells, as $CD4^+$ T_H17 cells from AHR-deficient mice fail to produce IL-22 when exposed to AHR ligands38, 39. Thus, it is plausible that T-bet expression has a synergistic effect on AHR-mediated induction of IL-22.

We detected endogenous T-bet binding in the region located 2kb upstream of the first *Rorc* exon in non-skewed and T_H1 cells, but not T_H17 cells. Interestingly, differentiation of T_H cells down the T_H1 pathway resulted in markedly reduced expression of Runx1. Conversely, culturing T_H cells in the presence of T_H17-polarizing cytokines resulted in suppression of T-bet protein expression. This is not surprising since TGF-β has a negative effect on T-bet expression, and IFN-γ, the most potent inducer of T-bet expression, is neutralized under *in*

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vitro T_H17 polarizing conditions. Thus, the lack of T-bet binding to the *Rorc* promoter in committed T_H17 cells could be explained by substantially reduced expression of T-bet in this T_H subset.

We did not detect any significant effects of T-bet over-expression on *Rorc* promoter activity in luciferase assays. Evidence that T-bet acts as a direct transcriptional repressor or can recruit co-repressors to these promoters is currently lacking. However, T-bet can exert a negative regulatory effect on gene expression by blocking the activity of competing transcription factors15,26, 27. Our understanding of the transcriptional regulation of RORγt is still incomplete. It has been reported that the expression of RORγt is substantially reduced in *Irf4*$^{-/-}$ and *Stat3*$^{-/-}$ $CD4^+$ cells31, 40–42. Runx1 induces RORγt expression and Batf is important for the maintenance of RORγt expression in stimulated T c ells30, 32. It is currently unknown whether regulation of *Rorc* expression by Irf4 and Batf is mediated by direct binding of these transcription factors to the *Rorc* locus, but the binding sites for STAT3 in the *Rorc* and *Il17a* loci have been identified33. STAT3 binding to both the *Rorc* and *Il17a* loci was similar between *Tbx21*$^{-/-}$ and WT T_H cells. Hence, we focused on the transcriptional activity of Runx1 since we detected two Runx1 consensus sites in the proximity of the T-bet peak binding site located -2kb from the first exon of *Rorc* as shown by ChIP.

Based on the protein expression data and co-immunoprecipitation experiments, we propose that the functionally important interaction of T-bet and Runx1 occurs in uncommitted T_H cells. We mapped the function of T-bet in interacting with Runx1, repressing Runx1 activity, and suppressing T_H17 differentiation program to Tyr^{304}. Our preliminary data indicate that this tyrosine is not phosphorylated and that the protein is stably expressed in HEK293 cells and primary T cells. In DNA pull-down assays, the T-bet$_{Y304F}$ mutant failed to bind to DNA. Although the Tyr^{304} residue is important for T-bet-DNA interaction, our results indicate that this interaction is not essential for T-bet's ability to block Runx1. WT T-bet was still able to block Runx1 binding to its target site in a RORγt promoter bearing a mutated T-bet half-site. Thus, the T-bet-Runx1 protein-protein interaction is the major mechanism by which T-bet blocks Runx1 activity by sequestering Runx1 away from the RORγt promoter.

In both infectious and autoimmune diseases, T cell-mediated responses are characterized by the presence of cells co-expressing IL-17A and IFN-γ, the so called IFN-γ^+ T_H17 cell. T cell clones from MBP-peptide immunized mice were genetically either T-bet$^+$-RORγt$^-$ or T-bet$^+$-RORγt$^+$ 28. T-bet$^+$-RORγt$^+$ cells were very responsive to exogenous cytokines such as IL-12 and IL-23, which influenced the relative levels of T-bet and RORγt, and shifted cytokine production towards IFN-γ or IL-17A, respectively28. The unstable phenotype of T_H17 cells is not restricted to CNS-specific autoimmunity. IL-17F reporter mice in a transfer model of colitis demonstrated that IFN-γ-producing $CD4^+$ T cells can emerge fromT_H17 committed cells during T cell driven inflammation. This transition of T_H17 cells into IFN-γ-producing cells was STAT4- and T-bet-dependent21. In the context of these recent findings, it is tempting to propose that this T-bet-mediated transition of T_H17 cells into a "T_H1-like" subset is controlled partly by T-bet-mediated interference with Runx1 transcriptional activity. Ectopic expression of T-bet in T_H17 cells results in the suppression of *Rorc*.

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However, in fully differentiated T_H17 cells, which already express RORγt, T-bet could still interfere with RORγt transcriptional activity by sequestering its co-activator, Runx1. Thus, over-expression of Runx1 in T_H17 cells overcame the inhibitory effect of T-bet and completely restored IL-17A production. Although Runx1 fully restored IL-17A production, the expression of *Rorc* was only partially increased. These results indicate that there are additional mechanism(s) by which T-bet inhibits *Rorc* gene expression. One potential mechanism could be via IL-12 signaling inducing repressive epigenetic changes of the *Rorc* locus, which are dependent on T-bet and STAT4[43]. Thus, T-bet re-expression in T_H17 cells turns off RORγt expression through the sequestration of Runx1 and through the introduction of epigenetic changes resulting in expression of T_H1 signature genes and acquisition of the "T_H1- like" phenotype by T_H17 cells.

METHODS

Mice

Tbx21$^{-/-}$, *Tbx21*$^{-/-}$ IL-23R.GFP and IL-23R.GFP mice (all C57BL/6 background) were housed at Harvard School of Public Health and were handled in accordance with guidelines from the Center for Animal Resources and Comparative Medicine (ARCM) at Harvard Medical School. *Tbx21*$^{-/-}$ rtTA$^+$ TrTB$^+$ mice were housed at College of Pharmacy; Ewha Womans University, Soul, S. Korea. *Ifng*$^{-/-}$ mice were purchased from Jackson Laboratories. *Cd4*^{cre} and *Tbx21*$^{F/F}$ mice were provided by Dr. Christopher B. Wilson (The Bill and Melinda Gates Foundation) and Dr. Steven L. Reiner (the University of Pennsylvania), respectively.

Plasmids

Constitutively active STAT3 (pRc/CMV STAT3-C) was obtained from Addgene (submitted by Dr. J.E. Darnell)[44]. pMCsIg-EV, pMCsIg-Runx1 and pMCsIg-Runx1-DN retroviral plasmids were generously provided by Dr. Warren Strober (NIAID, NIH).

CD4$^+$ T helper differentiation *in vitro*

Tbx21$^{-/-}$, *Ifng*$^{-/-}$ and WT CD4$^+$ T cells were stimulated for 48 h with anti-CD3 antibody (2 μg/ml) in the presence of irradiated splenocytes at a ratio of 5:1. CD4$^+$ T cells were cultured under T_H0 conditions (200 U/ml of hIL-2; NCI BRB Preclinical Repository) or differentiated into T_H1 cells with hIL-2 (200 U/ml), mIL-12 (10 ng/ml; Peprotech) and anti-mIL-4 antibody (10 μg/ml; clone 11B11; BioXCell) or into T_H17 cells by the addition of hTGF-β (2 ng/ml; R&D Systems), mIL-6 (20 ng/ml; R&D Systems), anti-mIL-4 (10 μg/ml) and anti-mIFN-γ (10 μg/ml; clone XMG1.2; BioXCell) antibodies for 5 days. T_H17+IL-23 cells were cultured in the presence of hTGF-β, mIL-6, anti-IL-4 and anti-IFN-γ antibodies for the first 48 hours of activation after which T_H17 cells were cultured in the presence of IL-23 (10 ng/ml; R&D Systems).

Isolation and functional analysis of CNS mononuclear cells

EAE was induced in 8 – 10 week old mice as described previously[23]. CNS-infiltrating cells were re-stimulated with PMA+I for 4 h before performing intracellular cytokine staining for IFN-γ (XMG1.2; BD Pharmingen)and IL-17A (TC11-18H10; BD Pharmingen). For ELISA

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and RT-PCR analysis, CNS-derived $CD4^+$ T cells were purified using CD4 negative selection kit (Stem Cell Technologies). Purified $CD4^+$ T cells (>95% purity) were pooled from 4–5 mice/group and cells were stimulated for 4 h with PMA+I. The RT-PCR primer sequences are listed in the Supplementary Table 1.

Retroviral transduction

Naïve ($CD62L^{hi}CD25^{lo}$) $CD4^+$ T cells were transduced with indicated retroviruses 24 h after activation. Transduced cells were cultured for 5 days under T_H17 conditions, and the sorted GFP^+ cells were tested for cytokine production after 4 h of PMA+I stimulation. For transduction of committed T_H17 cells, naïve $CD4^+$ T cells were cultured under T_H17 conditions for 6 days. T_H17 cells were activated on plate-bound anti-CD3/CD28 plates and transduced with retroviruses for 24 h under T_H17 conditions. Transduced cells were cultured for additional 48 h under T_H17 conditions. The sorted GFP^+ were stimulated with PMA+I for 4 h prior to functional analysis.

Doxycycline inducible T-bet transgenic T_H17 *in vitro* cultures

Twenty-four hours after anti-CD3/CD28 activation, T-bet was induced in T_H cells by the addition of 0.5 μg/ml of doxycycline to the T_H17 culture media. Twenty-fours after T-bet induction with doxycycline, lysates were analyzed for the expression of *Tbx21* and *Rorc* by RT-PCR. In addition, cells were immunoblotted with anti-T-bet and anti-RORγt antibody (clone B2D; eBioscience).

ChIP

Naïve cells were sorted from spleens and lymph nodes of *Tbx21*$^{-/-}$ and WT mice and differentiated under T_H0, T_H1 and T_H17 polarizing conditions as described above. Differentiated T_H cells were used in ChIP assay after 6 h PMA+I stimulation. The ChIP assay was performed as described previously45. The primers sets used for real-time PCR to quantitate the ChIP enriched DNA are listed in the Supplementary Table 2.The antibodies used for ChIP were polyclonal rabbit anti-T-bet (clone 9856) and anti-Stat3 (C-20; Santa Cruz) antibodies.

Luciferase assays

The mouse *Rorc(T)* promoterwas cloned into pGL3-basic plasmid upstreamof the firefly luciferase gene (Promega). To study the effects of T-bet and Runx1 on the *Rorc* promoter activity, HEK293 cells were transfected with *Rorc*-luc plasmid, increasing concentrations of Runx1,T-bet or pCDNA3.1- (empty vector control) and pRL-TK (Promega) using Fugene (Roche). To determine the dose-dependent effect of T-bet on Runx1 activity, HEK293 cells were transfected with *Rorc*-luc plasmid, Runx1, increasing concentration of T-bet and pRL-TK. Fireflyand *Renilla* luciferase activities were measured 48 h aftertransfection with the Dual Luciferase System (Promega).

Coimmunoprecipitation

HEK293 cells were transiently transfected with WT T-bet, or T-bet point mutants, Myc-Runx1 or empty vector using Fugene (Roche). 48 h after transfection, cells were lysed in

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TNT lysis buffer (1% triton X-100, 50mM Tris pH 7.5, 200mM NaCl, 1mM DTT, protease and phosphatase inhibitors). For endogenous Runx1 and T-bet coimmunoprecipitations, 3~4×10^8 of differentiated T_H0, T_H1 and T_H17 cells were stimulated for 6 h with PMA+I after which cells were lysed in TNT lysis buffer. Whole cell lysates were immunoprecipitated withthe anti-Runx1 antibody (ab23980; Abcam) or IgG and protein A-sepharose coupled beads at 4°C. Immune complexes were immunoblotted using polyclonal rabbit anti-T-bet antibody. In the reverse co-immunoprecipitation assay, T-bet was immunoprecipitated using polyclonal rabbit anti-T-bet antibody (H-210; Santa Cruz) and separated immune complexes were immunoblotted with polyclonal rabbit anti-Runx1 antibody (ab23980; Abcam). To minimize interference of Runx1 or T-bet detection by the heavy chains of the immunoprecipitating T-bet, Trueblot ULTRA kit (eBioscience) was used for interaction analysis of Runx1 with T-bet in the reverse co-immunoprecipitation assay.

DNA binding assay

DNA binding assay was performed as described previously46. HEK293 cells were transfected with Myc-Runx1 expression plasmid in the absence or presence of increasing concentrations of T-bet expression vector. 150 μg of nuclear protein was incubated with 50 nt biotinylated probe containing wild-type, mutant T-bet binding site or mutant Runx1 binding site [– 2kb of exon 1 encoding *Rorc(T)*] plus streptavidin-agarose (Invitrogen) for 1 hour at 4°C in Binding buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 1 mg/ml BSA, 20 μg/ml poly dI/dC plus protease inhibitors). Streptavidin-beads were washed in Binding buffer, and bound proteins were immunoblotted for over-expressed Myc-Runx1 and T-bet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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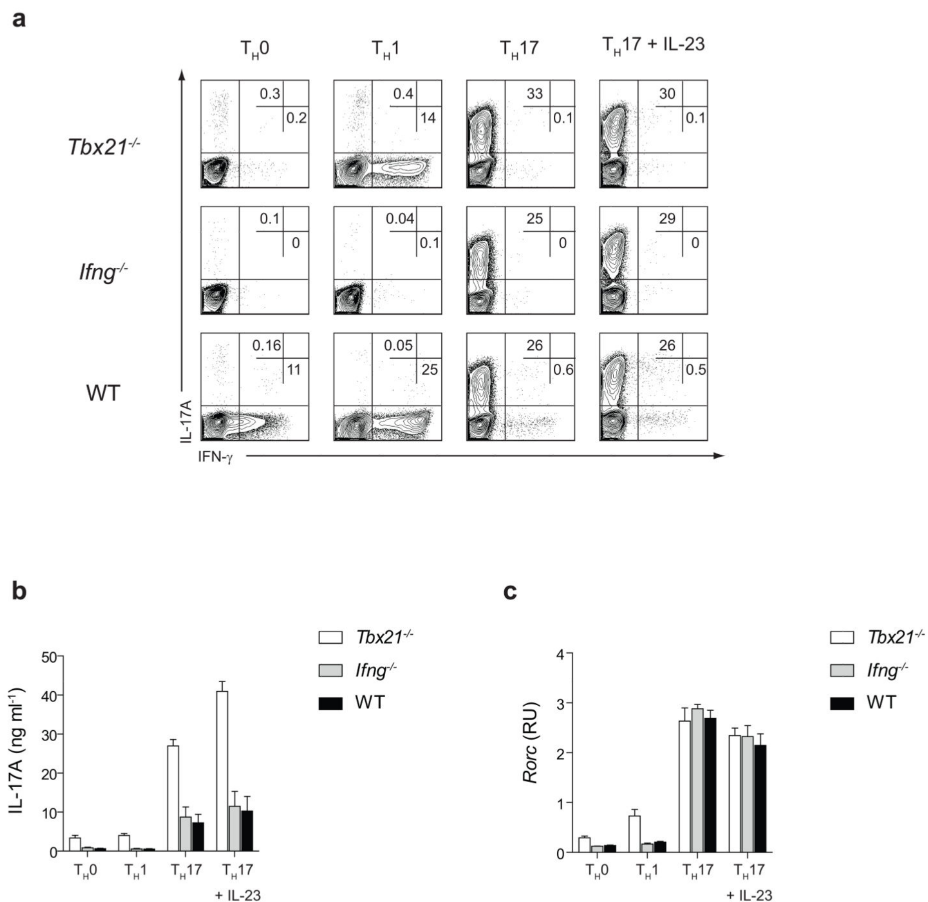

Figure 1.

T-bet deficiency promotes IL-17A production *in vitro* independently of IFN-γ. (**a**) Flow cytometry analyzing the IL-17A and IFN-γ production following 4 h stimulation with phorbol ester + ionomycin (PMA+I). *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ and wild-type CD4$^+$ T cells were cultured in the presence of IL-2 (T_H0 cells), IL-2 + IL-12 + anti-IL-4 antibody (T_H1 cells), TGF-β + IL-6 + anti-IL-4 + anti-IFN-γ antibodies (T_H17 cells) or TGF-β + IL-6 + anti-IL-4 + anti-IFN-γ antibodies followed by IL-23 treatment (T_H17+IL-23 cells). Following five days of *in vitro* differentiation, T_H cells were stimulated with PMA+I for 4 hours prior to intracellular cytokine staining with anti-IL-17A and anti-IFN-γ antibodies. (**b**) The amount of IL-17A in the supernatants of PMA+I stimulated T_H0, T_H1 and T_H17 and T_H17+IL-23 cells was determined by ELISA. The data are representative of four independent experiments (**a–b**). (**c**) *Rorc* mRNA expression in *Tbx21*$^{-/-}$ and WT T_H0, T_H1 and T_H17 and T_H17+IL-23 cells was determined by RT-PCR after 4 h stimulation with PMA+I. The data represent mean ± s.e.m. of two independent experiments.

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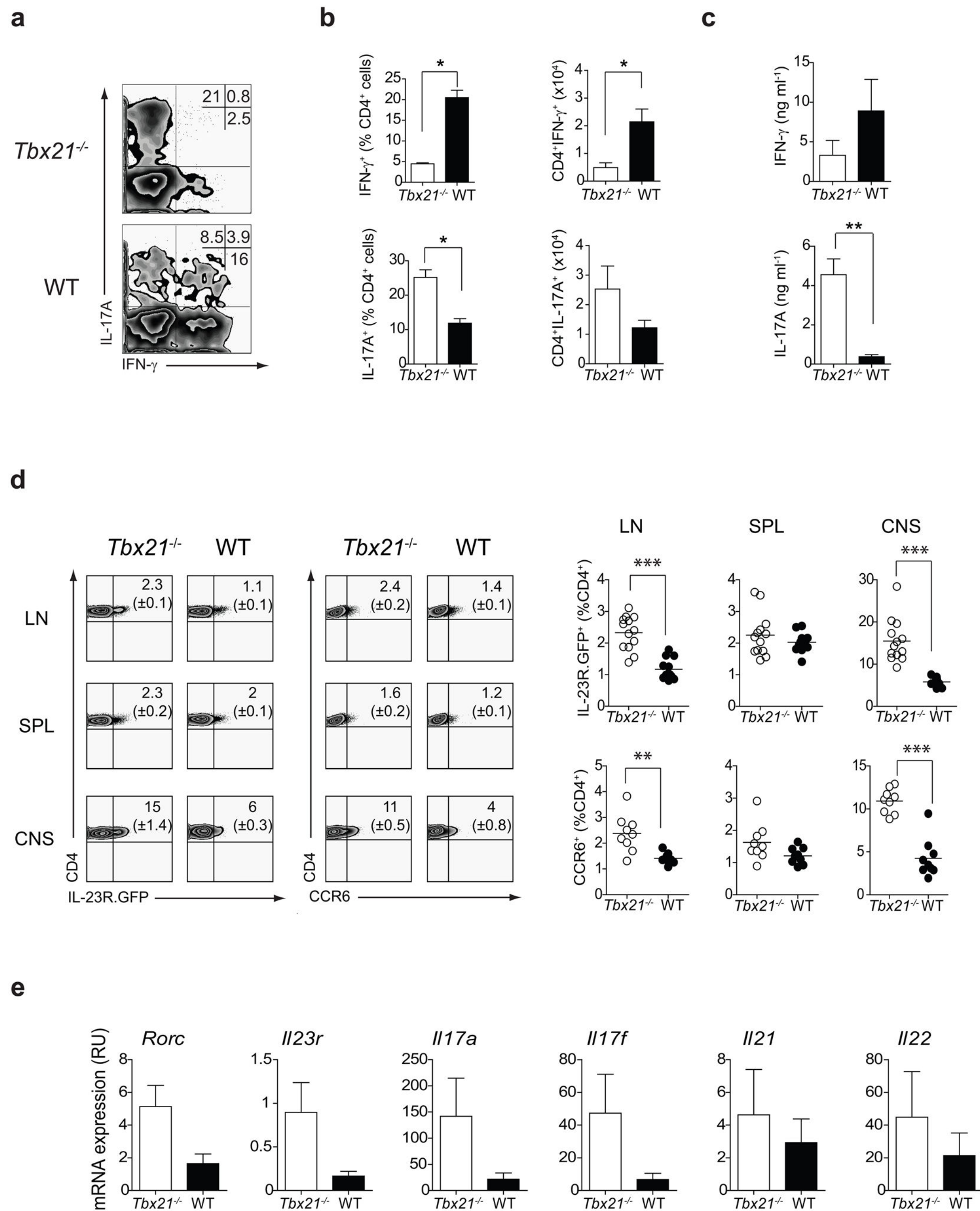

Figure 2.

T-bet deficiency promotes T_H17 responses in the CNS during EAE. (**a**) IL-17A and IFN-γ production by CNS-infiltrating $CD4^+$ lymphocytes on day 17 post-immunization with $MOG_{35–55}$ plus CFA. Numbers illustrate the percent positive cells in the $CD4^+$ gate. (**b**) Quantification of IL-17A- and IFN-γ producing $CD4^+$ cells in the CNS of *Tbx21*$^{-/-}$ and WT mice on day 17 post-immunization. (**c**) IL-17A and IFN-γ produced by purified CNS-infiltrating $CD4^+$ T cells as determined by ELISA. The concentration of cytokines was normalized to 1×10^6 cells/ml. The bars represent mean ± s.e.m. of three independent experiments. (**d**) Cells were prepared from *Tbx21*$^{-/-}$ IL-23R.GFP and WT-IL-23R.GFP

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mice on day 17 after EAE induction and analyzed by flow cytometry. Graphs summarize IL-23R and CCR6 expression (percent of the $CD4^+$ gate) in three independent experiments (n=3–4 mice per group in each experiment). (**e**) Expression of T_H17 signature genes in $CD4^+$ T cells purified from the CNS of *Tbx21*$^{-/-}$ and WT mice on day 17 post-immunization. Cells were pooled from 4 mice per group and were stimulated with PMA+I for 4 h before RNA extraction. The bars represent mean ± s.e.m. of three independent experiments. Statistical analysis was performed using Student-t test; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.0001$.

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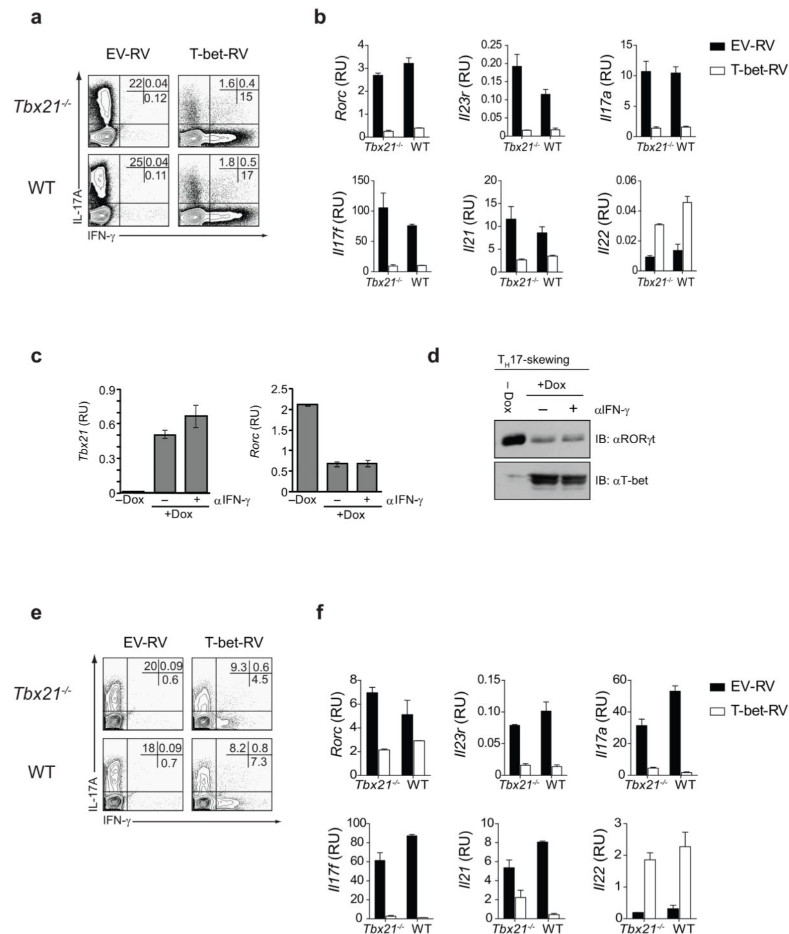

Figure 3.

T-bet expression in naïve T_H precursors and fully differentiated T_H17 cells inhibits T_H17 response. (**a**) Flow cytometry of IL-17A and IFN-γ expression by naïve $CD4^+$ T cells transduced with empty retrovirus or T-bet expressing retrovirus under T_H17 polarizing conditions. Intracellular cytokine staining was performed on sorted GFP^+ cells after a 4 h PMA+I stimulation. (**b**) Real-time PCR analysis of *Rorc*, *Il23r*, *Il17a*, *Il17f*, *Il21* and *Il22* mRNA expression in naïve $CD4^+$ T cells transduced with empty retrovirus or T-bet expressing retrovirus under T_H17 polarizing conditions. (**c**) *Tbx21* and *Rorc* mRNA expression and (**d**) T-bet and RORγt protein expression in untreated and doxycyline treated *Tbx21*$^{-/-}$ $rtTA^+TrTB^+$ transgenic T_H precursor cells activated under T_H17 polarizing conditions in the absence or presence of anti- IFN-γ antibody. (**e**) Flow cytometry of IL-17A and IFN-γ expression by fully differentiated T_H17 cells transduced with empty retrovirus or T-bet expressing retrovirus under T_H17 polarizing conditions. Intracellular cytokine staining was performed following a 4 h PMA+I stimulation. (**f**) Real-time PCR analysis of T_H17 signature genes in fully differentiated T_H17 cells transduced with empty retrovirus or T-bet expressing retrovirus in the presence of T_H17 polarizing cytokines. The data in (**a–f**) are representative of three independent experiments.

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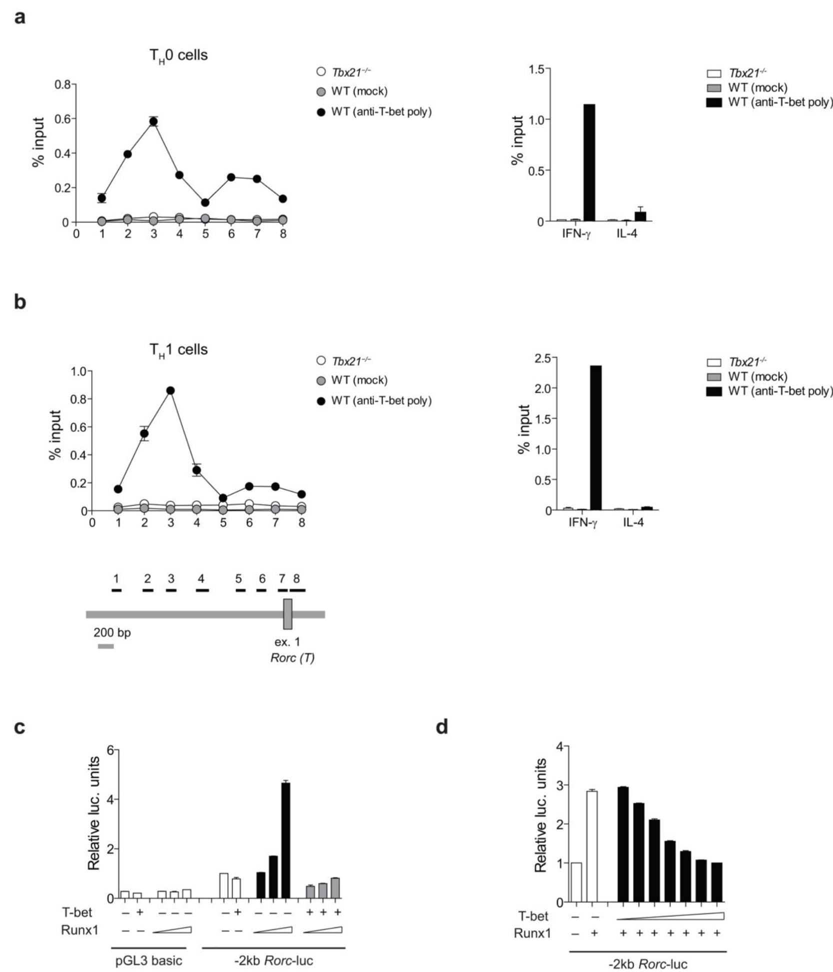

Figure 4.

T-bet blocks Runx1-mediated transactivation of the *Rorc* promoter. (**a–b**) T-bet binding to the *Rorc* promoter in T_H0 and differentiated T_H1 cells after 6 h PMA+I stimulation, as assessed by ChIP. In all experiments, the *Ifng* promoter and *Il4* promoter were included as positive and negative controls, respectively. As specificity controls, ChIP was performed using pre-immune serum and *Tbx21*$^{-/-}$ cells. Fold enrichment was calculated by determining the ratios of the amount of immunoprecipitated DNA tothat of the input sample. The ChIP assays were repeated three times. (**c**) *Rorc* reporter plasmid was constructed from a 2 kb fragment of the mouse *Rorc* promoter. HEK293 cells were transfected with empty pGL3 basic plasmid or *Rorc*-luc reporter construct, pRL-TK, increasing concentration of Runx1 in the absence or presence of T-bet. (**d**) HEK293 cells were transfected with the *Rorc*-luc reporter construct, pRL-TK, Runx1 in the absence or presence of increasing concentration of T-bet. Luciferase activity was measured by Dual Luciferase reporter assay and relative luciferase activity was normalized to pRL-TK for transfection efficiency and empty vector control. Bars represent mean ± s.e.m. of duplicate samples. The graphs are representative of three (**c**) and two (**d**) independent experiments.

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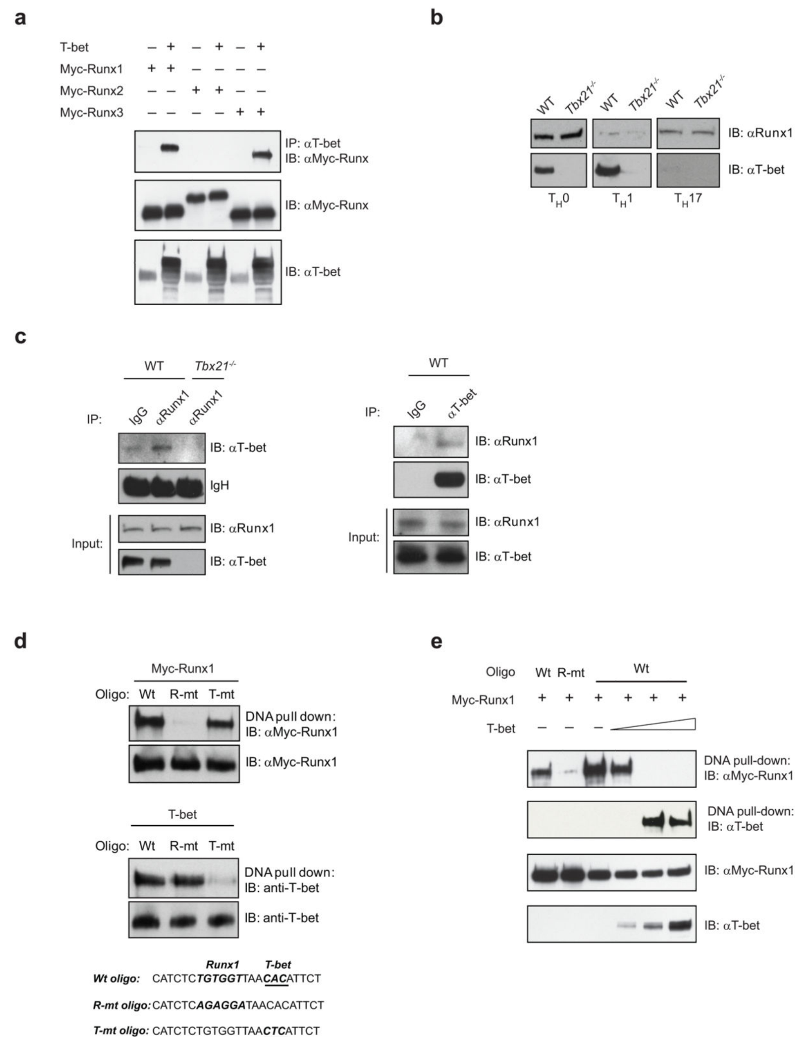

Figure 5.

T-bet interacts with Runx1. (**a**) HEK293 cells were transfected with empty vector, T-bet and Myc-tagged Runx1, Runx2 and Runx3 expression vectors as indicated. Lysates were immunoprecipitated with anti-T-bet polyclonal antibody followed by immunoblotting with anti-Myc antibody. (**b**) Runx1 and T-bet protein expression in *Tbx21*$^{-/-}$ and WT T_H0, T_H1 and T_H17 cells after 6 h stimulation with PMA+I. (**c**) Endogenous interaction between Runx1 and T-bet in non-polarized T_H0 cells. Cell lysates of *Tbx21*$^{-/-}$ and WT T_H0 cells were immunoprecipitated with a control, anti-Runx1 or anti-T-bet antibodies and immunoblotted with anti-T-bet or anti-Runx1 antibody. (**d**) A DNA pull-down assay with a Wt oligo (containing wild-type Runx1 and T-bet binding sites), R-mt oligo (containing mutated Runx1 binding site) or T-mt oligo (containing mutated T-bet binding site) in the presence of Myc-Runx1 or T-bet. (**e**) 293HEK cells were transfected with a Wt or R-mt oligo and Runx1 in the absence or presence of increasing doses of T-bet (0.1, 0.5 and 1 μg). Oligo-bound proteins were immunoblotted for over-expressed Myc-Runx1 and T-bet. The data are representative of one - two independent experiments.

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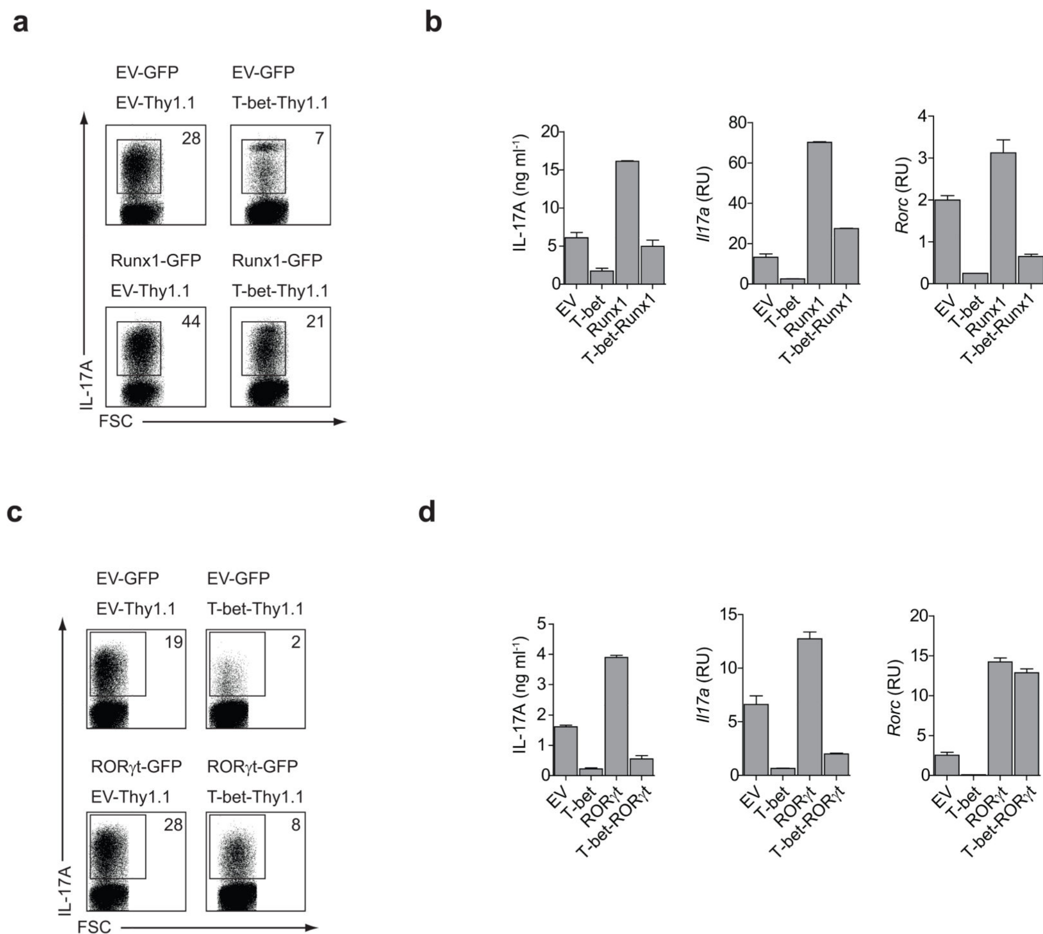

Figure 6.

Runx1 over-expression restores IL-17A production in T-bet expressing T_H17 cells. **(a)** $CD4^+$ T cells were transduced with retroviruses expressing GFP, Thy1.1, Runx1-GFP and/or T-bet-Thy1.1 within 24 hours of activation. Cells were cultured under T_H17 polarizing conditions for 5 days and stimulated with PMA+I for 4 h prior to intracellular cytokine staining for IL-17A. Numbers indicate the percentage of IL-17A producing cells within GFP^+/Thy1.1$^+$ gate. (**b**) $CD4^+$ T cells were transduced with various combinations of retroviruses as described in (**a**). Following 5 days of *in vitro* differentiation under T_H17 polarizing conditions, GFP^+-Thy1.1$^+$ cells were sorted and stimulated with PMA+I for 4 h prior to the analysis of IL-17A production and the *Rorc* gene expression. (**c**) Activated $CD4^+$ T cells were transduced with retroviruses expressing GFP, Thy1.1, RORγt-GFP and/or T-bet-Thy1.1. The transduced cells cultured under T_H17 conditions for 5 days were stimulated with PMA+I for 4 h prior intracellular cytokine staining for IL-17A. The numbers indicate percentage of IL-17A producing cells within GFP^+/Thy1.1$^+$ gate. (**d**) Cells were transduced under T_H17 condition as described in (**c**). IL-17A production was measured by ELISA and RT-PCR and *Rorc* mRNA transcripts were analyzed by RT-PCR. The results in **(a–d)** are representative of two independent experiments.

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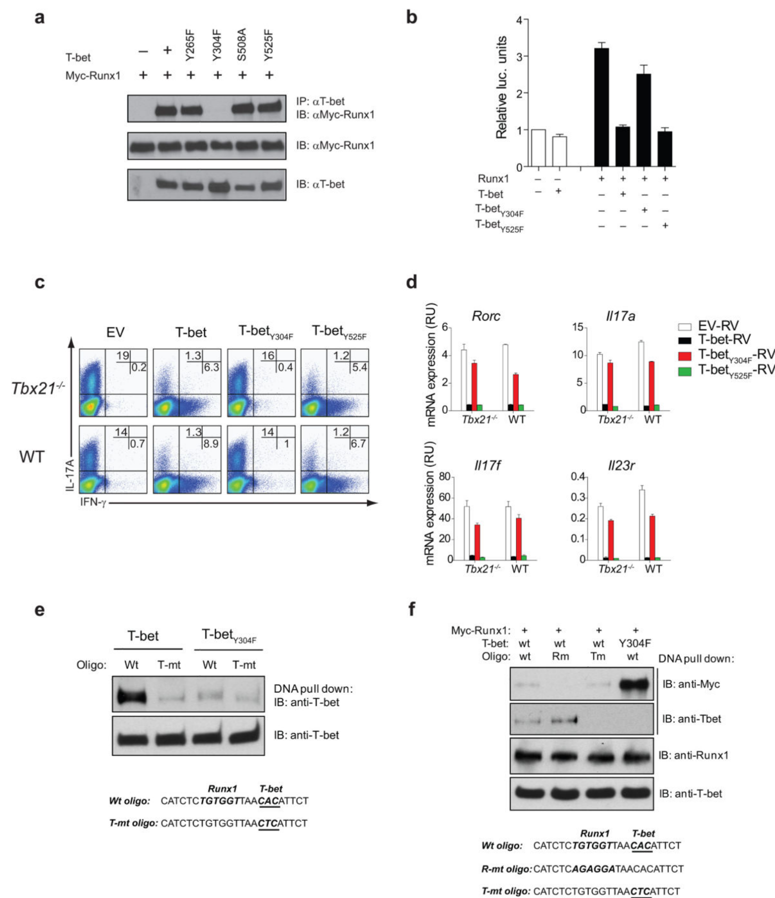

Figure 7.

T-bet Tyr^{304} is essential for T-bet-Runx1 interaction and for inhibiting the T_H17 differentiation program. (**a**) Interaction of Runx1 with WT T-bet or T-bet point mutants in co-immunoprecipitation experiments in HEK293 cells. (**b**) HEK293 cells were transfected with *Rorc*-luc reporter construct, pRL-TK, Runx1 in the absence or presence of WT T-bet or T-bet point mutants as indicated. (**c**) Flow cytometry analyzing IL-17A and IFN-γ expression by naïve CD4$^+$ T cells transduced with empty vector, WT T-bet, or T-bet point mutants under T_H17 polarizing conditions. Numbers in quadrants illustrate the percent positive cells in the CD4$^+$ gate. (**d**) RT-PCR analysis of T_H17 signature genes by CD4$^+$ T cells transduced with empty vector, WT T-bet, or T-bet point mutants under T_H17 conditions. (**e**) 293HEK cells were transfected with WT T-bet or T-bet$_{Y304F}$ mutant in the presence of a wild-type or T-bet mutant oligo. Oligo-bound proteins were immunoblotted for T-bet. (**f**) 293HEK cells were transfected with Myc-tagged Runx1, WT T-bet or T-bet$_{Y304F}$ mutant in the presence of wild-type oligo, Runx1 mutant oligo or T-bet mutant oligo as indicated. Oligo-bound proteins were immunoblotted with anti-Myc and anti-T-bet antibodies. The data are representative of three independent experiments (**ad**) and one experiment (**e–f**).