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Induction and molecular signature of pathogenic T_H17 cells

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Abstract

Interleukin 17 (IL-17)-producing T_H17 cells are often present at the sites of tissue inflammation in autoimmune diseases, which has lead to the conclusion that T_H17 are main drivers of autoimmune tissue injury. However, not all T_H17 cells are pathogenic, in fact T_H17 generated with TGF-β1 and IL-6 produce IL-17 but do not readily induce autoimmune disease without further exposure to IL-23. Here we show that TGF-β3, produced by developing T_H17 cells, is dependent on IL-23, which together with IL-6 induces highly pathogenic T_H17 cells. Moreover, TGF-β3-induced T_H17 cells are functionally and molecularly distinct from TGF-β1-induced T_H17 cells and possess a molecular signature that defines pathogenic effector T_H17 cells in autoimmune disease.

Upon antigenic stimulation, naïve $CD4^+$ T cells activate, expand and differentiate into different effector phenotypes [1]. T helper type 1 (T_H1) cells, induced by the transcription factor *Tbx21* (T-bet) produce interferon-γ (IFN-γ), interleukin 2 (IL-2) and lymphotoxin (LT) and were shown to be crucial for clearing intracellular pathogens [2, 3]. In contrast, T_H2 cells that are generated by the transcription factor GATA-3 produce IL-4, IL-5, IL-13 and were shown to be critical for clearing extracellular pathogens [4, 5]. An exaggerated T_H1 response against self-antigens was implicated in inducing autoimmune diseases [6]. However, the loss of IFN-γ or IFN-γ receptor (IFN-γR) did not induce resistance to developing autoimmune diseases [7, 8], in fact, mice deficient for these proteins were found to be highly susceptible to autoimmununity [7, 8]. Paradoxically, loss of the T_H1-specific transcription factor, T-bet, made these mice resistant to multiple autoimmune diseases including experimental autoimmune encephalomyelitis (EAE), an animal model of the human disease multiple sclerosis (MS) [9]. This raised the issue of what is the role of T-bet in inducing EAE,

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AUTHOR CONTRIBUTIONS

Y.L., A.A. designed the study, performed the experiments, analyzed the data, and wrote the manuscript. A.P., S.X., S.K., M.K. C.W did *in vitro* experiments. N.Y., F.J.Q., A.R. did the bioinformatics analysis of gene expression data. R.A.S did the immunohistochemical analysis. D.H provided discussion. V.K.K. and A.A. supervised the study and edited the manuscript.

because the production of IFN-γ is not required for conferring encephalitogenicity to effector T_H1 cells.

T_H17 cells, which have been characterized as an additional effector T cell subset that produce IL-17A, IL-17F, IL-21 and IL-22, have been suggested to be the critical driver of autoimmune tissue inflammation [10–14]. T_H17 cells were observed to be expressed at the sites of tissue inflammation and have been associated with the induction of many human autoimmune diseases including psoriasis, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), type 1 diabetes and MS [14]. T_H17 cells are differentiated by a combination of TGF-β1, IL-6 and IL-1 cytokines, which induces RORγt, a transcription factor required for their generation [10, 12, 13, 15]. Whereas TGF-β1 plus IL-6 can induce T_H17 cells, exposure to another cytokine, IL-23, was shown to be crucial for their stabilization and for their ability to induce autoimmune tissue inflammation in EAE[16–18]. IL-23R polymorphism has been genetically linked to many human autoimmune diseases including psoriasis, IBD and ankylosis spondylitis [19, 20]. Exposure to IL-23 was shown to reduce the levels of the anti-inflammatory cytokine IL-10 in developing T_H17 cells, thus making these cells pathogenic [21]. However, this also raised the question whether there are cytokines or effector molecules dependent on IL-23 that make them pathogenic to induce inflammation in autoimmune disease.

Although T_H17 cells were thought to be pathogenic[11, 22], accumulating data indicates the existence of non-pathogenic IL-17 producing T_H17 cells [23, 24]. It remains unclear whether there is a differential requirement for their induction and what makes a T_H17 cell pathogenic or nonpathogenic. Recent studies have shown that GM-CSF (CSF-2), which is produced by T_H17 and transactivated by RORγt, is required for conferring pathogenicity to T_H17 cells [25, 26]. Indeed, GM-CSF-deficient mice are highly resistant to the induction of EAE [25, 27]. However, it remains unclear why T-bet$^{-/-}$ mice are resistant to the induction of EAE and other autoimmune diseases, suggesting that T-bet must have additional roles in the induction of pathogenic T_H17 cells in EAE [24]. T-bet is expressed in T_H17 cells[24], but whether it plays a role in inducing pathogenic function of T_H17 cells has not been addressed.

In this study we have identified an endogenous cytokine, TGF-β3, which is specifically produced by developing T_H17 cells in an IL-23 dependent manner that is important for driving pathogenic T_H17 phenotype. Whereas TGF-β1 plus IL-6 differentiate naïve T cells into T_H17 cells, these T cells are not pathogenic unless they are further exposed to IL-23. We now show that IL-23 is critical for enhancing expression and/or maintaining the endogenous levels of TGF-β3 in developing T_H17 cells. In fact, differentiation of T_H17 cells in the presence of TGF-β3 and IL-6 makes T_H17 cells pathogenic without any need for further exposure to IL-23. Using these subsets of pathogenic and non-pathogenic T_H17 cells, we have identified a molecular signature associated with pathogenic T_H17 cells.

Results

TGF-β3 induction in T_H17 cells

TGF-β1 and IL-6 are required for differentiation of T_H17 cells[10, 12, 13]. However, T_H17 cells induced by TGF-β1 plus IL-6 are not pathogenic, unless they are further exposed to IL-23 for a prolonged period of time [21, 22]. In the EAE model, only adoptive transfer of myelin oligodendrocyte peptide (amino acid 35–55 ($MOG_{35–55}$)) specific TCR transgenic $CD4^+$ T cells (2D2) that were differentiated with TGF-β1-IL-6-IL-23 results in the induction of severe disease while T cells differentiated with TGF-β1-IL-6 develop mild to no disease, with majority of the recipient mice recovering rapidly within 24 days after the onset of clinical disease (Fig. 1a and Table 1). To investigate the role of IL-23 in the induction of pathogenic T_H17 cells, we undertook detailed temporal microarray analysis that revealed a

strong induction of TGF-β3 in developing T_H17 cells exposed to TGF-β1-IL-6-IL-23 (Supplementary Fig. 1a), which was dependent on IL-23R signaling (Supplementary Fig. 1b). Quantitative RT-PCR confirmed the induction of TGF-β3 in T_H17 cells (Fig. 1b). Naïve T cells activated in the presence of TGF-β1-IL-6 or TGF-β1-IL-6-IL-23 both expressed TGF-β3. However, IL-23R-deficient $CD4^+$ T cells under TGF-β1-IL-6-IL-23 condition failed to enhance or maintain TGF-β3 expression (Fig. 1c). IL-23 was not required for the initial induction of TGF-β3, which was induced by IL-6 (Supplementary Fig. 1c), but the presence of IL-23R was required for maintaining TGF-β3 expression (Fig. 1c).

It remains unknown if cells of the hematopoietic origin can produce TGF-β3. To investigate TGF-β3 protein expression, we generated TGF-β3-YFP fate reporter mice (TGF-$\beta3^{Cre}R26R^{eYFP}$) by crossing mice that express the Cre recombinase in the TGF-β3 locus (TGF-$\beta3^{Cre}$) [28] with reporter mice expressing eYFP under the Rosa26 promoter. Analysis of lymphoid tissue from the TGF-$\beta3^{Cre}R26R^{eYFP}$ mice revealed that TGF-β3 was produced in $CD4^+$, $CD8^+$, γσ T and B cells (Fig. 1d), but not by cells of the myeloid lineage ($CD11b^+$ and/or $CD11c^+$ cells) (data not shown). This observation suggested that TGF-β3 is endogenously produced by the hematopoietic cells. To address whether *in vivo* differentiating T_H17 cells induces TGF-β3 expression and determine whether TGF-β3 expression is enhanced in response to IL-23, we immunized TGF-$\beta3^{Cre}R26R^{eYFP}$ mice with $MOG_{35–55}$ emulsified in complete Freund's adjuvant (CFA) and reactivated T cells in the presence of IL-23 in a recall assay. A small percentage of IL-17 expressing and non-expressing T cells produced TGF-β3 upon activation, but exposure to IL-23 largely restricted TGF-β3 expression to IL-17-producing T_H17 cells. This was true in both $CD4^+$ (Fig. 1e) and non-$CD4^+$ T cell (Supplementary Fig. 1c) subsets. We also addressed whether TGF-β3 is co-produced by IFN-γ producing T_H1 cells, which are also induced by immunization with $MOG_{35–55}$+CFA. IFN-γ producing T cells did not co-express TGF-β3 (Fig. 1e). This was true for non-$CD4^+$ T cells as well (Supplementary Fig. 1d), suggesting that TGF-β3 may be mostly expressed by IL-17 producing cells. In addition, deletion of TGF-$\beta3^+$ cells *in vivo* in TGF-$\beta3^{Cre}R26R^{DTR}$ mice (TGF-$\beta3^{Cre}$ mice crossed to mice expressing the diphtheria toxin receptor (DTR) under the Rosa26 promoter) that were immunized with $MOG_{35–55}$+CFA resulted in selective ablation of IL-17^+ cells, while IFNγ^+ T cell numbers remained unchanged upon diphtheria toxin (DT) treatment (Fig. 1f). $CD4^+$ T cells derived from TGF-$\beta3^{fl/fl}$ mice in which TGF-β3 was deleted through the retroviral expression of a GFP-Cre, but not control retrovirus treated T cells, showed severely compromised IL-17 production in T_H17 differentiation conditions (Fig. 1g). These findings reveal that T_H17 cells produce TGF-β3 endogenously and that exposure to IL-23 largely restricted the expression of TGF-β3 to IL-17-producing cells.

TGF-β3-induced T_H17 cells are highly pathogenic

To determine whether TGF-β3 can induce T_H17 cells, we polarized naive $CD4^+$ T cells into T_H17 cells with either TGF-β1-IL-6 or TGF-β3-IL-6 and compared the expression of IL-17 and other T_H17 associated molecules. TGF-β3-IL-6-induced T_H17 cells expressed comparable amount of IL-17 as TGF-β-IL-6-induced T_H17 cells (Fig. 2a,b). Similar to TGF-β1-induced T_H17 cells, TGF-β3-induced T_H17 cells expressed *Rorc, Il17a, Il17f*, but no *Ifnγ* mRNA (Fig. 2c). However, TGF-β3-induced T_H17 cells had significantly higher expression of *Il23r* and *Il22* when compared to TGF-β1-induced T_H17 cells (Fig. 2d). The higher expression of IL-23R protein in TGF-β3-induced T_H17 cells was confirmed in IL-23R-GFP reporter mice[16] (Fig. 2e).

Because IL-23 induces T_H17 cells to become pathogenic and IL-23 can enhance and maintain expression of TGF-β3, we investigated the pathogenicity of TGF-β3-IL-6-induced T_H17 cells. T_H17 cells generated from naive 2D2 TCR transgenic (Tg) T cells with either TGF-β1-IL-6 or TGF-β3-IL-6 were transferred into naive recipients and development of

EAE was monitored. In contrast to TGF-β1-IL-6-induced T_H17 cells, which transferred a very mild disease (Fig. 2f and Table 1), TGF-β3-IL-6-induced T_H17 cells transferred EAE with a high disease incidence, severity and mortality (Fig. 2f and Table 1). Histo-pathological quantification of CNS lesions showed significantly more parenchymal and meningeal inflammatory lesions in the TGF-β3-IL-6 groups in comparison to the recipients of TGF-β1-IL-6 T_H17 cells (Fig. 2g), suggesting that TGF-β3-induced T_H17 cells are highly pathogenic.

We next addressed how TGF-β1 and TGF-β3 can induce such a difference in the pathogenicity of T_H17 cells, considering they both have been suggested to signal through a common TGF-βRII subunit[29, 30]. Mice expressing a dominant negative (DN) for of TGF-βRII (here called TGF-βRII DN mice) were previously shown to be to have reduced generation of T_H17 cells and to be protected from EAE [13]. We tested whether TGF-β3, similar to TGF-β1, requires TGF-βRII for the development of T_H17 cells. To address this, we generated TGF-βRII DN × IL-17A-eGFP reporter mice and $CD4^+$ T cells from these mice were differentiated with either TGF-β1-IL-6 or TGF-β3-IL-6 conditions. Both TGF-β1 and TGF-β3 were unable to induce the differentiation of T_H17 cells in TGF-βRII DN T cells (Supplementary Fig. 2a), suggesting that both TGF-β1 and TGF-β3 signal through TGF-βRII for T_H17 differentiation (Fig. 2d–f).

To test the possibility that TGF-βRII transmits different downstream signals upon ligation with TGF-β1 or TGF-β3, we performed a comprehensive TGF-β signaling PCR array analysis for T_H17 cells generated with either TGF-β1 or TGF-β3. This array allowed us to determine the expression of approximately 90 known signaling molecules downstream of TGF-βRII and showed that TGF-β1 and TGF-β3 induced different expression profiles of target genes downstream of TGF-βRII engagement (Supplementary Fig. 2b). In particular, TGF-β3-IL-6-differentiated T_H17 cells induced higher expression of Smad1 and Smad5 and lower expression of Smad3 when compared to TGF-β1/IL-6-induced T_H17 cells (Fig. 2h). Consistent with the PCR array data, TGF-β3 induced less Smad2 and Smad3 phosphorylation than TGF-β1, while phosphorylation of Smad 1 and Smad5 was greatly enhanced (Fig. 2i, Supplementary Fig. 2c). Taken together, these data indicate that TGF-β3-IL-6-induced T_H17 cells were functionally distinct from TGF-β1-IL-6-induced T_H17 cells, and that the difference in the functionality of T_H17 cells was supported by the differential signaling induced by TGF-β1 and TGF-β3 through TGF-βRII.

TGF-β3-IL-6 and IL-1β-IL-6-IL-23 T_H17 cells are comparable

TGF-β-independent T_H17 cells, which are induced by a combination of IL-1β/IL-6/IL-23 and are highly pathogenic compared to TGF-β1/IL-6-derived T_H17 cells, have been recently described [31]. We therefore compared the ability of TGF-β3-IL-6 and IL-1β-IL-6-IL-23 pathogenic T_H17 cell populations to transfer EAE. T_H17 cells differentiated with either TGF-β3-IL-6 or IL-1β-IL-6-IL-23 showed comparable IL-17 protein (Fig. 3a) and IL-23R mRNA (Fig. 3b) expression. Because the IL-23-IL-23R axis is essential for the development of pathogenic T_H17 cells [16–18], we tested whether the high expression of IL-23R contributed to the pathogenicity of TGF-β3-IL-6-or IL-1β-IL-6-IL-23-differentiated T_H17 cells. Adoptively transferred 2D2 T cells differentiated into T_H17 cells with TGF-β3-IL-6 or IL-1β-IL-6-IL-23 into wild-type recipients transferred EAE with similar severity and incidence. Transfer of IL-23R-deficient 2D2 T cells failed to induce EAE in both TGF-β3-IL-6 and IL-1β-IL-6-IL-23 differentiating condition (Fig. 3c and Table 1). This indicates that IL-23-IL-23R pathway is absolutely critical for the development of pathogenic T_H17 cells induced by either TGF-β3-IL-6 or IL-1β-IL-6-IL-23.

Further analysis of CNS-infiltrating T cells showed that TGF-β3-IL-6-induced T_H17 cells maintained higher IL-17 expression (~25%) and did not produce as much IFN-γ as IL-1β-

IL-6-IL-23 induced T_H17 cells (~15%) (data not shown). To address if TGF-β3 is involved in EAE induction following active immunization, we immunized mice with $MOG_{35–55}$+CFA, which is known to induce EAE by generating $MOG_{35–55}$-specific T_H1 and T_H17 cells. $CD4^+$ T cells infiltrating the CNS in $MOG_{35–55}$+CFA immunized mice showed a significantly higher expression of TGF-β3 in mice that developed the disease (score 2–3) compared to mice with no clinical signs of disease (score 0), even though there was infiltration of $CD4^+$ T cells in the CNS of these mice (Fig. 3d). Local blockade of TGF-βs by adding neutralizing anti-TGF-β1,2,3 antibody in the immunizing emulsion was shown to prevent the development of T_H17 cells and EAE [32], suggesting an *in vivo* role of TGF-β in the development of T_H17 cells and the disease. However, the study did not reveal which TGF-β was required for the development T_H17 cell, since anti-TGF-β1,2,3 neutralizes all three forms of TGF-β proteins. We therefore tested whether neutralizing TGF-β3 locally affects the development of EAE. The inclusion of anti-TGF-β3 antibody into the $MOG_{35–55}$+CFA emulsion at the time of induction significantly inhibited the development of EAE (Fig. 3e and Table 1), suggesting that endogenous TGF-β3 contributes to the induction of EAE. Taken together, these data indicate that the pathogenic T_H17 cells induced by TGF-β3 have a pathogenic and functional phenotype comparable to that of recently described IL-1β/IL-6/IL-23 T_H17 cells.

The transcriptional signature of pathogenic T_H17 cells

To characterize the molecular state associated with the different functionally of TGF-β1 and TGF-β3-induced T_H17 cells, we measured the mRNA profiles of *in vitro* differentiated T_H17 cells using whole genome microarrays. We compared the gene expression profiles of naïve $CD4^+$ T cells differentiated into T_H17 cell under six different polarizing conditions: IL-β1-IL-6, IL-β1-IL-6-IL-23, TGF-β1-IL-6, TGF-β1-IL-6-IL-23, TGF-β3-IL-6 and TGF-β3-IL-6-IL-23. Activated T_H0 cells with the same TCR specificity that produce IL-4 and IFN-γ were used as controls. We found 434 genes that were differentially expressed, up-regulated or down-regulated at least 2 fold in T_H17 cells in comparison to control T_H0 cells (Fig. 4a). Across these 434 genes, the transcriptional profile of T_H17 cells differentiated with IL-1β-IL-6 or IL-1β-IL-6-IL-23 was similar to that of T_H17 cells differentiated with TGF-β3-IL-6 or TGF-β3-IL-6-IL-23, whereas the T_H17 cells differentiated with TGF-β1-IL-6 or TGF-β1-IL-6-IL-23 had a distinct transcriptional profile (Fig. 4a). Direct comparison of the transcriptional profiles of T_H17 cells differentiated with TGF-β1-IL-6 or TGF-β3-IL-6, as these two different differentiation conditions led to significant differences in the pathogenicity of T_H17 cells, revealed 233 genes that were differentially expressed (Supplementary Table 1). Based on their biological function, we selected a representative subset of 23 genes (Fig. 4b) and validated their differential expression by qPCR (Fig. 4c).

TGF-β3-IL-6-induced T_H17 cells were characterized by the higher expression of transcripts that could be broadly divided into 3 different modules: cytokines/chemokines (*Cxcl3, Ccl4, Ccl5, Ccl3, Csf2, Il3, Il22* and *Casp1*, which is involved in the generation of mature IL-1β), transcription factors (*Tbx21* and *Stat4*), and effector molecules (*Gzmb, Lag3* and *Lglas3* (encoding Galectin-3)). In addition, TGF-β3-IL-6-induced T_H17 cells had downregulated, while TGF-β1-IL-6-induced T_H17 cells had upregulated, molecules that are broadly associated with immune regulation (regulatory module), including *Il10* and molecules involved in the regulation of IL-10 production (*Ahr* and *Maf*), *Il9, Il1rn* and the transcription factor *Ikzf3* (Aiolos) (Fig. 4c).

Based on this molecular signature, we compared the profiles of each of these T_H17 populations (Fig. 4d). Principal component analysis (PCA) revealed 3 groups: group I includes TGF-β1-IL-6-induced T_H17 cells, regardless of whether they were exposed to IL-23. These cells show a mild or no pathogenic phenotype *in vivo* (Fig. 1a) and express little or no IL-23R (Fig. 2d,e). Group II includes T_H17 cells induced with TGF-β3-IL-6 and

IL-1β-IL-6. These cells are highly pathogenic *in vivo* (Figs. 2f) and express high levels of IL-23R (Fig. 2d,e). Group III includes T_H17 cells induced with TGF-β3/IL-6/IL-23 and IL-1β/IL-6/IL-23, which also expressed high levels of IL-23R (Fig. 3b). Similar results were obtained when PCA was performed based on the expression of all 233 genes that are differentially expressed between TGF-β1-IL-6-induced and TGF-β3-IL-6-induced T_H17 cells (Fig. 4e). These results show that TGF-β3-IL-6-induced T_H17 cells are closely related to IL-1β-IL-6-induced T_H17 cells, and differ from TGF-β1-IL-6-induced by T_H17 cells. The 23-gene transcriptional signature of T_H17 cells defined here provides a means to identify key molecules involved in pathogenicity of T_H17 cells.

TGF-β3 overcomes lack of pathogenicity in Tbx21$^{-/-}$ T_H17 cells

Our microarray analysis showed that TGF-β3-IL-6-induced T_H17 cell expressed relatively higher level of the transcription factor *Tbx21* (T-bet) (Fig. 4b). Because exposure of T_H17 cells to IL-23 was shown to induce the expression of T-bet [33], we generated TGF-β1-IL-6-IL-23-induced T_H17 cells from 2D2 *Tbx21*$^{-/-}$ and wild type T cells. *Tbx21*$^{-/-}$ T_H17 cells induced higher expression of IL-17A as compared to wild-type T_H17 cells (Fig. 5a), however 2D2 *Tbx21*$^{-/-}$$T_H17$ failed to transfer EAE, whereas wild-type 2D2 T_H17 cells induced disease with high incidence and severity (Fig. 5b). This data indicates that T-bet plays a key role in inducing pathogenic functions of T_H17 cells, and this is independent on IL-17 production. Because TGF-β3 is required for the induction of pathogenic signatures in T_H17 cells, we tested whether the inability of T-bet-deficient T cells to induce disease was due to a decrease in TGF-β3 expression. In fact, TGF-β3 expression was decreased in T-bet-deficient T_H17 cells (Fig. 5c). Conversely, retroviral over-expression of T-bet enhanced the expression of TGF-β3 (Fig. 5d). We next tested whether compensating for TGF-β3 in T-bet-deficient T_H17 cells could induce EAE in an adoptive transfer system. Polarization of 2D2 *Tbx21*$^{-/-}$ CD4$^+$ T cells in the presence of TGF-β3-IL-6, but not TGF-β1-IL-6-IL-23 or TGF-β1-IL-6, overcame the requirement of T-bet and were encephalitogenic (Fig. 5e and Table 1). Histological analysis showed that adoptively transferred TGF-β3-IL-6-induced 2D2 *Tbx21*$^{-/-}$ cells produced CNS lesions in the meninges and the parenchyma, while TGF-β1-IL-6-induced 2D2 *Tbx21*$^{-/-}$ cells produced no detectable lesions after transfer (Fig. 5f). T-bet-deficient T cells produced relatively higher amounts of IL-17 when differentiated with TGF-β1-IL-6-IL-23 or TGF-β3-IL-6 (Fig. 5g). Previous reports have suggested a requirement for IFN-γ-producing cells to breach the blood brain barrier in order to allow entry of IL-17 producing cells into the CNS [34]. Analysis of peripheral or CNS isolated CD4$^+$ T cells at the peak of disease revealed that TGF-β3-IL-6-induced 2D2 cells, but not 2D2 *Tbx21*$^{-/-}$ cells, expressed IFN-γ. This suggests that independent of IFN-γ production, TGF-β3-IL-6-induced 2D2 *Tbx21*$^{-/-}$ T_H17 cells were pathogenic *in vivo* (Supplementary Fig. 3). These observations suggest that T-bet is part of the pathogenic T_H17 signature and may regulate the endogenous expression of TGF-β3. Loss of pathogenicity in T-bet–deficient T_H17 cells can be overcome by exogenous TGF-β3 and IL-6. It remains unclear whether T-bet is directly or indirectly required for the induction and/or maintenance of TGF-β3.

Discussion

Although T_H17 cells have been associated with induction of autoimmunity, emerging data suggests that not all T_H17 cell are pathogenic and exposure to IL-23 is crucial for their ability to induce autoimmunity [16–18, 22]. In this paper we show that TGF-β3 endogenously produced by developing T_H17 cells plays a critical role in inducing pathogenic T_H17 cells and T-bet a transcription factor normally associated with Th1 development is critical for the generation of pathogenic T_H17 cells.

While IFNγ$^{-/-}$ and IFNγR$^{-/-}$ mice are highly susceptible to developing EAE [7, 8] mice lacking T-bet are protected from almost all autoimmune disease [9, 24]. Our data begins to

address this paradox by showing that T-bet is part of the pathogenic signature expressed by a subset of T_H17 cells. Although it remains unclear how T-bet confers encephalitogenic potential of T_H17 cells, we show that in the absence of T-bet, T_H17 cells are not able to induce endogenous TGF-β3. Whether T-bet is directly transactivating TGF-β3 or is indirectly modulating other receptors/factors in T_H17 cells is not clear at this stage. IL-23 signaling was shown to induce T-bet expression in T_H17 cells and T-bet has been suggested to induce IL-23R expression[35], suggesting a feed-forward loop between IL-23R-signaling and T-bet expression. Because T-bet expression in T_H17 cells, it has been postulated that T_H17 cells might produce IFN-γ and therefore, IL-17-IFN-γ double producers are the main pathogenic T cells that induce autoimmune disease. Our data suggests that the requirement for T-bet expression in pathogenic T_H17 cells is independent of IFN-γ production, because IFN-γ-deficient T_H17 cells induced with TGF-β3/IL-6 were still capable of inducing EAE (data not shown). One possible reason why we observe T-bet expression in TGF-β3/IL-6 induced T_H17 cells is that, unlike TGF-β1, which suppresses T-bet [36] and IL-23R[37], TGF-β3 promotes expression of these molecules, promoting the stability and pathogenicity of T_H17 cells.

In addition to T-bet, several other factors were associated with the pathogenic signature of T_H17, including GM-CSF, IL-23R and IL-7R. GM-CSF (*csf2*), which is transactivated by RORγt, was recently described as the key cytokine involved in the pathogenicity T_H17 cells [25, 26] and our transcriptional analysis independently identified GM-CSF as a component of pathogenic T_H17 cells. Indeed, GM-CSF-deficient mice are highly resistant to EAE and GM-CSF-deficient T_H17 cells are incapable of transferring EAE [25, 26]. This is consistent with our data that pathogenic T_H17 cells induced by TGF-β3/IL-6 express GM-CSF. However, it remains to be determined whether TGF-β3/IL-6-induced T_H17 cells require GM-CSF for encephalitogencity. Similarly, IL-23R is the key component of the pathogenic T_H17 signature. IL-23R-deficient mice are resistant to EAE and IL-23R-deficient T_H17 cells are unable to transfer EAE [16]. The IL-7-IL-7R pathway has also been implicated in development of T_H17 cells and genetic data link IL-7R to susceptibility to multiple sclerosis in patients [16, 38–41]. However, the role of IL-7-IL-7R axis in the induction of pathogenic potential of TGF-β3/IL-6-induced T_H17 cells remains to be determined. Whether IL-7 simply expands pathogenic T_H17 cells or induces key molecules required for pathogenicity of T_H17 cells has not been addressed as of yet. A systematic analysis of each of the molecules belonging to the pathogenic signature of T_H17 cells will allow the identification of key nodes in the development of pathogenic T_H17 cells.

Acquisition of a pathogenic phenotype by T_H17 cells is not only the consequence of the upregulation of molecules that mediate pathology, but also of the downregulation of a number of other genes. We found that genes generally associated with immune-regulation, such as IL-10, Ahr and c-Maf, were significantly downregulated in pathogenic T_H17 cells. Ahr and c-Maf are induced by TGF-β1/IL-6 conditions[42–44], and they work together to transactivate IL-10 production [45]. Failure to express these genes in TGF-β3/IL-6-induced T_H17 cells might explain why these cells become highly pathogenic. Conversely, it will be interesting to determine whether T_H17 cells induced by TGF-β1/IL-6 in the absence of Ahr, cMaf or IL-10 expression can attain pathogenic functions. IL-10 has been previously associated with non-pathogenic T_H17 cells and exposure to IL-23 was shown to downregulate IL-10 production and increase their encephalitogenicity [21, 22]. In addition to IL-10, nonpathogenic T_H17 cells produced IL-1R antagonist (IL-1RN) and sIL-6R, both of which would presumably inhibit IL-1 and IL-6 signaling and thus further suppress differentiation of pathogenic T_H17 cells. However, the relationship between IL-1R antagonism and IL-10 production by T_H17 cells remains unclear at this point. It remains to be determined whether different subsets of T_H17 cells, some that produces IL-17 and IL-10 and other that produces IL-17 and GM-CSF and express T-bet have evolved to clear

different types of pathogens. Indeed, observations in human T_H17 cells suggests that IL-17$^+$IL-10$^+$ T_H17 cells are specific for *S. aureus* infection whereas IL-17$^+$IFN-γ$^+$ T_H17 cells are specific for *C.albicans* infection [46]. Similarly, our study suggests a pathogenic versus non-pathogenic subtypes of T_H17 cells, and their development may be contingent on the type of TGF-β present during the initial differentiation stage.

Existing data suggest that both pathogenic and non-pathogenic T_H17 cells exist in the repertoire. While the initial differentiation of IL-17-producing cells is not dependent on exposure to IL-23, it is now clear that IL-23 is required for attaining full pathogenic potential in T_H17 cells by maintenance and stabilization of the T_H17 phenotype, suppression of IL-10 production, promotion of GM-CSF production and other effector molecules and maintenance of TGF-β3 expression. The fact that enhancement and maintenance of TGF-β3 are dependent on IL-23 exposure is especially interesting given that TGF-β1/IL-6-differentiated T_H17 cells cannot transfer disease, yet when exposed to IL-23 they acquire pathogenic potential and induce autoimmune disease. Thus, it is likely that the IL-23-dependent TGF-β3 production in TGF-β1/IL-6-differentiated T_H17 cells is required for the induction of full pathogenic phenotype in T_H17 cells to induce autoimmunity.

In summary, we report a critical role for TGF-β3 cell autonomously produced by T_H17 cells in inducing pathogenic T_H17 cells. Whereas both TGF-β1 and TGF-β3 can induce T_H17 cells, TGF-β3 induced T_H17 cells differ from TGF-β1 induced T_H17 cells in multiple ways. TGF-β3 induced T_H17 cells are not only pathogenic in inducing EAE but also very potent in inducing colitis in the adoptive transfer model (data not shown), supporting a broader role of TGF-β3 in inducing highly pathogenic and proinflammatory T_H17 cells. Since TGF-β3 induces highly pathogenic T_H17 in both EAE and colitis, TGF-β3 may provide a useful target for regulating tissue inflammation in multiple autoimmune diseases.

Materials and Methods

Mice

C57BL/6 wild-type (wt), TGF-βRII DN mice were from the Jackson Laboratory. IL-17A-GFP.KI mice were purchased from Biocytogen LLC (Worcester, MA). IL-23R-GFP.KI and IL-23R$^{-/-}$ mice were generated as described [16]. TGF-β3$^{fl/fl}$ mice were generously provided by Dr. Ramireddy Bomireddy of University of Arizona. TGF-β3-YFP reporter mice were generated by crossing TGF-β3^{Cre} [28] mice with Rosa26^{YFP}. TGF-β3-Rosa^{DTR} were generated by crossing TGF-β3^{Cre} [28] to Rosa26^{DTR} mice. Wt, 2D2, 2D2xIL-23R$^{-/-}$, 2D2xT-bet$^{-/-}$, T-bet$^{-/-}$, IL-23R-GFP.KI, IL-17A-GFP.KI mice[10] were housed and maintained in a conventional pathogen-free facility at the Harvard Institute of Medicine in Boston, MA. All experiments were performed in accordance to the guidelines outlined by the Harvard Medical Area Standing Committee on Animals at the Harvard Medical School (Boston, MA).

Cell sorting, flow cytometry, p-smad and intracellular cytokine staining (ICC)

Cells were sorted with anti-CD4-PerCP, anti-CD62L-APC, anti-CD44-PE antibodies (all Biolegend, CA). Cells were stimulated with phorbol 12-myristate 13-aceate (PMA) (50ngml^{-1}, Sigma-aldrich, MO) and ionomycin (1μgml^{-1}, Sigma-aldrich, MO) and a protein transport inhibitor containing monensin (Goligistop) (BD Biosciences) for four hours prior to detection with staining with antibodies. Surface markers were stained in PBS with 1% FCS for 20 min in room temperature, then subsequently fixed in Cytoperm/Cytofix (BD Biosciences), permeabilized with Perm/Wash Buffer (BD Biosciences) and stained with cytokine antibodies (anti-IFN-γ-APC anti-IL-17A-alexa fluora 488 ab, all from Biolegend) diluted in Perm/Wash buffer as described previously [10]. For psmad signaling flow

cytometry, LN and SP cells from C57BL/6 wt mice were labeled with CD4 microbeads (Miltenyi Biotec, MA) then purified via the Automacs (Miltenyi Biotec, MA). Cells were stimulated in serum free media and serum starved overnight then subsequently cultured with either TGF-β1 or TGF-β3 (2ng/ml) for 30 min. Cells were fixed with Cytoperm/Cytofix (BD Biosciences), permeabilized with Perm/Wash Buffer (BD Biosciences), then stained with psmad2&3, psmad1&5, and isotype control antibodies (all Cell Signaling, MA). Data was collected using FACS Calibur or LSR II (Both BD Biosciences), then analyzed using Flow Jo software (Treestar, OR) [16, 47].

Active and passive EAE induction and disease analysis

For active EAE induction, mice were immunized with 100μg $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) in CFA injected subcutaneously, mice received pertussis toxin (List Biological Laboratory, CA) via intraperitoneally (i.p.). For local blockade of TGF-β3, 100ng/ml anti-TGF-β3 antibody (R&D, MN) was incorporated into the emulsion, and mice received 200ng of pertussis toxin on day 0 and 2. For passive EAE induction, cells were differentiated and adoptively transferred as previously described [16, 22]. Animals were monitored and scored daily for development of EAE according to the following criteria: Animals were monitored daily for the development of classical and atypical signs of EAE according to the following criteria: 0, no disease; 1, decreased tail tone or mild balance defects; 2, hind limb weakness, partial paralysis or severe balance defects that cause spontaneous falling over; 3, complete hind limb paralysis or very severe balance defects that prevent walking; 4, front and hind limb paralysis or inability to move body weight into a different position; 5, moribund state[22].

Analysis of CNS infiltrating mononuclear cells

At the peak of disease, mice were sacrificed for analysis of CNS infiltrating cells. Mice were perfused through the left ventrical of the heart with cold PBS. The brain and the spinal cord were flushed out with PBS by hydrostatic pressure. CNS tissue with minced with a sharp razor blade and digested with collagenase D (2.5mg/ml, Roche Diagnostics, IN) and DNaseI (1mg/ml, Sigma, MO) at 37°C for 20 minutes. Mononuclear cells were isolated by passing the tissue through a cells strainer (70um), followed by a percoll gradient (37% and 70%) centrifugation. Mononuclear cells in the interphase was removed, washed and resuspended in culture medium for analysis by intracellular cytokine staining.

In vitro T-cell differentiation

$CD4^+$ T cells were purified from spleen and lymph nodes using anti-CD4 microbeads (Miltenyi Biotech) then further sorted for naïve $CD4^+$ $CD62L^{hi}$ $CD44^{low}$ T cells. Sorted cells were activated with plate bound anti-CD3 (2μg/ml) and anti-CD28 (2μg/ml) in the presence of cytokines (rmIL-23: R&D Systems, all other cytokines: Miltenyi Biotec). Anti-TGF-β3 (R&D Biosystems) were neutralized at 30μg/ml. For T_H17 differentiation: 2ng/mL rhTGF-β1 and rhTGF-β3 (Miltenyi Biotec), 25ng/mL rmIL-6 (Miltenyi Biotec), 20ng/ml rmIL-23 (R&D Biosystems), and 20ng/ml rmIL-1β (Miltenyi Biotec). Cells were cultured for 4 days and harvested for RNA, intracellular cytokine staining, and for flow cytometry.

Cytokine analysis (ELISA) and real time PCR

Cytokines in culture supernatants were collected on day 4 and determined by enzyme-linked immunosorbent assay (ELISA) as previously described [10]. On day 4 (unless otherwise noted) post culture, RNA was extracted with RNeasy kit (Qiagen), reverse transcribed using iscript cDNA synthesis kit (Bio-rad, CA), and was analyzed by quantitative RT-PCR with vii7 Real-time PCR systems (Applied Biosystems) for the gene of interest. Primer/probe mixtures were purchased from Applied Biosystems. The comparative threshold cycle

method and an internal control (GAPDH) were use for normalization of the target genes. The list of primer and probes from Applied Biosystems: *Cxcl3*: Mm01701838_m1, *Il3*: Mm00439631_m1, *Csf2*: Mm01290062_m1, *Il1rn*: Mm01337566_m1, *Lrmp*: Mm00493168_m1, *Gzmb*: Mm00442834_m1, *Tbx21*: Mm00450960_m1, *Il17a*: Mm00439618_m1, *Il17f*: Mm00521423_m1, *Ifng*: Mm01168134_m1, *Ahr*: Mm00478932_m1, *Il9*: Mm00434305_m1, *Il10*: Mm00439614_m1, *Maf*: Mm02581355_s1, *Il22*: Mm00444241_m1, *Il23r*: Mm00519943_m1, *Rorc*: Mm00441144_g1, *Tgfb1*: Mm01178820_m1, *Tgfb2*: Mm00436955_m1, *Tgfb3*: Mm01307950_m1, *GAPDH*: 4352339E

TGF-β PCR array

TGF-β/BMP PCR array kit was purchased from Sabiosciences and cells were processed using manufacturer's protocol. Naïve CD4 T cells ($CD44^{neg}$ $CD62L^{+}$ $CD4^{+}$) were sorted using BD Biosciences FACAria Cell sorter and cultured *in vitro* under TGF-β1/IL-6 or TGF-β3/IL-6 conditions. After 24 hours, cells were harvested and total RNA was purified using RNeasy mini kit (Qiagen, CA). RNA was converted to cDNA using manufacturer's cDNA sythesis kit, RT^2 First Strand Kit (Sabiosciences, CA). SYBR Green qPCR Master mix provided by the manufacturer was used and plated in pre-coated 96 well PCR array kit. RT-PCR was performed using the vii7 real time PCR system (Applied Biosystems, CA). Raw data was uploaded onto the manufacturer's website and analyzed.

Histopathologic analysis

Mice were sacrificed 30 days after transfer and the CNS fixed in 10% neutral-buffered formalin and processed routinely for paraffin embedment. Inflammatory foci (>10 mononuclear cells) were quantified in the meninges and parenchyma by a pathologist in a double blind manner, such that disease status of the mice as well as the cell transfer groups were not revealed.

Microarray analysis

Wt naïve T cells were sorted and activated for 4 days with plate bound anti-CD3, anti-CD28 alone, or with the indicated cytokines. RNA was purified using the RNeasy kit (Qiagen, RD), amplified using the Ovation Biotin RNA Amplification and Labeling System (NuGEN, CA). The cDNA (10μg) was fragmented, labeled, and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix). The data was normalized using the GenePattern software [48] with the Robust Multi Array (RMA) algorithm [49]. Since the data was collected in two batches (SOM), we use the COMBAT software to remove batch effects [50]. For our clustering analysis (Fig. 4a) we only considered probe sets that show at least two fold change (up or down) compared with the no-cytokine (T_H0) state in at least one of the conditions (572 probe sets corresponding to 434 genes; when several repeats were available, we take the average fold change over all repeats). We clustered the genes using average linkage hierarchical agglomerative clustering.

To directly compare the gene expression profiles of TGF-β1/IL-6 and TGF-β3/IL-6 T_H17 cells we considered genes that showed at least a 1.5 fold change (up or down) between these two conditions. From the 233 genes that showed at least a 1.5 fold change (Supplementary Table 1), we selected 23 representative genes based on their biological function for further validation and principal component analysis (PCA). Similar results were obtained when PCA was performed using all 233 genes that showed at least a 1.5 fold change or only the 23 representative genes (Fig. 4d and e).

Statistics

GraphPad Prism 4.0 was used for statistical analysis (linear regression with 95% confidence interval, and unpaired, two-tailed Student's *t* test). Differences were considered statistically significant when $p < 0.05$. * denotes. *p<0.05, **p<0.01, ***p<0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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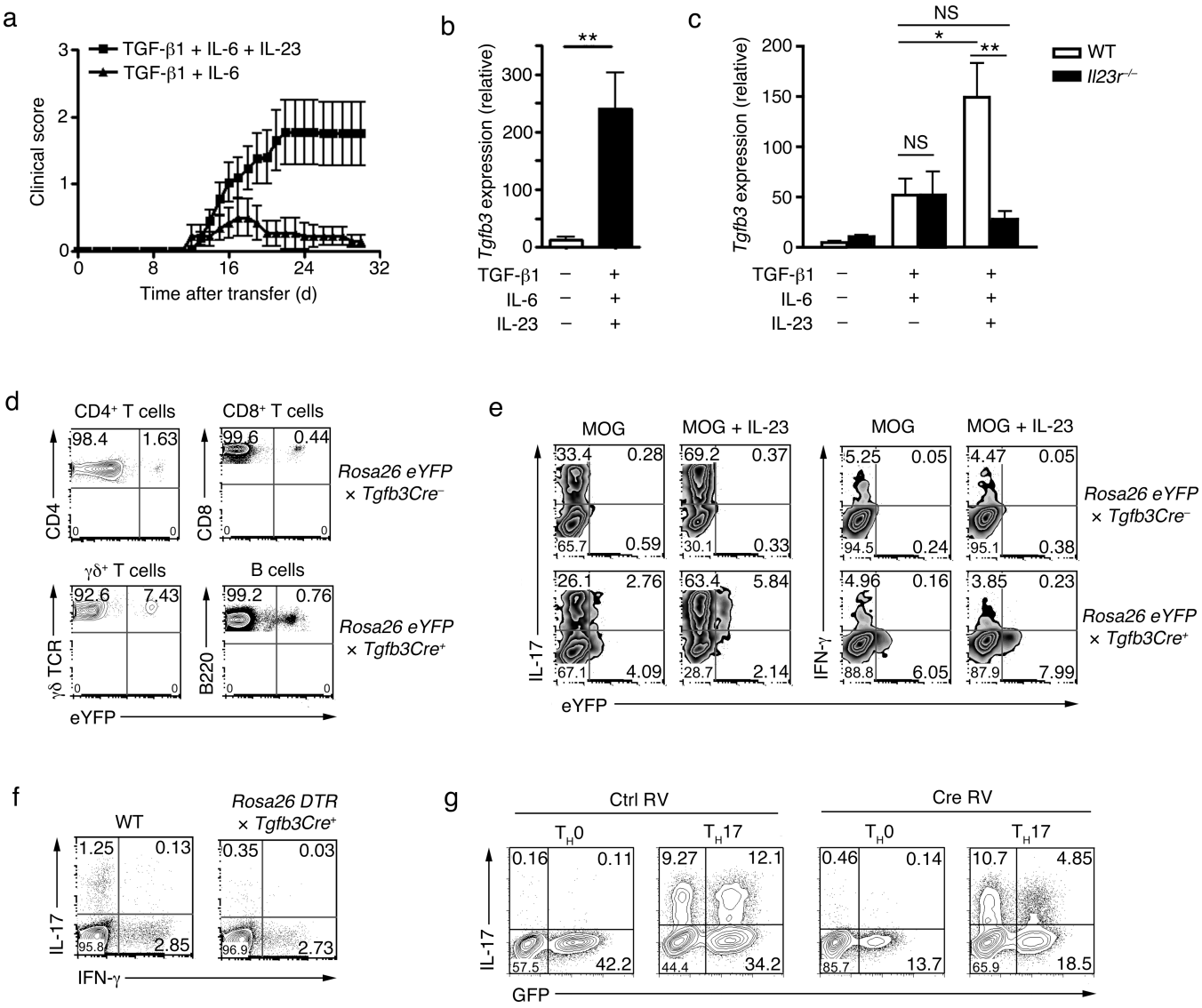

Figure 1. The induction of TGF-β3 in T_H17 cells

(**a**) Mean clinical scores (disease incidence) in wild-type recipients 30 days after transfer of naïve $CD4^+$ T cells (5×10^6 cells) from 2D2 transgenic mice that were differentiated *in vitro* with TGF-β1-IL-6-IL-23 or TGF-β1-IL-6. (**b**) Quantitative RT-PCR analysis of *Tgfb3* mRNA in naïve $CD4^+$ T cells differentiated for four day *in vitro* with TGF-β1-IL-6-IL-23 or no cytokines. (**c**) Quantitative RT-PCR analysis of *Tgfb3* mRNA in naïve $CD4^+$ T cells from wild-type (WT) or *Il23r*$^{-/-}$ mice differentiated *in vitro* with TGF-β1-IL-6-IL-23 or no cytokines. (**d**) Flow cytometry analysis of TGF-β3-YFP expression in $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells and B cells from the lymph nodes and spleen of TGF-$\beta3^{cre}$x$Rosa26^{yfp}$ mice. (**e**) Intracellular cytokine staining showing co-expression of TGF-β3-YFP with either IL-17 or IFN-γ in naïve $CD4^+$ T cells from $MOG_{35–55}$+CFA immunized TGF-$\beta3^{cre}$x$Rosa26^{yfp}$ mice stimulated with PMA+ionomycin four days post *in vitro* culture with or without IL-23. (**f**) Intracellular cytokine staining showing IL-17 and IFN-γ expression in PMA+ionomycin *ex-vivo* stimulated splenocytes from $MOG_{35–55}$-immunized TGF-$\beta3^{Cre+}$x$Rosa^{DTR}$ and TGF-$\beta3^{Cre-}$x$Rosa^{DTR}$ littermate control (WT) mice treated with diptheria toxin on day 4 post-immunization. (**g**) IL-17 expression in naïve TGF-$\beta3^{fl/fl}$ $CD4^+$

T cells cultured *in vitro* with IL-1β-IL-6-IL-23 (T_H17) or no cytokines (T_H0) then retrovirally transduced with Cre-GFP to delete TGF-β3. All data are a representative of more than three independent experiments with similar results. Statistical significance of *$p<0.05$, **$p<0.01$, or ***$p<0.001$ is indicated for the RT-PCR data. Error bars indicate mean ± s.d.

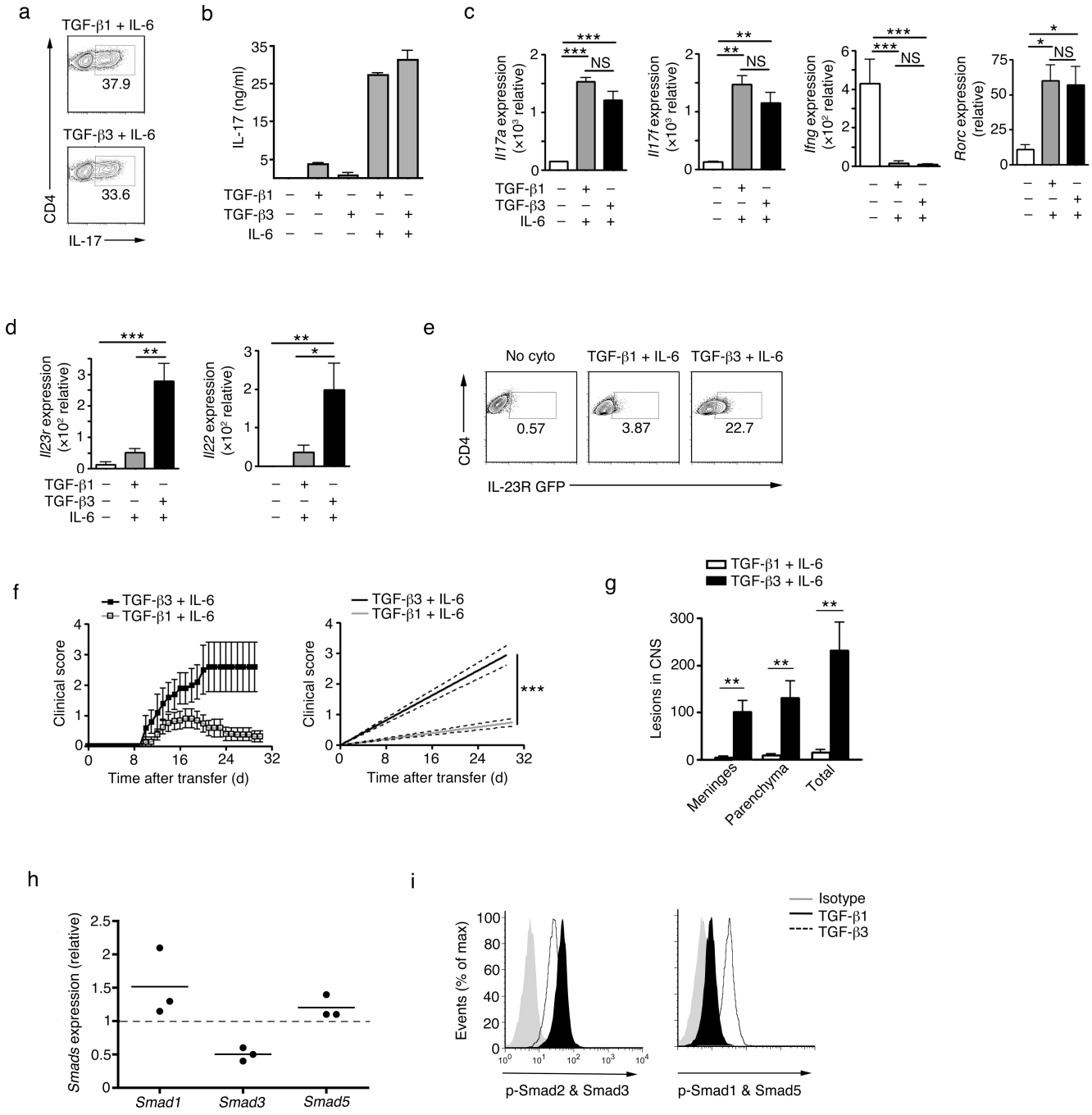

Figure 2. TGF-β3-induced T_H17 cells are highly pathogenic in inducing autoimmunity

(**a**) Intracellular cytokine staining showing IL-17 expression of PMA+ionomycin stimulated naïve CD4$^+$ T cells from C57BL/6 (wt) mice differentiated *in vitro* with TGF-β1-IL-6 or TGF-β3-IL-6 for four days. (**b**) ELISA showing IL-17 production from supernatants of naïve CD4$^+$ T cells from C57BL/6 (wt) mice stimulated *in vitro* with TGF-β1, TGF-β3, TGF-β1-IL-6, TGF-β3-IL-6 for four days. (**c,d**) Quantitative RT-PCR of *Rorc, IL-17a, IL-17f, IFNγ*, *Il23r* and *Il22* mRNA expression from wt naïve CD4$^+$ T cells stimulated in vitro with TGF-β1-IL-6, TGF-β3-IL-6 or no cytokines for four days. (**e**) Flow cytometry analysis of IL-23R-GFP expression of naive CD4$^+$ T cells from IL-23R-GFP knock-in mice

differentiated *in vitro* with TGF-β1-IL-6, TGF-β3-IL-6 or no cytokines for four days (**f**) Mean clinical scores (disease incidence) in wild-type recipients 30 days after transfer of naïve $CD4^+$ T cells (5×10^6 cells) from 2D2 transgenic mice that were differentiated *in vitro* with TGF-β1-IL-6 or TGF-β3-IL-6. Statistical analysis by linear regression curve was performed and graphed including the 95% confidence band of the regression line (***$p<0.001$) (**g**) Quantification of CNS lesions in the meninges and parenchyma from recipient mice sacrificed on day 30 (**$p<0.01$). Data is pooled from three independent experiments (n=6). (**h**) TGF-β signaling PCR array analysis of Smad 1, 3, 5 from wt naïve $CD4^+$ T cells differentiated with TGF-β1-IL-6 or TGF-β3-IL-6 for 24 hours. cDNA was prepared and processed according to manufacturer's instruction (Sabioscience, CA). The graph illustrates the relative fold change in the Smads of TGF-β3-IL-6 condition compared to TGF-β1-IL-6. The dotted line represents the normalized expression of TGF-β1-IL-6 set at one for each of the Smads. The data is pooled from three independent replicates. (**i**) Flow cytometry analysis showing p-smad1&5 or p-smad3 expression from wt naïve $CD4^+$ T cells stimulated with TGF-β1 or TGF-β3 for 30 minutes. Adoptive transfer data from (**f**) is a representative of four independent experiments. All data are a representative of more than three independent experiments, unless otherwise mentioned, with similar results. Statistical significance of *$p<0.05$, **$p<0.01$, or ***$p<0.001$ is indicated for the RT-PCR data. Error bars indicate mean ± s.d.

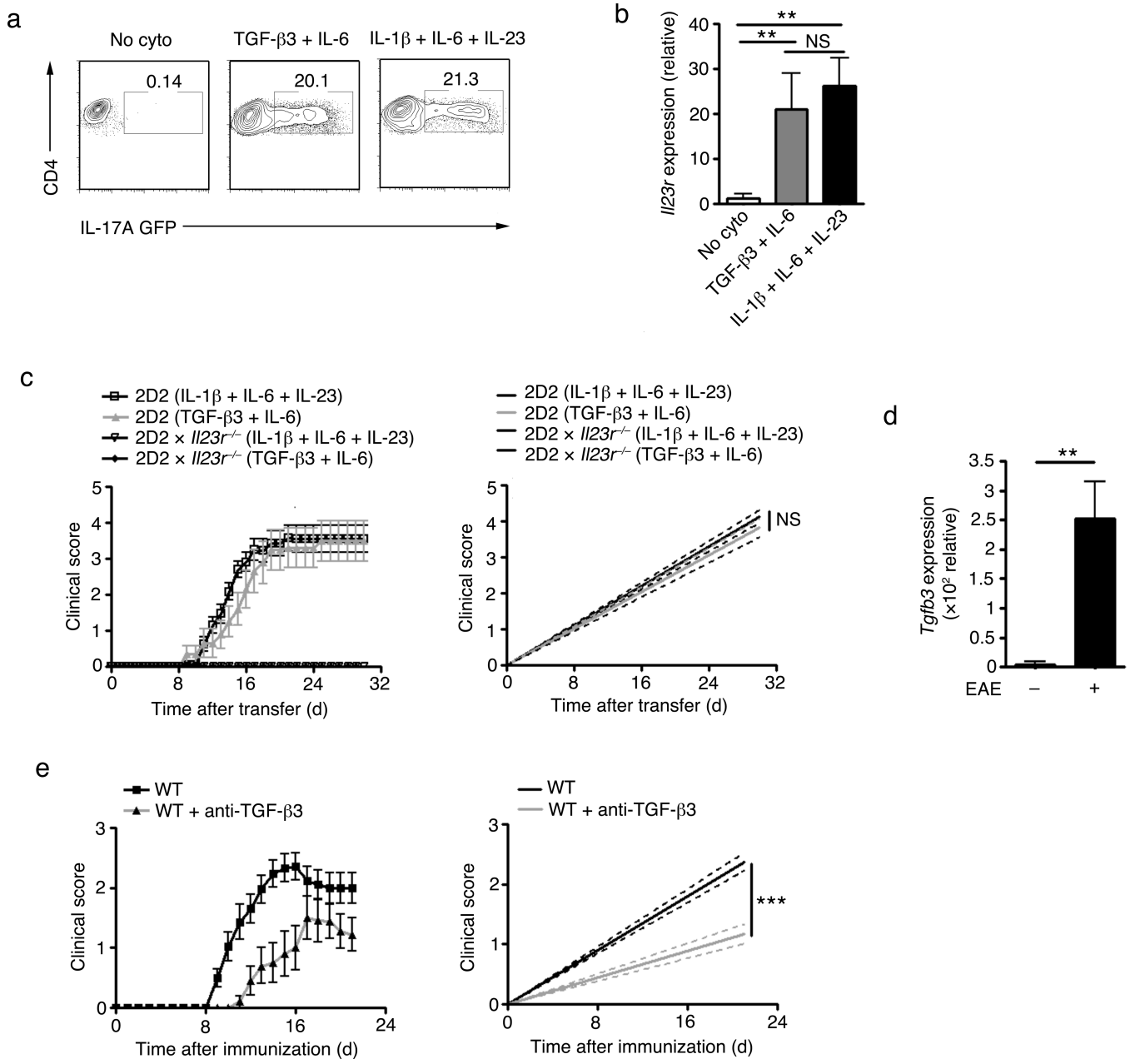

Figure 3. The pathogenicity of TGF-β3–IL-6 T_H17 cells are highly comparable to IL-1β-IL-6-IL-23 T_H17 cells

(**a**) Flow cytometry analysis showing IL-17-GFP expression in naïve $CD4^+$ T cells from the IL-17-GFP knock-in mice differentiated *in vitro* with IL-1β-IL-6-IL-23, TGF-β3-IL-6, or no cytokines for four days. (**b**) Quantitative RT-PCR analysis of *Il23r* mRNA expression from naïve $CD4^+$ T cells from wt mice differentiated in vitro with IL-1β-IL-6-IL-23, TGF-β3-IL-6, or no cytokines for four days. (**c**) Mean clinical scores (disease incidence) in wild-type recipients 30 days after transfer of naïve $CD4^+$ T cells (5×10^6 cells) from 2D2 transgenic or 2D2xIL-23$R^{-/-}$ mice that were differentiated *in vitro* with IL-1β-IL-6-IL-23 or TGF-β3-IL-6. Statistical analysis by linear regression curve was performed and graphed including the 95% confidence band of the regression line. (ns) denotes not significant. (**d**) Quantitative RT-PCR showing *Tgfb3* mRNA expression of CNS-infiltrating $CD4^+$ T cells isolated from

$MOG_{35–55}$+CFA+pertussis toxin (PT) immunized wt mice with either score 2–3 (peak of disease) or score 0 (no disease). Figure represents data of twelve individual mice pooled from three independent experiments. (**e)** Mean clinical scores (disease incidence) in wt mice immunized with $MOG_{35–55}$+CFA+PT with either isotype control or anti-TGF-β3 neutralizing antibody in the emulsion then monitored for 21 days. Statistical analysis by linear regression curve was performed and graphed including the 95% confidence band of the regression line. Adoptive transfer data from (c) is pooled data from three independent experiments (n=8) and (e) is pooled data from two independent experiments (n=10). Cumulative data on the number of animals used are shown in Table 1. All *in vitro* experiments are a representative of at least three independent experiments unless specified differently. Error bars indicate mean ± s.d. (**p<0.01). (ns) denotes not significant.

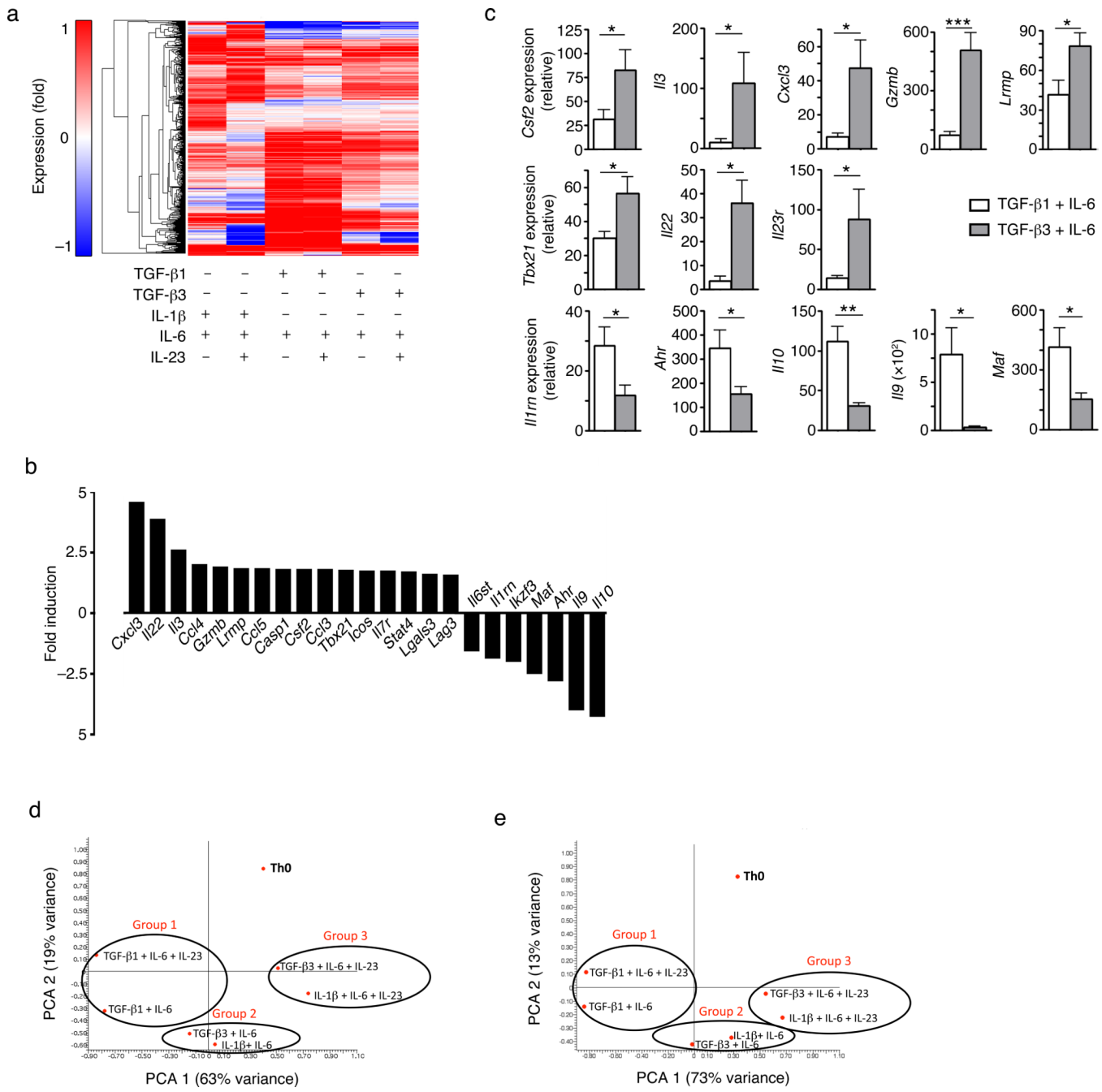

Figure 4. Identification of transcriptional signature for pathogenic T_H17 cells

(**a**) A heat map of the microarray analysis showing differential expression of 434 genes that were up- or down-regulated (>2 fold; compared with T_H0 cells) from wt naïve $CD4^+$ T cells differentiated *in vitro* with IL-1β-IL-6, IL-1β-IL-6-IL-23, TGF-β1-IL-6, TGF-β1-IL-6-IL-23, TGF-β3-IL-6, or TGF-b3-IL-6-IL-23 for 60 hours. (**b**) Bar graph representing analysis of 23 genes selected from a pool of 233 targets that had greater than 1.5 fold difference between TGF-β3-IL-6 and TGF-β1-IL-6 differentiated T_H17 cells. Data is represented as a fold change of TGF-β3-induced T_H17 cells over TGF-β1-induced T_H17 cells. Up-regulated genes denote a "pathogenic signature" from TGF-β3-induced T_H17 cells while down-regulated genes are "nonpathogenic signature" from TGF-β1-induced T_H17

cells. (**c**) Quantitative RT-PCR analysis showing mRNA expression of several targets highlighted in (b) from wt naïve $CD4^+$ T cells differentiated *in vitro* with TGF-β1-IL-6 or TGF-β3-IL-6 for four days. Error bars indicate mean ± s.d., *p<0.05, **p<0.01, ***p<0.001). Data is pooled from at least four independent experiments. (**d,e)** Principal component analysis (PCA) of the 23 selected genes (d) or 233 genes (e) that were differentially expressed and had greater than 1.5 fold difference between the TGF-β3-IL-6 and TGF-β1-IL-6 differentiated T_H17 groups.

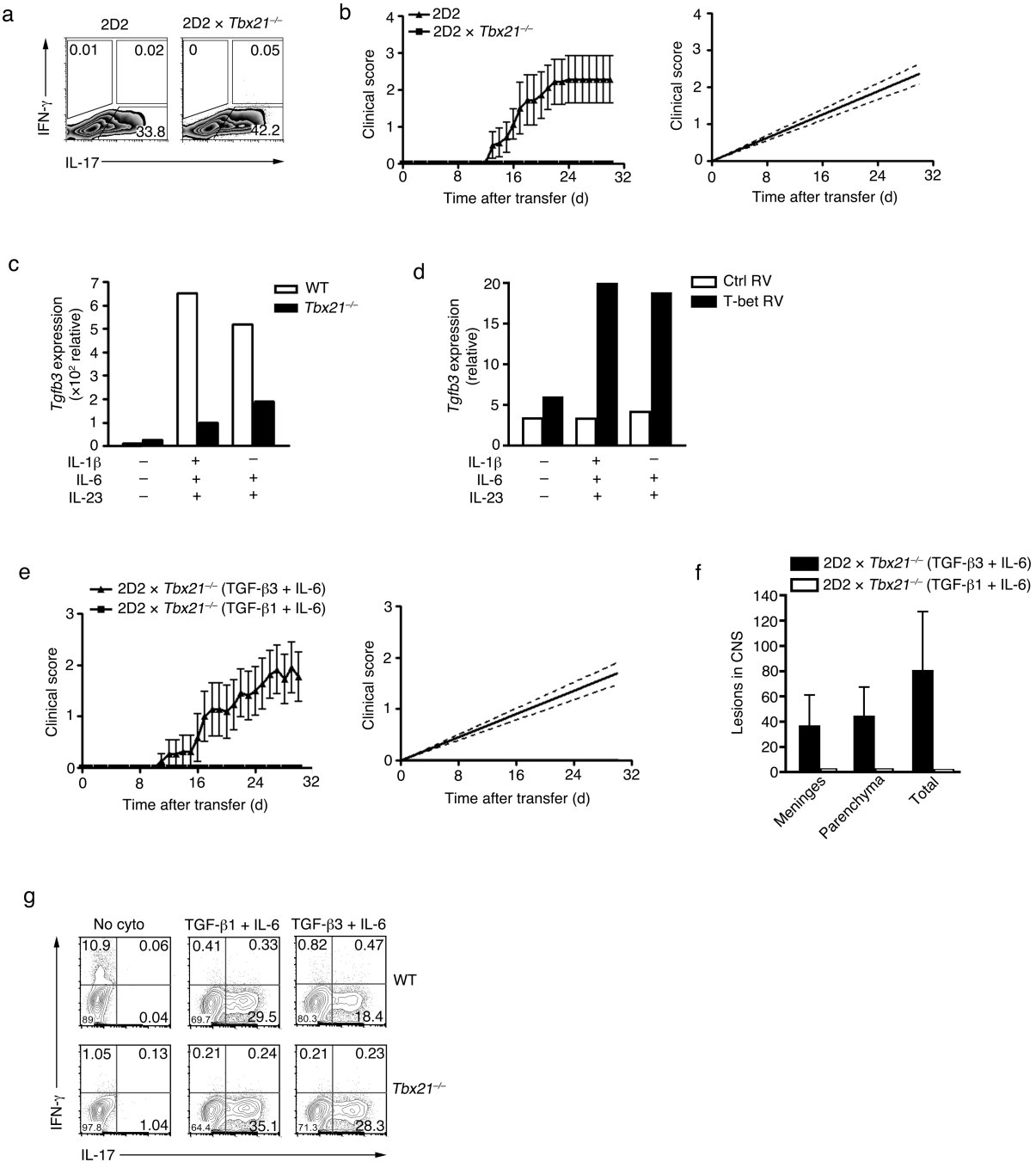

Figure 5. Exogenous TGF-β3 can overcome the absence of T-bet

(**a**) Intracellular cytokine staining showing IL-17A and IFN-γ expression in naïve CD4$^+$ T cells from 2D2 or 2D2 Tbx21$^{-/-}$ mice stimulated with PMA+ionomycin after *in vitro* differentiation with TGF-β1-IL-6-IL-23 for four days. (**b**) Mean clinical scores (disease incidence) in wild-type recipients 30 days after transfer of naïve CD4$^+$ T cells (5×10^6 cells) from 2D2 transgenic or 2D2 Tbx21$^{-/-}$ mice that were differentiated *in vitro* with TGF-β1-IL-6-IL-23. Analysis by linear regression curve was performed and graphed including the 95% confidence band of the regression line. Data is pooled from two independent experiments (n=7). (**c**) Quantitative RT-PCR analysis of *Tgfb3* mRNA expression from

Tbx21$^{-/-}$ or wt naïve CD4^{+} T cells differentiated *in vitro* with IL-1β-IL-6-IL-23, IL-6-IL-23 or no cytokines for four days (**d**) Quantitative RT-PCR of *Tgfb3* mRNA expression of wt CD4^{+} T cells differentiated *in vitro* with IL-1β-IL-6-IL-23, IL-6-IL-23 or no cytokines and retrovirally over-expressed with T-bet-RV or empty-vector-RV (both Thy1.1 marker) for four days. (**e**) Mean clinical scores (disease incidence) in wild-type recipients 30 days after transfer of naïve CD4^{+} T cells from 2D2 Tbx21$^{-/-}$ mice (5×10^{6} cells) that were differentiated *in vitro* with TGF-β1-IL-6 or TGF-β3-IL-6. Analysis by linear regression curve was performed and graphed including the 95% confidence band of the regression line. Data is pooled from three independent experiments (n=11). (**f**) Quantification of CNS lesions in the meninges and parenchyma from recipient mice sacrificed on day 30 (***$p<0.001$). Data was pooled from three independent experiments, n=4. (**g**) Intracellular cytokine staining of IFNγ and IL-17 expression from naïve wt and Tbx21$^{-/-}$ CD4 T cells differentiated *in vitro* with TGF-β1-IL-6, TGF-β3-IL-6, or no cytokines for four days. All *in vitro* experiments are representative of at least three experiments with similar results. Adoptive transfer experiments are pooled from two to three independent experiments and cumulative data on the number of animals used are shown in Table 1.

Table 1

Induction of EAE following adoptive transfer of MOG specific T_H17 cells and active immunization.

Mice	Groups	Disease incidence (%)	Mean score α	Mean day of onset α	Mortality (%)	Statistics	
2D2	IL-1β+IL-6+IL-23	20/21 (95%)	3.7±0.29	12.2±0.35	10/21 (48%)		ns
2D2	TGF-β3+IL-6	13/15 (86%)	3.5±0.35	12.7±0.92	4/15 (26%)	p<0.001	
2D2	TGF-β1+IL-6	10/17 (58%)	2.6±0.45	14.1±1.77	0/17 (0%)		
2D2	TGF-β1+IL-6+IL-23	11/21 (52%)	3.6±1.53	14.8±2.40	5/21 (23%)	p<0.001 χ	
2D2xIL-23R$^{-/-}$	IL-1β+IL-6+IL-23	0/8 (0%)	n/a	n/a	0/8 (0%)	p<0.001 β	
2D2xIL-23R$^{-/-}$	TGF-β3+IL-6	0/8 (0%)	n/a	n/a	0/8 (0%)	p<0.001 β	
Wt	Isotype control	19/22 (86%)	2.4±0.25	9±0.83	0/22 (0%)	p<0.001	
Wt	Anti-TGF-β3	710 (70%)	1.6±0.37	9.8±2.2	0/10 (0%)		
2D2xTbx21$^{-/-}$	TGF-β3+IL-6	8/11 (73%)	2.8±1.13	18.9±4.58	1/11 (1%)	p<0.001	
2D2xTbx21	TGF-β1+IL-6	0/11 (0%)	n/a	n/a	0/11 (0%)		
2D2xTbx21	TGF-β1+IL-6+IL-23	0/7 (0%)	n/a	n/a	0/7 (0%)	p<0.001 β	

The transferred 2D2 CD4$^+$ cells were cultured in the presence of various combinations of IL-23, TGF-β, IL-1β, and IL-6.

Active EAE was induced with MOG35-55/CFA plus pertussis toxin.

Disease incidence, mean day of onset, and mortality are reported as number of mice affected/total number of mice

α Mean calculated only from mice that developed clinical signs of experimental autoimmune encephaltomyelitis.

β Compared to 2D2 controls with indicated cytokine conditions

χ Compared to 2D2 (TGF-β1+IL-6+IL-23) to 2D2 (TGF-β1+IL-6) conditions

n/a : Denotes: not applicable

ns: Denotes: not significant