Highly polarized Th17 cells induce EAE via a T-bet independent mechanism

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In the MOG_{35-55} induced EAE model, autoreactive Th17 cells that accumulate in the central nervous system acquire Th1 characteristics via a T-bet dependent mechanism. It remains to be determined whether Th17 plasticity and encephalitogenicity are causally related to each other. Here, we show that IL-23 polarized T-bet$^{-/-}$ Th17 cells are unimpaired in either activation or proliferation, and induce higher quantities of the chemokines RANTES and CXCL2 than WT Th17 cells. Unlike their WT counterparts, T-bet$^{-/-}$ Th17 cells retain an IL-17^{hi}IFN-γ^{neg-lo} cytokine profile following adoptive transfer into syngeneic hosts. This population of highly polarized Th17 effectors is capable of mediating EAE, albeit with a milder clinical course. It has previously been reported that the signature Th1 and Th17 effector cytokines, IFN-γ and IL-17, are dispensable for the development of autoimmune demyelinating disease. The current study demonstrates that the "master regulator" transcription factor, T-bet, is also not universally required for encephalitogenicity. Our results contribute to a growing body of data showing heterogeneity of myelin-reactive T cells and the independent mechanisms they employ to inflict damage to central nervous system tissues, complicating the search for therapeutic targets relevant across the spectrum of individuals with multiple sclerosis.

See accompanying article by O'Connor et al. and commentary by Spath and Becher

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Introduction

EAE is a CD4$^+$ T-cell-mediated autoimmune disease of the central nervous system (CNS), widely used as an animal model of multiple sclerosis (MS). Despite substantial progress in elucidating pathogenic pathways that drive EAE, the mechanisms employed by autoreactive T cells to initiate inflammatory demyelination and,

hence, the effector functions that are critical for their encephalitogenicity, are largely unknown. We and others have previously shown that IL-12-polarized Th1 and IL-23-polarized Th17 cells specific for the same myelin antigen are independently capable of inducing EAE following adoptive transfer into naïve syngeneic hosts [1,2]. Surprisingly, full blown disease occurs in the absence of the signature Th1 and Th17 cytokines, IFN-γ, and IL-17A/F, either alone or in combination [3–5]. More recently, the master regulatory transcription factor, T-bet, was identified as a critical molecule in the programming of encephalitogenic Th17 as well as Th1 cells [6]. T-bet was originally described as a driver of

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Th1 differentiation via direct activation of the IFN-γ gene and upregulation of the IL-12 receptor β2 chain [7, 8]. Gocke et al. [9] subsequently reported that T-bet could also promote the differentiation of autoimmune effector Th17 cells by inducing IL-23 receptor expression.

Several laboratories have established a role for T-bet in the plasticity of Th17 cells, particularly in their acquisition of Th1-like characteristics to become so called "ex-Th17" cells [10–12]. Fate mapping experiments using IL-17 reporter mice demonstrated that the majority of CD4$^+$ T cells infiltrating the CNS of C57BL/6 mice actively immunized with a peptide of myelin oligodendrocyte glycoprotein (MOG$_{35-55}$) are ex-Th17 cells [13, 14]. This observation has led some investigators to speculate that the plasticity of myelin-reactive Th17 cells is causally related to their acquisition of encephalitogenic properties. If they are correct then T-bet would be critical for the development of EAE based on its role in facilitating the transition of myelin reactive Th17 cells into ex-Th17 cells. In the current study we directly assess the requirement of T-bet expression in IL-23 polarized, myelin-reactive T cells for the adoptive transfer of EAE. We find that, unlike their WT counterparts, autoreactive T-bet$^{-/-}$ cells resist conversion to an ex-Th17 phenotype upon in vitro or in vivo reactivation. Moreover, these stable Th17 cells trigger the accumulation of myeloid cells in the spleen and CNS, thereby retaining the ability to induce EAE in WT as well as RAG2-deficient hosts.

Results

MOG$_{35-55}$ immunized T-bet$^{-/-}$ mice generate a Th17 biased response and succumb to EAE

The master transcription factor, T-bet, has been implicated in the pathogenesis of EAE and MS [15–18]. We revisited the role of T-bet in EAE by comparing the clinical courses of C57BL/6 T-bet$^{-/-}$ and WT mice following subcutaneous immunization with an emulsion of MOG$_{35-55}$ in CFA and intraperitoneal injection of inactivated *Bordetella pertussis* toxin. Ninety percent of T-bet$^{-/-}$ mice succumbed to moderate to severe EAE, although disease onset was slightly delayed compared with that of their WT counterparts (Fig. 1A). Examination of cytokine expression by CNS mononuclear cells pooled from representative mice in each group, and by splenocytes harvested from individual mice at peak EAE, revealed skewing toward an IL-17$^+$IFN-γ^- profile in the T-bet$^{-/-}$ cohort (Fig. 1B and C). Splenocytes from immunized T-bet$^{-/-}$ mice produced significantly higher levels of IL-17 and lower levels of IFN-γ than splenocytes from WT mice in response to in vitro challenge with MOG$_{35-55}$ (Fig. 1D).

IL-23 polarized T-bet$^{-/-}$ Th17 cells are phenotypically stable in vitro and in vivo

Collectively, these data suggested that in the absence of T-bet, inflammatory demyelination was mediated by myelin-reactive

Th17 cells that resist conversion to ex-Th17 cells. In support of this hypothesis, MOG-primed T-bet$^{-/-}$ CD4$^+$ T cells predominantly exhibited an IL-17$^+$IFN-γ^- profile following a 96 h culture with antigen plus recombinant IL-23 and IL-1β, while a significant percent of WT CD4$^+$ T cells cultured under the same conditions were IL-17$^-$IFN-γ^+ (Fig. 2A). IL-23 polarized T-bet$^{-/-}$ CD4$^+$ T cells maintained a Th17 phenotype upon secondary challenge with MOG$_{35-55}$ alone (Fig. 2B and C). Multiplex bead immunoassays revealed increased levels of RANTES and CXCL2 in supernatants from primary cultures of T-bet$^{-/-}$ Th17 cells by comparison with WT Th17 cells (Fig. 2D). Conversely, the concentration of the IFN-γ-induced chemokine CXCL9 was relatively low in supernatants from T-bet$^{-/-}$ Th17 cell cultures. T-bet$^{-/-}$ cells also expressed GM-CSF at a lower frequency than WT cells during primary culture (Fig. 2A). However, T-bet$^{-/-}$ and WT Th17 cells secreted comparable quantities of GM-CSF upon secondary challenge (Supporting Information Fig. 1). T-bet$^{-/-}$ and WT Th17 cells produced similar quantities of other cytokines and chemokines implicated in EAE pathogenesis, including IL-1α, IL-6, and G-CSF (Fig. 2D). The majority of T-bet$^{-/-}$ Th17 cells upregulated activation markers and proliferated in response to antigen to a similar extent as their WT counterparts (Fig. 2E), indicating that their failure to acquire Th1 characteristics was not a consequence of insufficient antigen presentation or TCR engagement. The fact that a relatively high percentage of T-bet$^{-/-}$ cells expressed a CD44$^+$CD69$^+$CD25$^+$CD62L^{neg} profile could reflect a less differentiated state [19].

We next compared the stability of MOG-primed, IL-23 polarized T-bet$^{-/-}$ and WT CD4$^+$ CD45.2$^+$ T cells in vivo following transfer into naïve CD45.1 congenic hosts. Spleens harvested from the recipients of T-bet$^{-/-}$ donor cells contained a higher frequency of MOG$_{35-55}$-specific IL-17 producers and a lower frequency of MOG$_{35-55}$-specific IFN-γ producers than spleens from recipients of WT donor cells (Fig. 3A). These stable T-bet$^{-/-}$ Th17 cells induced EAE in 85–90% of hosts, although disease severity was reduced compared with recipients of WT cells (Fig. 3B). IL-23 polarized T-bet$^{-/-}$ Th17 cells did not express FoxP3 and did not mitigate EAE severity when cotransferred with WT Th17 effectors (data not shown).

FACS analysis of spinal cord mononuclear cells at peak disease indicated that the majority of infiltrating CD45.2$^+$ T-bet$^{-/-}$ donor cells were IL-17$^+$IFN-γ^-, while the majority of infiltrating CD45.2$^+$ WT donor cells were IL-17$^-$IFN-γ^+ (Fig. 3C).

T-bet$^{-/-}$ Th17 cells transfer EAE to hosts lacking endogenous B or T lymphocytes

Although T-bet$^{-/-}$ donor cells were enriched for the CD4$^+$ T-cell subset prior to transfer, we entertained the possibility that immunocompetent host T cells had been activated by contaminating donor APCs bearing MOG$_{35-55}$/class II complexes. Therefore, we repeated the adoptive transfer experiments using RAG2$^{-/-}$ recipients. Consistent with the results obtained in immunocompetent hosts, RAG2$^{-/-}$ mice were susceptible to disease induced by
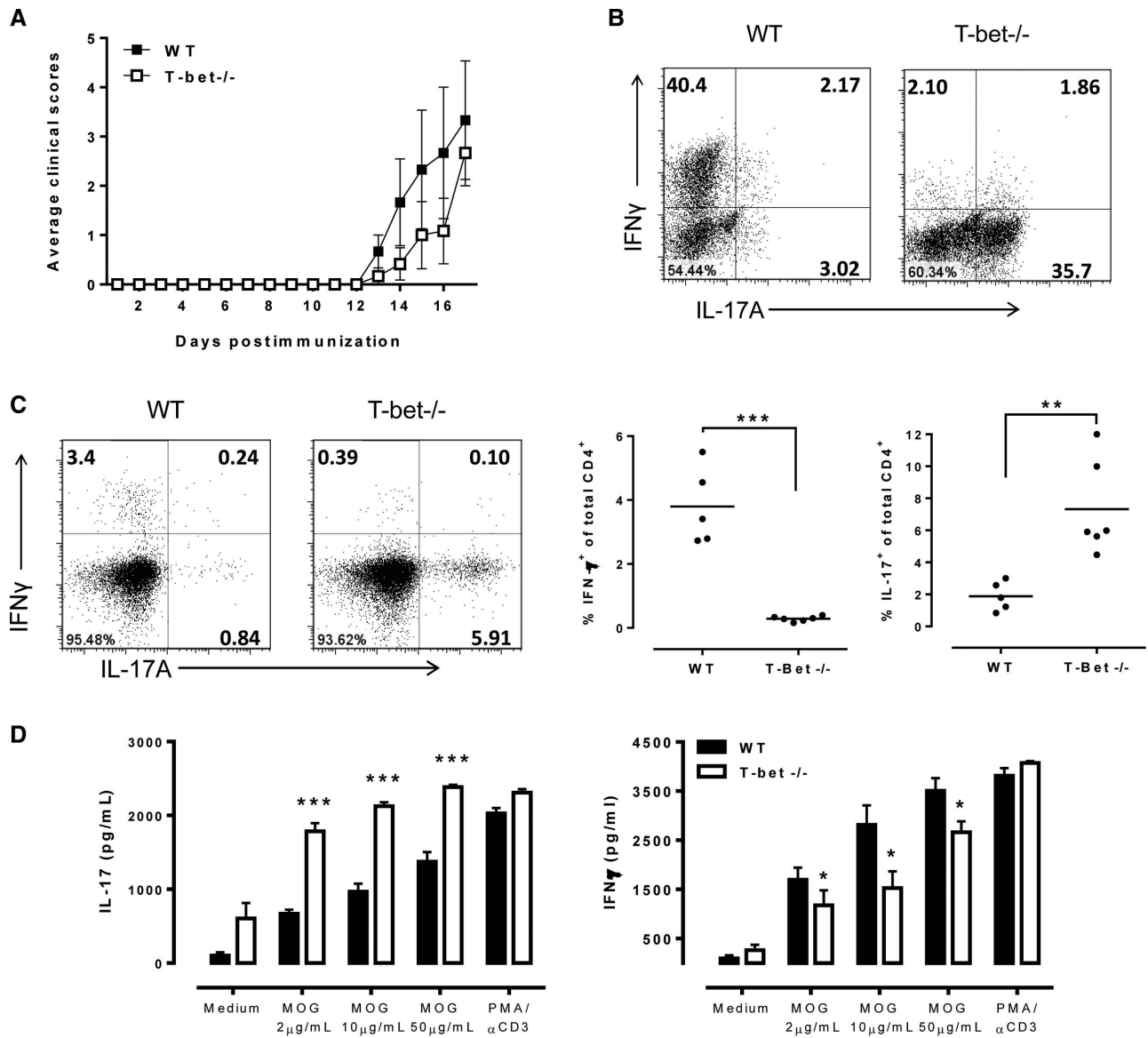
Figure 1. MOG_{35-55} primed T-bet$^{-/-}$ mice mount an enhanced Th17 response and succumb to EAE. EAE was induced in WT and T-bet$^{-/-}$ C57BL/6 mice via immunization with MOG_{35-55} in CFA and administration of inactivated pertussis toxin. (A) WT (closed squares) and T-bet$^{-/-}$ (open squares) mice were rated on a daily basis for degree of neurological disability by an examiner blinded to genotype. Clinical scores for each cohort were averaged over two experiments with a total of 8–10 mice/group. (B) CNS m cells and (C) splenocytes were harvested at peak EAE. Pooled CNS mononuclear cells, or splenocytes harvested from individual mice, were analyzed by intracellular staining and flow cytometry following stimulation with PMA and ionomycin for 6 h. Representative flow cytometry plots are gated on CD4$^+$ T cells. Each symbol in the right panel in (C) represents the percent of cytokine positive cells among splenocytes from an individual mouse. Data shown are from one experiment representative of three independent experiments performed. Bar represents mean. (D) Splenocytes obtained from MOG_{35-55}-immunized WT (closed bars) and T-bet$^{-/-}$ (open bars) mice were cultured with either MOG_{35-55} at the indicated concentrations, PMA and anti-CD3, or medium only. Supernatants were collected at 96 h and subjected to sandwich ELISA to measure cytokine levels. The data are shown as mean +SEM of five mice per group and are from one experiment representative of four experiments performed. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to WT control, unpaired, two-tailed Student's t-test.

IL-23 polarized T-bet$^{-/-}$ donor cells (Fig. 3D). At peak disease, a very high percent of the T-bet$^{-/-}$ cells that had accumulated in the CNS of RAG2$^{-/-}$ recipients were IL-17$^+$IFN-γ^- (Fig. 3E and F, left panel). Similarly, the frequency of IL-17$^+$IFN-γ^- T-bet$^{-/-}$ cells was significantly higher than that of WT donor Th17 cells in the spleen (Fig. 3F, right panel). IFN-γ single-producing T-bet$^{-/-}$ cells

were virtually undetectable either in the spleen or the CNS. In contrast, IL-17$^-$IFN-γ^+ cells were more numerous than IL-17$^+$IFN-γ^- cells among the WT donor population in both the periphery and the CNS. Spleens of RAG2$^{-/-}$ mice that received T-bet$^{-/-}$ donor cells were disproportionately enlarged, primarily due to a local expansion of myeloid cells (Fig. 3G, right panel). There was no

A

B

C

D

E
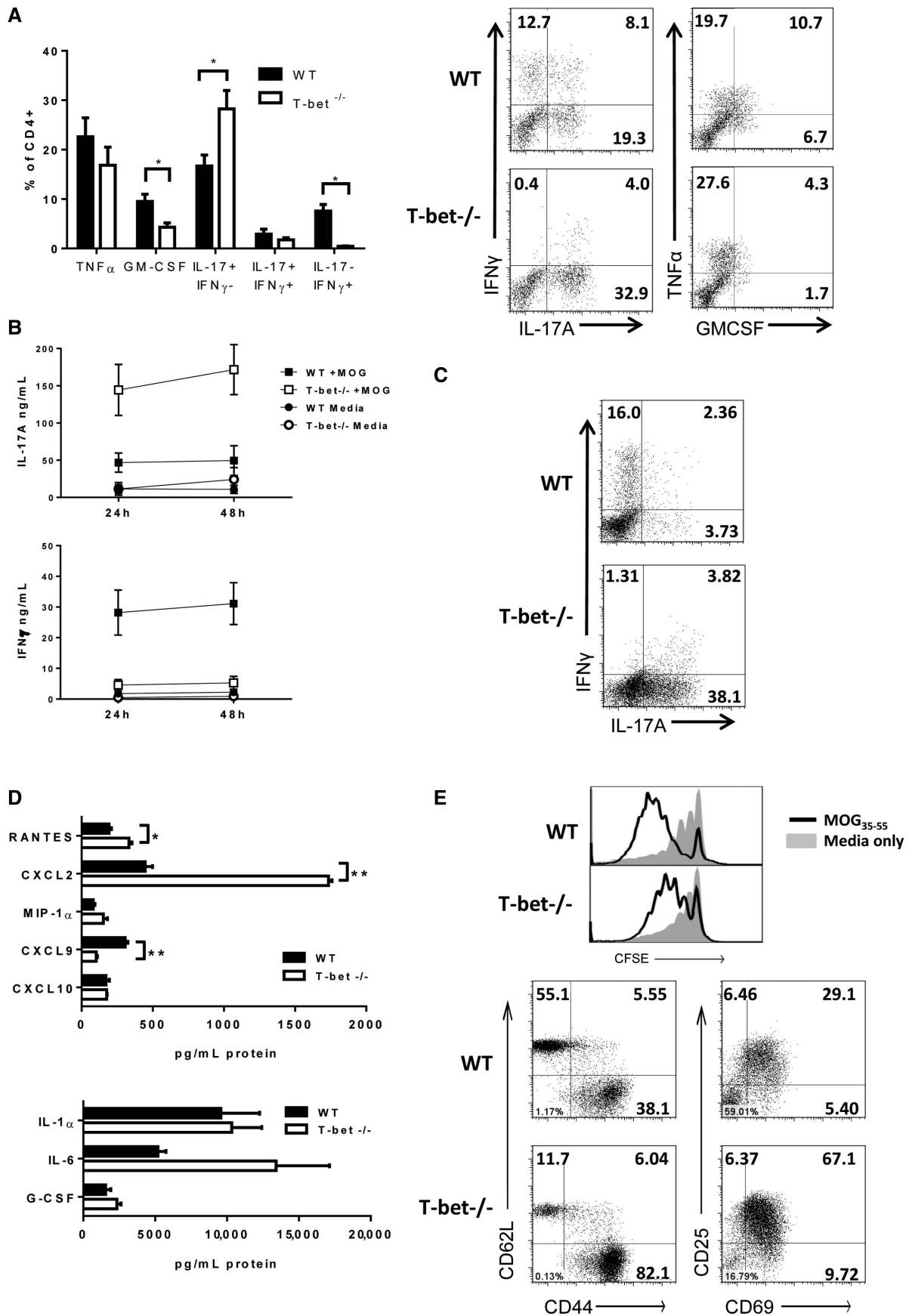
difference in the absolute numbers of CD4$^+$CD3$^+$ T cells, granulocytes or monocytes infiltrating the spinal cords of T-bet$^{-/-}$ or WT hosts (Fig. 3G, left panel).

Discussion

MS is a heterogeneous disease characterized by diversity in both the clinical course and in responsiveness to individual therapeutic agents. At present, no biomarkers have been identified that can guide the selection of an optimal disease modifying regimen. Strategies to manage MS are complicated by the observation that distinct myelin-reactive Th-cell subsets can induce inflammatory demyelination via independent cellular and molecular pathways [1]. Therefore it is not surprising that signature Th1 and Th17 cytokines are dispensable for the manifestation of EAE [3–5]. The identification of a molecule that is critical for encephalitogenicity, irrespective of Th effector phenotype, would serve as an ideal therapeutic target. The transcription factor T-bet has been proposed as a candidate therapeutic target in MS, based on its nonredundant roles in Th1 differentiation and in Th17 plasticity. However, in the current study we show that IL-23 polarized myelin-reactive Th17 cells can mediate autoimmune demyelination without expressing T-bet or converting into Th1 ("ex-Th17") cells. Consistent with our findings, Duhen et al. [20] recently reported that T-bet deficiency confined to CD4$^+$ T cells does not confer resistance against EAE induced by active immunization with MOG peptide emulsified in CFA. We found that stable T-bet$^{-/-}$ Th17 cells maintain the capacity to produce GM-CSF, and induce augmented production of CXCL2, each of which has been implicated in EAE pathogenesis [21–24]. In ongoing studies we are investigating whether compensatory upregulation of these factors drives the accumulation of myeloid cells (Ly6G$^+$ granulocytes in particular) in the spleens of the recipients of T-bet$^{-/-}$ Th17 donor cells. Engagement of alternative chemokine/cytokine pathways could underlie the preserved encephalitogenicity of myelin-reactive T-bet$^{-/-}$ Th17 cells.

We consistently found that MOG-specific T-bet$^{-/-}$ Th17 cells induce a milder course of EAE than their WT counterparts. This could be due to reduced production of the pro-inflammatory factor GM-CSF, as we observed in primary cultures of T-bet$^{-/-}$ and WT CD4$^+$ T cells (Fig. 2A). However, we detected similar frequencies of GM-CSF$^+$ cells among T-bet$^{-/-}$ and WT donor cells harvested from the CNS and peripheral lymphoid tissues of adoptive transfer recipients with EAE (Fig. 3F and data not shown). Furthermore, MOG-primed, Th17 polarized T-bet$^{-/-}$ and WT cells produce comparable amounts of GM-CSF upon secondary challenge (Supplementary Information Fig. 1). The diminished potency of T-bet$^{-/-}$ donor cells could also be secondary to a failure to express adhesion molecules, such as P-selectin ligand, and chemokine receptors, such as CXCR3, that facilitate efficient CNS trafficking [25]. The delay in clinical onset that we observed following adoptive transfer of T-bet$^{-/-}$ effectors into RAG2$^{-/-}$ hosts (Fig. 3D) is consistent with that hypothesis. Finally, our experiments revealed differences in the composition of myeloid cells that were mobilized and recruited by T-bet$^{-/-}$ versus WT effector cells (Fig. 3G and data not shown) that could be responsible for differences in EAE severity. Each of the above possibilities is currently under investigation in our laboratory.

In conclusion, the current study contributes to a growing body of data that demonstrates that multiple parallel immunopathogenic pathways can potentiate autoimmune neuroinflammation, and it suggests that disease-modifying therapies might need to be customized based on immune profiling. 02

Materials and methods

Mice

Eight to 12-week-old C57BL/6 WT, CD45.1 congenic, T-bet$^{-/-}$, and RAG2$^{-/-}$ mice were obtained from the Jackson Laboratory and housed in microisolator cages under specific pathogen-free conditions. T-bet$^{-/-}$ and RAG2$^{-/-}$ mice were subsequently bred in our facility. All animal protocols were approved by the University Committee on Use and Care of Animals.

Induction of EAE

Mice were injected subcutaneously with 100 μg MOG$_{35-55}$ (MEVGWYRSP-FSRVVHLYRNGK; Biosynthesis) in complete Freund's adjuvant (Difco). For induction of EAE by active immunization, inactivated Bordetella pertussis toxin was administered intraperitoneally on days 0 and 2.

◄ **Figure 2.** IL-23 polarized T-bet$^{-/-}$ Th17 cells are stable in vitro. WT and T-bet$^{-/-}$ mice were immunized with MOG$_{35-55}$/CFA. Ten to 14 days later, single cell suspensions of draining LN cells were cultured with MOG$_{35-55}$ in the presence of IL-23, IL-1α, anti-IFN-γ, and anti-IL-4. (A) At 96 h cells were harvested, washed, and stimulated with PMA and ionomycin for 6 h prior to for intracellular staining and flow cytometry. The percentages of WT (black bars) and T-bet$^{-/-}$ (white bars) CD4$^+$ T cells that expressed cytokines as indicated were averaged over seven experiments with 4–6 donors/group/experiment (left). Representative flow cytometry plots are gated on CD4$^+$ T cells. (B) Following 96 h primary culture, LN cells were washed and restimulated with T-cell-depleted syngeneic splenocytes pulsed with MOG$_{35-55}$. Supernatants collected 24 and 48 h later were analyzed by ELISA. Data are shown as mean +SEM of three samples from three independent experiments. (C) LN cells from primed mice were cultured for 96 h in the presence of MOG$_{35-55}$ and Th17 polarizing factors after which they were rested in the absence of antigen or recombinant cytokines for an additional 96 h. They were then washed and restimulated with T-cell-depleted splenocytes and MOG$_{35-55}$. Cells were harvested 24 h later for intracellular staining followed by flow cytometry. Representative flow cytometry plots are gated on CD4$^+$ T cells. (D) Supernatants from cells cultured for 96 h as in (A) were analyzed by the Luminex protein assay. Data are shown as mean + SEM of two samples from two experiments. (E) CFSE dilution (top) and activation status (bottom) of cells cultured as in (C). Cells were stained with CFSE prior to rechallenge with MOG$_{35-55}$ (solid line) or media alone (shaded) and analyzed by flow cytometry 6 days later. Activation marker expression was assessed 24 h after rechallenge. Histograms are gated on CD4$^+$ T cells. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to WT controls, unpaired two-tailed Student's t-test.
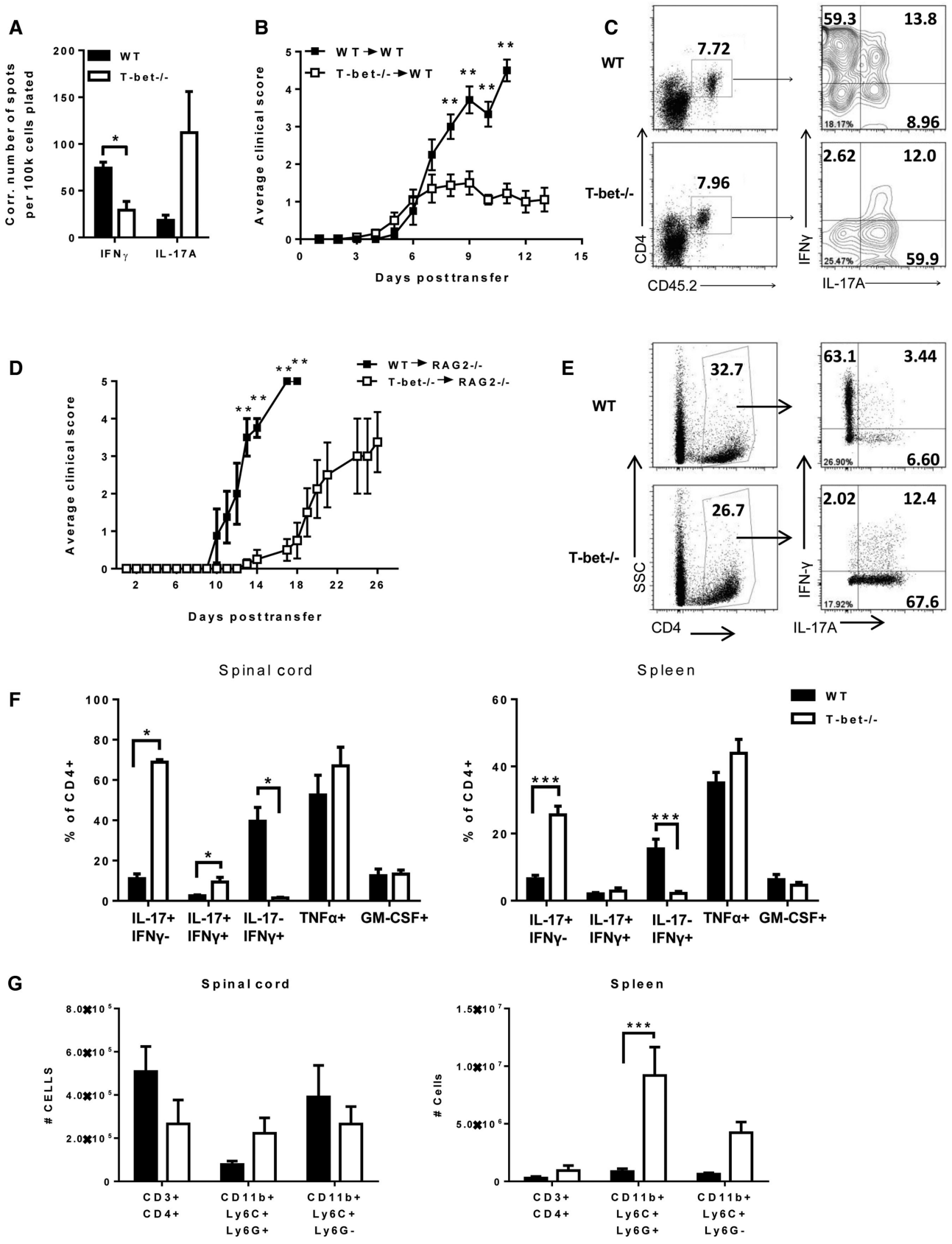
For induction of EAE by adoptive transfer, draining lymph nodes were harvested 10–14 days postimmunization, homogenized, and passed through a 70 μm cell strainer (BD Falcon). LNCs were cultured in vitro with MOG_{35-55} (50 μg/mL) under conditions favorable to the generation of Th17 cells (rmIL-23, 8 ng/mL; rm IL-1α, 10 ng/mL; anti-IFN-γ (clone XMG1.2), 10 μg/mL; anti-IL-4 (clone 11B11), 10 μg/mL). A total of 2×10^6 $CD4^+$ T cells were injected intraperitoneally, and mice were observed daily for signs of EAE as described previously [24].

Flow cytometry

Spinal cords were harvested at peak disease, homogenized in DNase (1 mg/mL) and collagenase A (2 mg/mL) and incubated for 30 min at 37°C. Mononuclear cells were isolated over a 30/70% Percoll gradient (GE Healthcare). Splenocytes were passed through a 70-μm cell strainer, ACK lysed and washed twice prior to analysis. For intracellular staining, cells were stimulated with PMA (50 ng/mL) and ionomycin (2 μg/mL) in the presence of brefeldin A (10 μg/mL) for 6 h or with MOG_{35-55} for 24 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin prior to incubation with flourochrome-conjugated antibodies. Flow cytometry was performed using a BD FacsCanto II.

ELISA

Splenocytes were cultured with or without MOG_{35-55} (50 μg/mL) in a 96 well plate (2×10^6 cells/well). Supernatants were collected at serial time points and analyzed by sandwich ELISA according to the manufacturer's protocol (eBioscience). In some experiments, cell culture supernatants were analyzed using luminex protein array according to the manufacturer's instructions (Millipore).

ELISPOT assay

The frequency of antigen-specific cytokine producers was determined following culture for 24 h in 96-well filtration plates (Millipore), with or without 50 μg/mL MOG_{35-55}. Antibodies from eBioscience were: anti-IL-17 (TC11–18H10), biotinylated anti-IL-17 (TC11–8H4), IFN-γ (AN18), and biotinylated anti-IFN-γ (R4–6A2). Streptavidin–alkaline phosphatase (Southern Biotech) and an alkaline phosphatase substrate kit (Vector Laboratories) were used to identify trapped cytokine. Spots were counted using the CTL ImmunoSpot Analyzer (Cellular Technology) with ImmunoSpot software, and the number of spots in the medium-only wells subtracted to generate the data shown.

Statistical analysis

Statistical analyses were performed using GraphPad Prism statistical analysis software. Group differences were analyzed by unpaired, two-tailed Students t-test. p-values of 0.05 or less were considered significant.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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◄ **Figure 3.** IL-23 polarized T-bet$^{-/-}$ Th17 cells are encephalitogenic. (A–C) IL-23 polarized T-bet$^{-/-}$ and WT $CD4^+$ T cells were adoptively transferred into naïve CD45.1 congenic hosts (5×10^6 $CD4^+$ T cells/recipient). (A) Splenocytes harvested from hosts at peak disease were subjected to IL-17 and IFN-γ ELISpot assays. Background spots (detected in wells that were not pulsed with antigen) were subtracted to generate the Ag-specific data shown. (B) Adoptive transfer recipients of WT (closed symbols) or T-bet$^{-/-}$ (open symbols) MOG-reactive Th17 cells were rated on a daily basis for degree of neurological disability. The data shown are from one experiment with 4–6 mice/group representative of three performed. (C) CNS mononuclear cells obtained at peak disease were analyzed for intracellular cytokine expression by flow cytometry following stimulation with PMA and ionomycin for 6 h. Representative flow cytometry plots are gated on $CD4^+CD45.2^+$ donor T cells. (D–G) WT (closed symbols) or T-bet$^{-/-}$ (open symbols) Th17 cells were transferred into naïve RAG2$^{-/-}$ hosts (5×10^6 $CD4^+$ T cells/recipient). (D) Recipients were monitored on a daily basis and scored for degree of neurological disability. (E, F, G) CNS mononuclear cells and (F, G) splenocytes were harvested at peak disease and analyzed for (E, F) intracellular cytokine expression and (G) surface marker expression by flow cytometry. Representative flow cytometry plots for intracellular cytokine expression are gated on $CD4^+$ donor T cells. Monocytes were defined as $CD11b^{pos}Ly6C^{int-hi}Ly6G^{neg}$. Granulocytes were defined as $CD11b^{pos}Ly6C^{int}Ly6G^{pos}$. (D, F, G) Data are shown as mean + SEM of four mice per group from one experiment representative of three performed. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to WT controls, unpaired two-tailed Student's t-test.

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