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Tbet or Continued RORγt Expression Is Not Required for Th17-Associated Immunopathology

Verena Brucklacher-Waldert,* Cristina Ferreira,* Silvia Innocentin,* Shraddha Kamdar,† David R. Withers,‡ Marika C. Kullberg,‡ and Marc Veldhoen*†

The discovery of Th17 cell plasticity, in which CD4+ IL-17–producing Th17 cells give rise to IL-17/IFN-γ double-producing cells and Th1-like IFNγ+ ex-Th17 lymphocytes, has raised questions regarding which of these cell types contribute to immunopathology during inflammatory diseases. In this study, we show using Helicobacter hepaticus-induced intestinal inflammation that IL-17ACre−or Rag1Cre-mediated deletion of Tbx21 has no effect on the generation of IL-17AIFN-γ double-producing cells, but leads to a marked absence of Th1-like IFNγ+ ex-Th17 cells. Despite the lack of Th1-like ex-Th17 cells, the degree of H. hepaticus-triggered intestinal inflammation in mice in which Tbx21 was excised in IL-17–producing or Rag1-expressing cells is indistinguishable from that observed in control mice. In stark contrast, using experimental autoimmune encephalomyelitis, we show that IL-17ACre−mediated deletion of Tbx21 prevents the conversion of Th17 cells to IL-17AIFN-γ double-producing cells as well as Th1-like IFN-γ+ ex-Th17 cells. However, IL-17ACre−mediated deletion of Tbx21 has only limited effects on disease course in this model and is not compensated by Ag-specific Th1 cells. IL-17ACre−mediated deletion of Rorc reveals that RORγt is essential for the maintenance of the Th17 cell lineage, but not immunopathology during experimental autoimmune encephalomyelitis. These results show that neither the single Th17 subset, nor its progeny, is solely responsible for immunopathology or autoimmunity. The Journal of Immunology, 2016, 196: 4893–4904.

The immune system needs to rapidly and robustly respond to pathogenic threats, whereas inappropriate responses to benign stimuli must be avoided. For a long time, the CD4+expressing Th cells that orchestrate adaptive immune responses were thought to consist of two subsets, the Th type 1 (Th1) and Th type 2 (Th2) cells (1). Regulatory T cells (Treg) were identified based on their ability to prevent autoimmunity (2) and were able to reduce the activity of both Th1 and Th2 subsets, thereby upholding the paradigm of two ultimate effector lineage fates. However, in recent years, this paradigm has undergone substantial revision. Upon activation, Ag-inexperienced CD4+ T cells can differentiate into multiple lineages, including Th1, Th2, Treg, Th17, Th9, and follicular Th cells (Tfh) (3). The development of these Th subsets is determined by the local environment, and especially, but not exclusively, the cytokines present (4, 5).

Th subsets are largely defined by the signature cytokines they produce and their lineage-associated transcription factors. Thus, Th1 cells are characterized by their expression of the cytokine IFN-γ and the transcription factor T box expressed in T cells (Tbet) (6). However, Th subsets also play a prominent role in aberrant immunity. Although Th1 cells were initially thought to be critical in autoimmune disorders such as rheumatoid arthritis, type 1 diabetes, and multiple sclerosis, the focus rapidly shifted to Th17 cells being involved in these diseases (10, 11).

Shortly after the first description of Th17 cells, CD4+ T cells producing both IL-17 and IFN-γ (Th1/Th17 or IL-17/IFN-γ double producers) were discovered in both humans and mice (12, 13), their frequency sometimes outnumbering IL-17 or IFN-γ single producers (14). These IL-17/IFN-γ double-producing cells coexpress RORγt and Tbet (15–17). Detailed studies in mice revealed not only the presence of IL-17/IFN-γ double producers (16, 18, 19), but the existence of IFNγ+ ex-Th17 cells. Using a fate reporter system in which Th17 cells are perma-

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observed that in vitro polarized Tbet-deficient Th17 cells or Tbet-deficient CD4+ T cells maintain a high pathogenic potential (30, 31). In this study, we investigated whether the Th17 cell lineage and its Tbet- and IFN-γ-expressing progeny are directly responsible for immunopathology during inflammatory responses associated with the Th17 cell lineage. We used two models of inflammation, experimental autoimmune encephalomyelitis (EAE) and the Helicobacter hepaticus typhlocolitis model, to examine whether conversion of Th17 cells into Th1-like cells (defined by the expression of Tbet and IFN-γ, and absence of ROsy, IL-17A, and IL-17F) is necessary for immunopathology. The use of an IL-17A-Cre mouse (20) enabled us to demonstrate using Rag1<sup>Cre</sup>-or Rag1<sup>Cre</sup>−mediated excision of similar genes. We show that the IL-17A-mediated excision of similar genes. We show that the IL-17A-induced colitis without reducing immunopathology. During EAE both IL-17F/IFN-γ double producers and Th17–derived Th1-like cells are markedly reduced after IL-17A-Cre–mediated Thb21<sup>−</sup> deletion but this only modestly reduced immunopathology. Finally, we demonstrate using Rag1<sup>Tbet−</sup>, Rag1<sup>ΔRosa</sup>−, and IL-17A<sup>ΔROsy</sup>− mice that neither Tbet cell conversion toward Th1-like cells, long-term maintenance of Th17 cells, nor Tbet expression in lymphocytes is essential for the induction of EAE. Together, our findings imply that T cell–associated pathogenicity may not be solely attributed to the Tbet- and IFN-γ–expressing progeny of the Th17 cell lineage.

**Materials and Methods**

**Mice**

C57BL/6, IL-17A<sup>−/−</sup> Rosa<sup>stop-tdRFP</sup> (20), IL-17A<sup>Cre</sup> Tbx21<sup>−/−</sup> Rosa<sup>stop-tdRFP</sup> IL-17A<sup>Cre</sup> cmosesodermin (Eomes<sup>Cre</sup>), IL-17A<sup>Cre</sup> Rosa<sup>stop-tdRFP</sup>, Rag1<sup>Cre</sup> Rosa<sup>stop-tdRFP</sup> (32), Rag1<sup>Tbet−</sup> Tbx21<sup>−/−</sup> Rosa<sup>stop-tdRFP</sup> Rag1<sup>Cre</sup> Rosa<sup>stop-tdRFP</sup> (33), and IFN-γ<sup>Fyp</sup> [Yeti; yellow-enhanced transcript for IFN-γ (34)], all on the C57BL/6 strain, were bred at the Babraham Institute. IL-17A<sup>Cre</sup> Rorγt<sup>−/−</sup>Rosa<sup>stop-tdRFP</sup> were bred at the University of Birmingham. Tbx21<sup>−/−</sup> and Eomes<sup>Cre</sup> (35) were obtained from S. Reiner (Department of Microbiology and Immunology and Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York, NY), Rorγt<sup>Cre</sup> (36) from A. McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.), and Rorγ<sup>Cre</sup> from JAX Laboratories. All animals were bred and maintained under specific pathogen-free conditions, and experiments were conducted in accordance with the United Kingdom Scientific Procedures Act (1986) under Project Licenses authorized by the United Kingdom Home Office and local ethical review committees (EAE, Babraham; H. hepaticus, York). Animals employed tested negative for Abs to specific agents from Sigma-Aldrich). Cells were stained with anti-CD4, anti-CXCR5, with 80% Percoll. After centrifugation at 600 g for 60 min, the cell strainer, centrifuged, resuspended in 40% Percoll, and overlayed with 80% Percoll. After centrifugation at 600 g for 20 min at 37°C, LP cells were recovered from the 40/80% interface and resuspended in medium.

For cytokine profiles, cells were stimulated for 4 h with 500 ng/ml PdBU (Becton Dickinson) and 500 ng/ml ionomycin (EAE experiments) or 10 ng/ml PMA and 1 μg/ml ionomycin (H. hepaticus experiments) in the presence of brefeldin A (all agents from Sigma-Aldrich). Cells were stained with anti-CD4, anti-CD3, anti-CD44, anti-CD3, and anti-PD1 (EAE experiments) or anti-CD4 and anti-CD3 (H<sub>e</sub> experiments) and a fixable viability dye, followed by intracellular staining with anti–IL-17A, anti–IL-17F, anti–IFN-γ, anti–TNF, anti–GM-CSF (all BioLegend) (EAE experiments) or anti–IL-17A, anti–IFN-γ, and anti–Tbet (all from eBioscience) (H<sub>e</sub> experiments). The proportion and absolute numbers of T cells were determined by including counting beads (Spherotech). I-<sub>A</sub>MOG85-95 tetramer was obtained through the National Institutes of Health Tetrimer Facility and used according to their guidelines.

**Clinical signs**

The p values were calculated with a two-tailed Student t test. Differences were considered statistically significant with p < 0.05. Significance is indicated as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

**Results**

Immunopathology coincides with appearance of Th17-derived IFN-γ producers

We have previously reported in EAE that Th17–derived Th1-like cells become the dominant T cell population in the CNS (20). To
examine the role of Tbet in Th17 cells and their Th1-like progeny in pathology, we made use of two models in which Th17 to Th1-like cell conversion has been established: EAE and the *H. hepaticus* typhlocolitis model (16, 20). Using IL-17A<sup>Cre</sup> Rosa26<sup>sno-stop-dRFP</sup> lineage-reporter mice (from hereon called IL-17A<sup>WT</sup>) in which IL-17 is lineage marked by RFP and IFN-γ coincides with the onset and maintenance of clinical disease.

To examine the importance of the Th17 to Th1-like cell conversion of Th17 to Th1 is not required IL-17– or IFN-γ–produced CD4<sup>+</sup> T cells in steady state conditions in the large intestine, the proportions of Th17 cell–derived IL-17/IFN-γ progeny have converted to an IL-17/IFN-γ (peak) in secondary lymphoid tissues, peritoneal cavity, blood, and lymph nodes (Fig. 1A). Under steady state conditions in the large intestine, the proportions of IL-17– or IFN-γ–produced CD4<sup>+</sup> T cells average at 3–4%, indicating that the enhanced frequencies of cytokine-positive CD4<sup>+</sup> T cells observed in the intestine are induced following *H. hepaticus* inoculation (data not shown) (16). To gain insight into the generation and distribution of these three subpopulations, we performed a kinetic analysis upon EAE induction. We analyzed IL-17 and IFN-γ expression in RFP<sup>+</sup> CD4<sup>+</sup> T cells of IL-17A<sup>WT</sup> mice in draining (inguinal) lymph nodes, the peritoneal cavity, blood, spleen, lungs, and the CNS before MOG<sub>35-55</sub> immunization, at day 6 prior to onset of clinical signs (presymptom), and at day 17 during established EAE (peak). In secondary lymphoid tissues, peritoneal cavity, blood, and lung, the fraction of Th17 cells (IL-17 single positive) within the RFP<sup>+</sup>CD4<sup>+</sup> T cell compartment increased during the presymptomatic phase, and regressed at the peak of the disease in favor of IL-17/ IFN-γ double-producing cells and IFN-γ–producing ex-Th17 cells (Fig. 1B). In the CNS, all three cell populations were detectable at their highest levels at the peak of the disease (Fig. 1B). We confirmed that RFP<sup>+</sup> cells were the majority of CD4<sup>+</sup> T cells present in the CNS at the peak of the disease, and 46.1% ± 17.4% of these RFP<sup>+</sup> cells were IFN-γ single-producing ex-Th17 cells (data not shown). The total number of Th17 cells in inguinal lymph nodes (iLN) declined during peak of clinical EAE symptoms, whereas in all other organs total Th17 cell numbers were highest at the peak of the disease (Fig. 1B). IL-17/IFN-γ double-producing T cell numbers remained low before disease onset, and increased during the peak clinical phase in all organs (Fig. 1B). Numbers of ex-Th17 cells, with a Th1-like profile (RFP<sup>+</sup>, IFN-γ<sup>-</sup>, IL-17<sup>+</sup>), increased during peak clinical scores in all organs (Fig. 1B) and also outnumbered bona fide Th1 cells in the iLNs, blood, spleen, and CNS (data not shown) (20). Thus, the majority of CD4<sup>+</sup> T cells present in the target organ during EAE in IL-17A<sup>WT</sup> mice are Th17 cells, and their progeny have converted to an IL-17/IFN-γ double-producing or Th1 cell-like phenotype. The appearance of these latter two subpopulations coincides with the onset and maintenance of clinical disease.

To determine which cell population harbors Ag-specific T cells, we used MOG<sub>35-55</sub> MHC-II tetramer staining in IL-17A<sup>Cre</sup> Rosa26<sup>sno-stop-dRFP</sup> IFN-γ<sup>-YFP</sup> mice (from hereon called IL-17A<sup>WT</sup>IFN-γ<sup>-YFP</sup>) in which IL-17 is lineage marked by RFP and IFN-γ protein expression reported via eYFP (20, 41). We determined the proportion of MOG<sub>35-55</sub> MHC-II tetramer-positive cells within activated CD4<sup>+</sup>CD44<sup>HI</sup> T cells that were negative for RFP and eYFP, single positive for either, or positive for both. In line with an important role for Th17 cells in the initiation of EAE (11), the majority of Ag-specific CD4<sup>+</sup> T cells in the CNS was found within the Th17 cells (eYFP<sup>+</sup>) and the Th17 cell–derived IL-17/IFN-γ double or IFN-γ single producers (eYFP<sup>+</sup>RFP<sup>+</sup>), but not in bona fide Th1 cells (eYFP<sup>+</sup>RFP<sup>-</sup>) (Fig. 1C).

For *H. hepaticus*–induced intestinal pathology, full conversion of Th17 to Th1 is not required

To examine the importance of the Th17 to Th1-like cell conversion for the onset and progression of immunopathology, we generated mice in which Tbx21 is conditionally deleted upon IL-17 expression and in which IL-17–producing T cells are permanently marked by RFP (IL-17A<sup>Cre</sup> Tbx21<sup>fl/fl</sup> Rosa26<sup>sno-stop-dRFP</sup>, from hereon called IL-17A<sup>Tbet</sup>). The IL-17A<sup>Tbet</sup> mice exhibited normal gross development and were born according to a Mendelian distribution (data not shown). Moreover, we confirmed efficient and specific Tbx21 excision in RFP<sup>+</sup> cells from IL-17A<sup>Tbet</sup> mice by PCR (data not shown). The in vitro differentiation potential of naïve CD4<sup>+</sup> T cells toward the Th1 or Th17 cell lineages as well as their proliferation were similar in IL-17A<sup>Tbet</sup> and IL-17A<sup>WT</sup> control mice (Fig. 2A, 2B), demonstrating that polarization toward Th17 and Th1 cells was not affected by the IL-17A<sup>Cre</sup>–mediated removal of Tbx21.

Th17-derived Th1-like cells have been detected in *H. hepaticus*–induced intestinal inflammation in which their generation correlates with the development of pathology (16). To examine the role of Tbet in Th17 to Th1 conversion in this model, IL-17A<sup>WT</sup> and IL-17A<sup>Tbet</sup> mice were given *H. hepaticus* plus anti–IL-10R mAb to induce typhlocolitis, and colonic inflammation was examined 2 wk later. To exclude the potential initiation of Th17 to Th1 cell conversion prior to Tbx21 excision giving rise to IL-17/IFN-γ double-producing T cells, we also included in these experiments Rag1<sup>Cre</sup> Rosa26<sup>sno-stop-dRFP</sup> and Rag1<sup>Cre</sup> Tbx21<sup>fl/fl</sup> Rosa26<sup>sno-stop-dRFP</sup> mice (from hereon called Rag1<sup>WT</sup> and Rag1<sup>Tbet</sup> mice). Rag1<sup>Tbet</sup> mice allowed us to study the influence of Rag1–mediated Tbx21 excision on the development of *H. hepaticus*-induced pathology and Th17 conversion. Our findings demonstrate that the degree of colonic pathology was indistinguishable between *H. hepaticus*–anti–IL-10R–treated IL-17A<sup>WT</sup>, IL-17A<sup>Tbet</sup>, Rag1<sup>WT</sup>, and Rag1<sup>Tbet</sup> mice (Fig. 2C). Within the CD4<sup>+</sup> T cells not derived from Th17 cells (RFP<sup>+</sup> cells), we did not observe any differences in proportion or cell numbers expressing IL-17 or IFN-γ between IL-17A<sup>Tbet</sup> and IL-17A<sup>WT</sup> controls (Fig. 2D, 2E). As expected, very few RFP<sup>+</sup> CD4<sup>+</sup> T cells were observed in Rag1<sup>WT</sup> and Rag1<sup>Tbet</sup> mice (Fig. 2E). When examining the RFP<sup>+</sup> population, the percentage of LP IL-17 single-producing Th17 cells was significantly enhanced in IL-17A<sup>Tbet</sup> compared with IL-17A<sup>WT</sup> animals and in Rag1<sup>Tbet</sup> compared with Rag1<sup>WT</sup> mice (Fig. 2D). However, upon IL-17A<sup>Cre</sup>–mediated Tbx21 deletion, CD4<sup>+</sup> T cells in mLN and LP failed to fully switch to Th1-like cells (Fig. 2F), and both the percentage and number of RFP<sup>+</sup> Th1-like cells were almost absent (90% reduction) in *H. hepaticus*–anti–IL-10R–treated IL-17A<sup>Tbet</sup> compared with IL-17A<sup>WT</sup> mice (Fig. 2D, 2E). A similar picture was observed in the LP of Rag1<sup>Tbet</sup> mice (Fig. 2D–F). It could be possible for bona fide Th1 cells to compensate for the reduction in Th17-derived Th1-like cells in the IL-17A<sup>Tbet</sup> mice. However, we found no difference in IFN-γ–producing CD4<sup>+</sup> RFP<sup>+</sup> cell proportions or numbers between this strain and IL-17A<sup>WT</sup> mice (Fig. 2D, 2E). That Tbx21 excision had worked efficiently in the two ΔTbet strains was confirmed by flow cytometry showing the absence of Tbet staining in IFN-γ and IL-17/IFN-γ–producing CD4<sup>+</sup> RFP<sup>+</sup> populations of IL-17A<sup>Tbet</sup> and Rag1<sup>Tbet</sup> mice, but the presence of Tbet in the CD4<sup>+</sup> RFP<sup>+</sup> cells from IL-17A<sup>WT</sup> and Rag1<sup>WT</sup> animals (Fig. 2G, 2H). Together, these data indicate that colonic immunopathology during *H. hepaticus*-induced typhlocolitis does not depend on the generation of Tbet- and IFN-γ–expressing ex-Th17 cells or the activity of Tbet; however, a role for IL-17/IFN-γ double producers cannot be excluded.

Conditional deletion of Tbet prevents Th17 to Th1 cell conversion in EAE

The role of specific CD4<sup>+</sup> Th subsets in EAE pathogenesis remains poorly understood with conflicting findings in the literature
The Th17 cell lineage dominates during inflammation. IL-17A fate-reporter mice (IL-17A<sup>WT</sup>) were subjected to MOG/CFA administration to induce EAE or given <i>H. hepaticus</i> (<i>Hh</i>) plus anti-IL-10R mAb to induce typhlocolitis, and the cytokine-secreting phenotype of Th17 lineage-positive (RFP<sup>+</sup>) CD4<sup>+</sup> cells was assessed at different time points. (A) Representative intracellular flow cytometry plots for IFN-γ and IL-17 of gated RFP<sup>+</sup>CD4<sup>+</sup>T cells during EAE (day 17) in iLN and CNS, or during <i>H. hepaticus</i> colitis (day 14) in mLN and large intestinal LP. (B) Dynamics of Th17 cell–derived populations as a proportion of RFP<sup>+</sup>CD4<sup>+</sup>T cells (<i>upper panels</i>) and their absolute numbers (<i>lower panels</i>) in indicated tissues during EAE induction (PEC, peritoneal exudate cells). Naive = prior to MOG/CFA administration, presym = presymptomatic (day 6), and peak = peak of clinical score (day 17). Values represent average ± SEM, n = 4/time point. (C) Representative staining for I-Ab/MOG<sub>38-49</sub> (<i>top flow panels</i>) and average distribution (<i>bottom panel</i>) in indicated T cell populations as proportion of CD4<sup>+</sup>CD44<sup>hi</sup>T cells harvested from the CNS of IL-17A<sup>WT</sup>IFN-γ<sup>eYFP</sup> mice at day 17 after EAE induction. Data are representative of two independent experiments (average ± SEM, n = 6). ***p < 0.001.
we found an enhanced proportion and a 3-fold increase in the number of IL-17 (and IL-17F) single-producing cells in the CNS of IL-17A\textsuperscript{ΔTbet} mice 17 d post-MOG peptide administration, indicating a greater stability of the IL-17–producing Th17 cell profile upon Tbx21 excision (Figs. 3B, 3C, 4D). Consistent with the findings in \textit{H. hepaticus}-induced colitis, Th17-derived IFN-γ+ Th1-like cells were absent in IL-17A\textsuperscript{ΔTbet} mice compared with IL-17A\textsuperscript{WT} controls at this same time point after EAE induction.
(Fig. 3A–C). However, in marked contrast to \textit{H. hepaticus}-induced colitis, both the proportion and number of IL-17/IFN-\(\gamma\) double-producing T cells were reduced (>95\%) during EAE (Fig. 3A–C). As Eomes can be an important mediator of T cell IFN-\(\gamma\) expression and T cell cytotoxicity (44), we next used mice in which Eomes is conditionally deleted in IL-17-expressing cells (IL-17A\(^{\Delta\text{Eomes}}\) mice). Our data show that the proportions of IL-17– and IFN-\(\gamma\)–producing T cells were indistinguishable in IL-17A\(^{\Delta\text{Eomes}}\) and IL-17A\(^{\Delta\text{Tbet}}\) control mice (Fig. 3A). Together, these results demonstrate that Tbet, but not Eomes, is required for the efficient conversion of Th17 cells to IL-17/IFN-\(\gamma\) double-producing and IFN-\(\gamma\) single-producing CD4\(^+\) T cells in the EAE model.

As Th17 cells have also been shown to convert to Tfh in the intestine (45), we next analyzed Th17-derived Tfh cells in Peyer’s patches in nonimmunized IL-17A\(^{\Delta\text{Tbet}}\) versus IL-17A\(^{\Delta\text{Tbet}}\) mice. Th17-derived Tfh cells (identified as CD4\(^+\)RFP\(^{+}\)CXCR5\(^{+}\)PD-1\(^{+}\) cells) were detected in similar proportions in Peyer’s patches of IL-17A\(^{\Delta\text{Tbet}}\) controls and IL-17A\(^{\Delta\text{Tbet}}\) mice (Fig. 3D), together suggesting that Tbet is not required for the conversion of Th17 cells to Tfh cells. Furthermore, it highlights that Tbet is not required for the conversion of Th17 cells per se, but only for the generation of IFN-\(\gamma\)–producing Th1-like cells. These results indicate that the IL-17A\(^{\Delta\text{Tbet}}\) mouse is a promising model to specifically study the role of Th17 to Th1 plasticity in autoimmunity and infection.

\textit{Tbet-deficient Th17 cell populations have an altered cytokine profile}

Because Th17 cells and IL-17/IFN-\(\gamma\) double-producing T cells have been implicated in the pathogenesis of EAE, we analyzed the cytokine profile of the Th17 cell lineage in the presence or absence of Tbet. Seventeen days after induction of EAE, the CD4\(^+\) T cell populations present in the CNS were analyzed for their cytokine profile by flow cytometry. As expected, inflammatory cytokines assayed, with the exception of GM-CSF, were enriched within the RFP\(^+\) Th17 cell lineage compared with the RFP\(^-\) population (Fig. 4A–C) (20). No significant difference in cytokine production by the RFP\(^-\) non-Th17 lineage-derived cells was found between IL-17A\(^{\Delta\text{Tbet}}\) and IL-17A\(^{\Delta\text{Tbet}}\) hosts (Fig. 4A–C).

The prevention of Th17 to Th1 cell conversion in IL-17A\(^{\Delta\text{Tbet}}\) mice resulted in changes in cytokine profiles of the Th17 cell–derived populations. IL-17F–expressing cells were significantly increased in the Tbet-deficient Th17 cell population (Fig. 4A, 4D). This increase in number was found in all RFP\(^+\) Th17 cell–derived populations independent of their IL-17 expression profile. The proportion and number of GM-CSF–expressing Th1 cells, a cytokine strongly associated with autoimmunity and required for the induction of EAE (46), were significantly altered in the Th17 cell population from IL-17A\(^{\Delta\text{Tbet}}\) mice compared with controls. Thus, in the absence of Tbet, there were more IL-17F\(^+\) GM-CSF\(^-\) RFP\(^+\) cells, whereas in the presence of Tbet there were more IL-17F\(^+\) GM-CSF\(^+\) RFP\(^+\) cells (Fig. 4B).

This increase in number was found in all RFP\(^+\) Th17 cell–derived populations independent of their IL-17 expression profile. The proportion and number of GM-CSF–expressing CD4\(^+\) T cells was reduced, the total number of cells expressing GM-CSF was not significantly altered when the Th17 cell subset was Tbet sufficient or deficient (Fig. 4D). Expression of TNF followed a similar pattern as GM-CSF. In the absence of Tbet, there were more IL-17F\(^+\) TNF\(^+\) RFP\(^+\) cells, whereas in the presence of Tbet there were more that have lost their IL-17 expression (IL-17\(^{-}\) TNF\(^{+}\) RFP\(^{+}\)) cells (Fig. 4C). The combination of RFP\(^+\) cells expressing both IL-17 and TNF was significantly higher in the IL-17A\(^{\Delta\text{Tbet}}\) mice compared with the...
IL-17A<sup>WT</sup> controls (Fig. 4C). The overall proportion of TNF-producing cells was, however, not significantly different in CD4<sup>+</sup> T cells from IL-17A<sup>ΔTbet</sup> mice compared with IL-17A<sup>WT</sup> controls (76.5 ± 6) (Fig. 4D). These results suggest that TNF and GM-CSF expression do not depend on the expression of Tbet and may precede the conversion of Th17 to Th1-like cells. Furthermore, it shows that Tbet expression alters the combination of cytokines simultaneously expressed by the same T cell, but does not affect the total number of TNF- or GM-CSF–producing CD4<sup>+</sup> T cells present in the CNS.

**Th17 to Th1 cell conversion is not required for EAE pathogenesis**

As IL-17/IFN-γ double-producing T cells have been associated with autoimmune and inflammatory pathology (19, 21, 47, 48), we next investigated the susceptibility of the IL-17A<sup>ΔTbet</sup> mouse to EAE. We found no difference with respect to timing of EAE onset between IL-17A<sup>ΔTbet</sup> and IL-17A<sup>WT</sup> controls (Fig. 5A). However, the maximum clinical scores were reduced in IL-17A<sup>ΔTbet</sup> hosts (Fig. 5A). Tbet and other factors implicated in immunopathology, such as GM-CSF, are not exclusively expressed by the Th17 cell lineage, so we next assessed the susceptibility of Rag1<sup>ΔTbet</sup> mice in which Tbet was conditionally deleted via Rag1-Cre in all lymphocytes. In this case, Rag1<sup>ΔTbet</sup> mice showed a more pronounced reduction in EAE susceptibility, with later onset and lower maximum clinical score than IL-17A<sup>WT</sup>, Rag1<sup>WT</sup> controls, and IL-17A<sup>ΔTbet</sup> mice (Fig. 5A). This finding indicates that blocking Th17 to Th1 cell conversion as well as de novo Th1 cell differentiation had a more pronounced impact on reducing EAE pathogenesis than removal of Tbet in IL-17–expressing cells only. However, we cannot exclude an additional role for Tbet in other lymphocytes that once expressed Rag1. As we observed in the IL-17A<sup>ΔTbet</sup> animals, Rag1<sup>ΔTbet</sup> mice showed an increased proportion and number of IL-17–producing cells and a marked decrease in the proportion and number of IFN-γ–producing CD4<sup>+</sup> T cells in the CNS at day 17 post-MOG immunization (Fig. 5B). Although no difference in the proportion of GM-CSF–producing T cells was observed in Rag1<sup>ΔTbet</sup> mice compared with Rag1<sup>WT</sup> controls, the number of GM-CSF–producing CD4<sup>+</sup> T cells in the CNS was reduced in the former animals (Fig. 5B). The reduction in IFN-γ– and GM-CSF–producing...
T cells in the CNS of Rag1<sup>+</sup> mice correlated with reduced maximum EAE scores (Fig. 5A).

We subsequently investigated whether the block in Th17 to Th1 cell conversion was maintained long-term in vivo. Seventeen days following MOG<sub>35–55</sub> immunization of IL-17A<sup>WT</sup> and IL-17A<sup>Dtbet</sup> mice, RFP<sup>+</sup> CD4<sup>+</sup> T cells were isolated from the draining iLN and transferred to Rag2<sup>2/2</sup> hosts. Upon subsequent MOG<sub>35–55</sub>/CFA immunization of the recipient mice, a delayed onset of EAE was observed in the group receiving IL-17A<sup>Dtbet</sup>RFP<sup>+</sup>CD4<sup>+</sup> T cells, although equally high clinical scores were observed in both host groups (Fig. 5C).

Furthermore, the majority of IL-17A<sup>WT</sup> control cells had converted to a Th1-like IFN-γ–expressing phenotype (Fig. 5D), whereas IL-17A<sup>Dtbet</sup> cells remained stable in their IL-17–expressing profile and did not express IFN-γ (Fig. 5D). The distribution of TNF and GM-CSF was also similar to that seen in the respective donor mice (Fig. 4B, 4C), with the majority of GM-CSF– and TNF-producing cells found among the IFN-γ–producing cells in IL-17A<sup>WT</sup> controls, but within IL-17–producing cells in IL-17A<sup>Dtbet</sup> cells (Fig. 5D).

Although the majority of CD4<sup>+</sup> T cells encountered in the CNS in both IL-17A<sup>Dtbet</sup> and IL-17A<sup>WT</sup> control mice were originally derived from the Th17 cell subset, as we reported before in IL-17A<sup>WT</sup> control mice (20), it was possible that Ag specificity could have risen in the bona fide Th1 cell population. However, MOG<sub>38–49</sub> tetramer staining in IL-17A<sup>Dtbet</sup>IFN<sup>γ</sup>eYFP mice at day 17 after EAE induction revealed that Ag specificity remained within the RFP<sup>+</sup> populations, as previously seen in IL-17A<sup>WT</sup>IFN-γeYFP control mice (Fig. 1C), but was particularly enriched within the few remaining IL-17/IFN-γ double producers (Fig. 5E).

**RORγt is required to maintain Th17 cells**

Th17 cells rely on the RORα and especially RORγt for their differentiation (9, 49). Hence, the absence of RORγt prevents...
the differentiation of Th17 cells and susceptibility to EAE (9, 49). However, it is not clear whether these orphan receptors remain important for Th17 cell maintenance. This is of particular importance for potential therapeutic targeting of Th17 cells in inflammatory disorders. Thus, we isolated naive CD4+ T cells from Rag1CreRorcfl/fl Rosa26stopRORyt (herefrom called Rag1ARORyt), IL-17ACre RORytfl/fl Rosa26stopRFP (from hereon called IL-17AARORyt), and their respective Rag1WT and IL-17AWT controls, and differentiated the cells in vitro toward the Th17 subset. In vitro polarization of naive CD4+ T cells from Rag1ARORyt and IL-17AARORyt cells into Th1 or Th17 was indistinguishable from their respective controls (Fig. 6A). Moreover, IL-17A-expressing cells compared with controls (Fig. 6E). The reduction in GM-CSF–producing T cells remaining in IL-17AARORyt mice did not significantly delay the onset or final clinical score of EAE, but resulted in a minor delay in disease progression (Fig. 6C). Detailed analysis of the CD4+ T cell compartment in the CNS of IL-17AARORyt mice revealed a significant reduction (71% ± 12%) in RFP+CD4+ T cells compared with that observed in IL-17AWT controls, a number that contributed to the reduced total numbers of CNS-infiltrating T cells in the former strain (Fig. 6D). The limited number of RFP+ CD4+ T cells remaining in IL-17AARORyt mice did not express IL-17 or IL-17F, but did produce IFN-γ, with reduced proportions of TNF and GM-CSF–positive cells compared with controls (Fig. 6E). The reduction in GM-CSF–producing cells in IL-17AARORyt mice was not compensated by GM-CSF production from the RFP+ T cell CNS infiltrate (Fig. 6F). As a result, the total number of CD4+ T cells producing IL-17, IL-17F, GM-CSF, and TNF was significantly reduced in the IL-17AARORyt mice (Fig. 6G). However, no significant difference in numbers of total IFN-γ–producing CNS-infiltrating T cells was found between IL-17AWT and IL-17AARORyt mice (Fig. 6G). These data indicate that Th17 cells require RORyt not only for their initial generation, but also for their IL-17 production and long-term survival. Importantly, once Th17 cells have been generated, the excision of Rorc did not significantly affect the clinical outcome of EAE despite the significant reduction in IL-17– and GM-CSF–producing CD4+ T cells.

Discussion

The mechanism underlying the pathogenicity of T cells and the identity of CD4+ T cells instrumental for the onset and maintenance of immunopathology, especially those inducing EAE, are still debated in the literature. In this study, we demonstrate that Th17 cells and their Tbet and IFN-γ-expressing progeny are the predominant populations of T cells present in EAE and H. hepaticus-induced typhlocolitis, in line with their established role as potent effector cells contributing to immunity and immunopathology (10, 11). To our knowledge, for the first time, we assessed the influence of the excision of Tbet, Eomes, RORα, and RORyt, in all lymphocytes or in IL-17–expressing cells only, in the development of immunopathology in vivo. We show that neither the IFN-γ–producing Th17 cell progeny (ex-Th17 and IL-17IFN-γ double producers in the case of EAE, or ex-Th17 cells in the case of H. hepaticus colitis) nor long-term Th17 cell maintenance (in the case of EAE) is essential for the establishment of T cell–mediated immunopathology.

Numerous studies have shown that distinct populations of T cell subsets have the capacity to induce pathology upon adoptive transfer into lymphopenic or T cell–sufficient hosts, with different types of EAE as a result (50, 51). However, criticism has been raised that these cells, often bearing an Ag-specific TCR and polarized in vitro with a mix of cytokines, may not accurately recapitulate the phenotype of in vivo generated effector T cells. Our study did not make use of TCR transgenic mice or the transfer of in vitro cultured cells. Instead, we employed conditional deletion, either Rag1Cre– or IL-17ACre–mediated, of genes of interest and tracked the Th17 population and its progeny with an RFP lineage marker. We show that the generation of IL-17/IFN-γ double-producing T cells requires the expression of Tbx21 in Th17 cells during EAE. In stark contrast to the EAE model, we further demonstrate that Tbet is not an absolute requirement for the generation of these double-producing lymphocytes, as these cells were readily found in H. hepaticus typhlocolitis in Rag1Tbet and IL-17AΔTbet mice. This may highlight the different microenvironments present in the intestine compared with the CNS, providing different cues enabling the development of double-producing T cells. Furthermore, it re-emphasizes the high degree of plasticity of Th17 cells and the extraordinary tailored response of the immune system, depending on microorganisms encountered, as well as the site of inflammation.

IL-17/IFN-γ double-producing T cells have been found during active colitis in mice and humans (18, 21, 52, 53); however, their contribution to intestinal pathology is largely unknown. A recent study by Harbour et al. (24) using adoptive transfer into lymphopenic hosts of in vitro polarized Th17 cells from Tbx21−/− mice showed that these cells were unable to induce colitis, despite unaffected in vivo generation of IL-17/IFN-γ double-producing cells in the recipients. In contrast, we have previously demonstrated that IL-17/IFN-γ double-positive T cells isolated from the large intestine of H. hepaticus-infected colitic mice are able to induce colitis upon transfer to H. hepaticus-infected Rag2−/− mice (16), indicating that ex vivo IL-17/IFN-γ double-producing lymphocytes isolated from Tbet-sufficient mice can induce intestinal pathology. Moreover, as shown in the current study, IL-17ACre– or Rag1Cre–mediated excision of Tbx21 in cells once expressing IL-17 or Rag1 had no effect on the number of IL-17/IFN-γ– double-producing cells, nor on the severity of immunopathology in H. hepaticus colitis. Hence, in vivo polarized cells or those encountering specific cues associated with particular pathogens such as H. hepaticus may directly contribute to colitis, independently of their ability to express Tbet. Of note, Rag1Cre–mediated excision of Tbx21 did result in the presence of IL-17/IFN-γ double-producing cells in the absence of bona fide Th1 cell development (6), indicating that double producers are most likely Th17 cell derived.

The generation of Th17-derived Th1-like cells, which have lost the expression of IL-17, was dependent on the presence of Tbet in both the H. hepaticus typhlocolitis and EAE models. Importantly, the excision of Tbx21 in IL-17–producing cells had no impact on H. hepaticus-induced intestinal pathology, indicating that, in this model, Th17 cell transition to IFN-γ–producing Th1-like cells is not absolutely required for colitis development. These findings are in contrast to those by Harbour et al. (24), who concluded that Tbet expression by Th17 cells is required for their transition to Th1-like cells and for mediating transfer colitis. Among possible explanations for this discrepancy is the use of different colitis models and the use of mice in which Tbx21 is excised in vivo upon IL-17 or Rag1 expression in our study versus the use of in vitro differentiated Th17 cells from Tbet-deficient mice in the report by Harbour et al. (24). In contrast to the findings in the H. hepaticus
FIGURE 6. Th17 cell maintenance is not required for EAE immunopathology. (A) Flow cytometry for IFN-γ and IL-17 from naive T cells from Rag1WT and Rag1ΔRORα mouse lines polarized in vitro toward Th1 or Th17 cells. (B) Clinical EAE scores of Rag1WT and Rag1ΔRORα mouse lines immunized with MOG/CFA (n = 6-7). (C) Clinical EAE scores of IL-17AWT and IL-17AΔRORγt mouse lines immunized with MOG/CFA (n = 8/group). (D) Numbers of total CD4+ T cells, RFP+ Th17 lineage-positive, and RFP- Th17 lineage-negative cells present in the CNS of IL-17AWT controls (white bars) or IL-17AΔRORγt mice (gray bars) upon EAE induction at day 17 (averages ± SEM, n = 6). (E and F) Flow cytometry for indicated cytokines on RFP+ Th17 lineage-positive (E) or RFP- lineage-negative (F) cells obtained from IL-17AWT and IL-17AΔRORγt mice undergoing EAE, as shown in (C). (G) Numeric presence of total CD4+ T cells expressing indicated cytokines in the CNS of IL-17AWT controls (white bars) or IL-17AΔRORγt (gray bars) during EAE at day 17 (averages ± SEM, n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.
colitis model, IL-17–mediated deletion of Tbx21 had a mild reducing impact on EAE. These results are in line with recent studies indicating that pathogenicity of IFN-γ-producing T cells is independent or partially dependent on Tbet (22, 31, 54). Two studies made use of in vitro stimulated and adoptively transferred Ag-specific T cells from Tbx21-deficient mice or CD4Cre-mediated Tbx21 excision (22, 31). Duhnen et al. (54) used gene knockout mice or CD4Cre-mediated gene excision, affecting several lineages and cell types, including CD8 T cells. We extended these observations by employing Tbx21 excision specifically in IL-17-producing cells, and conclude that the absence of Tbet in vivo differentiated Th17 cells has limited impact on immunopathology in the intestine and CNS. In line with CD4Cre-mediated or germline deletion of Eomes, we failed to observe effects of Eomes on Th17 cell polarization, plasticity, or immunopathology in the EAE model (data not shown). The removal of Tbx21 in all lymphocytes, through Rag1Cre-mediated deletion, resulted in a much more pronounced reduction of EAE scores, although clinical symptoms were not completely ameliorated. In this case, both bona fide Th1 cells as well as Th17-derived Th1-like cells were largely absent, yet some immunopathology was still observed. This suggests that even an interplay between Th1- and Th17-derived Th1-like cells is not essential for the development of EAE.

In the current study, to our knowledge, we addressed for the first time whether the maintenance of Th17 cells or their progeny is important for the immunopathology observed in EAE. We found a minor contribution of RORα+ cells in EAE, in line with a more essential role of RORγt in Th17 cell differentiation (9, 49). Moreover, the excision of Rorc after the generation of Th17 cells resulted in rapid loss of Th17 cells, in line with results from pharmacological inhibition of RORγt (55). This reveals an important role for RORγt in maintaining Th17 cells after their generation in addition to their differentiation. The remaining Th17-derived cells exclusively produced IFN-γ, in line with their loss of Rorc that is required for the Th17 lineage program, including the expression of IL-17 and IL-17F (9). Interestingly, despite the significant reduction of cells expressing IL-17, the marked loss of Th17 cell progeny, and cells expressing GM-CSF in the CNS of IL-17AΔRORγt mice, the onset and pathology of EAE were only mildly affected.

Although no significant reduction in EAE upon IL-17AΔCre-mediated Tbx21 or Rorc excision was observed, this does not exclude a role for Th17 cells in the initiation of EAE. We found that both MOG Ag specificity and the majority of other cytokines implicated in EAE pathogenicity, such as IFN-γ, TNF, and GM-CSF, were found within the Th17 cell–derived lineages. Upon deletion of Tbet within the Th17 subset, the MOG Ag specificity did remain within the Th17 cell lineage. Although MOG Ag is not the only Ag involved in EAE, it suggests that Th17 cell polarization at the initiation of EAE is sufficient to enable entry to the CNS (56). Moreover, it is clear that factors implicated in immunopathology, such as TNF and GM-CSF, are not exclusive for the Th17 cell–derived lineage found in the CNS. Cytokine profiles were altered within the Th17 cell subsets upon Tbet deletion, modifying the combinations of cytokines secreted by the same T cell. It also remains possible that the absence of Tbet does allow for a partial conversion of Th17 cells, but without terminating IL-17 expression or initiating the Tbet transcriptional program such as IFN-γ expression. Although combinations of cytokines produced by the same cell, such as TNF in combination with either IL-17 or IFN-γ that result in distinct cellular responses (57, 58), were altered upon excision of Tbx21 or Rorc, the effect on immunopathology was limited. Because GM-CSF has been shown to be necessary for the development of EAE (46, 59, 60), the alteration of T cell populations producing GM-CSF/TNF in combination with IL-17 or IFN-γ may not significantly impact on immunopathology.

Extensive studies to find the pathogenicity factor(s) have focused on the Th17 cell subset with potential novel mediators of pathology reported (61). However, inflammation is characterized by diversity in cell subsets, mediators, as well as clinical course and drug responsiveness (51). Important cytokines in the Th1 and Th17 cell axis, with the exception of IL-6 and IL-23, have been reported to be dispensable for the induction and clinical disease progression of EAE (51, 62, 63). Our work implies that a focus on a particular Th subset during the pathology phase of disease may be of limited clinical benefit. In summary, our results contribute to a growing body of evidence that immunopathology cannot be attributed to a single lineage of Th cells. Instead, it is likely that multiple Th cell lineages and immune cell types contribute to immunopathology. Until the identification of a lineage-independent pathogenicity factor, disease-modifying therapies may need to continue to be targeted more broadly.

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Disclosures

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