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Research Article Immunology

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IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis

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IL-17–producing CD8+ T (Tc17) cells are detectible in multiple sclerosis (MS) lesions; however, their contribution to the disease is unknown. To identify functions of Tc17 cells, we induced EAE, a murine model of MS, in mice lacking IFN regulatory factor 4 (IRF4). IRF4-deficient mice failed to generate Tc17 and Th17 cells and were resistant to EAE. After adoptive transfer of WT CD8+ T cells and subsequent immunization for EAE induction in these mice, the CD8+ T cells developed a Tc17 phenotype in the periphery but could not infiltrate the CNS. Similarly, transfer of small numbers of WT CD4+ T cells alone did not evoke EAE, but when transferred together with CD8+ T cells, IL-17–producing CD4+ (Th17) T cells accumulated in the CNS and mice developed severe disease. Th17 accumulation and development of EAE required IL-17A production by CD8+ T cells, suggesting that Tc17 cells are required to promote CD4+ T cell–mediated induction of EAE. Accordingly, patients with early-stage MS harbored a greater number of Tc17 cells in the cerebrospinal fluid than in peripheral blood. Our results reveal that Tc17 cells contribute to the initiation of CNS autoimmunity in mice and humans by supporting Th17 cell pathogenicity.

Introduction

Multiple sclerosis (MS) is an incurable inflammatory autoimmune disease of the CNS that affects several million people worldwide. The murine model of MS, EAE, can be induced by activation or adoptive transfer of CD4+ Th cells that recognize myelin antigens and cross the blood-brain barrier. Activation of autoreactive Th cells is, therefore, believed to be important for the induction, maintenance, and regulation of inflammatory demyelination in EAE and MS (1). Several lines of evidence indicate that Th17 cells, which can produce IL-17A, IL-17F, IL-21, and IL-22, are involved in the onset and maintenance of EAE (2). Previously, we and others have described the essential role of IFN regulatory factor 4 (IRF4), a member of the IRF family of transcription factors (3, 4), for Th17 cell differentiation and EAE (5–8).

Although CD8+ T cells are also present in MS lesions, their role in the disease is unclear (1). Conflicting evidence from studies of EAE suggests pathogenic (9, 10) or beneficial (11, 12) functions of these cells. Recently, an IL-17–producing CD8+ T cell subpopulation, termed Tc17, was described in mice and humans (13–16). Compared with canonical CTLs, Tc17 cells exert many less cytotoxic effector functions, due to their greatly diminished levels of the T-box transcription factor Eomesodermin (Eomes), of IFN-γ, and of the cytolytic molecule granzyme B. Tc17 cells are detectable in MS lesions (17) and in the CNS and LNs of mice during EAE (16), but their function remained undefined.

In this study, we analyzed (a) molecular requirements for Tc17 differentiation, (b) function of Tc17 cells during EAE, and (c) their presence in patients with early-stage MS. We show that IRF4 is pivotal for differentiation of Tc17 cells in vitro and in vivo during CNS autoimmunity. Using IRF4-deficient mice, we demonstrate a previously unknown cooperation of Tc17 and Th17 cells for the induction of EAE. The pathogenic interplay requires IL-17A but not CCR6 competence by CD8+ T cells and CCR6 but not IL-17A sufficiency by CD4+ T cells. Along with the in vivo data, we demonstrate a direct, cell contact–mediated helper activity of Tc17 cells for Th17 differentiation in vitro. Furthermore, increased numbers of Tc17 are detectable in cerebrospinal fluid (CSF) from patients with early-stage MS, suggesting their contribution to disease progression in humans.

Results

IRF4 governs Tc17 differentiation by balancing the levels of RORγt, Eomes, and Foxp3. As a prerequisite for our concept to use *Irf4*$^{-/-}$ mice in order to study the role of CD8+ T cells during EAE, we first analyzed the dependence of Tc17 differentiation on IRF4. Therefore, we primed CD8+ T cells from *Irf4*$^{+/+}$ (WT) or *Irf4*$^{-/-}$ mice under conditions favoring CTL differentiation or with IL-6 and TGF-β added alone or in combination (Tc17 condition) and found that IRF4 was mandatory for the development of Tc17 cells, as determined by intracellular staining (Figure 1A). Consistent with the defect in IL-17 production, the mRNA levels

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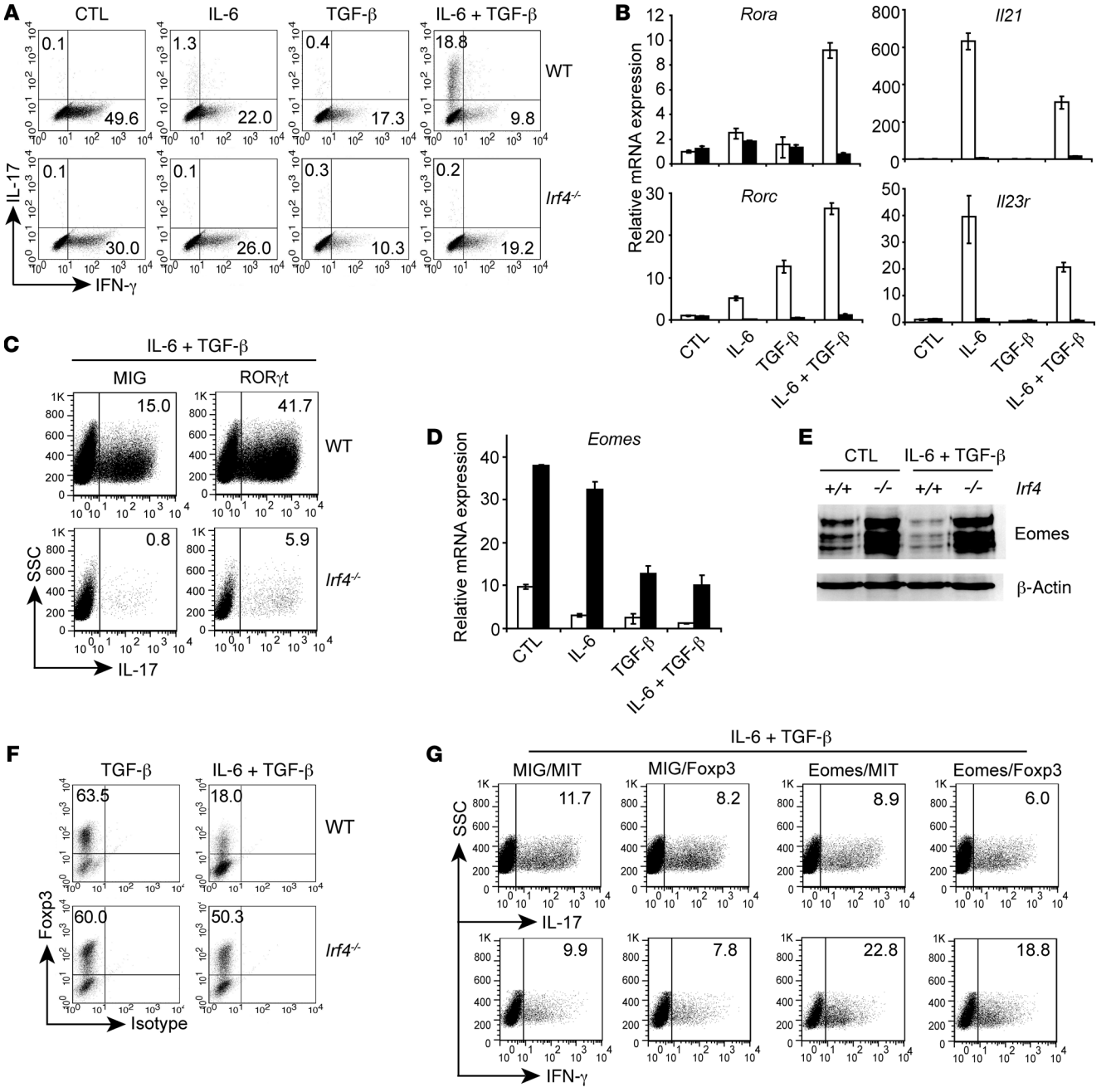

Figure 1

Tc17 differentiation depends on IRF4. (**A** and **F**) Purified WT and *Irf4*$^{-/-}$CD8$^+$ T cells activated via CD3/28 plus cytokines as indicated were stained for IFN-γ, IL-17, or Foxp3. (**B** and **D**) mRNA expression of the indicated genes analyzed by quantitative real-time RT-PCR in *Irf4*$^{-/-}$ (black) or WT (white) CD8$^+$ T cells. Data (± SD) represent PCR duplicates. (**C** and **G**) WT or *Irf4*$^{-/-}$CD8$^+$ T cells transduced with retroviruses expressing RORγt-GFP (RORγt), Eomes-VP16-GFP (Eomes), Foxp3-Thy1.1 (Foxp3), or the control viruses MSCV-GFP (MIG) or MSCV-Thy1.1 (MIT) activated as indicated and stained for IL-17 or IFN-γ. Analyses were performed on a (**C**) GFP$^+$ or (**G**) Thy1.1$^+$GFP$^+$ gate. (**E**) Western blot for Eomes and β-actin in WT and *Irf4*$^{-/-}$CD8$^+$ T cells after 72 hours of activation. (**A**, **C**, **F**, and **G**) Numbers represent percentages of positive cells. (**A**–**G**) Data are representative of 3 independent experiments.

for factors characteristic for Tc17 differentiation (14–16), such as RORγt (*Rorc*) and RORα (*Rora*) as well as the IL-23 receptor (*Il23r*) and the cytokine *Il21*, were strongly diminished in *Irf4*$^{-/-}$ CD8$^+$ T cells (Figure 1B). To analyze whether this block in Tc17 differentiation was caused by defective induction of RORγt, we overexpressed RORγt in WT and *Irf4*$^{-/-}$CD8$^+$ T cells cultured under Tc17 conditions. Forced expression of RORγt led to strongly enhanced IL-17 production in WT cells and, albeit at a markedly lower level, also in *Irf4*$^{-/-}$ cells (Figure 1C). Thus, RORγt is necessary but not sufficient to restore the Tc17 phenotype in *Irf4*$^{-/-}$ cells and additional mechanisms, such as interplay with other transcription factors, are likely to be relevant.

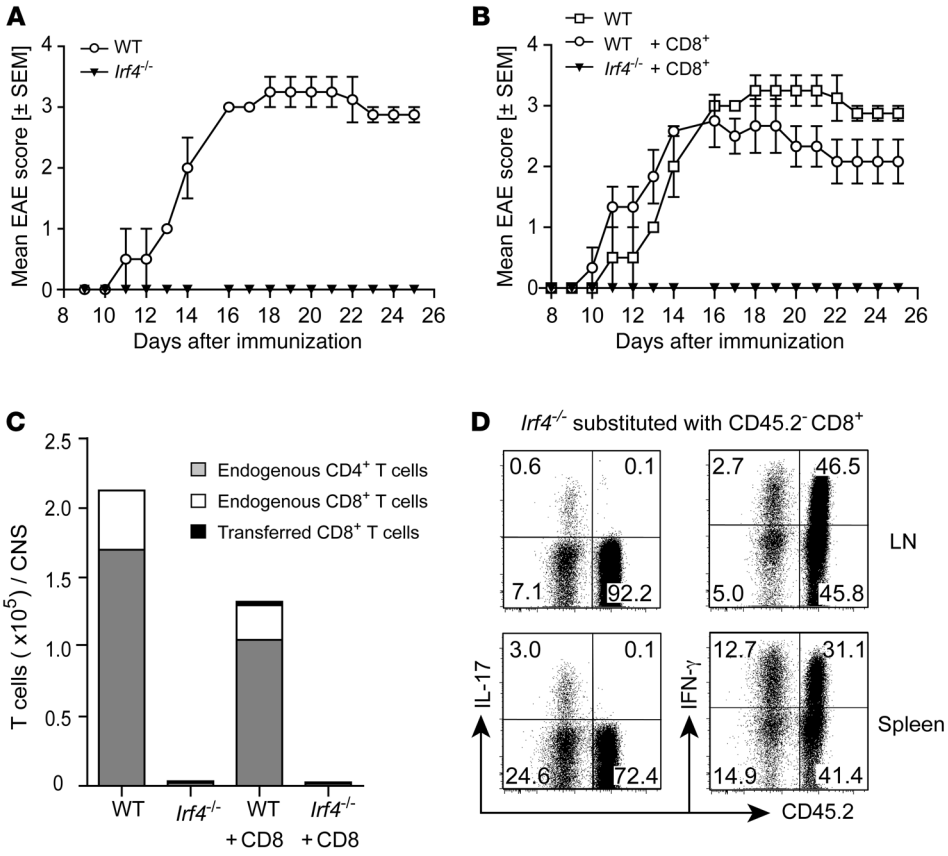

Figure 2
Tc17 cells do not migrate into the CNS in an IRF4-deficient environment. (**A** and **B**) Mean clinical scores (± SEM) of $MOG_{37–50}$-induced EAE in WT mice ($n = 4$) and *Irf4*$^{-/-}$ mice ($n = 4$) (**A**) without or (**B**) with transfer of congenic 10^7 CD45.2$^-$CD8$^+$ WT T cells. (**C**) Absolute T cell number per CNS (endogenous or transferred, averages of pooled cells of 4 mice). (**D**) CD8$^+$ gate of cells from LNs and spleens of *Irf4*$^{-/-}$ mice substituted with 10^7 CD45.2$^-$CD8$^+$ WT T cells stained for CD8, CD45.2, IL-17, or IFN-γ at day 19 after immunization. Numbers represent percentages of cells in the respective quadrant. (**A**–**D**) Data are representative of 6 independent experiments.

We and others have previously published that the amounts of the CTL-specific transcription factor Eomes (18) negatively correlated with Tc17 development (16, 19, 20). Notably, the expression of Eomes at the mRNA and protein level (Figure 1, D and E) was markedly enhanced in *Irf4*$^{-/-}$ cells as compared with that in WT CD8$^+$ T cells, even under Tc17 conditions.

In CD4$^+$ T cells, IL-6 acts as a switch factor between Th17 and Treg cells by upregulating RORγt and RORα and by suppressing TGF-β induced Foxp3 (2). When we tested Foxp3 expression, we found that, similar to CD4$^+$ T cells (5), culture with TGF-β induced high Foxp3 expression in WT and *Irf4*$^{-/-}$CD8$^+$ T cells, whereas addition of IL-6 downregulated Foxp3 in WT but not *Irf4*$^{-/-}$CD8$^+$ T cells (Figure 1F).

Because the protein levels for Eomes and Foxp3 were upregulated in *Irf4*$^{-/-}$CD8$^+$ T cells (Figure 1, E and F), we examined whether enhanced expression of these transcription factors influences IL-17 production. Therefore, WT CD8$^+$ T cells were infected with viruses expressing Eomes-GFP and/or Foxp3-Thy1.1 and cultured under Tc17 conditions. Compared with infection with control viruses, forced expression of either Eomes or Foxp3 suppressed the frequencies of IL-17–positive cells to a limited extent, while transduction with Eomes considerably increased the percentage of IFN-γ–positive cells, as expected (18). Coexpression of Eomes and Foxp3 led to an additive inhibition of IL-17 production (Figure 1G), while the high IFN-γ production induced by Eomes alone was not significantly influenced by Foxp3 coexpression. The effects on the amounts of IL-17 and IFN-γ in doubly infected cells were then analyzed based on the relative intensities of GFP and Thy1.1 expression, representing the relative expression of Eomes and Foxp3, respectively. Importantly, within the same culture reduction of IL-17 and enhancement of IFN-γ production, both correlated with the ratio of Eomes and Foxp3 expression (Supplemental Figure 1, A–C; supplemental material available online with this article; doi:10.1172/JCI63681DS1), strongly implying that Eomes and Foxp3 suppress IL-17 production cell-intrinsically in a quantitative manner. Thus, the increase of Eomes and Foxp3 expression in *Irf4*$^{-/-}$CD8$^+$ T cells, together with reduced amounts of RORγt and RORα, cumulatively contribute to impaired *Irf4*$^{-/-}$ Tc17 differentiation.

We confirmed the role of IRF4 during Tc17 differentiation also in WT CD8$^+$ T cells after transient knockdown using siRNA. Along with reduction of IRF4 protein amounts (Supplemental Figure 2A), nucleofection with IRF4-specific siRNA (IRF4si), but not control scrambled siRNA, substantially reduced expression of *Rorc* mRNA and frequencies of IL-17–producing cells (Supplemental Figure 2, B and C). Conversely, the expression of Eomes, IFN-γ, and Foxp3 was elevated. Together, these results point to a central role of IRF4 in balancing the levels of the transcription factors Eomes, Foxp3, and RORγt during Tc17 differentiation.

Contribution of IRF4 and Tc17 cells to the induction of EAE. To explore the IRF4 requirement for Tc17 differentiation in vivo, we used an EAE model characterized by the presence of Tc17 cells (16). WT but not *Irf4*$^{-/-}$ mice immunized with the myelin oligodendrocyte glycoprotein amino acids 37–50 ($MOG_{37–50}$) peptide developed severe EAE (Figure 2A) accompanied by demyelination as well as prominent CNS infiltration with CD3$^+$ and MAC3$^+$ cells (Supplemental Figure 3A). Moreover, CD8$^+$ T cells able to produce IL-17 and/or IFN-γ were present in LNs and CNSs of diseased WT mice (Supplemental Figure 3B). In contrast, almost no cell infiltrates

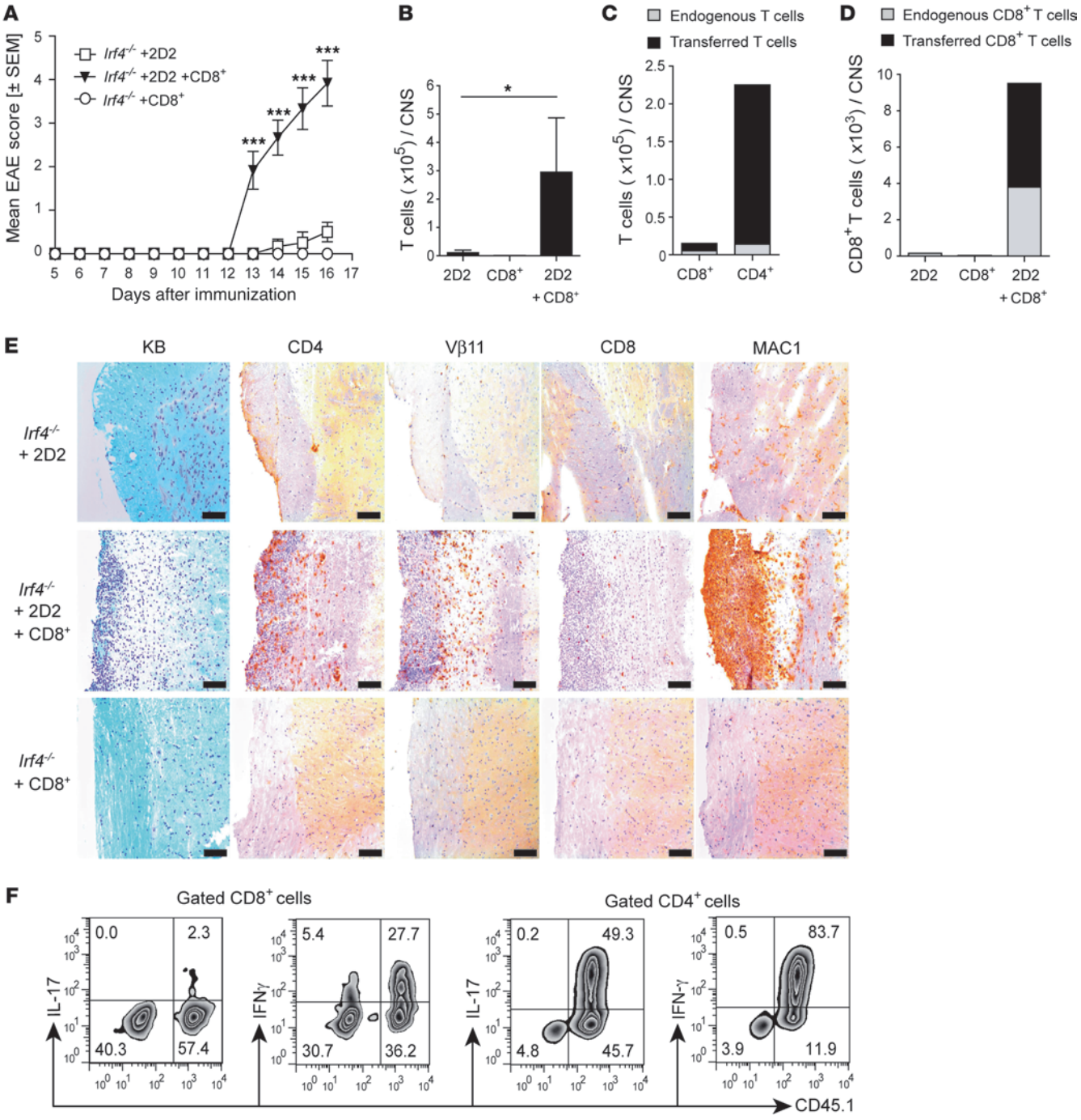

Figure 3

CD8+ T cells mutually interact with CD4+ T cells to induce EAE. (**A**) Mean EAE scores (± SEM) combining 2 independent experiments of $MOG_{37–50}$-immunized *Irf4*$^{-/-}$ mice ($n = 6$) that received sorted congenic 2.5×10^6 CD45.1+CD44^{lo}CD8+ and/or 10^4 CD62L^{hi}CD45.1+ 2D2 T cells. *P* values were calculated comparing the scores of *Irf4*$^{-/-}$ mice transferred with 2D2 cells alone or in combination with CD8+ T cells. (**B** and **D**) Absolute numbers in the CNSs of *Irf4*$^{-/-}$ mice of (**B**) T cells (mean ± SEM, $n = 4$) or (**D**) CD8+ T cells after transfer of 2D2 or CD8+ T cells alone or in combination. (**C**) Absolute numbers of CD8+ compared with CD4+ T cell numbers after cotransfer of 2D2 and CD8+ T cells. (**C** and **D**) Averages of pooled cells of 4 mice at day 15 after immunization. (**E**) Histology of spinal cords at day 15 after immunization. Immunochemically stained cells were detected as red-brown foci. Scale bar: 100 μm. KB, Klüver-Barrera. (**F**) Flow cytometry of gated CD4+ or CD8+ CNS cells after PMA/ionomycin restimulation. Numbers represent percentages of cells in the respective quadrant. (**A**–**F**) The experiments were repeated 4 times with consistent results. *$P < 0.05$; ***$P < 0.001$.

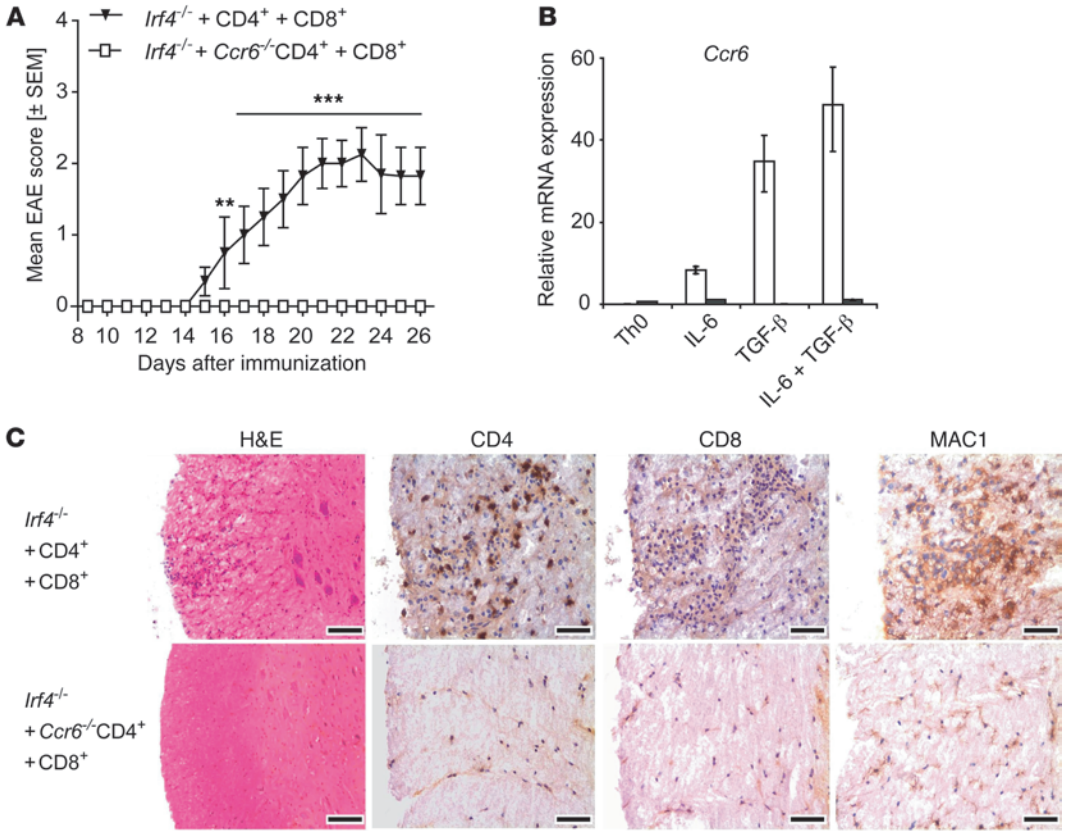

Figure 4

CD4+ T cells require CCR6 to cooperate with CD8+ T cells for infiltration into the CNS. (**A**) Mean clinical scores (± SEM) of $MOG_{37–50}$-immunized *Irf4*$^{-/-}$ mice (n = 4) substituted with 10^7 WT CD45.2$^-$CD8$^+$ T plus 2.5 × 10^6 WT CD45.2$^+$CD4$^+$ T cells or *Ccr6*$^{-/-}$CD45.2$^+$CD4$^+$ T cells. P values were calculated comparing the scores of *Irf4*$^{-/-}$ mice transferred with WT CD8$^+$ T cells plus either WT CD4$^+$ or *Ccr6*$^{-/-}$CD4$^+$ T cells. (**B**) qRT-PCR for *Ccr6* mRNA in *Irf4*$^{-/-}$ (black) or WT (white) CD4$^+$ T cells stimulated for 2 days as described. Data (± SD) of PCR duplicates. (**C**) Histology of spinal cords at day 26 after immunization: H&E staining (scale bar: 100 μm); immunochemically stained cells were detected as brown foci (scale bar: 50 μm). (**A**–**C**) The experiments were repeated twice with consistent results. $^{**}P < 0.005$; $^{***}P < 0.001$.

and no demyelination were found in the CNSs of *Irf4*$^{-/-}$ mice and the few CD8$^+$ T cells barely produced cytokines (Supplemental Figure 3, A and B), while LNs contained only IFN-γ–producing CD8$^+$ T cells but not Tc17 cells. Thus, despite being nonlymphopenic, *Irf4*$^{-/-}$ mice are characterized by loss of Tc17 differentiation and resistance to EAE after immunization with $MOG_{37–50}$.

To analyze the contribution of WT CD8$^+$ T cells to the pathogenesis of EAE, we transferred congenic WT CD45.2$^-$CD8$^+$ T cells into CD45.2$^+$*Irf4*$^{-/-}$ or control CD45.2$^+$ WT mice. This transfer had no substantial effect on the disease course or CNS pathology in WT mice (Figure 2B and Supplemental Figure 3A). Remarkably, *Irf4*$^{-/-}$ mice did not develop EAE or CNS pathology, even after transfer of WT CD8$^+$ T cells (Supplemental Figure 3A). Consistent with clinical outcome, only negligible numbers of endogenous and transferred T cells were detectable in the CNSs of *Irf4*$^{-/-}$ mice (Figure 2C). In contrast, we found high numbers of transferred CD8$^+$ T cells in lymphatic organs of *Irf4*$^{-/-}$ mice. Of these, a substantial fraction produced IL-17 or IFN-γ (Figure 2D), while endogenous *Irf4*$^{-/-}$CD8$^+$ T cells again produced only IFN-γ, but not IL-17. Hence, WT CD8$^+$ T cells differentiate into Tc17 cells within *Irf4*$^{-/-}$ lymphatic organs after immunization with $MOG_{37–50}$, but do not migrate into the CNS and are not sufficient to cause autoimmune CNS inflammation.

CD8$^+$ T cells help Th17 cells during induction of EAE. A previous report in a viral model described CD4$^+$ T cell dependence of CD8$^+$ T cell mobilization into infected tissue (21). Such a supportive CD4$^+$ T cell component could be absent in *Irf4*$^{-/-}$ mice due to their defect in Th17 differentiation (5, 6). To determine whether antigen-specific help provided by IRF4-competent CD4$^+$ T cells is required for migration of Tc17 cells into the CNS, we first transferred titrated numbers of either 2D2 T cells, which are transgenic for a MOG-specific Vβ11$^+$ T cell antigen receptor (TCR) (22), or polyclonal naive CD4$^+$ T cells and evaluated disease induction in *Irf4*$^{-/-}$ mice. For 2D2 cell transfers, we found that we were still at a saturating level (elucidated by disease outcome) with 10^5 cells. Because our aim was to analyze a contribution of CD8$^+$ T cells to EAE development, we then applied subpathogenic numbers of the respective CD4$^+$ T cells, which either failed to induce EAE (transfer of polyclonal CD4$^+$ T cells; Supplemental Figure 4A) or caused delayed disease (transfer of 2D2 cells; Figure 3A). Due to the lower frequency of antigen specificity, we injected polyclonal CD8$^+$ T cells at high numbers as compared with antigen-specific 2D2 cells. Importantly, when subpathogenic numbers of 2D2 or WT CD4$^+$ T cells were cotransferred with WT CD8$^+$ T cells, the susceptibility of *Irf4*$^{-/-}$ mice to EAE was restored (Figure 3A and

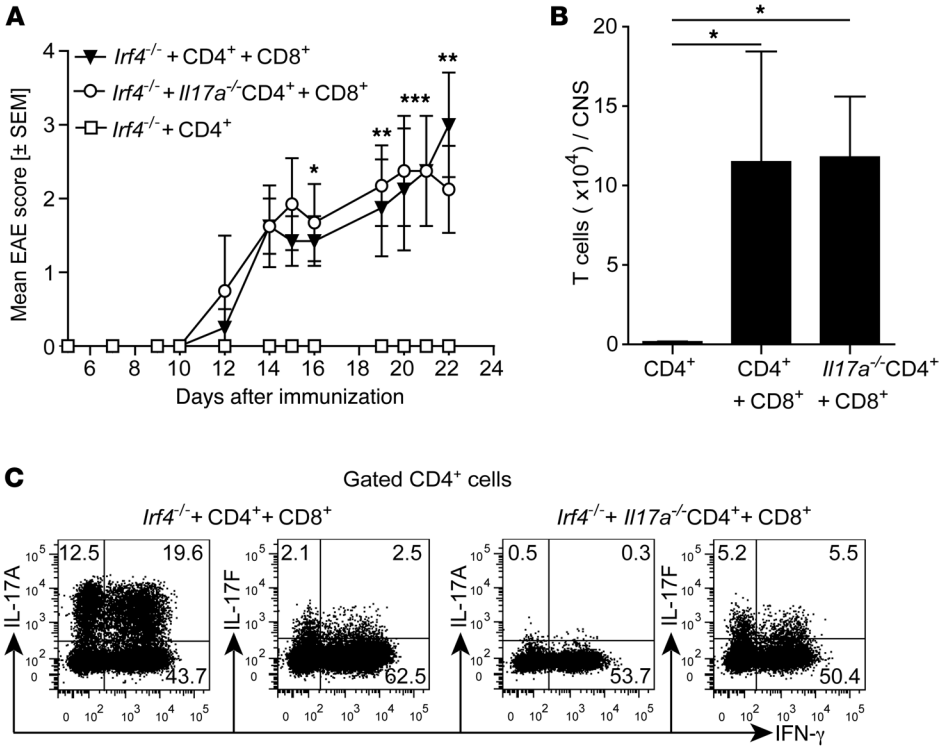

Figure 5
IL-17A competence of CD4+ T cells is not required for their pathogenicity. (**A**) Mean clinical scores (± SEM) of $MOG_{37–50}$-immunized *Irf4*$^{-/-}$ mice (n = 4) substituted with 2.5 × 10^6 WT CD4+ T cells or *Il17a*$^{-/-}$CD4+ T cells with or without 10^7 WT CD8+ T cells. P values were calculated comparing the scores of *Irf4*$^{-/-}$ mice transferred with WT CD4+ T cells alone or WT CD8+ T cells plus *Il17a*$^{-/-}$CD4+ cells. (**B**) Absolute numbers of T cells (mean ± SEM, n = 4) in the CNSs of *Irf4*$^{-/-}$ mice after transfer of WT CD4+ T cells alone or of WT CD8+ T cells in combination with either WT CD4+ or *Il17a*$^{-/-}$CD4+ T cells. (**C**) Flow cytometry of gated CD4+ CNS cells stained for IL-17A, IL-17F, or IFN-γ. Numbers represent percentages of cells in the respective quadrant. (**A**–**C**) The experiments were repeated twice with consistent results. *P < 0.05; **P < 0.005; ***P < 0.001.

Supplemental Figure 4A), a finding accompanied by prominent T cell infiltration and pathology in the CNS (Figure 3, B–E, and Supplemental Figure 4, B–D). Notably, cotransfer of CD4+ cells licensed not only transferred but also endogenous *Irf4*$^{-/-}$CD8+ cells to enter the CNS (Figure 3D).

Most surprisingly, however, cotransferred CD4+ T cells (injected at lower numbers) outnumbered by far the CD8+ T cells (injected at higher numbers) among the CNS-invading cell population (Figure 3C and Supplemental Figure 4D). These findings were confirmed by histological analysis of spinal cord sections: mostly Vβ11+CD4+ 2D2 T cells accompanied by MAC1+ cells infiltrated the CNSs of *Irf4*$^{-/-}$ mice after cotransfer of 2D2 and CD8+ T cells (Figure 3E). Furthermore, a high frequency of the transferred CD45.1+ WT 2D2 cells or the polyclonal CD45.1+ WT CD4+ T cells and a considerable fraction of the transferred CD45.1+ WT CD8+ cells in the CNSs of diseased *Irf4*$^{-/-}$ mice produced IL-17 (Figure 3F and Supplemental Figure 4E). Together, these findings argue for a previously unappreciated help of CD8+ cells for Th17 cells to infiltrate the CNS and initiate EAE. In accordance with our data, CD8-deficient mice developed significantly milder EAE than WT mice (9). In further accordance, antibody-mediated depletion of CD8+ T cells in WT mice ameliorated disease severity, and conversely, injection of both CD8+ and CD4+ T cells into *Rag1*$^{-/-}$ mice evoked stronger disease as compared with single cells transfers (Supplemental Figure 5, A and B).

Interestingly, when transferred at high numbers, CD4+ T cells were encephalitogenic by themselves, with kinetics, cytokine profile, and histopathology similar to that induced by cotransfer of low numbers of CD4+ T cells and CD8+ T cells (Supplemental Figure 6, A–D).

Th17 cells require CCR6 to cooperate with CD8+ T cells for infiltration into the CNS. The chemokine receptor CCR6 has been shown to be essential for the first wave of antigen-specific Th17 cell migration into the CNS and induction of autoimmune inflammation (23, 24). Therefore, we next tested whether CD4+ T cells require CCR6 to be able to cooperate with CD8+ T cells for infiltrating the CNS. In contrast to WT CD4+ T cells, cotransfer of *Ccr6*$^{-/-}$CD4+ T cells and WT CD8+ T cells did not evoke EAE in *Irf4*$^{-/-}$ mice, and, accordingly, very low numbers of CD4+, CD8+, and MAC1+ cells infiltrated the CNS (Figure 4, A and C, and Supplemental Figure 7C). However, ex vivo cytokine analyses demonstrated IL-17–producing CD8+ and CD4+ T cells in the spleens of substituted *Irf4*$^{-/-}$ mice (Supplemental Figure 7, A and B), confirming their potential for type 17 differentiation in vivo. Consistent with the loss of the Th17 phenotype of *Irf4*$^{-/-}$CD4+ T cells (5–7) and a recent publication (25), we confirmed that IRF4 is essential for CCR6 expression by CD4+ T cells (Figure 4B). Thus, IRF4-deficient CD4+ T cells, at least partially, can not mediate CD8+ T cell migration into the CNS due to their strongly diminished CCR6 expression.

In contrast to the disease induced by cooperating CD4+ and CD8+ T cells, high numbers of CD4+ T cells failed to require CCR6 for the induction of disease, because transfer of *Ccr6*$^{-/-}$ or WT CD4+ T cells evoked similar disease course in *Irf4*$^{-/-}$ mice (Supplemental Figure 8, A–C). Accordingly, WT and *Ccr6*$^{-/-}$ mice developed similar disease course after immunization with $MOG_{37–50}$ (Supplemental Figure 8D). Thus, these data reveal 2 different ways to induce EAE: one requires CCR6 on CD4+ cells to cooperate with CD8+ T cells, while the other operates at high numbers of CD4+ T cells and acts independently of CCR6.

Th17 cell–derived IL-17A is not required for EAE induction by cooperating CD8+ T cells and Th17 cells. Because high frequencies of IL-17–producing CD4+ T cells infiltrated the CNSs of *Irf4*$^{-/-}$ mice after cotransfer of CD4+ and CD8+ T cells, we investigated whether the production of this Th17 signature cytokine by CD4+ T cells has an impact on disease development. *Il17a*$^{-/-}$CD4+ T cells cooperated with CD8+ T cells to the same extent as WT CD4+ cells for the

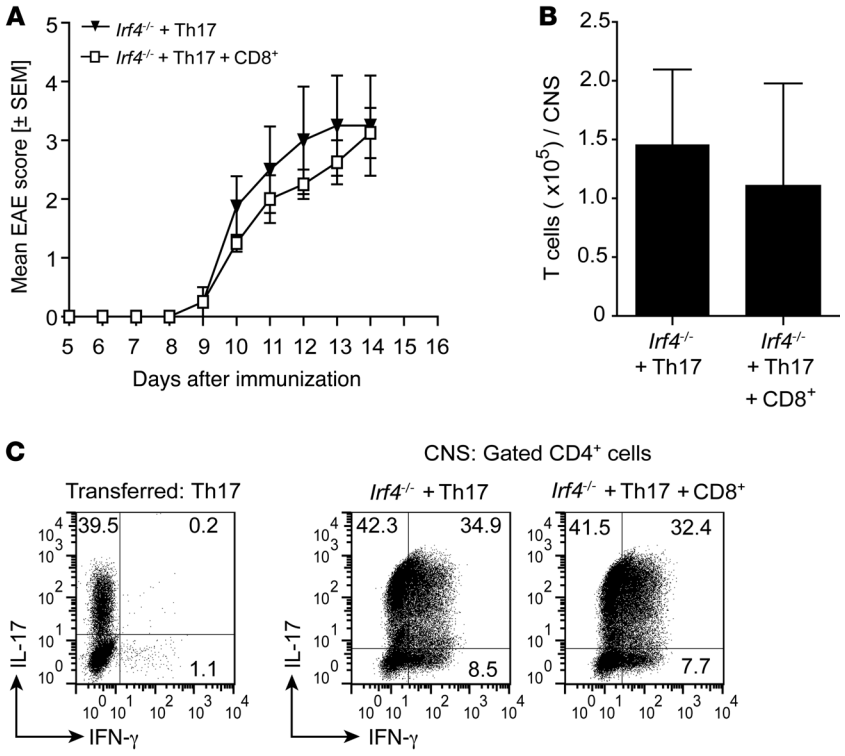

Figure 6
CD8+ T cells fail to accelerate pathogenicity of committed Th17 cells. (**A**) 2D2 cells were polarized under Th17 conditions in vitro for 3 days. Thereafter, *Irf4*$^{-/-}$ mice (n = 4) were substituted with these cells either alone or in combination with WT 2.5 × 10^6 $CD44^{lo}CD8^+$ T cells and subsequently immunized with $MOG_{37–50}$. Mean clinical scores (± SEM) are shown. (**B**) Absolute numbers of T cells (mean ± SEM, n = 4) in the CNSs of *Irf4*$^{-/-}$ mice after transfer of Th17 cells alone or in combination with $CD44^{lo}CD8^+$ T cells. (**C**) Flow cytometry of transferred in vitro–differentiated 2D2 cells polarized under Th17 conditions and of gated CD4+ CNS cells stained for IL-17A or IFN-γ. Numbers represent percentages of cells in the respective quadrant. (**A**–**C**) The experiments were repeated twice with consistent results.

induction of EAE as determined by severe paralysis and massive T cell infiltration in the CNS (Figure 5, A and B). As anticipated, the CNS-invading WT but not the *Il17a*$^{-/-}$CD4+ T cells were able to produce IL-17A, while both of them were positive for the other Th17-marker cytokine, IL-17F (Figure 5C). Possibly due to compensatory mechanisms, *Il17a*$^{-/-}$CD4+ T cells produced more IL-17F than their WT counterparts. Therefore, IL-17A produced by CD4+T cells is not required for the cooperation of CD4+ and CD8+ T cells during induction of EAE, probably because *Il17a*$^{-/-}$CD4+ T cells invading the CNS still developed a Th17-like phenotype, as determined by their ability to produce IL-17F.

Committed Th17 cells do not require help of CD8+ T cells for their pathogenicity. To evaluate whether CD8+ T cells influence the pathogenicity of already committed Th17 cells, we transferred in vitro–differentiated Th17 cells alone or in combination with CD8+ T cells. In contrast to uncommitted CD4+ T cells, low numbers of differentiated Th17 cells already induced disease in *Irf4*$^{-/-}$ mice by themselves, and cotransfer of CD8+ T cells failed to influence disease course and severity (Figure 6). These data suggest that CD8+ T cells enhance the pathogenicity of CD4+ cells during initiation of the disease by influencing Th17 phenotype development.

IL-17A but not CCR6 competence of CD8+ T cells promotes Th17 cell encephalitogenicity. According to the "2-waves hypothesis," the flux of cells into the CNS during the second wave should be CCR6 independent (23). To evaluate whether CD8+ T cell migration meets this criterion, we cotransferred 2D2 T cells and *Ccr6*$^{-/-}$CD8+ T cells into *Irf4*$^{-/-}$ mice. Cotransfer of 2D2 and either WT or *Ccr6*$^{-/-}$CD8+ T cells caused nearly identical onset of disease and CNS infiltration by T and MAC1+ cells (Figure 7, A and C). Thus, in contrast to CD4+ T cells, CCR6 expression by CD8+ T cells is not essential for induction of disease, suggesting that CD8+ T cells enter the CNS in the CCR6-independent second wave of autoimmune inflammation.

Next, we evaluated whether CD8+ T cells need IL-17A, and therefore belong to the Tc17 subset, to accelerate encephalitogenicity of CD4+ T cells. Strikingly, *Il17a*$^{-/-}$CD8+ T cells did not provide help for the pathogenicity of 2D2 T cells, as shown by clinical onset of EAE and tissue infiltration by CD4+, CD8+, or MAC1+ cells (Figure 7, B and C). To provide more direct evidence for the supportive role of Tc17 cells for Th17 pathogenicity, we performed cotransfers of in vitro skewed WT and *Il17a*$^{-/-}$ Tc17 cells together with 2D2 T cells. In agreement with data obtained with cotransfers of naive CD8+ T cells, WT but not *Il17a*$^{-/-}$ Tc17 cells cooperated with CD4+ T cells for EAE induction (Supplemental Figure 9, A and B). Consistent with clinical outcome, in the CNSs of diseased mice we found high numbers of T cells and the ratio of CD4+ to CD8+ T cell numbers was similar to that observed by cotransfer of naive CD8+ T cells (Supplemental Figure 9, C and D, and Figure 3C). CD4+ T cells were the dominating population characterized by strikingly high IL-17 production (Supplemental Figure 9, D and E). As expected CNS-infiltrating CD8+ T cells produced high amounts of IL-17; however, in vivo they acquired additionally the ability to coproduce IFN-γ (Supplemental Figure 9E).

These data imply that IL-17A produced by Tc17 cells is required to render CD4+ cells more encephalitogenic and support the previously published idea of the importance of IL-17A during the initiation of EAE (26–28). Moreover, this result suggests that the inability of *Irf4*$^{-/-}$CD8+ T cells to potentiate the pathogenicity of CD4+ T cells is at least partially explained by their defective Tc17 differentiation.

Tc17 cells but not their supernatants trigger the Th17 differentiation program. Given that WT but not *Il17a*$^{-/-}$ Tc17 cells provided help for stronger pathogenicity of Th17 cells, we investigated whether Tc17 cells can directly promote Th17 differentiation in vitro. To this end, we cocultured WT or *Il17a*$^{-/-}$ Tc17 cells or their supernatants

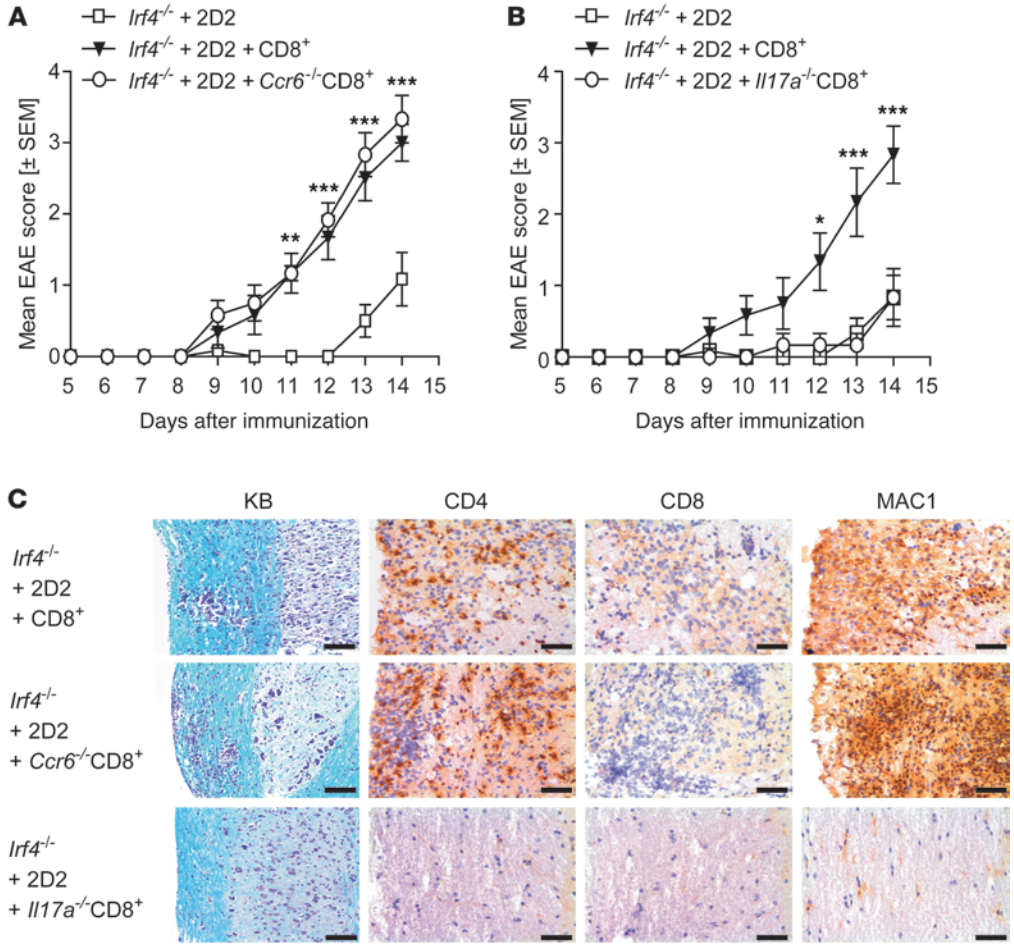

Figure 7

IL-17A competence of CD8+ T cells accelerates CD4+ T cell encephalitogenicity. (**A** and **B**) Mean EAE scores (± SEM) combining 2 independent experiments of $MOG_{37–50}$-immunized *Irf4*$^{-/-}$ (n = 6) mice that received sorted 2.5 × 10^6 $CD44^{lo}CD8^+$ cells from WT, *Ccr6*$^{-/-}$, or *Il17a*$^{-/-}$ mice and/or 10^4 $CD62L^{hi}$ 2D2 T cells. P values were calculated comparing (**A**) the scores of *Irf4*$^{-/-}$ mice transferred with 2D2 alone or 2D2 in combination with *Ccr6*$^{-/-}$CD8+ T cells and (**B**) the scores of mice transferred with 2D2 T cells in combination with WT CD8+ T cells or in combination with *Il17a*$^{-/-}$CD8+ T cells. (**C**) Histology of spinal cords at day 13 after immunization: Klüver-Barrera staining (scale bar: 100 μm); immunochemically stained cells were detected as brown foci (scale bar: 50 μm). (**A**–**C**) The experiments were repeated twice with consistent results. *P < 0.05; **P < 0.005; ***P < 0.001.

(SNs) with purified CD4+ T cells in the presence of anti-CD3/CD28 stimulation. In order to directly verify the role of soluble IL-17A, exogenous recombinant murine IL-17A (rmIL-17A) was added to cocultures containing *Il17a*$^{-/-}$ Tc17 cells. After 3 days of coculture, CD4+ T cells were FACS sorted and either analyzed directly for their mRNA expression profiles or restimulated to assess cytokine production in the SNs.

Tc17 cells themselves, but neither their SNs nor rmIL-17A, induced the type 17 transcriptional program in CD4+ T cells. Surprisingly, the induction of *Rorc*, *Il21*, and *Il23r* mRNA was independent of the Tc17 cell capacity to produce IL-17A (Figure 8A). In striking contrast, WT but not *Il17a*$^{-/-}$ Tc17 cells promoted expression of IL-17A itself in CD4+ T cells at the mRNA and protein level (Figure 8, A and B). To evaluate the possibility that SNs from *Il17a*$^{-/-}$ Tc17 cells possess any inhibitory activity on the IL-17A production by CD4+ T cells, we cocultured WT Tc17 cells with CD4+ T cells in the presence of SN from *Il17a*$^{-/-}$ Tc17 cells. However, *Il17a*$^{-/-}$ Tc17 cell SNs did not influence Tc17 cell–mediated IL-17A production by CD4+ T cells (Supplemental Figure 10). Taken together, these results indicate that in vitro WT and *Il17a*$^{-/-}$ Tc17 cells support the Th17 differentiation program via direct cell contact. In contrast, IL-17A competence of Tc17 cells is required for production of IL-17A itself in CD4+ T cells.

An important control for these conclusions was that similar amounts of IL-17A had been present in the respective culture settings. Therefore, we confirmed that SNs harvested after the coculture period contained approximately similar quantities of IL-17A in wells with WT Tc17 cells or with *Il17a*$^{-/-}$ Tc17 cells plus exogenous IL-17A (Figure 8C). The highest amounts of IL-17A were detectible in control CD4+ T cultures containing exogenous IL-17A but no Tc17 cells, although this condition lacked IL-17A–inducing activity (Figure 8B). These data suggest that the promotion of IL-17A production in CD4+ T cells requires both direct contact and IL-17A competence by Tc17 cells.

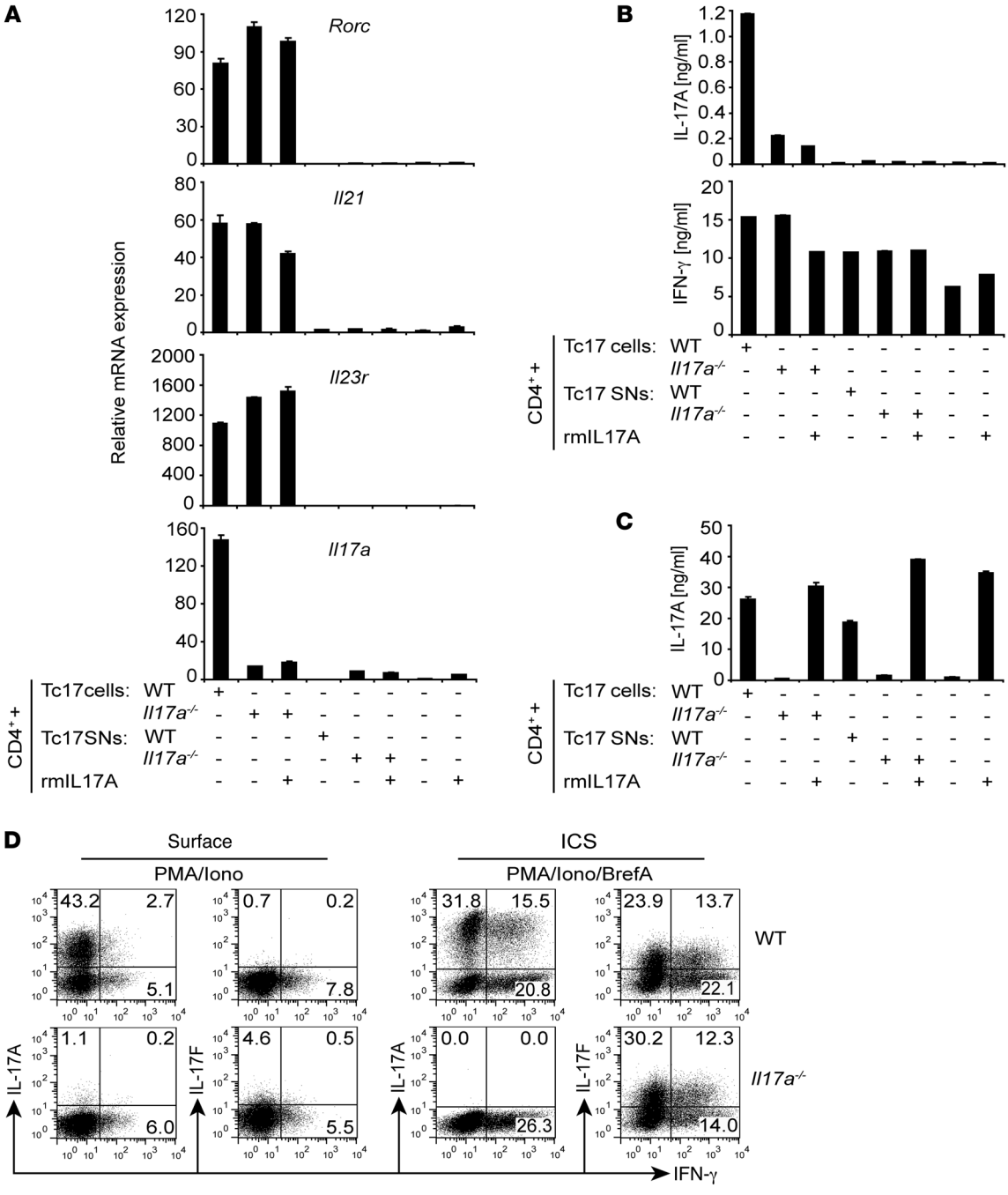

Figure 8

Tc17 cells promote Th17 differentiation via direct cell contact in vitro. (**A**) Purified CD4+ T cells were mixed 1:1 with in vitro–differentiated WT or *Il17a*$^{-/-}$ Tc17 cells or their SNs with or without exogenous rmIL-17A and stimulated via CD3/CD28. After 72 hours, CD4+ T cells were sorted, and mRNA expression of the indicated genes was analyzed by qRT-PCR. Data (± SD) of PCR duplicates. (**B**) ELISA for IL-17A and IFN-γ produced by sorted CD4+ T cells after restimulation for 24 hours with anti-CD3. (**C**) ELISA for IL-17A in SNs of 72 hours cocultures as described for **A**. (**B** and **C**) Data (± SD) of ELISA duplicates. (**D**) WT or *Il17a*$^{-/-}$ Tc17 cells differentiated in vitro for 96 hours were restimulated with PMA/ionomycin in the presence or absence of brefeldin A and stained for IL-17A, IL-17F, and IFN-γ either on their surface (left) or intracellularly (ICS) after permeabilization (right). Numbers represent percentages of cells in the respective quadrant. (**A**–**D**) Data are representative of two independent experiments.

Recently, it has been reported that Th17 cells express surface IL-17A (29). Similarly to Th17 cells, high frequencies of WT Tc17 cells expressed surface IL-17A (Figure 8D). In contrast, IL-17F was almost absent from the surface of these cells but was easily detectable intracellularly. As expected, *Il17a*$^{-/-}$ Tc17 cells expressed IL-17A neither on the surface nor intracellularly. Probably, membrane IL-17A contributes to the promotion of IL-17 production by CD4+ T cells in a direct cell-cell interac-

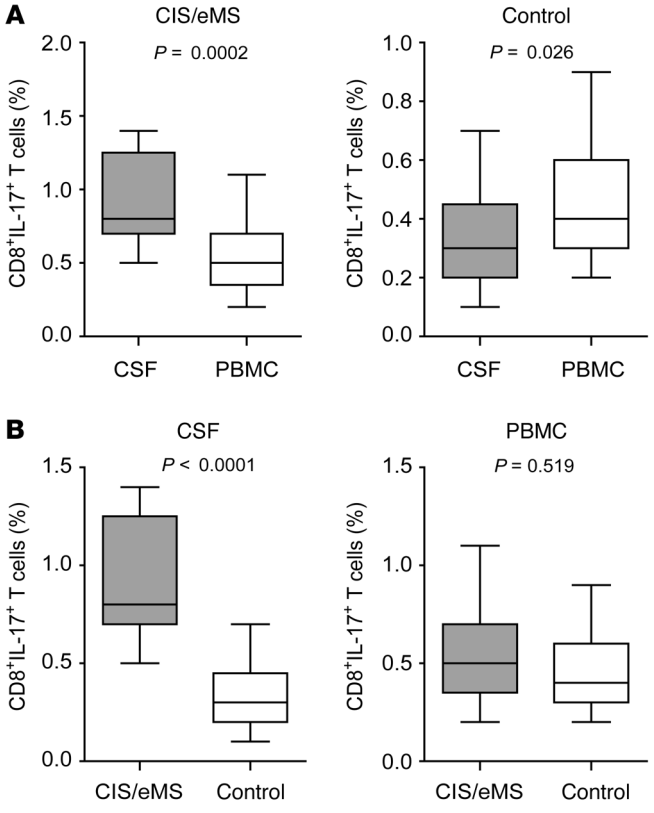

Figure 9
Frequency of Tc17 cells in peripheral blood and CSF of control patients and patients with early-stage MS. (**A** and **B**) IL-17+ cells among gated CD8+ T cells from CSF and peripheral blood (PBMCs) obtained from patients with early-stage MS (CIS/eMS, $n = 17$) as well as from patients with noninfectious headache (control, $n = 17$). PBMC and CSF lymphocytes were restimulated with CytoStim human. Thereafter, the cells were stained for surface CD8 and then fixed, permeabilized, and stained for intracellular IL-17. The box plots depict the minimum and maximum values (whiskers) and the upper and lower quartiles (top and bottom edges of the box). The median is identified by a line inside the box. The length of the box represents the interquartile range. *P* values were calculated with Mann-Whitney *U* test.

tion, because *Il17a*$^{-/-}$ Tc17 cells lack this activity, even in the presence of exogenous soluble IL-17A. As (a) IL-17A competence by Tc17 cells is required for the onset of EAE and (b) their IL-17A competence is not required for induction of Th17 differentiation in vitro, the EAE-promoting activity of Tc17 cells can not entirely be explained by their capacity to directly induce Th17 cell differentiation. Rather, they probably need IL-17A to act on the local environment, e.g., to increase production of innate cytokines that in turn may augment generation of pathogenic Th17 cells.

Tc17 cells in CSF and peripheral blood of patients with MS. To determine whether the findings reported above in the mouse EAE model are compatible with the human disease, we analyzed the presence of Tc17 cells in patients with MS. We included in our study 17 patients at an early-stage of MS (clinical data are summarized in Supplemental Table 1), of which 11 suffered from a clinically isolated syndrome suggestive for MS (CIS) and 6 from early MS (eMS). In contrast to the control group that suffered from noninfectious headache (control), patients with early-stage MS (CIS/eMS) had higher frequencies of Tc17 cells within CD8+ T cells from CSF than from peripheral blood (Figure 9A). Furthermore, we found significantly higher percentages of Tc17 cells in CSF of patients with early-stage MS compared with control patients (Figure 9B). In contrast, the frequencies of Tc17 cells in peripheral blood of control patients and patients with early-stage MS did not differ (Figure 9B). This finding demonstrates selective enrichment of Tc17 cells in the CSF of patients with early-stage MS, suggesting that, also in humans, Tc17 cells contribute to the initiation of CNS autoimmunity.

Discussion

Previous reports have described the necessity of the transcription factor IRF4 for the development of the CD4+ T cell subsets Th2 (30, 31), Th17 (5, 6, 8), and Treg (32). Here, we extend these studies to CD8+ T cells and show that IRF4 is also critical for Tc17 differentiation. These results combined with our previous data on CD4+ T cells (5, 6) point to the central role of IRF4 for type 17 differentiation.

Complementary to the results obtained in CD4+ T cells (5, 6), we found increased levels of Foxp3 in *Irf4*$^{-/-}$CD8+ T cells cultured under Tc17 conditions and demonstrated that Foxp3 inhibits IL-17 production also in CD8+ T cells. Concomitantly, the amounts of Eomes were upregulated in *Irf4*$^{-/-}$CD8+ T cells and Eomes inhibited IL-17 production in CD8+ T cells, in support of the previously proposed repression of the Tc17 program by a combination of Eomes and T-bet (13) and the recently published Eomes-mediated suppression of Th17 differentiation by its direct binding to the *Rorc* and *Il17a* promoters (33). Together with greatly impaired levels of RORγt and RORα, our data point to the central role of IRF4 in CD8+ T cells in balancing the levels of transcription factors responsible for Tc17, Treg (34), and CTL differentiation.

In the absence of IRF4, Tc17 development is also abolished in vivo. We showed this in an EAE model in *Irf4*$^{-/-}$ mice, induced by immunization with the MOG_{37-50} peptide and characterized by the presence of Tc17 cells in the CNS (16). We found a lack of Tc17 cells in these mice, which correlated with complete EAE resistance. Surprisingly, adoptive transfer of WT CD8+ T cells was not sufficient to restore EAE susceptibility, although Tc17 development of the transferred cells was readily detectable in LNs and spleens of *Irf4*$^{-/-}$ mice. Thus, Tc17 development of WT CD8+ T cells occurs in an *Irf4*$^{-/-}$ environment, but differentiated Tc17 cells are not sufficient on their own to infiltrate the CNS; rather, they require support from an IRF4-competent environment. This situation was reminiscent of the reported need for CD4+ T cell help to mobilize CD8+ T lymphocytes to the site of a viral infection (21). Indeed, we show that cotransfer of CD8+ T cells and few CD4+ T cells, which by themselves induced either no or strongly delayed onset of disease, caused early onset of EAE in *Irf4*$^{-/-}$ mice, accompanied by presence of transferred CD8+ and CD4+ T cells in the CNS.

Apparently, endogenous *Irf4*$^{-/-}$CD4+ T cells were unable to support CD8+ T cell CNS migration, demonstrating an essential role for IRF4 within both CD4+ and CD8+ T cells during onset of EAE. Within CD4+ T cells, a contribution of IRF4 is to regulate CCR6 expression because *Irf4*$^{-/-}$CD4+ T cells expressed diminished amounts of *Ccr6* mRNA and *Ccr6*$^{-/-}$CD4+ T cells were unable to

cooperate with WT CD8$^+$ T cells for encephalitogenicity. These features combined with the loss of the entire Th17 phenotype (5, 6) probably caused the defect of *Irf4*$^{-/-}$CD4$^+$ T cells to enable CD8$^+$ T cell migration into the CNS. Thus, together with previously published data (23), our results argue for dependence of the CD8$^+$ T cell recruitment into the CNS on a "first-wave" infiltration by CCR6- and IRF4-competent CD4$^+$ T cells, while CD8$^+$ T cells migrate in a second wave CCR6 independently. This concept is corroborated by our finding that CCR6 expression by CD8$^+$ T cells was not necessary for their copathogenic function. Consistent with our results, the α4 integrin, a subunit of very late antigen-4 (VLA-4), has been defined as a major contributor of CD8$^+$ T cell entry into CNS (35).

In contrast to the necessity for CCR6 expression by CD4$^+$ T cells during cotransfer with CD8$^+$ T cells, when applied at high numbers CD4$^+$ T cells did not require CCR6 for induction of EAE in *Irf4*$^{-/-}$ mice. Moreover, *Ccr6*$^{-/-}$ mice developed a similar disease course after immunization with $MOG_{37–50}$ as compared with WT controls. Thus, in our system, EAE induced by cooperating CD4$^+$ and CD8$^+$ T cells requires CCR6 expression by CD4$^+$ T cells, in agreement with previous data (23), whereas EAE induced by high numbers of CD4$^+$ T cells is CCR6 independent. Apparently, CD4$^+$ T cells can react to different conditions by using alternative mechanisms for CNS invasion, e.g., via the integrins α4 or αL (CD11a, a subunit of LFA-1), as suggested recently (36, 37). Importantly, however, this CCR6-independent invasion of CD4$^+$ T cells operates only when they are available at high numbers. When translated to the situation in humans, such a high frequency of reactive CD4$^+$ T cells presumably relates to a later stage of the disease when multiple events already occurred and a strong CD4$^+$ T cell immunity exists. In contrast, the herein characterized cooperation of CD4$^+$ and CD8$^+$ T cells probably refers to the onset of the disease, when antigens for CD4$^+$ cells are limiting but for CD8$^+$ cells are present, particularly when the disease will be triggered by a viral infection.

Interestingly, cotransfer of small numbers of WT CD4$^+$ T cells and high numbers of WT CD8$^+$ T cells resulted in more CD4$^+$ than CD8$^+$ T cells detectable in the CNS. Because very low numbers of CD4$^+$ T cells were found in the CNS after transfer of CD4$^+$ T cells alone, our data suggest a previously not appreciated unconventional "reverse" help, namely of CD8$^+$ T cells to support CNS infiltration by CD4$^+$ T cells. We characterize the ability to produce IL-17A by CD8$^+$ T cells as an important quality contributing to this help, because only IL-17A–competent naive CD8$^+$ T cells or committed Tc17 cells accelerated CD4$^+$ T cell pathogenicity, resulting in severe disease and accumulation of high numbers of IL-17–producing CD4$^+$ T cells in the CNSs of EAE-diseased mice. Given that IL-17A was described as the hallmark of Tc17 cells, our results suggest that it is the Tc17 effector subset that accounts for the cooperation with CD4$^+$ T cells.

The requirement for IL-17A–producing CD8$^+$ T cells explains, at least in part, why endogenous *Irf4*$^{-/-}$CD8$^+$ cells were not able to enhance CD4$^+$ T cell pathogenicity. The importance of IL-17A for initiation of autoimmunity has already been established (27, 38, 39). Our study extends these previous reports by linking IL-17A production to CD8$^+$ T cells and specifically to the Tc17 subset in order to potentiate CD4$^+$ T cell CNS pathogenicity. In contrast, IL-17A competence was not required for the CD4$^+$ T cell part of this cooperation. Thus, the cellular origin of IL-17A is relevant for pathogenicity. Our data suggest that CD8$^+$ T cells and especially the Tc17 subset are a sufficient and necessary IL-17A source, at least in our EAE model of co-operating CD4$^+$ and CD8$^+$ T cells. Consistent with the importance of IL-17A during initiation of autoimmunity, CD8$^+$ T cells enhanced the pathogenicity of uncommitted CD4$^+$ T cells, probably by enhancing their Th17 phenotype, whereas they failed to influence EAE mediated by already in vitro committed Th17 cells.

CNS-invading IL-17A–deficient CD4$^+$ T cells developed a similar Th17 phenotype to that of WT CD4$^+$ T cells, because they produced IL-17F at even enhanced amounts. Apparently, these "Th17-like" cells still kept their pathogenicity, because their effect was comparable to that of WT CD4$^+$ T cells and because endogenous *Irf4*$^{-/-}$CD4$^+$ cells were unable to replace them, most likely due to their defect in the entire Th17 differentiation program. Together, these data support the idea that, during induction of EAE by cooperating CD4$^+$ and CD8$^+$ cells, IL-17A is mainly an effector molecule of CD8$^+$ T cells, while in CD4$^+$ T cells, it can be viewed as a marker of pathogenic Th17 cells (40). Nevertheless, IL-17A produced by CD4$^+$ T cells may contribute to pathogenicity at later stages, e.g., by facilitating leukocyte trafficking across the blood-brain barrier and inflammation within the CNS (40).

To analyze whether Tc17 cells directly transmit signals that activate CD4$^+$ T cells, we performed in vitro coculture assays. Our results demonstrate a cell contact–dependent but IL-17A–independent interaction between Tc17 and CD4$^+$ T cells that induces the Th17 transcriptional profile. In contrast, enhanced production of IL-17A itself by CD4$^+$ T cells required IL-17A competence of Tc17 cells, and this activity could not be replaced by exogenous soluble IL-17A. Probably, surface IL-17A expressed by Tc17 cells contributed to their IL-17A–inducing function during the direct CD4$^+$/CD8$^+$ T cell interaction. Surface IL-17A has been previously described on human and mouse CD4$^+$ T cells isolated from the CNSs of EAE-diseased animals (29).

Because, for pathogenicity in vivo, IL-17A competence is required in CD8$^+$ cells and specifically in Tc17 cells but not in CD4$^+$ T cells, we suggest that besides the direct CD4$^+$/CD8$^+$ interaction, Tc17 cells exert an additional IL-17A–dependent indirect effect to induce EAE. An amplifying function of IL-17A during Th17-mediated autoimmunity has been suggested before (26, 39) and was attributed to stimulation of innate immune cells to produce the Th17 driving cytokines IL-1β, IL-6, or IL-23. We assume that during EAE, IL-17A–producing Tc17 cells promote pathogenicity of Th17 cells also via a similar indirect mechanism, e.g., by activating APCs.

Based on our data, we propose 2 sequential steps during EAE induction by cooperating CD4$^+$ and CD8$^+$ T cells. The first event is crucial to endow CD4$^+$ T cells with stronger pathogenicity and requires direct cell contact among Tc17 cells, CD4$^+$ T cells, and probably also APCs. During this initial process, Tc17 cells might promote directly and indirectly, via IL-17A–dependent APC activation, the differentiation of CD4$^+$ T cells toward the pathogenic Th17 phenotype and thus regulate the first wave of CD4$^+$ cell migration into the CNS via CCR6 (23). These Th17 cells in turn facilitate the CCR6-independent migration of Tc17 cells in the second wave. The exact details of the in vivo cooperation between IL-17A–competent CD8$^+$ T cells and CCR6-competent CD4$^+$ T cells during autoimmune CNS inflammation deserve further exploration.

We think that the herein described support of Tc17 cells for the initiation of Th17-mediated disease uncovered by T cell transfers into *Irf4*$^{-/-}$ mice also applies to a WT situation. This reasoning is supported by several findings obtained by our group and others.

First, CD8$^+$ T cell–deficient mice develop significantly milder EAE as compared with WT controls (9). Accordingly, we found that antibody-mediated depletion of CD8$^+$ T cells also ameliorates disease severity. Second, even in the lymphopenic *Rag1*$^{-/-}$ environment, which is associated with homeostatic proliferation of transferred cells, CD8$^+$ T cells significantly enhanced CD4$^+$-mediated EAE. Probably because of the homeostatic proliferation and therefore increased activation, already low numbers of CD4$^+$ T cells caused paralysis in *Rag1*$^{-/-}$ mice, in contrast to experiments performed in *Irf4*$^{-/-}$ mice, suggesting that the nonlymphopenic *Irf4*$^{-/-}$ environment is a better model for studying the influence of transferred T cells for initiation of EAE. Furthermore, we found under in vitro conditions, which are independent of the genetic environment, a supportive function of Tc17 cells for Th17 differentiation. In this setting, Tc17 cells via cell-cell contact directly provided help for the development of the type 17 transcriptional profile in CD4$^+$ T cells and for the production of the cytokine IL-17A, which is associated with EAE severity. Finally, and most importantly for human MS, previous reports have already demonstrated the presence of Tc17 cells in the lesions of patients with MS (17). Herein, we found enrichment in Tc17 cell frequencies in the CSF of patients with early-stage MS. This indicates that, also in humans, Tc17 cells are involved in the initiation of disease, perhaps when a cooperation of Tc17 and Th17 cells in the periphery has already occurred and Tc17 cells start to enter the CNS. This finding also suggests selective expansion and CNS recruitment of Tc17 cells in early-stage MS and that targeting of Tc17 cells may be of relevance for therapy in eMS.

Methods

Mice. WT C57BL/6 mice were purchased from The Jackson Laboratory. *Irf4*$^{-/-}$, *Ccr6*$^{-/-}$ (41), *Rag1*$^{-/-}$, 2D2 mice expressing a transgenic TCR specific for MOG$_{35-55}$ (22), and CD45.1$^+$ mice on the C57BL/6 background were bred at the animal facility of the Biomedical Research Center, University of Marburg. *Il17a*$^{-/-}$ mice were provided by Y. Iwakura, Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan. All mouse experiments were approved by the local government.

Patients. Patients included persons with CIS (n = 11, 64.7%) and with eMS (n = 6, 35.3%). None of the patients received any immunosuppressant or immunomodulation drug therapy prior to bio-sampling. They all suffered from their first clinical demyelinating attack and were diagnosed according to the McDonald criteria (42). Patients lacking prove of dissemination in time were classified as CIS (43); those who showed concurrent dissemination in space and time were classified as eMS. An additional 17 patients with noninfectious headache who underwent diagnostic lumbar puncture were included as controls. None of the latter had any symptoms or signs of systemic infection. They all were otherwise healthy. The clinical characteristics of patients and their controls are summarized in Supplemental Table 1.

T cell purification, in vitro stimulation, and fluorescence staining. CD8$^+$ or CD4$^+$ T cells were purified by negative magnetic cell sorting (MACS, Miltenyi Biotech) from spleens and LNs of 8- to 12-week-old mice and primed with plate-bound αCD3 (3 μg/ml; clone 145-2C11) and soluble αCD28 mAb (0.5 μg/ml; clone 37.51) in the presence of recombinant human IL-2 (50 U/ml; Novartis), and αIFN-γ (5 μg/ml, clone XMG1.2) (CTL conditions). Some cultures received in addition 0.5 ng/ml recombinant human TGF-β1 (R&D Systems), 20 ng/ml rmIL-6 (Peprotech), or combinations of these stimuli (Tc17 conditions). 72 hours later, cells were restimulated with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence or absence of 5 μg/ml brefeldin A (all from Sigma-Aldrich) for 4 hours, after which IL-17A–, IL-17F– and IFN-γ–producing cells (αIL-17A, clone eBio17B7; αIL-17F, clone eBio18F10; αIFN-γ clone XMG1.2; all from eBioscience) were analyzed by intracellular or surface staining, as described previously (30), on a FACSCalibur machine using the FlowJo software (Tree Star). Isotype staining revealed specificity of the IL-17 and IFN-γ stainings. For Foxp3 detection, the Foxp3 Staining Kit (αFoxp3; FJK-16s; eBioscience) was used.

EAE induction and ex vivo flow cytometry. EAE was induced in sex- and age-matched WT *Irf4*$^{+/+}$ and *Irf4*$^{-/-}$ mice on the C57BL/6 background by subcutaneous injection of 200 μg MOG$_{37-50}$ peptide (44) (VGWYRSPFSRVVHL; synthesized by R. Volkmer, Charité, Berlin, Germany) emulsified in CFA (containing 500 μg *M. tuberculosis* H37RA; Difco) together with i.p. administration of 200 ng pertussis toxin (Sigma-Aldrich) on days 0 and 2. Disease severity was scored daily on a scale of 0 to 5 (0, no paralysis; 1, limp tail; 2, limp tail and partial hind leg paralysis; 3, complete hind leg paralysis; 4, tetraparesis; and 5, moribund). For transfer experiments, MACS-purified CD45.1$^+$CD8$^+$ T cells (10^7 per mouse) or CD62L^{hi}CD4$^+$ T cells (2.5×10^6 per mouse; CD45.1$^+$, CD45.2$^+$, CD45.2$^+$*Ccr6*$^{-/-}$, or CD45.2$^+$*Il17a*$^{-/-}$) isolated from naive donor mice were injected i.p. into recipients 1 day before immunization. Alternatively, FACS-sorted CD44^{lo}CD8$^+$ T cells (2.5×10^6 per mouse; CD45.1$^+$, CD45.2$^+$, CD45.2$^+$*Ccr6*$^{-/-}$, or CD45.2$^+$*Il17a*$^{-/-}$) or MACS-purified CD62L^{hi}CD4$^+$ T cells from 2D2 transgenic mice on the CD45.1$^+$ or CD45.2$^+$ background (low numbers, 10^4, or high numbers, 10^5 per mouse) isolated from naive donor mice were transferred i.p. into *Irf4*$^{-/-}$ recipients 1 day before immunization. For CD4$^+$ and CD8$^+$ T cell depletion, WT mice were injected i.p. twice, on days 3 and 6 after immunization with 300 μg monoclonal antibody against CD4 (clone YTS191) or CD8 (clone YTS169) or control immunoglobulin (rat IgG). The preparation of LN cells, splenocytes, and CNS lymphocytes was performed as previously described (5). CNS cells (pooled from the mice of one group or analyzed for each mouse individually), LN cells, or spleen cells (analyzed for each mouse individually) were restimulated in vitro with 50 ng/ml PMA and 1 μg/ml ionomycin for 4 hours in the presence of 5 μg/ml brefeldin A. Viable cells (LIVE/DEAD Fixable Aqua Dead Cell Stain; Invitrogen) were analyzed for surface expression of CD8a (53–6.7; eBioscience), CD4 (RM4-5; Biolegend), CD45.2 (clone 104; BD), or CD45.1 (A20; eBioscience). After fixation/permeabilization, cells were stained for IL-17A, IL-17F, and IFN-γ or with the respective isotype controls. Data were acquired with an LSRII, AriaIII, or FACSCalibur (BD Biosciences) and analyzed with the FlowJo software. Cell sorting was performed on a MoFlo (Beckman-Coulter) using the Summit-software (DakoCytomation).

Human PBMCs, staining, and FACS analysis. PBMCs were isolated from peripheral blood (on Ficoll-1077 density; Biochrom), and CSF leukocytes were collected after centrifugation; then these cells were restimulated for 6 hours with CytoStim human (Miltenyi Biotech). After 2 hours of stimulation, brefeldin A was added for the next 4 hours. Thereafter, the cells were stained for surface CD8 (SK1, BD Pharmingen), and then fixed, permeabilized, and stained for IL-17A (clone CZ8-23G1) using the Inside Stain Kit (both from Miltenyi Biotech). Staining procedures were performed as described previously (45). Data were acquired with a FACSCalibur machine and analyzed with CellQuest software.

Quantitative real-time PCR. Total RNA was extracted from CD8$^+$ or CD4$^+$ T cells at day 2 of priming or after FACS sorting from CD4$^+$ cells at day 3 of coculture with Tc17 cells. For RNA isolation, the High Pure RNA Isolation Kit (Roche) was used. cDNA was synthesized with oligo(dT) primers using the RevertAid First-Strand cDNA Synthesis Kit (MBI Fermentas), and gene expression was examined with an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using the SYBR Green I qPCR Core Kit (Eurogentec). Levels of each gene were normalized to hypoxanthine-guanine phosphoribosyl transferase (*Hprt1*) expression, and relative fold differences were calculated. The lowest experimental value was set to 1. The primer sets have been described previously (6, 16, 27). The following primer pair for *Ccr6* was used: forward, 5′-TCCATCATCATCTCAAGCCCTAC-3′, and reverse, 5′-CGTGATGGGCTCTGAGACAGA-3′.

Nucleofection. WT CD8$^+$ T cells were nucleofected immediately after purification with or without IRF4si or scrambled siRNA preparations. These siRNAs were prepared by IBA. Their sequences were previously described (5). Nucleofection of 10^7 cells in 100 μl of mouse T cell Nucleofector solution (Amaxa) was performed using 500 pMol total of siRNAs and the W001 program of the Nucleofector II machine (Amaxa). The nucleofected cells were primed under Tc17 conditions. The cells were harvested after 48 hours for mRNA preparation and after 72 hours for intracellular staining of Foxp3, IL-17, and IFN-γ.

Immunoblotting. For the IRF4 immunoblots, whole cell lysates were prepared after 1 day of in vitro stimulation, and for Eomes immunoblots, whole cell lysates were prepared after 3 days of in vitro stimulation. Immunoblotting was performed as described previously (6). Briefly, proteins were fractionated by SDS/PAGE, transferred to nitrocellulose membrane, immunoblotted with anti-IRF4 (M-17; sc6059; Santa Cruz Biotechnology) or anti-TBR2/Eomes (ab23345; Abcam) antibodies, and reprobed with antibodies to β-actin (A2066; Sigma-Aldrich).

Retroviral transduction. The retroviral vector pMSCV containing Eomes-VP16-IRES-GFP (46) (Eomes) and the empty control vector containing IRES-GFP (MIG) were a gift from S.L. Reiner (University of Pennsylvania, Philadelphia, Pennsylvania, USA). The retroviral vectors containing Foxp3 and RORγt have been described previously (6). The gene encoding Foxp3 (GenBank accession no. NM_054039.1) was amplified by PCR using published primers (47), control sequenced, digested by *Not*I and *Sal*I, and cloned into the retroviral vector MSCV-IRES-Thy1.1 (gift from V. Heissmeyer, GSF-Institute of Molecular Immunology, Munich, Germany) containing the internal ribosome entry site–regulated (IRES-regulated) gene for mouse Thy1.1. WT or *Irf4*$^{-/-}$CD8$^+$ T cells were infected with the retroviruses as described previously (6, 16) and stimulated via CD3/28 under the conditions indicated in the experiments. On day 3, the cells were restimulated with PMA/ionomycin and then analyzed for GFP, Thy1.1, IL-17, and IFN-γ expression by flow cytometry.

Histological analysis. Histology of spinal cords was performed on serial sections (3 μm) from paraffin-embedded — or from cryostat — sections (10 μm) of shock frozen tissue, as described previously (48). Antibodies against MAC1 (M1/70, a gift from M. Simon, MPI, Freiburg, Germany), CD4 (RM4-5; BD), Vβ11 (RR3-15; BD), and CD8a (53-6.7; BD) for cryostat sections and CD3 (CD3-12AbD; Serotec) and MAC3 (M3/84; BD) for paraffin sections were detected with biotinylated goat anti-rat IgG (Southern Biotechnology) and visualized with the Vectastain Kit (Vector Laboratories). Furthermore, H&E or Klüver-Barrera stainings were performed.

Coculture experiments. CD8$^+$ T cells from WT or *Il17a*$^{-/-}$ mice were differentiated under Tc17 conditions for 96 hours as described above, some of them were restimulated for 24 hours with immobilized αCD3 (5 μg/ml) to generate Tc17-conditioned SNs. Purified CD4$^+$ T cells (5×10^5) were mixed 1:1 with differentiated WT or *Il17a*$^{-/-}$ Tc17 cells or their SNs (final dilution: 30%) and stimulated with immobilized αCD3 (1 μg/ml) and soluble αCD28 (5 μg/ml) in 1 ml volume. Exogenous rmIL-17A (100 ng/ml) was added to some cocultures. After 72 hours of coculture, IL-17 concentrations were determined by ELISA (R&D), and CD4$^+$ T cells were separated from the Tc17 cells by flow cytometric cell sorting (>98.5% purity) using an AriaIII (BD Biosciences) and processed for mRNA purification or restimulated with plate-bound αCD3 mAb (5 μg/ml). Culture SNs were analyzed by ELISA (R&D Systems).

Statistics. For clinical scores, differences between groups were evaluated by 2-way ANOVA test with Bonferroni's post-hoc test. For cell numbers in the CNS, differences between 2 cell populations were evaluated by 2-tailed Student's *t* test. The differences between patients with CIS/eMS and control patients were evaluated using 2-sided Mann-Whitney *U* test. Calculations were performed using GraphPad Prism software (GraphPad Software Inc.). *P* values of less than 0.05 were considered significant.

Study approval. All patients and controls gave their written informed consent after the University of Marburg IRB approval (no. 126/00) for these experiments. Animal experiments were approved by the local committees (RP Gießen and TLLV Bad Langensalza, Germany).

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