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

Magdalena Huber,1 Sylvia Heink,2 Axel Pagenerstecher,3 Katharina Reinhard,1 Josephine Ritter,1 Alexander Visekruna,1 Anna Guralnik,1 Nadine Bollig,1 Katharina Jeltsch,1 Christina Heinemann,2 Eva Wittmann,4 Thorsten Buch,5 Olivia Prazeres da Costa,6 Anne Brüstle,6 Dirk Brenner,6,7 Tak W. Mak,6,8 Hans-Willi Mittrücker,9 Björn Tackenberg,10 Thomas Kamradt,2 and Michael Lohoff1

1Institute for Medical Microbiology and Hygiene, University of Marburg, Marburg, Germany. 2Institute for Immunology, Jena University Hospital, Jena, Germany. 3Department Neuropathology and 4Institute for Immunology, University of Marburg, Marburg, Germany. 5Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany. 6Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada. 7Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar, Technische Universität München, Munich, Germany. 8The Campbell Family Cancer Research Institute, Ontario Cancer Institute, University Health Network, Toronto, Ontario, Canada. 9Institute for Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. 10Clinical Neuroimmunology Group, Department of Neurology, University of Marburg, Marburg, Germany.

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+ T 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 LN 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 of IFN-γ, and of the cytolytic molecule granzyme B. Tc17 cells are...
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.
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 \( \text{Irf4}^{-/-} \) 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 ROR\( \gamma \) and ROR\( \alpha \) and by suppressing TGF-\( \beta \) induced Foxp3 (2). When we tested Foxp3 expression, we found that, similar to CD4+ T cells (5), culture with TGF-\( \beta \) induced high Foxp3 expression in WT and \( \text{Irf4}^{-/-} \) CD8+ T cells, whereas addition of IL-6 downregulated Foxp3 in WT but not \( \text{Irf4}^{-/-} \) CD8+ T cells (Figure 1F).

Because the protein levels for Eomes and Foxp3 were upregulated in \( \text{Irf4}^{-/-} \) 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-\( \gamma \)–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-\( \gamma \) production induced by Eomes alone was not significantly influenced by Foxp3 coexpression. The effects on the amounts of IL-17 and IFN-\( \gamma \) 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-\( \gamma \) 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 \( \text{Irf4}^{-/-} \) 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-\( \gamma \), 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 ROR\( \gamma \) during Tc17 differentiation.

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 \( \text{Irf4}^{-/-} \) 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) \( \text{CD8}^{+} \) gate of cells from LNs and spleens of \( \text{Irf4}^{-/-} \) mice substituted with 10^{7} CD45.2+CD8+ WT T cells stained for CD8, CD45.2, IL-17, or IFN-\( \gamma \) at day 19 after immunization. Numbers represent percentages of cells in the respective quadrant. (A–D) Data are representative of 6 independent experiments.
Figure 3
CD8+ T cells mutually interact with CD4+ T cells to induce EAE. (A) Mean EAE scores (± SEM) combining 2 independent experiments of MOG35–55-immunized Irf4−/− mice (n = 6) that received sorted congenic 2.5 × 10^6 CD45.1+CD44loCD8+ and/or 10^4 CD62LhiCD45.1+ 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.
and no demyelination were found in the CNSs of \textit{Irf4–/–} mice and the few CD8\(^+\) T cells barely produced cytokines (Supplemental Figure 3, A and B), while LNs contained only IFN-\(\gamma\)–producing CD8\(^+\) T cells but not Tc17 cells. Thus, despite being nonlymphopenic, \textit{Irf4–/–} mice are characterized by loss of Tc17 differentiation and resistance to EAE after immunization with MOG37–50.

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\(^+\) \textit{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, \textit{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 \textit{Irf4–/–} mice (Figure 2C). In contrast, we found high numbers of transferred CD8\(^+\) T cells in lymphatic organs of \textit{Irf4–/–} mice. Of these, a substantial fraction produced IL-17 or IFN-\(\gamma\) (Figure 2D), while endogenous \textit{Irf4–/–} CD8\(^+\) T cells again produced only IFN-\(\gamma\), but not IL-17. Hence, WT CD8\(^+\) T cells differentiate into Tc17 cells within \textit{Irf4–/–} lymphatic organs after immunization with MOG37–50, but do not migrate into the CNS and are not sufficient to cause autoimmune CNS inflammation.

\textit{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 \textit{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 V\(\beta\)11\(^+\) T cell antigen receptor (TCR) (22), or polyclonal naive CD4\(^+\) T cells and evaluated disease induction in \textit{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 \textit{Irf4–/–} mice to EAE was restored (Figure 3A and
Irf4–/– mice evoked stronger disease as compared to WT CD4+ T cells, while the other operates at high numbers of CD4+ T cells to induce EAE: one requires CCR6 on CD4+ cells to cooperate with CD8+ T cells for infiltration of 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–/– 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 B; 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 induction of disease, while IL-17A competence of CD4+ T cells is not required for their pathogenicity.

---

**Figure 5**

IL-17A competence of CD4+ T cells is not required for their pathogenicity. (A) Mean clinical scores (± SEM) of MOG35–55-immunized Irf4–/– mice (n = 4) substituted with 2.5 × 10⁶ WT CD4+ T cells or Il17a–/–CD4+ T cells with or without 10⁷ WT CD8+ T cells. P values were calculated comparing the scores of Irf4–/– mice after transfer of 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.
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 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.

**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 or Ccr6–/–CD8+ T cells and subsequently immunized with MOG35–55. 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 CD4+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.
IL-17A competence of CD8+ T cells accelerates CD4+ T cell encephalitogenicity. (A and B) Mean EAE scores (± SEM) combining 2 independent experiments of MOG37–50-immunized Irf4−/− (n = 6) mice that received sorted 2.5 × 10^6 CD44^loCD8+ 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.
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<sup>_−/−</sup> 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<sup>+</sup> T cells in a direct cell-cell interac-
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/eMS) and 6 from early MS (eMS). In contrast to the control (noninfectious headache, control), 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.

**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.

**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 RORyt 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 MOG35–50 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–/– CD8+ 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, Cer6−/− mice developed a similar disease course after immunization with MOG35-55 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−/− CD8+ 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 Ifnγ–/– mice, suggesting that the nonlymphopenic Ifnγ–/– 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 and Th17 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. Ifnγ–/–, Ccr6–/– (41), Rag1–/–, 2D2 mice expressing a transgenic TCR specific for M OG35,55 (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 proof of dissemination in space and time were classified as CIS (43); those who showed concurrent dissemination to the McDonald criteria (42). Patients lacking proof of dissemination in time were classified as eMS. An additional 17 patients with eMS (2.5 × 106 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+CD8– T cells (2.5 × 106 per mouse; CD45.1+, CD45.2+, CD45.2+ Ccr6–/–, or CD45.2+ Il17a–/–) or MACS-purified CD62L+CD4+ T cells from 2D2 transgenic mice on the CD45.1+ or CD45.2+ background (low numbers, 106; or high numbers, 107 per mouse) isolated from naive donor mice were transferred i.p. into Ifnγ–/– 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 CD8α (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, IFN-γ and Foxp3; FJK-16s; ebioscience) was used. Stainings were acquired 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 (eBioscience) was used. A FACSCalibur machine using the FlowJo software (Tree Star). Isotype staining revealed specificity of the IL-17 and IFN-γ stainings.

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 phosphoribosyltransferase (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′-TCTCCATCATACCTCTCAAGGCTAC-3′; and reverse, 5′-CTGATGGGCTCTGAGACAGA-3′.
Nucleofection. WT CD8+ T cells were nucleofected immediately after purifi-
ication with or without IRF4si or scrambled siRNA preparations. These siRNA
were prepared by IBA. Their sequences were previously described (9). Nucleofection of 106 cells in 100 μl of mouse T cell Nucleofector solu-
tion (Amasia) was performed using 500 pMol total of siRNAs and the WO01 program of the Nucleofector II machine (Amasia). 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 IF4m immunoblots, whole cell lysates were prepa-
red after 1 day of in vitro stimulation, and for Eomes immunoblots, whole cell lysates were prepared after 3 days of in vitro stimulation. Immuno-
blotting 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) was a gift from S.L. Reiner (University of Pennsylvania, Philadelphia, Pennsylvania, USA). The retroviral vectors containing Foxp3 and RORe were described previously (6). The gene encoding Foxp3 (GenBank accession no. NM_054039.1) was amplified by PCR using published primers (47), clone sequenced, digested by NotI and SalI, and cloned into the retroviral vector MSCV-IRES-Thy1.1 (gift from V. Heiss-
meier, GSF-Institute of Molecular Immunology, Munich, Germany) con-
taining the internal ribosome entry site–regulated (IRES-regulated) gene for mouse Thy1.1. WT or Il17a–/– CD8+ T cells were infected with the retrovi-
dres as described previously (6, 16) and stimulated via CD3/28 under the conditions indicated in the experiments. On day 3, the cells were restimu-
lated with PMA/ionomycin and then analysed 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 (RAM45+SBD), Vβ11 (R3–15+B), and CD8a (53–6.7; BD) for cytotoxic T cells sections and CD3 (CD3+12AB; Serotec) and MAC3 (M3/84; BD) for para-
affin 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 differ-
entiated 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 × 104) were mixed
with differentiated WT or Il17a–/– Tc17 cells or their SNs (final dilu-
tion: 30%; and stimulated with immobilized αCD3 (1 μg/ml) and soluble
αCD28 (5 μg/ml) in 1 ml volume. Exogenous rIL-17A (100 ng/ml) was added to some cocultures. After 72 hours of coculture, IL-17 concentra-
tions 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 restimu-
lation 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 Stu-
dent’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 con-
sent 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).

Acknowledgments

We thank Y. Iwakura for providing us with the IL-17A–defi-
cient mouse strain. We thank Hartmann Raifer and Gavin Giel for cell sorting. We thank Markus Hofer, Cornelia Brendel, and Katrin Kohse for advice and experimental support as well as Bär-
el Camara, Ginette Bortolussi, and Heike Geissel for technical support. This work was supported by Deutsche Forschungsgemeinschaft (grant HU 1824/2-1 to M. Huber; Behring-Röntgen-Stiftung and SFB/TR22 to M. Lohoff), Klinikum Marburg-Giesen (Projekt Nr. 16/2009 MR), Gemeinnützige Hertie-Stiftung (1.01.1/08/003), and LOEWE grant Tumor and inflammation (county of Hessen, Germany).

Received for review March 2, 2012, and accepted in revised form October 4, 2012.

Address correspondence to: Magdalena Huber or Michael Lohoff, Institute for Medical Microbiology and Hygiene, University of Marburg, Hans-Meierwein-Straße 2, 35033 Marburg, Germany. Phone: 49.6421.5866455; Fax: 49.6421.5866420; E-mail: magdalena.huber@staff.uni-marburg.de (M. Huber), lohoff@med. uni-marburg.de (M. Lohoff).

2. Korn T, Betteli E, Oukka M, Kuchroo VK. IL-17 and T(H)-17 cells require interferon-regulatory factor 4 (IRF-4) for cell different-
3. Chen Q, et al. IRF-4-binding protein inhibits inter-
leukin-17 and interleukin-21 production by con-
5. Bettini M, Rosenthal K, Evavalod BD. Pathogenic MOG-reactive CD8+ T cells require MOG-re-
active CD4+ T cells for sustained CNS inflam-
8. Pearce EL, et al. Control of effector CD8+ T cell

research article

The Journal of Clinical Investigation http://www.jci.org Volume 123 Number 1 January 2013 259


