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Astrocyte-Restricted Ablation of Interleukin-17-Induced Act1-Mediated Signaling Ameliorates Autoimmune Encephalomyelitis

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SUMMARY

Interleukin-17 (IL-17) secreted by T helper 17 (Th17) cells is essential in the development of experimental autoimmune encephalomyelitis (EAE). However, it remains unclear how IL-17-mediated signaling in different cellular compartments participates in the central nervous system (CNS) inflammatory process. We examined CNS inflammation in mice with specific deletion of Act1, a critical component required for IL-17 signaling, in endothelial cells, macrophages and microglia, and neuroectoderm (neurons, astrocytes, and oligodendrocytes). In Act1-deficient mice, Th17 cells showed normal infiltration into the CNS but failed to recruit lymphocytes, neutrophils, and macrophages. Act1 deficiency in endothelial cells or in macrophages and microglia did not substantially impact the development of EAE. However, targeted Act1 deficiency in neuroectoderm-derived CNS-resident cells resulted in markedly reduced severity in EAE. Specifically, Act1-deficient astrocytes showed impaired IL-17-mediated inflammatory gene induction. Thus, astroctyes are critical in IL-17-Act1-mediated leukocyte recruitment during autoimmune-induced inflammation of the CNS.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease in which T lymphocytes reactive to myelin antigens initiate an inflammatory response in the central nervous system (CNS) leading to demyelination and subsequent axonal injury (Becher et al., 2006; Gold et al., 2006; Sospedra and Martin, 2005). Experimental autoimmune encephalomyelitis (EAE) is an animal model commonly used to study the immunopathogenesis of multiple sclerosis (Stromnes and Goverman, 2006). EAE is induced by either immunization of animals with myelin antigens or the adoptive transfer of myelin antigen-specific T cells. EAE can be subdivided into an initiation stage involving activation and expansion of myelin-specific T cells in the periphery, which then cross the blood brain barrier (BBB), an effector stage involving reactivation of myelin-specific T cells in the CNS, resulting in cytokine-induced chemokine expression in CNS-resident cells, thereby mediating recruitment of hematogenously derived inflammatory cells, and a stage of remission and repair in which the immune response is down-regulated (McFarland and Martin, 2007; Steinman, 2001).

Whereas T helper 1 (Th1) cells were shown to play a critical role in the initiation of inflammatory responses in CNS (Agrawal et al., 2006; Bettelli et al., 2004; Korn et al., 2009; Yang et al., 2009), Th2 cells were considered as counterinflammatory (Butti et al., 2008; Kleinschek et al., 2007; Ramírez and Mason, 2000). This Th1-Th2 cell paradigm was challenged by the fact that lack of interferon-γ (IFN-γ) leads to increased EAE phenotype (Cua et al., 2003; Ferber et al., 1996). Importantly, both Th1 and Th17 cells can independently induce EAE possibly through different mechanisms (Lees et al., 2008; Kroenke et al., 2008; Stromnes et al., 2008; Park et al., 2005), and even Th2 cells can lead to a mild atypical form of EAE (Das et al., 1997; Jäger et al., 2009; Steinman, 2008). Th17 cells are generated as a discrete lineage after priming in the presence of the cytokines TGF-β and IL-6 and acquisition of encephalitogenicity after proliferation in the presence of IL-23 (Cua et al., 2003; Mangan et al., 2006; Veldhoen et al., 2006). Th17 cells are now recognized at least as one of the major mediators of tissue damage in EAE (Haak et al., 2009; Yang et al., 2009; Steinman, 2009). EAE is markedly suppressed in mice lacking IL-17 or IL-17 receptor, and IL-17-specific inhibition attenuates inflammation indicating that IL-17-mediated signaling plays a critical role in the effector stage of EAE (Gonzalez-García et al., 2009; Komiyama et al., 2006; Park et al., 2005; Harrington et al., 2005; Veldhoen et al., 2006). However, the precise mechanism by which IL-17 participates in EAE development and pathogenesis remains unclear.

A major function of IL-17 involves coordination of local tissue inflammation through upregulation of proinflammatory and neutrophil-mobilizing cytokines and chemokines including IL-6, G-CSF, TNF-α, IL-1, CXCL1(KC), CCL2(MCP-1), CXCL2(MIP-2), CCL7(MCP-3), and CCL20(MIP-3A) as well as matrix metalloproteases (MMPs) to allow activated T cells to penetrate the extracellular matrix (Awane et al., 1999; Weaver et al., 2005; Jovanovic et al., 1998). Several new studies have shown that IL-17

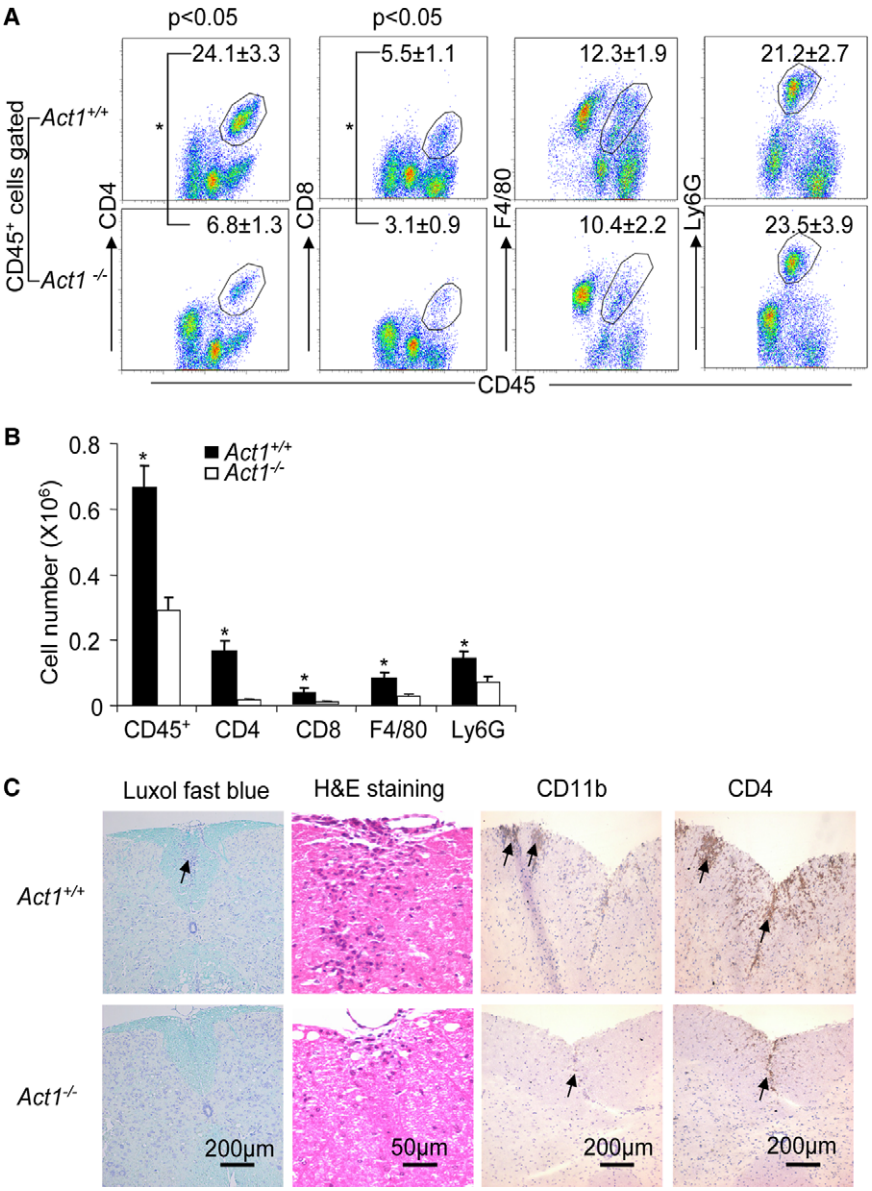

Figure 1. Act1 Deficiency Impairs Inflammatory Cell Infiltration in the CNS

(A and B) Immune cell infiltration in the brains of MOG 35-55-immunized wild-type and *Act1*$^{-/-}$ mice (n = 7, 7 days after disease onset) was analyzed by flow cytometry. Error bars, SEM. *p < 0.05.

(C) Luxol fast blue, hematoxylin and eosin, and anti-CD11b and CD4 staining of spinal cords of wild-type and Act1-deficient mice at peak of disease. Data are representative of three independent experiments. See also Figure S1.

signals through a heteromeric receptor complex, consisting of IL-17R (IL-17RA) and IL-17RC, which are single-pass transmembrane proteins expressed by a variety of cells including astrocytes and microglia (Inoue et al., 2006; Kolls and Lindén, 2004; Toy et al., 2006; Trajkovic et al., 2001). The important questions are whether and how these CNS-resident cells respond to IL-17 to mediate inflammatory response in the central nervous system leading to demyelination and subsequent axonal injury.

Both IL-17RA and IL-17RC belong to a newly defined SEFIR protein family with a conserved sequence segment called SEFIR in their cytoplasmic domain (Novatchkova et al., 2003). We recently described the signaling molecule Act1 as a key component in IL-17 signaling (Qian et al., 2007). Act1 contains two TRAF binding sites: a helix-loop-helix domain at the N terminus and a coiled-coil domain at the C terminus (Li et al., 2000; Qian et al., 2002; Leonardi et al., 2000). Act1 contains a SEFIR domain in its coiled-coil region at the C terminus and therefore Act1 is a member of the SEFIR protein family (Novatchkova et al., 2003). Upon IL-17 stimulation, Act1 is recruited to IL-17R through the IL-17R conserved cytoplasmic SEFIR domain, which is followed by recruitment of the kinase TAK1 and E3 ubiquitin ligase TRAF6 that mediate "downstream" NF-κB activation. Act1 deficiency results in reduced EAE severity in the presence of normal activation and expansion of encephalitogenic Th17 T cells, indicating that IL-17-induced Act1-mediated signaling plays a critical role in the effector stage of EAE development. However, it remains unclear how IL-17-mediated signaling in different cellular compartments participates in CNS inflammation during EAE. In the current study, we aimed to define the specific cell types that are critical for IL-17-dependent autoimmune inflammation of the CNS. By cell type-specific deletion of Act1, we showed that Act1 deficiency in the CNS-resident cells originated from neuroectodermal cells (neurons, astrocytes, and oligodendrocytes) delayed the onset and reduced the severity of EAE induced by either active immunization with the encephalitogenic MOG 35-55 peptide or by adoptive transfer of MOG-specific Th17 T cells.

RESULTS

Act1 Is Required for the Effector Stage of Th17 Cell- but Not Th1 Cell-Induced EAE

Our previous studies have shown that the onset and severity of EAE are greatly reduced in mice lacking Act1, the key adaptor molecule of IL-17R (Figure S1 available online). Act1-deficient (*Act1*$^{-/-}$) mice had fewer CNS inflammatory cells, including neutrophils, macrophages, CD4$^+$, and CD8$^+$ T cells compared to wild-type control mice (Figures 1A and 1B). Furthermore, histological examination revealed abundant CD4$^+$ T cells and CD11b$^+$ leukocytes in the meninges and in the spinal cord parenchyma, accompanied by severe demyelination in the white matter in wild-type mice (Figure 1C). In contrast,

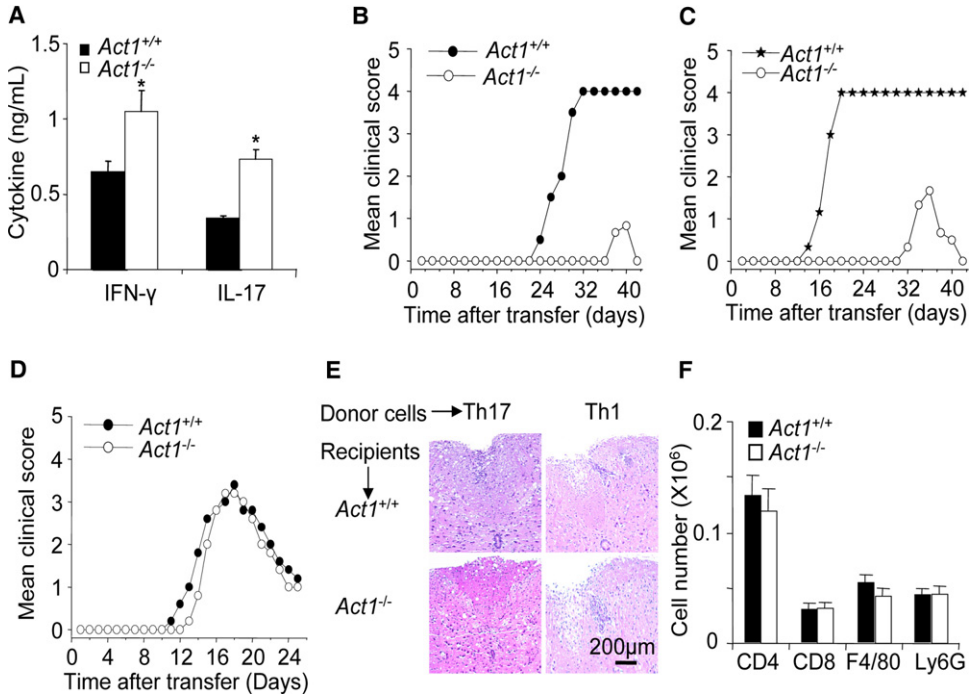

Figure 2. Act1 Is Required for the Effector Stage of Th17 Cell- but Not Th1 Cell-Induced EAE

(A) Draining lymph node cells from wild-type mice and *Act1*$^{-/-}$ mice 10 days after immunization with MOG35-55 were restimulated with MOG35-55 in vitro for 4 days, followed by ELISA of IL-17, IFN-γ. Error bars, SEM; n = 5 mice per group. *p < 0.05.

(B–D) Primed MOG 35-55-specific T cells (10 days) from (B) wild-type or (C) *Act1*$^{-/-}$ mice were restimulated with MOG 35-55 in vitro in the presence of recombinant IL-23 for 5 days and then transferred to naive wild-type or *Act1*$^{-/-}$ mice. Graph represents the average clinical score after T cell transfer.

(D) Primed MOG 35-55-specific wild-type T cells (10 days) were restimulated with MOG 35-55 in vitro in the presence of recombinant IL-12 and anti-IL-23p19 for 5 days and then transferred to naive wild-type and *Act1*$^{-/-}$ mice. Graph represents the average clinical score after T cell transfer.

(E) H&E staining of the spinal cords of wild-type and *Act1*$^{-/-}$ mice transferred with Th1 and Th17 cells and sacrificed 7 days after the onset of disease.

(F) Immune cell infiltration in the brains of wild-type and *Act1*$^{-/-}$ mice transferred with wild-type Th1 cells (n = 5, 7 days after disease onset) was analyzed by flow cytometry.

Error bars, SEM. Data are representative of three independent experiments. See also Figure S2.

inflammatory cells were reduced in spinal cords from *Act1*$^{-/-}$ mice coincident with minimum signs of demyelination (Figure 1C). The absence of Act1 therefore leads to impaired CNS inflammation, resulting in reduced demyelination and subsequent axonal injury.

Supernatants from primed lymph node cells of *Act1*$^{-/-}$ mice showed increased production of Th1 (IFN-γ) and Th17 (IL-17) cell-associated cytokines relative to primed wild-type mice (Figure 2A). Consistent with this finding, intracellular staining showed increased numbers of IFN-γ- and IL-17-producing CD4^{+} T cells in the primed lymph node cells of *Act1*$^{-/-}$ mice than that in wild-type mice. IL-17-producing γδ T cells were also increased in the primed lymph node cells of *Act1*$^{-/-}$ mice compared to that in wild-type mice (Figure S2A). The impact of Act1 deficiency on IFN-γ- and IL-17-producing CD4^{+} and γδ T cells in periphery might be related to the hyper T cell-dependent immune responses previously observed in the *Act1*$^{-/-}$ mice (Qian et al., 2007), although the detailed mechanism remains unclear. Importantly, activated MOG 35-55-specific Th1 (Qian et al., 2007) and Th17 cells derived from primed *Act1*$^{-/-}$ mice were both fully encephalitogenic because they were able to passively transfer EAE in wild-type recipients (Figures 2B–2D; Figures S2B and S2C). Thus, *Act1*$^{-/-}$ mice had no defect in the activation of MOG 35-55-specific Th17 or Th1 cells.

Both wild-type and *Act1*$^{-/-}$ MOG 35-55-specific Th17 cells induced EAE in wild-type recipients. However, the onset and severity of EAE were greatly reduced in *Act1*$^{-/-}$ recipients, independent of the genotype of the transfer population (Figures 2B and 2C). These results demonstrate that Act1 is required for the effector stage of EAE induced by Th17 cells. However, recent studies and the results described above indicate that Th1 cells can also induce EAE. To show that Act1 only acts through Th17 cells and not Th1 cells, the activated MOG-specific Th1 cells polarized from wild-type mice were tested for EAE induction. *Act1*$^{-/-}$-recipient mice of Th1 cells exhibited similar onset and severity of EAE as wild-type recipients, indicating that Act1 deficiency has no obvious impact on the effector stage of Th1 cell-induced EAE (Figures 2D–2F).

Th17 Cells Infiltrate the CNS but Fail to Initiate a Pathogenic Inflammatory Cascade in *Act1*$^{-/-}$ Mice

Our results clearly indicate that Act1 deficiency does not impair peripheral activation of MOG 35-55-specific T cells, but rather affects functions within the CNS. Furthermore, Th17 cell

responses in the CNS are associated with recruitment of bone marrow-derived myeloid cells (Park et al., 2005; Komiyama et al., 2006; Harrington et al., 2005; Mangan et al., 2006; Kolls and Lindén, 2004; Veldhoen et al., 2006). We therefore investigated a role of Act1 in CNS recruitment of MOG-specific T cells as well as subsequent recruitment of other mononuclear cells. MOG-specific T cells were isolated from the lymph nodes of Thy1.1 C57BL/6 mice 10 days after MOG 35-55 immunization, polarized to Th17 cells, and transferred into Thy1.2$^+$ wild-type or *Act1*$^{-/-}$ mice. Similar numbers of Thy1.1$^+$CD4$^+$ donor T cells were detected in the brains of wild-type and *Act1*$^{-/-}$ brains at days 3 and 5, early after adoptive transfer. However, during the later phase at days 12 and 15, at the peak of the disease, significantly more Thy1.1$^+$CD4$^+$ T cells and monocytes accumulated in the brains of wild-type compared to *Act1*$^{-/-}$ mice (Figures 3A–3C). Overall, the vast majority of CNS-derived CD4$^+$ T cells were of donor Thy1.1 phenotype throughout disease induction (data not shown). We also examined the Thy1.1$^+$CD4$^+$ T cells in the spleen of wild-type and *Act1*$^{-/-}$ recipient mice after adoptive transfer. We detected more Thy1.1$^+$CD4$^+$ T cells in the spleen of the *Act1*$^{-/-}$ mice at day 5 and day 12 compared to that in wild-type control mice. By day 15, Thy1.1$^+$CD4$^+$ T cells disappeared from spleen. The temporal retention of Thy1.1$^+$CD4$^+$ T cells in the *Act1*$^{-/-}$ spleen might reflect the reduced recruitment of these cells to the *Act1*$^{-/-}$ CNS (Figure 3D).

These results indicate that Th17 cells can infiltrate the CNS of *Act1*$^{-/-}$ mice but fail to initiate an effective inflammatory response, presumably because of the inability to propagate IL-17-mediated signaling. IL-17-induced signature cytokines and chemokines were indeed markedly reduced in the spinal cord of the *Act1*$^{-/-}$ recipient mice at the peak of disease after adoptive transfer of MOG-specific Thy1.1$^+$ Th17 cells (Figure 3E). Of note, both CXCL1 and MMP9 associated with neutrophil recruitment, as well as IL-6 and CXCL12 involved in parenchymal leukocyte access, were reduced in *Act1*$^{-/-}$ recipients. As a result, the inflammatory amplification loop is blocked, leading to reduced recruitment of inflammatory cells.

It is important to note that in addition to Thy1.1$^+$CD4$^+$ IL-17-producing cells in the brain of the recipient mice at the peak of the disease, IL-17-producing Thy1.1$^+\gamma\delta$ T cells were also detected in the brain of these mice (Figures 3F and 3G). Consistent with the clinical signs of the mice (Figure 3A), the absolute numbers of IFN-γ- and IL-17-producing Thy1.1$^+$CD4$^+$ T cells and Thy1.1$^+\gamma\delta$ T cells were significantly reduced at the peak of the disease in the brain of *Act1*$^{-/-}$-recipient mice compared to that in the wild-type control mice. IL-17-producing CD4$^+$ (Th17) cells activated in the presence of IL-23 in the primary culture (Figure S2C) were relatively stable in vivo (Figures 3F and 3G), whereas the IL-17-producing Thy1.1$^+\gamma\delta$ T cells showed more plasticity in vivo, becoming IFN-γ and IL-17 double-positive cells or IFN-γ-producing cells (Figures 3F and 3G). In contrast, the majority of both CD4$^+$ T cells and $\gamma\delta$ T cells activated in the presence of IL-12 in the primary culture (Figure S2B) remain as IFN-γ-producing cells in the brain of the recipient mice, indicating their stability in vivo (Figure S2D). This result is consistent with the fact that Act1 deficiency did not show any impact on Th1 cell-mediated EAE, because these "Th1" cells remain as IFN-γ-producing cells in the brain after adoptive transfer (Figure S2D).

Act1 Deficiency in Recipient Mice Does Not Impact Proliferation or Survival of CNS Th17 Cells

We further investigated whether the reduced IL-17-induced cytokine and chemokine production in the CNS has any impact on CD4$^+$ T cell survival and/or proliferation. To test this possibility, MOG 35-55-specific Thy1.1$^+$ Th17 cells were adoptively transferred into Thy1.2 *Act1*$^{-/-}$ and wild-type recipient mice. Thy1.1$^+$ cells costained with Ki-67, indicating that the infiltrated Thy1.1$^+$ cells were in a proliferating state irrespective of their wild-type or *Act1*$^{-/-}$ environment in recipients (Figure 4A). Furthermore, the frequency of infiltrated CD4$^+$Brdu$^+$ cells in the spinal cord of wild-type mice was similar to that in *Act1*$^{-/-}$ mice (Figure 4B). Thus, the reduced accumulation of Thy1.1$^+$ cells in the CNS of *Act1*$^{-/-}$ mice at the peak of disease cannot be attributed to their reduced proliferation or survival, but rather reflects impaired chemokine- and/or cytokine-mediated recruitment of activated myeloid cells, which in turn propagate ongoing inflammation.

Endothelial-Derived Act1 Is Dispensable for EAE Development

Laminin is present in both endothelial basement membrane and parenchymal basement membrane (Bauer et al., 2009; Sixt et al., 2001) providing a tool to visualize inflammatory cells in the perivascular space. No differences were noted in the distribution of MOG 35-55-specific Thy1.1$^+$ Th17 cells in wild-type compared to Act1-deficient recipient mice (Figure 5A). Although some Thy1.1 cells were in close proximity to laminin, a large proportion had spread into the parenchyma. These results negated a role for Act1 in influencing access of myelin-specific T cells through the BBB into the CNS parenchyma. Nevertheless, previous studies have shown that the IL-17 receptor is expressed on BBB endothelial cells (Kebir et al., 2007), potentially influencing induction of chemokines or cytokines. To further explore whether endothelial cell-specific IL-17-induced Act1-mediated signaling impacts EAE pathogenesis, we generated endothelial cell-specific Act1-deficient mice. TIE2eCre transgenic mice (generated with the endothelial-specific TIE2e promoter) express Cre within endothelium of brain, heart, and liver, among others. Real-time PCR showed that Act1 expression was indeed abolished in PECAM-1$^+$ endothelial cells from the TIE2eCre*Act1*$^{fl/-}$ mice (Figure 5B). The onset and disease severity of EAE were similar in TIE2eCre*Act1*$^{fl/-}$ and TIE2eCre*Act1*$^{fl/+}$ mice after either immunization with MOG 35-55 or adoptive transfer with MOG 35-55-specific Th17 cells (Figures 5C and 5D). However, EAE induced by active immunization resulted in fewer CD4$^+$ and CD8$^+$ T cells in the CNS of the endothelial-specific Act1-deficient mice compared to control mice during the recovery phase of disease (Figure 5E). Therefore, despite moderate effects of endothelial-derived Act1 on the leukocyte infiltration, they were insufficient to have a substantial impact on clinical symptoms of Th17 cell-mediated EAE.

Macrophage- and Microglial-Derived Act1 Are Dispensable for EAE Development

Perivascular macrophages and microglia are important antigen-presenting cells in the CNS, which can also produce inflammatory cytokines and chemokines upon activation (Aloisi, 2001; Carson, 2002). Macrophages are one of the major effector cells

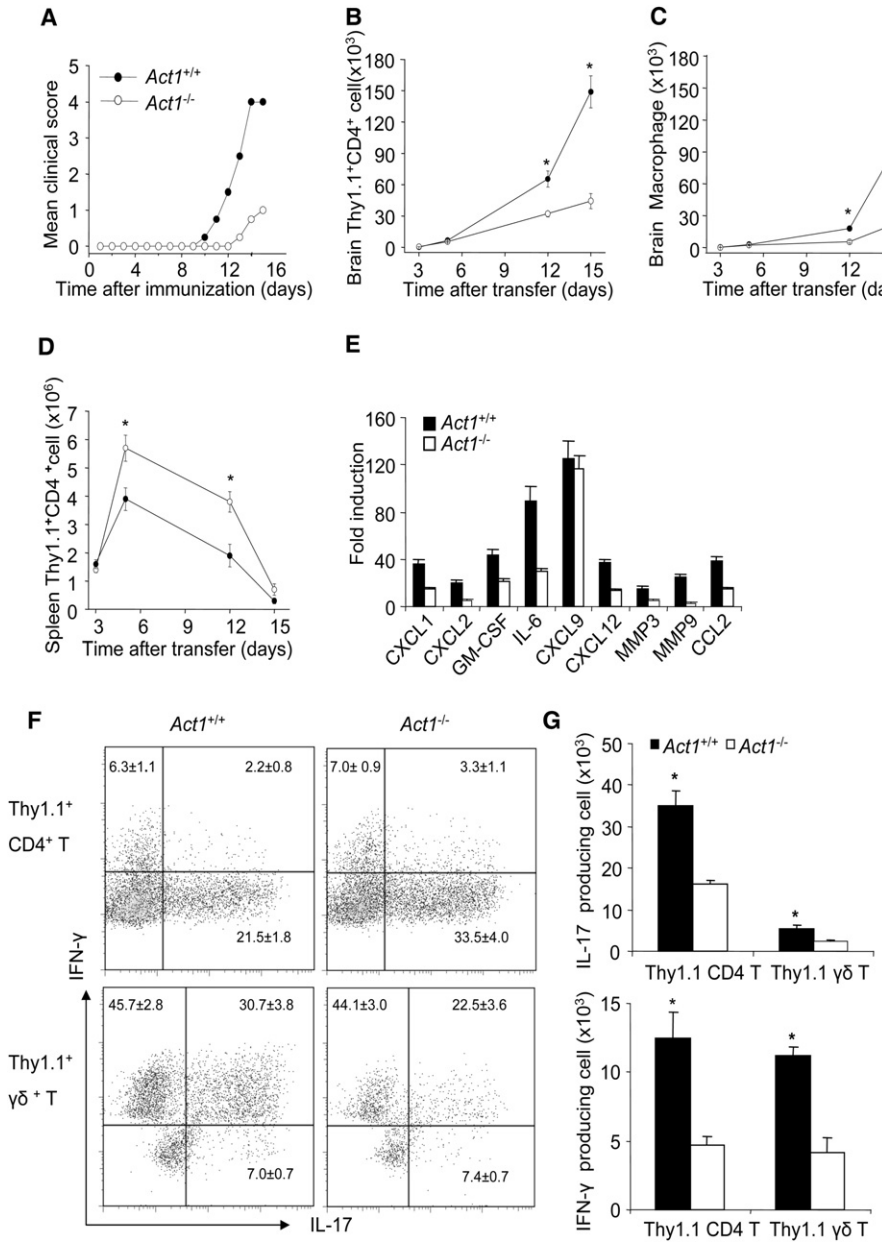

Figure 3. Th17 Cells Can Infiltrate CNS but Fail to Initiate Effective Inflammatory Cascade in *Act1*$^{-/-}$ Mice

(A) Mean clinical score of wild-type and *Act1*$^{-/-}$ mice adoptively transferred with MOG-specific Thy1.1$^+$ Th17 cells (n = 4/group). Lymph node cells harvested from Thy1.1 C57BL/6 mice 10 days after immunization with MOG 35-55 were polarized to Th17 cells as described in Figure 2 and transferred to wild-type and *Act1*$^{-/-}$ mice. Graph represents the average clinical score after T cell transfer.

(B–D) Immune cell infiltration in the (B, C) brain and (D) spleen of wild-type and *Act1*$^{-/-}$ mice transferred with MOG-specific Thy1.1$^+$ Th17 cells (n = 5/group, 3, 5, 12, and 15 days after T cell transfer) was analyzed by flow cytometry. Error bars, SEM; *p < 0.05.

(E) Real-time PCR analysis of relative expression of inflammatory genes as indicated in spinal cords of wild-type and *Act1*$^{-/-}$ mice transferred with MOG-specific Thy1.1$^+$ Th17 cells (n = 3, 15 days after T cell transfer) compared to the control samples from naive mice.

(F and G) Intracellular staining for the infiltrated IL-17-secreting or IFN-γ-secreting Thy1.1$^+$CD4$^+$ T cells and Thy1.1$^+$ γδ T cells in the brain of wild-type and *Act1*$^{-/-}$ mice transferred with MOG-specific Thy1.1$^+$ Th17 cells (n = 10/group, 15 days after T cell transfer). The frequency of IL-17$^+$ or IFN-γ$^+$ cells in Thy1.1$^+$CD4$^+$ cell population (top) and Thy1.1$^+$ γδ T cells (bottom) were shown in (F) and the corresponding absolute cell numbers were shown in (G).

See also Figure S2.

in CNS damage during EAE development. Because IL-17 responsiveness has been detected in both microglia and macrophages, it is critical to examine the importance of IL-17-induced Act1-mediated signaling in macrophages and microglial cells for the pathogenesis of EAE. CD11bCre transgenic mice (generated with the promoter for CD11b, an integrin expressed exclusively

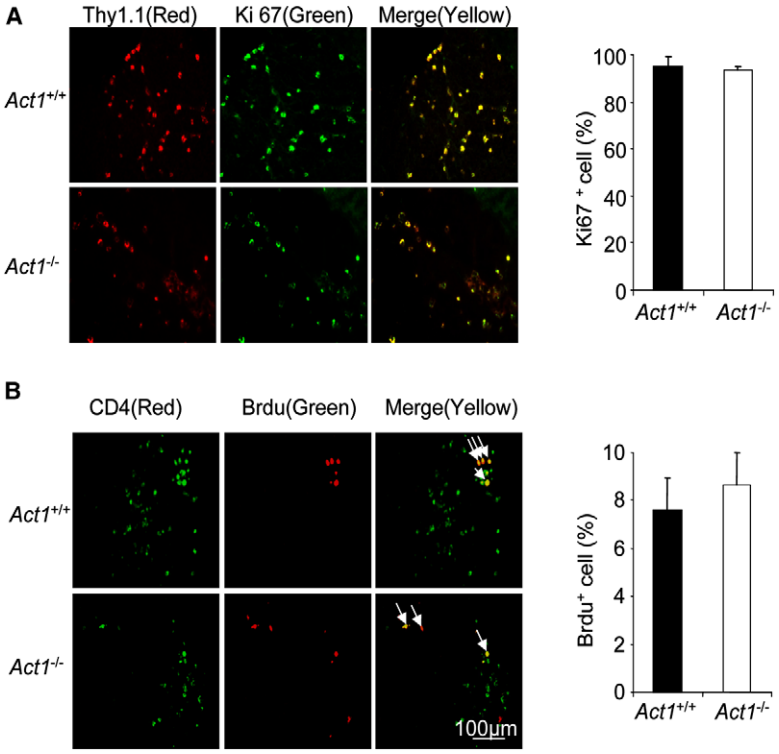

Figure 4. Act1 Deficiency in the Recipient Mice Does Not Impact on the Proliferation and Survival of the Infiltrated Th17 Cells

(A) Immunofluorescent staining for Ki67-positive Thy1.1 cells in the spinal cords of wild-type and Act1-deficient mice transferred with MOG-specific Thy1.1$^+$ Th17 cells (n = 4, 12 days after T cell transfer). Frozen sections of spinal cords collected from the recipient mice were stained with Thy1.1 (red) and Ki67 (green) antibodies. The presented frequency of Ki67$^+$ cells in total Thy1.1$^+$ cells in the spinal cord was an average of four spinal cord regions each group. Error bars, SEM.

(B) Brdu incorporation of infiltrated T cells in the spinal cords of wild-type and *Act1*$^{-/-}$ mice transferred with MOG-specific Thy1.1$^+$ Th17 cells (n = 4, 12 days after T cell transfer). On day 12 after adoptive transfer of MOG-specific Thy1.1$^+$ Th17 cells, wild-type and *Act1*$^{-/-}$ recipient were administrated with Brdu (i.p., 1 mg/mouse). Spinal cords were collected from the recipient mice 12 hr after Brdu administration and stained with Brdu antibody (red) and CD4 antibody (green). The presented percentage of Brdu-positive cells in total CD4$^+$ cells was an average of four spinal cord regions each group. Error bars, SEM.

in the myeloid lineage) express Cre in macrophages and microglia. CD11bCre*Act1*$^{+/-}$ mice were bred onto *Act1*$^{fl/fl}$ to generate control mice (CD11bCre*Act1*$^{fl/+}$) and macrophage- and microglia-specific Act1-deficient mice (CD11bCre*Act1*$^{fl/-}$). Real-time PCR showed that Act1 expression was abolished in bone marrow-derived macrophage and in microglia cultures derived from CD11bCre*Act1*$^{fl/-}$ mice (Figure 6A). Immunoblots confirmed the absence of Act1 expression in macrophages and demonstrated a negligible contribution of microglia-derived Act1 to overall protein expression in both brain and spinal cord (Figure 6B). Both immunization with MOG 35-55 and adoptive transfer of activated MOG 35-55-specific Th17 cells resulted in similar onset and disease severity of EAE in the CD11bCre*Act1*$^{fl/-}$ mice compared to control mice (Figures 6C and 6D). Interestingly, infiltrating Ly6G$^+$ neutrophils were reduced in CNS of CD11bCre*Act1*$^{fl/-}$ mice compared to that in control mice, whereas total numbers of F4/80$^+$ microglia and monocytes were not affected (Figure 6E). These results indicate that macrophage- and microglial-specific Act1 deficiency has some impact on the recruitment of polymorphonuclear neutrophils. Importantly, however, reduced neutrophils did not have a substantial impact on clinical signs of Th17 cell-mediated EAE pathogenesis.

Amelioration of EAE by Targeting Act1 in CNS-Resident Cells Derived from Neuroectodermal Cells

The results above revealed that Act1 signaling in either endothelial cells or myeloid cells is not the major source contributing to Th17 cell-mediated EAE pathogenesis. We therefore investigated the impact of other CNS-resident cells in Th17 cell-mediated EAE development and pathogenesis. The NesCre transgene mediates excision of LoxP-flanked sequences in early neuronal precursors during embryonic life, resulting in efficient target gene inactivation in all neuroectodermal cells of the CNS, including neurons, astrocytes, and oligodendrocytes (Graus-Porta et al., 2001; Tronche et al., 1999). For CNS-restricted ablation of Act1, the *Act1*$^{-/-}$ mice were first bred onto NesCre transgenic mice to generate NesCre*Act1*$^{+/-}$ mice. These mice were further bred onto Act1$^{fl/fl}$ mice to generate control mice (NesCre*Act1*$^{fl/+}$) and CNS-restricted Act1-deficient mice (NesCre*Act1*$^{fl/-}$). Immunoblot analysis showed that Act1 expression was completely ablated in astrocytes derived from NesCre*Act1*$^{fl/-}$ mice (Figure 7A). Moreover, Act1 expression was also dramatically reduced in the brains of the NesCre*Act1*$^{fl/-}$ mice (Figure 7A).

To determine the impact of CNS-restricted Act1 deficiency on EAE development, EAE was induced in NesCre Act1$^{fl/-}$ mice and control mice (NesCre*Act1*$^{fl/+}$) by active immunization with MOG 33-55. The onset of EAE was significantly delayed in NesCre*Act1*$^{fl/-}$ compared to that in control mice. Importantly, NesCre*Act1*$^{fl/-}$ mice exhibited greatly reduced disease severity (Figure 7B). Consistent with reduced clinical disease, mononuclear cell infiltrates were substantially decreased in white matter of spinal cords from NesCre*Act1*$^{fl/-}$ relative to control mice (Figure 7C). Control mice exhibited many CD4$^+$ T cells and CD11b$^+$ leukocytes in the meninges and in the spinal cord parenchyma, accompanied by histological signs of severe demyelination in the white matter (Figure 7C). In contrast, reduced inflammatory cells in spinal cords from NesCre*Act1*$^{fl/-}$ mice coincided with minimum signs of demyelination (Figure 7C). Therefore, the lack of Act1 in these CNS-resident cells leads to impaired infiltration of inflammatory cells into the CNS, resulting in reduced demyelination.

Consistent with the fact that Act1 deficiency does not impair the activation of MOG 35-55-specific T cells in EAE, supernatants from 10-day primed lymph node cells from both NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ control mice showed

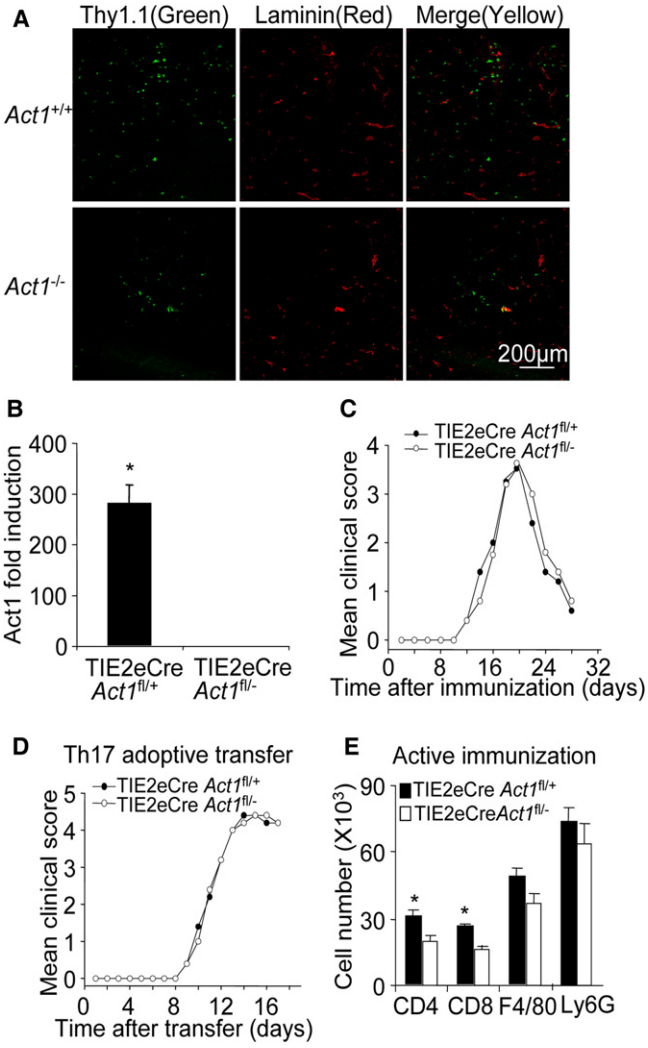

Figure 5. Endothelial-Derived Act1 Is Dispensable for EAE Development

(A) Immunofluorescent staining of $CD4^+$ T cells relative to the vasculature in the spinal cord. Spinal cords of wild-type and *Act1*$^{-/-}$ mice transferred with MOG-specific $Thy1.1^+$ Th17 cells (n = 3, 12 days after T cell transfer) were stained with CD4 (green) and pan-laminin (red) antibodies. The presented data are a representative of three independent experiments.

(B) Real-time PCR for the Act1 expression in $PECAM\text{-}1^+$ heart endothelial cells from endothelial-specific Act1-deficient (TIE2eCre*Act1*$^{fl/-}$) and control (TIE2eCre*Act1*$^{fl/+}$) mice.

(C) Mean clinical score of EAE in TIE2eCre*Act1*$^{fl/-}$ and TIE2eCre*Act1*$^{fl/+}$ induced by active immunization with MOG 35-55 (n = 5/group).

(D) Mean clinical score of EAE in TIE2eCre*Act1*$^{fl/-}$ and TIE2eCre*Act1*$^{fl/+}$ mice induced by MOG-specific wild-type Th17 cells.

(E) Flow cytometry analysis of immune cell infiltration in the brains of MOG 35-55 immunized TIE2eCre*Act1*$^{fl/-}$ and TIE2eCre*Act1*$^{fl/+}$ mice (n = 5, 14 days after disease onset).

Error bars, SEM; *$p < 0.05$.

comparable frequency of Th1 and Th17 cells (Figure S3). To confirm that the resistance of NesCre*Act1*$^{fl/-}$ mice to EAE is due to the the effector stage of EAE, both NesCre*Act1*$^{fl/+}$ and NesCre*Act1*$^{fl/-}$ mice were used as recipients of activated wild-type MOG 35-55-specific Th1 or Th17 cells. Whereas the Th1 cells induced a similar EAE phenotype in both groups of mice, the onset and severity of Th17 cell-induced EAE were greatly reduced in NesCre*Act1*$^{fl/-}$ compared to that in control mice (Figures 7D and 7E). The NesCre*Act1*$^{fl/-}$ mice had fewer inflammatory cells, including $CD4^+$ and $CD8^+$ T cells, macrophages, and neutrophils, infiltrating the CNS at peak of disease than did control mice after Th17 cell transfer (Figure 7E).

Impaired IL-17-Mediated Inflammatory Gene Induction in Astrocytes from CNS-Restricted Act1-Deficient Mice

During EAE, signature IL-17-responsive inflammatory genes (cytokines, chemokines, and matrix metalloproteinases) are significantly induced in CNS (Weaver et al., 2005; Gold et al., 2006; Becher et al., 2006). Whereas the expression of CXCL1, CXCL2, CCL20, CXCL-12, GM-CSF, IL-6, MMP3, and MMP9 was induced in control mice, the induction of these inflammatory genes was greatly reduced in NesCre*Act1*$^{fl/-}$ mice (Figure 7F). These results suggest that the impact of CNS-restricted Act1 deficiency in the induction of EAE was probably due to the defect of IL-17-induced inflammatory gene expression in these mice.

Because astrocytes have been shown to be responsive to IL-17 and play a major role in the production of cytokines and chemokines during EAE (Dong and Benveniste, 2001; Trajkovic et al., 2001), we examined astrocytes from NesCre*Act1*$^{fl/+}$ and NesCre*Act1*$^{fl/-}$ mice. IL-17-mediated NF-κB activation (phosphorylation and degradation of IκB) was abolished in astrocytes from NesCre*Act1*$^{fl/-}$ compared to that in control cells (Figure 7G). IL-17- and IL-17+TNF-induced inflammatory gene expression was reduced in Act1-deficient astrocytes as compared to that in control mice (Figure 7H; Figure S4A). Because previous studies reported that astrocytes are also responsive to IFN-γ (Croitoru-Lamoury et al., 2003; Halonen et al., 2006), we examined the gene expression profile of astrocytes in response to IL-17+TNF and IFN-γ+TNF. We found that distinct chemokines were induced by IL-17+TNF and IFN-γ+TNF in astrocytes. CXCL1, CXCL2, and CCL20 were specifically induced by IL-17+TNF in an Act1-dependent manner, whereas CXCL9, CXCL10, and CXCL11 were only induced by IFN-γ+TNF (but not by IL-17+TNF) in both wild-type and Act1-deficient astrocytes (Figure 7H). Taken together, these results implicate astrocytes as the probable source, contributing to the different mechanisms utilized by Th1 and Th17 cells to mediate EAE.

Neurons from wild-type mice had very weak response to IL-17 stimulation and the synergistic effect between IL-17 with TNF was not observed in these cells (Figure S4B). Because Act1 deficiency in endothelial or myeloid cells did not have substantial impact on EAE phenotype, it is important to determine whether they are indeed unresponsive to IL-17, similar to what was observed for neuron cells. We found that IL-17 stimulation alone failed to induce inflammatory gene expression in microglial or endothelial cells (Figures S4C and S4D). Although endothelial cells and microglia were highly responsive to TNF, IL-17 had modest effect only on some of the TNF-induced genes in these cells that might be compensated by other inflammatory cytokine signaling (such as TNF or IL-1 signaling) on these cells during EAE. Taken together, these results suggest that the reduction of IL-17 signaling in Act1-deficient astrocytes probably contributes to EAE resistance in Act1-deficient and NesCre*Act1*$^{fl/-}$ mice.

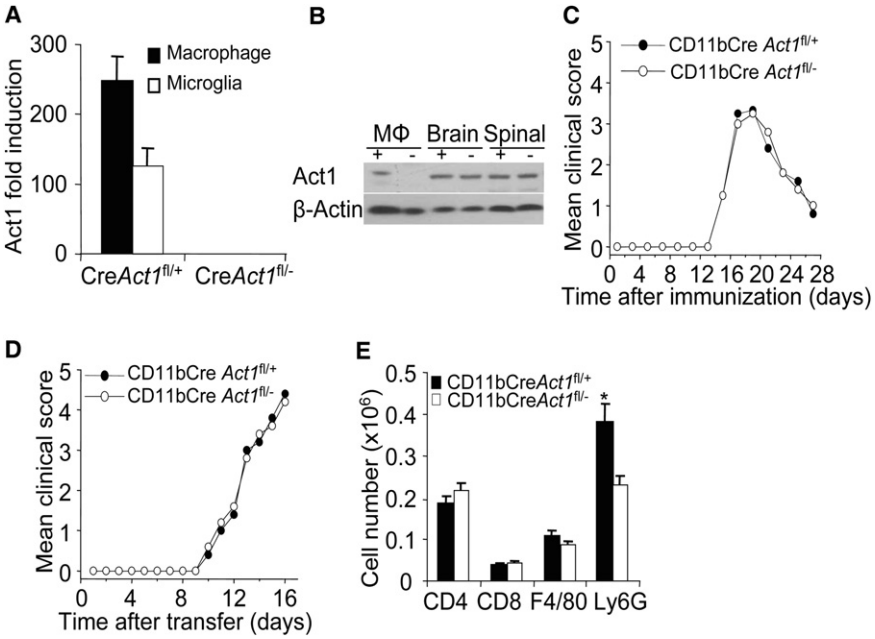

Figure 6. Macrophage- and Microglial-Derived Act1 Are Dispensable for EAE Development

(A) Real-time PCR for the Act1 expression in bone marrow-derived macrophages and microglia from the macrophage/microglia-specific Act1-deficient (CD11bCre*Act1*$^{fl/-}$) and control (CD11bCre*Act1*$^{fl/+}$) mice.

(B) Immunoblot analysis for the Act1 expression in bone marrow-derived macrophages from the macrophage- and microglia-specific Act1-deficient (CD11bCre*Act1*$^{fl/-}$) and control (CD11bCre*Act1*$^{fl/+}$) mice.

(C) Mean clinical score of EAE in CD11bCre*Act1*$^{fl/-}$ and CD11bCre*Act1*$^{fl/+}$ mice induced by active immunization with MOG 35-55 (n = 5/group).

(D) Mean clinical score of EAE in the CD11bCre*Act1*$^{fl/-}$ and CD11bCre*Act1*$^{fl/+}$ mice induced by MOG-specific Th17 cells.

(E) Flow cytometry analysis of immune cell infiltration in the brains of MOG 35-55 immunized CD11bCre*Act1*$^{fl/-}$ and CD11bCre*Act1*$^{fl/+}$ mice (n = 5, 7 days after disease onset).

Error bars, SEM; *p < 0.05.

DISCUSSION

Here we have addressed how Act1, the key adaptor molecule of IL-17-mediated signaling in different cellular compartments, participates in the inflammatory process in the CNS, leading to the pathogenesis of EAE. Importantly, we found that deletion of Act1 in the neuroectoderm-derived cells (including astrocytes, oligodendrocytes, and neurons), but not in endothelial cells or macrophages and microglia, delayed the onset and reduced the severity of Th17 cell-induced EAE. Furthermore, IL-17-mediated production of cytokines and chemokines was impaired in astrocytes from these CNS-restricted Act1-deficient mice, consistent with reduced recruitment of inflammatory cells in CNS of these mice and their resistance to EAE.

Previous studies have demonstrated the important role of activated CD4$^+$ Th17 cells for the effector stage of EAE development through the production of IL-17. Recent studies have shown that IL-17-producing γδ T cells were found at substantial frequency in the brain of mice with EAE (Sutton et al., 2009; Wohler et al., 2009). It has been proposed that IL-17-secreting γδ T cells cooperate with CD4$^+$ T cells in the development of EAE. We also detected IL-17-producing γδT cells in the primed lymph node cells and in the brain of mice with EAE. Regardless of the source of IL-17, IL-17 antibody attenuates the induction of chemokines, delays the onset of EAE, and reverses the progress of EAE (Park et al., 2005; Weaver et al., 2005; Gold et al., 2006; Becher et al., 2006). IL-17 or IL-17 receptor deficiency reduces the disease severity of EAE (Gonzalez-García et al., 2009; Komiyama et al., 2006; Park et al., 2005; Harrington et al., 2005; Veldhoen et al., 2006). Consistently, although activated wild-type and Act1-deficient MOG 35-55-specific Th17 cells (IL-17-producing T cells, activated in the presence of IL-23 in primary culture) induced EAE in wild-type recipient mice, the onset and severity of EAE were greatly reduced in Act1-deficient recipient mice, demonstrating that Act1 is indeed required for the effector stage of EAE induced by Th17 cells. It is important to note that similar onset and severity of EAE were induced in wild-type and Act1-deficient recipient mice after adoptive transfer of wild-type activated MOG-specific Th1 cells (IFN-γ-producing cells, activated in the presence of IL-12 in the primary culture), indicating that Act1 deficiency does not impact Th1 cell-mediated EAE. Taken together, these results confirm the critical role of IL-17-producing T cells in EAE development and pathogenesis and demonstrate the essential role of IL-17-induced Act1-mediated pathway in this disease process.

The important question is how IL-17-mediated signaling contributes to the effector stage of Th17 cell-induced EAE. The effector stage of EAE includes the recruitment and reactivation of neuroantigen-reactive CD4$^+$ T lymphocytes and subsequent inflammatory response within CNS. After priming in lymph nodes, antigen-specific Th17 cells traffic through the choroid plexus into the subarachnoid space (Wave 1) where they are reactivated (Reboldi et al., 2009; Ransohoff, 2009). As a consequence of productive T cell-APC interactions, Th17 cell signature cytokines, including IL-17, are produced and impinge on the adjacent CNS tissue. After activation of the parenchymal vasculature by this cytokine flux, perivascular leukocyte infiltrates accumulate (Wave 2), leading to the explosive inflammatory cascade associated with the onset of EAE. Th17 cells had been proposed to act either on glial cells (including astrocytes and microglia) or directly on endothelial cells to promote BBB disruption (Kebir et al., 2007; Das Sarma et al., 2009). We demonstrated that Act1 deficiency in endothelial or myeloid cells did not have a substantial impact on EAE phenotype. Instead, the deletion of Act1 in neuroectoderm cells abolished the induction of EAE, indicating the essential role of this particular cellular compartment in Th17 cell-mediated EAE.

Astrocytes are neuroectoderm cells whose direct contacts with the glial limitans and the cerebral vasculature enables them to couple inflammatory cytokine expression to invasion

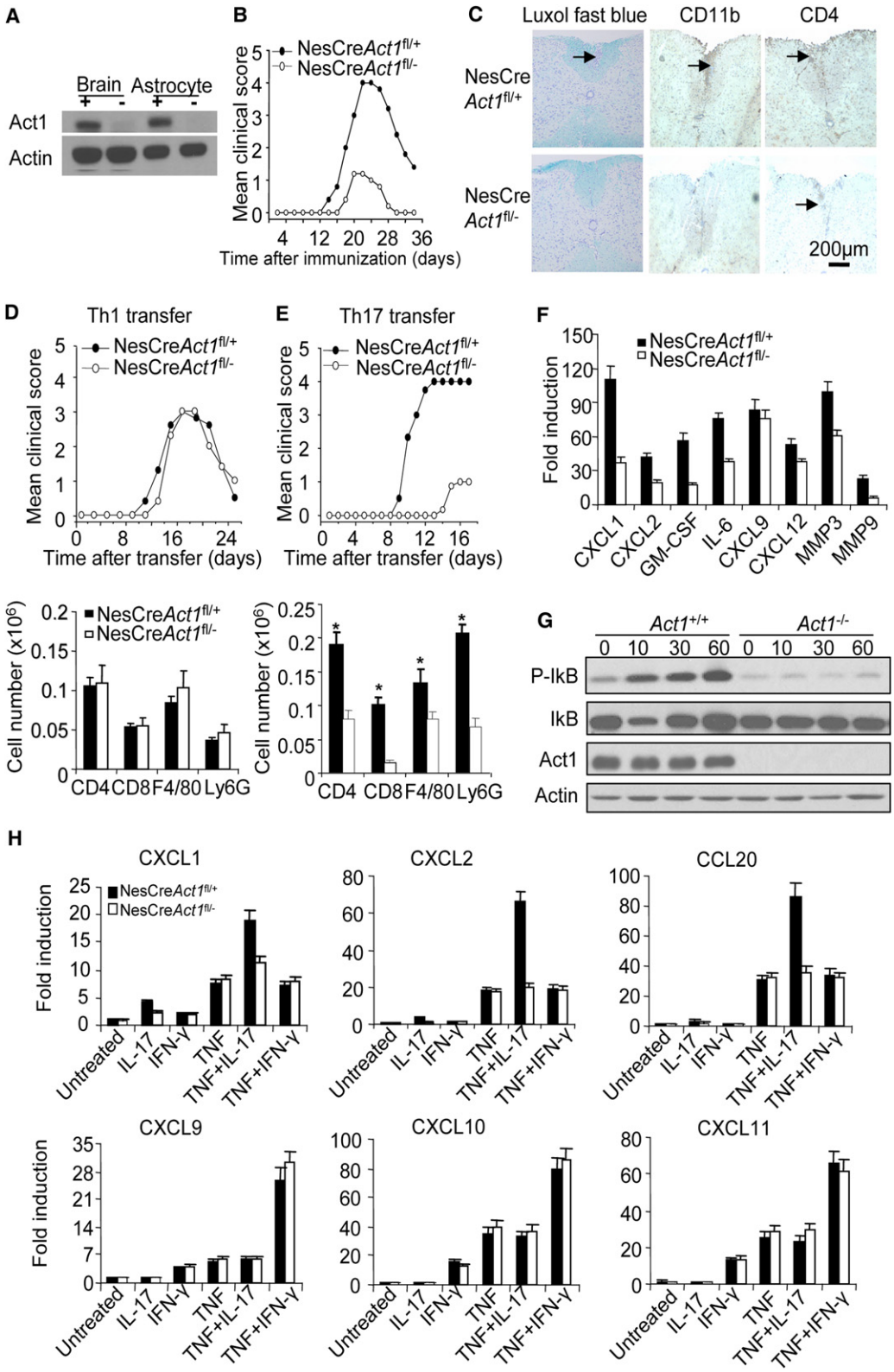

Figure 7. Amelioration of EAE by Targeting Act1 in CNS-Resident Cells Derived from Neuroectodermal Cells

(A) Immunoblot analysis for the Act1 expression in the brains and astrocytes from CNS-restricted Act1-deficient (NesCre*Act1*$^{fl/-}$) and control mice (NesCre*Act1*$^{fl/+}$).

(B) Mean clinical score of EAE in NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ mice induced by active immunization with MOG 35-55.

of the CNS by hematogenous leukocytes. IL-17 stimulation alone induced inflammatory gene expression (CXCL1 and GM-CSF) only in astrocytes but not in microglia or endothelial cells. Inflammatory cytokines, chemokines, and MMPs (including GM-CSF, CXCL12, CCL20, and MMP9) were synergistically induced by IL-17+TNF in astrocytes, but not in endothelial cells or microglia. Thus, astrocytes might directly respond to IL-17 from Th17 cells to produce cytokines and chemokines, contributing to Th17 cell-mediated EAE. Based on our findings, we propose that IL-17-induced Act1-mediated signaling cascades led to inflammatory gene expression in astrocytes, which is critical for the conversion of Wave 1 to Wave 2, contributing to Th17 cell-mediated pathogenesis of EAE. However, it is important to note that Act1 is also required other IL-17-related signaling, such as the IL-25 pathway (Swaidani et al., 2009; Claudio et al., 2009). Although IL-25 was shown to have an inhibitory impact on Th17 cell differentiation and function in an EAE model (Kleinschek et al., 2007), we found that astrocytes were not responsive to IL-25 stimulation (data not shown). The role of Act1 in CNS-resident cells is probably restricted to its signaling in the IL-17 pathway. Future studies are required to carefully compare and contrast the phenotypes of the CNS cell type-specific IL-17RC- and Act1-deficient mice.

We show that distinct chemokines were induced by IL-17+TNF and IFN-γ+TNF in astrocytes. Although CXCL1, CXCL2, and CCL20 were specifically induced by IL-17+TNF in an Act1-dependent manner, CXCL9, CXCL10, and CXCL11 were only induced by IFN-γ+TNF but not by IL-17+TNF in both wild-type and Act1-deficient astrocytes. Consistent with our finding, a recent study showed that IFN-γ-induced CXCL9, 10, and 11 were preferentially expressed in the CNS of mice injected with IL-12p70 (Th1 cell)-modulated T cells, whereas the neutrophil-attracting chemokines CXCL1 and CXCL2 were upregulated in the CNS of mice given IL-23 (Th17 cell)-modulated T cells (Kroenke et al., 2008). Taken together, it is plausible that astrocytes receive signals from both Th1 and Th17 cells to produce distinct sets of chemokines, contributing to Th1 cell- and Th17 cell-mediated EAE.

EXPERIMENTAL PROCEDURES

Mice

Act1-deficient mice in the C57BL/6J background were generated as described (Qian et al., 2004). B6.Cg-Tg(Nes-Cre)1Kln/J (NesCre transgenic), C57BL/6J, and B6.PL-Thy1a/CyJ (Thy1.1 B6) mice were purchased from Jackson laboratory. CD11bCre transgenic mice were provided by G. Kollias (Biomedical Sciences Research Centre "Alexander Fleming") (Boillée et al., 2006). TIE2eCre transgenic mice were a gift of X.-Y. Fu (National University of Singapore) (Kano et al., 2003). All mice used in the experiment were housed under specific-pathogen-free conditions. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Cleveland Clinic.

Induction and Assessment of EAE

Active EAE was induced and assessed as previously described (Qian et al., 2007). For passive EAE, Act1-deficient or wild-type control mice were injected with 3.0×10^7 polarized MOG 35-55-specific Th1 or Th17 cells/mouse 4 hr after 500 Rad sublethal irradiation. To prepare MOG-specific polarized T cell populations, drain lymph node cells were prepared from mice immunized as described above at day 10 postimmunization. Cells were cultured for 5 days with MOG 35-55 at a concentration of 25 μg/ml under Th1 cell (20 ng/ml rmIL-12 [R&D], 2 μg/ml αIL-23p19 [eBioscience]) polarizing conditions or Th17 cell (20 ng/ml rmIL-23 [R&D]) polarizing condition.

Isolation and Analysis of CNS Inflammatory Cells

Brains were homogenized in ice-cold tissue grinders and filtered through a 100 μm cell strainer, and the cells were collected by centrifugation at 400 × g for 5 min at 4°C. Cells were resuspended in 10 ml of 30% Percoll (Amersham Bioscience) and centrifuge onto a 70% Percoll cushion in 15 ml tubes at 800 × g for 30 min. Cells at the 30%–70% interface were collected and were subjected to flow cytometry. Fluorescence-conjugated CD4, CD8, CD11b, CD45, Ly6G, IL-17, IL-4, and IFN-γ monoclonal antibodies and isotype controls were purchased from BD Biosciences. F4/80 was obtained from Serotech.

Histological Analysis

For paraffin-embedded tissue, spinal cord removed from PBS-perfused mice was fixed in 10% formalin. Sections were stained with either hematoxylin and eosin (H&E) or luxol fast blue (LFB) to evaluate inflammation and demyelination, respectively. For frozen sections, spinal cords were embedded in OCT (Tissue-Tek) and snap frozen in liquid nitrogen. Sections (10 μm) were incubated with anti-CD4, anti-CD11b, anti-Thy1.1 (BD Biosciences), anti-laminin (Sigma). Antigens were visualized after incubation with HRP- or fluorescence-conjugated secondary antibodies (Molecular Probe).

Primary Neuronal Cell Cultures

Mixed glial culture was prepared from 1- to 2-day-old mice. In brief, brains freed of meninges were dissociated with 1 ml pipettes. Debris was removed by filtration with a 40 μm cell strainer (Falcon). Cells were cultured in DMEM plus 10% FBS supplemented with 50 μg/ml penicillin and 50 μg/ml streptomycin. For isolation of microglia, confluent monolayers were vigorously shaken between day 12 and 14, and the floating cells were collected and replated on 6-well plates and cultured for one more hour. Microglial cells attached to the plates and were collected by trypsin digestion. For gene expression experiments, microglial cells were further sorted by anti-CD11b microbeads and purity was >95%. Astrocytes stained with anti- glial fibrillary acidic protein (GFAP) (Santa Cruz) and purity was >90%. Neurons were prepared from the pups at E15. Brains were dissociated with 1 ml pipettes and the debris removed with a 70 μm cell strainer (Falcon). Cells were cultured in Neuronbasal media (Invitrogen) plus B-27 (Invitrogen) and 50 μg/ml penicillin and 50 μg/ml

(C) Luxol fast blue, anti-CD11b, and anti-CD4 staining of spinal cords of NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ mice after immunization with MOG 35-55 (n = 5, 7 days after disease onset). Data are representative of three independent experiments.

(D) Mean clinical score of Th1 cell-induced EAE (top) and flow cytometry analysis of immune cell infiltration in the brains (bottom) of NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ mice (n = 5, 15 days after T cell transfer).

(E) Mean clinical score of Th17 cell-induced EAE (top) and flow cytometry analysis of immune cell infiltration in the brains (bottom) of NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ mice (n = 5, 15 days after T cell transfer).

(F) Real-time PCR analysis of relative expression of inflammatory genes as indicated in spinal cords of NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ mice transferred with MOG-specific Th17 cells (n = 3, 15 days after T cell transfer) compared to the control samples from naive mice.

(G) Primary astrocytes from NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ mice were treated with 50 ng/ml IL-17 for the indicated times and analyzed by immunoblot with indicated antibodies.

(H) Differential regulation of chemokines by IL-17 and IFN-γ in astrocytes. Astrocytes from NesCre*Act1*$^{fl/-}$ and NesCre*Act1*$^{fl/+}$ mice were treated with 50 ng/ml IL-17, 10 ng/ml TNF-α, 10 ng/ml IFN-γ, medium alone, IL-17+TNF-α, and TNF-α+IFN-γ for 16 hr, followed by real-time PCR for the expression of inflammatory genes as indicated.

The data are presented as mean ± SEM from three independent experiments (*$p < 0.05$). See also Figures S3 and S4.

streptomycin. >90% of these cultured cells were positive for MAP2 (a marker for neurons, anti-MAP2 from Abcam).

ELISA

IL-17 and IFN-γ level were assayed by IL-17- or IFN-γ ELISA kit (R&D systems) according to the manufacturer's instruction.

Real-Time PCR

Total RNA was extracted from spinal cord and cultured astrocytes with TRIzol (Invitrogen) according to the manufacturer's instructions. All gene expression results are expressed as arbitrary units relative to expression of the gene encoding β-actin. Fold induction of gene expression in spinal cord after EAE induction was determined by dividing the relative abundance of experimental samples by the mean relative abundance of control samples from naive mice. Primer sequences are available in Supplemental Experimental Procedures.

Proliferation and Apoptosis Assay

Cell division was detected with the Brdu In Situ detection kit (BD Biosciences) according to manufacturer's instructions. The percentage of Brdu-positive cells in total $CD4^+$ cells was averaged of four spinal cord regions after double staining by anti-Brdu and anti-CD4. Ki67 antibody (Dako) was also used for proliferation analysis by immunohistochemical staining. The frequency of $Ki67^+$ cells in total $Thy1.1^+$ cells in the spinal cord represents an average of four spinal cord regions.

Statistics

The p values of clinical scores were determined by one-way multiple-range analysis of variance (ANOVA) for multiple comparisons. Other p values were determined by Student's t tests. Unless otherwise specified, all results are shown as mean and the standard error of the mean (mean ± SEM). A p value of < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.immuni.2010.03.004.

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REFERENCES

Agrawal, A., Dillon, S., Denning, T.L., and Pulendran, B. (2006). ERK1-/- mice exhibit Th1 cell polarization and increased susceptibility to experimental autoimmune encephalomyelitis. J. Immunol. *176*, 5788–5796.

Aloisi, F. (2001). Immune function of microglia. Glia *36*, 165–179.

Awane, M., Andres, P.G., Li, D.J., and Reinecker, H.C. (1999). NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. J. Immunol. *162*, 5337–5344.

Bauer, M., Brakebusch, C., Coisne, C., Sixt, M., Wekerle, H., Engelhardt, B., and Fässler, R. (2009). Beta1 integrins differentially control extravasation of inflammatory cell subsets into the CNS during autoimmunity. Proc. Natl. Acad. Sci. USA *106*, 1920–1925.

Becher, B., Bechmann, I., and Greter, M. (2006). Antigen presentation in autoimmunity and CNS inflammation: How T lymphocytes recognize the brain. J. Mol. Med. *84*, 532–543.

Bettelli, E., Sullivan, B., Szabo, S.J., Sobel, R.A., Glimcher, L.H., and Kuchroo, V.K. (2004). Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. J. Exp. Med. *200*, 79–87.

Boillée, S., Yamanaka, K., Lobsiger, C.S., Copeland, N.G., Jenkins, N.A., Kassiotis, G., Kollias, G., and Cleveland, D.W. (2006). Onset and progression in inherited ALS determined by motor neurons and microglia. Science *312*, 1389–1392.

Butti, E., Bergami, A., Recchia, A., Brambilla, E., Del Carro, U., Amadio, S., Cattalini, A., Esposito, M., Stornaiuolo, A., Comi, G., et al. (2008). IL4 gene delivery to the CNS recruits regulatory T cells and induces clinical recovery in mouse models of multiple sclerosis. Gene Ther. *15*, 504–515.

Carson, M.J. (2002). Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis. Glia *40*, 218–231.

Claudio, E., Sønder, S.U., Saret, S., Carvalho, G., Ramalingam, T.R., Wynn, T.A., Chariot, A., Garcia-Perganeda, A., Leonardi, A., Paun, A., et al. (2009). The adaptor protein CIKS/Act1 is essential for IL-25-mediated allergic airway inflammation. J. Immunol. *182*, 1617–1630.

Croitoru-Lamoury, J., Guillemin, G.J., Boussin, F.D., Mognetti, B., Gigout, L.I., Chéret, A., Vaslin, B., Le Grand, R., Brew, B.J., and Dormont, D. (2003). Expression of chemokines and their receptors in human and simian astrocytes: Evidence for a central role of TNF alpha and IFN gamma in CXCR4 and CCR5 modulation. Glia *41*, 354–370.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., et al. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature *421*, 744–748.

Das, M.P., Nicholson, L.B., Greer, J.M., and Kuchroo, V.K. (1997). Autopathogenic T helper cell type 1 (Th1) and protective Th2 clones differ in their recognition of the autoantigenic peptide of myelin proteolipid protein. J. Exp. Med. *186*, 867–876.

Das Sarma, J., Ciric, B., Marek, R., Sadhukhan, S., Caruso, M.L., Shafagh, J., Fitzgerald, D.C., Shindler, K.S., and Rostami, A. (2009). Functional interleukin-17 receptor A is expressed in central nervous system glia and upregulated in experimental autoimmune encephalomyelitis. J. Neuroinflammation *6*, 14.

Dong, Y., and Benveniste, E.N. (2001). Immune function of astrocytes. Glia *36*, 180–190.

Ferber, I.A., Brocke, S., Taylor-Edwards, C., Ridgway, W., Dinisco, C., Steinman, L., Dalton, D., and Fathman, C.G. (1996). Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J. Immunol. *156*, 5–7.

Gold, R., Linington, C., and Lassmann, H. (2006). Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. Brain *129*, 1953–1971.

Gonzalez-García, I., Zhao, Y., Ju, S., Gu, Q., Liu, L., Kolls, J.K., and Lu, B. (2009). IL-17 signaling-independent central nervous system autoimmunity is negatively regulated by TGF-beta. J. Immunol. *182*, 2665–2671.

Graus-Porta, D., Blaess, S., Senften, M., Littlewood-Evans, A., Damsky, C., Huang, Z., Orban, P., Klein, R., Schittny, J.C., and Müller, U. (2001). Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron *31*, 367–379.

Haak, S., Croxford, A.L., Kreymborg, K., Heppner, F.L., Pouly, S., Becher, B., and Waisman, A. (2009). IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. J. Clin. Invest. *119*, 61–69.

Halonen, S.K., Woods, T., McInnerney, K., and Weiss, L.M. (2006). Microarray analysis of IFN-gamma response genes in astrocytes. J. Neuroimmunol. *175*, 19–30.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., and Weaver, C.T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat. Immunol. *6*, 1123–1132.

Inoue, D., Numasaki, M., Watanabe, M., Kubo, H., Sasaki, T., Yasuda, H., Yamaya, M., and Sasaki, H. (2006). IL-17A promotes the growth of airway

epithelial cells through ERK-dependent signaling pathway. Biochem. Biophys. Res. Commun. *347*, 852–858.

Jäger, A., Dardalhon, V., Sobel, R.A., Bettelli, E., and Kuchroo, V.K. (2009). Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. J. Immunol. *183*, 7169–7177.

Jovanovic, D.V., Di Battista, J.A., Martel-Pelletier, J., Jolicoeur, F.C., He, Y., Zhang, M., Mineau, F., and Pelletier, J.P. (1998). IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. J. Immunol. *160*, 3513–3521.

Kano, A., Wolfgang, M.J., Gao, Q., Jacoby, J., Chai, G.X., Hansen, W., Iwamoto, Y., Pober, J.S., Flavell, R.A., and Fu, X.Y. (2003). Endothelial cells require STAT3 for protection against endotoxin-induced inflammation. J. Exp. Med. *198*, 1517–1525.

Kebir, H., Kreymborg, K., Ifergan, I., Dodelet-Devillers, A., Cayrol, R., Bernard, M., Giuliani, F., Arbour, N., Becher, B., and Prat, A. (2007). Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nat. Med. *13*, 1173–1175.

Kleinschek, M.A., Owyang, A.M., Joyce-Shaikh, B., Langrish, C.L., Chen, Y., Gorman, D.M., Blumenschein, W.M., McClanahan, T., Brombacher, F., Hurst, S.D., et al. (2007). IL-25 regulates Th17 function in autoimmune inflammation. J. Exp. Med. *204*, 161–170.

Kolls, J.K., and Lindén, A. (2004). Interleukin-17 family members and inflammation. Immunity *21*, 467–476.

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Sudo, K., and Iwakura, Y. (2006). IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J. Immunol. *177*, 566–573.

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V.K. (2009). IL-17 and Th17 cells. Annu. Rev. Immunol. *27*, 485–517.

Kroenke, M.A., Carlson, T.J., Andjelkovic, A.V., and Segal, B.M. (2008). IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. J. Exp. Med. *205*, 1535–1541.

Lees, J.R., Golumbek, P.T., Sim, J., Dorsey, D., and Russell, J.H. (2008). Regional CNS responses to IFN-gamma determine lesion localization patterns during EAE pathogenesis. J. Exp. Med. *205*, 2633–2642.

Leonardi, A., Chariot, A., Claudio, E., Cunningham, K., and Siebenlist, U. (2000). CIKS, a connection to Ikappa B kinase and stress-activated protein kinase. Proc. Natl. Acad. Sci. USA *97*, 10494–10499.

Li, X., Commane, M., Nie, H., Hua, X., Chatterjee-Kishore, M., Wald, D., Haag, M., and Stark, G.R. (2000). Act1, an NF-kappa B-activating protein. Proc. Natl. Acad. Sci. USA *97*, 10489–10493.

Mangan, P.R., Harrington, L.E., O'Quinn, D.B., Helms, W.S., Bullard, D.C., Elson, C.O., Hatton, R.D., Wahl, S.M., Schoeb, T.R., and Weaver, C.T. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature *441*, 231–234.

McFarland, H.F., and Martin, R. (2007). Multiple sclerosis: A complicated picture of autoimmunity. Nat. Immunol. *8*, 913–919.

Novatchkova, M., Leibbrandt, A., Werzowa, J., Neubüser, A., and Eisenhaber, F. (2003). The STIR-domain superfamily in signal transduction, development and immunity. Trends Biochem. Sci. *28*, 226–229.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat. Immunol. *6*, 1133–1141.

Qian, Y., Zhao, Z., Jiang, Z., and Li, X. (2002). Role of NF kappa B activator Act1 in CD40-mediated signaling in epithelial cells. Proc. Natl. Acad. Sci. USA *99*, 9386–9391.

Qian, Y., Qin, J., Cui, G., Naramura, M., Snow, E.C., Ware, C.F., Fairchild, R.L., Omori, S.A., Rickert, R.C., Scott, M., et al. (2004). Act1, a negative regulator in CD40- and BAFF-mediated B cell survival. Immunity *21*, 575–587.

Qian, Y., Liu, C., Hartupee, J., Altuntas, C.Z., Gulen, M.F., Jane-Wit, D., Xiao, J., Lu, Y., Giltiay, N., Liu, J., et al. (2007). The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. Nat. Immunol. *8*, 247–256.

Ramírez, F., and Mason, D. (2000). Induction of resistance to active experimental allergic encephalomyelitis by myelin basic protein-specific Th2 cell lines generated in the presence of glucocorticoids and IL-4. Eur. J. Immunol. *30*, 747–758.

Ransohoff, R.M. (2009). Immunology: In the beginning. Nature *462*, 41–42.

Reboldi, A., Coisne, C., Baumjohann, D., Benvenuto, F., Bottinelli, D., Lira, S., Uccelli, A., Lanzavecchia, A., Engelhardt, B., and Sallusto, F. (2009). C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nat. Immunol. *10*, 514–523.

Sixt, M., Engelhardt, B., Pausch, F., Hallmann, R., Wendler, O., and Sorokin, L.M. (2001). Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. J. Cell Biol. *153*, 933–946.

Sospedra, M., and Martin, R. (2005). Immunology of multiple sclerosis. Annu. Rev. Immunol. *23*, 683–747.

Steinman, L. (2001). Multiple sclerosis: A two-stage disease. Nat. Immunol. *2*, 762–764.

Steinman, L. (2008). A rush to judgment on Th17. J. Exp. Med. *205*, 1517–1522.

Steinman, L. (2009). Shifting therapeutic attention in MS to osteopontin, type 1 and type 2 IFN. Eur. J. Immunol. *39*, 2358–2360.

Stromnes, I.M., and Goverman, J.M. (2006). Active induction of experimental allergic encephalomyelitis. Nat. Protoc. *1*, 1810–1819.

Stromnes, I.M., Cerretti, L.M., Liggitt, D., Harris, R.A., and Goverman, J.M. (2008). Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. Nat. Med. *14*, 337–342.

Sutton, C.E., Lalor, S.J., Sweeney, C.M., Brereton, C.F., Lavelle, E.C., and Mills, K.H. (2009). Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity *31*, 331–341.

Swaidani, S., Bulek, K., Kang, Z., Liu, C., Lu, Y., Yin, W., Aronica, M., and Li, X. (2009). The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation. J. Immunol. *182*, 1631–1640.

Toy, D., Kugler, D., Wolfson, M., Vanden Bos, T., Gurgel, J., Derry, J., Tocker, J., and Peschon, J. (2006). Cutting edge: Interleukin 17 signals through a heteromeric receptor complex. J. Immunol. *177*, 36–39.

Trajkovic, V., Stosic-Grujicic, S., Samardzic, T., Markovic, M., Miljkovic, D., Ramic, Z., and Mostarica Stojkovic, M. (2001). Interleukin-17 stimulates inducible nitric oxide synthase activation in rodent astrocytes. J. Neuroimmunol. *119*, 183–191.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., and Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. *23*, 99–103.

Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., and Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity *24*, 179–189.

Weaver, A., Goncalves da Silva, A., Nuttall, R.K., Edwards, D.R., Shapiro, S.D., Rivest, S., and Yong, V.W. (2005). An elevated matrix metalloproteinase (MMP) in an animal model of multiple sclerosis is protective by affecting Th1/Th2 polarization. FASEB J. *19*, 1668–1670.

Wohler, J.E., Smith, S.S., Zinn, K.R., Bullard, D.C., and Barnum, S.R. (2009). Gammadelta T cells in EAE: early trafficking events and cytokine requirements. Eur. J. Immunol. *39*, 1516–1526.

Yang, Y., Weiner, J., Liu, Y., Smith, A.J., Huss, D.J., Winger, R., Peng, H., Cravens, P.D., Racke, M.K., and Lovett-Racke, A.E. (2009). T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. J. Exp. Med. *206*, 1549–1564.