Increased Th17 and Regulatory T Cell Responses in EBV-Induced Gene 3-Deficient Mice Lead to Marginally Enhanced Development of Autoimmune Encephalomyelitis

Jin-Qing Liu, Zhenzhen Liu, Xuejun Zhang, Yun Shi, Fatemeh Talebian, Joseph W. Carl, Jr., Chuan Yu, Fu-Dong Shi, Caroline C. Whitacre, Joanne Trgovcich and Xue-Feng Bai

*J Immunol* published online 2 March 2012
http://www.jimmunol.org/content/early/2012/03/02/jimmunol.1100106
Increased Th17 and Regulatory T Cell Responses in EBV-Induced Gene 3-Deficient Mice Lead to Marginally Enhanced Development of Autoimmune Encephalomyelitis

Jin-Qing Liu,* Zhenzhen Liu,* Xuejun Zhang,* Yun Shi,* Fatemeh Talebian,* Joseph W. Carl, Jr.,* Chuan Yu,* Fu-Dong Shi,§ Caroline C. Whitacre,§ Joanne Trgovcich,* and Xue-Feng Bai*

EBV-induced gene 3 (EBI3)-encoded protein can form heterodimers with IL-27P28 and IL-12P35 to form IL-27 and IL-35. IL-27 and IL-35 may influence autoimmunity by inhibiting Th17 differentiation and facilitating the inhibitory roles of Foxp3+ regulatory T (Treg) cells, respectively. In this study, we have evaluated the development of experimental autoimmune encephalomyelitis (EAE) in EBI3-deficient mice that lack both IL-27 and IL-35. We found that myelin oligodendrocyte glycoprotein peptide immunization resulted in marginally enhanced EAE development in EBI3-deficient C57BL/6 and 2D2 TCR-transgenic mice. EBI3 deficiency resulted in significantly increased Th17 and Th1 responses in the CNS and increased T cell production of IL-2 and IL-17 in the peripheral lymphoid organs. EBI3-deficient and -sufficient 2D2 T cells had equal ability in inducing EAE in Rag1−/− mice; however, more severe disease was induced in EBI3−/−Rag1−/− mice than in Rag1−/− mice by 2D2 T cells. EBI3-deficient mice had increased numbers of CD4+Foxp3+ Treg cells in peripheral lymphoid organs. More strikingly, EBI3-deficient Treg cells had more potent suppressive functions in vitro and in vivo. Thus, our data support an inhibitory role for EBI3 in Th17, Th1, IL-2, and Treg responses. Although these observations are consistent with the known functions of IL-27, the IL-35 contribution to the suppressive functions of Treg cells is not evident in this model. Increased Treg responses in EBI3−/− mice may explain why the EAE development is only modestly enhanced compared with wild-type mice.

EBI3 has also been reported to be associated with the IL-12 p35 to form a heterodimeric hematopoietin (2), which was later named IL-35. Although the expression pattern of IL-35 may differ in human (20), it is constitutively expressed in Foxp3+CD4+CD25+ regulatory T (Treg) cells in mice and contributes to their suppressive activity (4). IL-35-deficient (either deficient for EBI3 or P35) Treg cells have significantly reduced regulatory activity in vitro and fail to control homeostatic proliferation or cure inflammatory bowel disease in vivo (4). RIL-35 suppresses T cell proliferation (3, 4), Th17 differentiation, and experimental arthritis (3). Moreover, exogenous IL-35 or Treg-produced IL-35 has been shown to either expand Foxp3+ Treg cells (3) or to induce a population of Foxp3-negative iT R35 cells that mediates suppression via IL-35 but not the inhibitory cytokines IL-10 or TGF-β (21). Adoptively transferred iT R35 cells inhibited inflammatory bowel disease and experimental autoimmune encephalomyelitis (EAE) in mice (21). Thus, IL-35 is a novel cytokine with therapeutic effects against autoimmune diseases.

EBI3−/− mice are deficient for both IL-27 and IL-35, thus the deficiency of EBI3 could have additive effects on the development of autoimmune inflammation. However, no overt autoimmunity or inflammatory disease has been reported in EBI3−/− mice (22). The role of IL-27 in promoting EAE development has been mainly demonstrated in IL-27R−/− mice (12) and by IL-27 systemic injection (17). Recently, the EBI3 partner, P28-deficient mice have been shown to develop enhanced autoimmune inflammation in the CNS (23). Given the often distinct expression kinetics and the reported differences of the phenotypes between EBI3−/− and IL-27R−/− mice (24, 25), evaluation of autoimmune responses in EBI3−/− may offer a new opportunity to identify novel pathways that regulate autoimmune disease. In this study, we evaluated the EAE development in EBI3-deficient mice.

*Department of Pathology, The Ohio State University Medical Center, Columbus, OH 43210; †Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center, Phoenix, AZ 85013; and ‡Department of Virology, Immunology and Cancer Genetics, The Ohio State University, Columbus, OH 43210

Received for publication January 12, 2011. Accepted for publication January 17, 2012.

This work was supported in part by an R21 grant (A078266 to X.-F.B.) from the National Institutes of Health.

Address correspondence and reprint requests to Dr. Xue-Feng Bai, Department of Pathology, Ohio State University Medical Center, 129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210. E-mail address: Xue-Feng.Bai@osumc.edu

Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; EBI3, EBV-induced gene 3; MOG, myelin oligodendrocyte glycoprotein; p.i., postimmunization; qPCR, quantitative PCR; WT, wild-type.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/0000–0000.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100106
found that myelin oligodendrocyte glycoprotein (MOG) peptide immunization resulted in enhanced EAE development in EBI3-deficient C57BL6 mice and EBI3-deficient 2D2 TCR-transgenic mice. Enhanced EAE development in EBI3-deficient mice was associated with increased IL-2, Th17, Th1, and Treg responses. Increased numbers and enhanced suppressive function of Treg cells in EBI3−/− mice may explain why the EAE development is only modestly enhanced compared with wild-type (WT) mice.

Materials and Methods

Mice

C57BL6 mice were purchased from The Jackson Laboratory. 2D2 TCR-transgenic mice (28) were described previously (27). EBI3-deficient mice in the C57BL6 background have been described (22). 2D2 mice deficient for EBI3 (2D2EBI3−/−) were generated through breeding of 2D2 mice with EBI3−/− mice for two generations. PCR was used for identification of mice genotypes. The primers used were: EBI3 (forward, 5′-CTG ATG GGT CAC TTA CTC GCC TGC-3′ and reverse, 5′-ACG ACA TCA GGG TAT ATC AAG-3′; 2D2 (forward, 5′-CCC GGG CAA GGC TCA GCC ATG CTC-3′ and reverse, 5′-GGG GCC GCA ATT CCC AGA GAC ATC CCT-3′; EBI3−/−/2D2 (forward, 5′-AGT TCC AAG CTG CTC TTC-3′ and reverse, 5′-GCG GCC GCA ATT CCC AGA TGG-3′). All mice were maintained in the animal facilities of The Ohio State University. The animal facilities are fully accredited by American Association for Accreditation of Laboratory Animal Care.

Induction and assessment of EAE

MOG peptide 35–55 (MEVGWYRSPFSRVVHLYRNGK), purchased from MOG35–55 (MEVGWYRSPFSRVVHLYRNGK), purchased from Genemed Synthesis (San Antonio, TX), was used as the immunogen. Mice of 8–12 wk of age were immunized s.c. with 200 μg MOG peptide in CFA (containing 400 μg Mycobacterium tuberculosis) in a total volume of 100 μL. Mice also received 150 ng pertussis toxin (List Biological, Campbell, CA) in 200 μL PBS via the tail vein immediately after the immunization and again 48 h later. In some experiments, Rag1−/− or EBI3−/− Rag1−/− mice received 1 × 106 CD4+ T cells from 2D2 mice or 5 × 105 CD4+ T cells from C57BL6 mice. The recipient mice were then immunized for the induction of EAE, as described above. The mice were observed every day and scored on a scale of 0–5 with gradations of 0.5 for intermediate scores: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, moribund; and 5, death.

Histology

Mice were sacrificed by inhaling CO2. Lumbar spinal cords were removed and fixed in 10% formalin/PBS. Paraffin sections were prepared and stained with H&E in the histology core facilities of the Department of Pathology (The Ohio State University). Neurologic lesions were graded on each of the 10 cross sections per spinal cord, according to the following criteria: 0, no lesions; 1, ≤5 focal meningeal infiltrates; 2, >5 focal meningeal infiltrates; 3, up to 5 perivascular infiltrate foci in the parenchyma with involvement of <5% of the white matter; 4, 5–10 perivascular foci in the parenchyma or invasions involving 5–25% of the white matter; and 5, >10 perivascular foci or diffuse infiltration involving >25% of the white matter.

Isolation of total RNA from spleen, spinal cord, and quantitative PCR

Total RNA was isolated from spleens and spinal cords by using the TRIzol method (Invitrogen). The first-strand cDNA of each sample was synthesized using a reverse transcription kit (Invitrogen). Quantitative real-time PCR was performed using an ABI Prism 7900 HT sequence system (Applied Biosystems) with the Quantitect SYBR Green PCR kit (Quagen) in accordance with the manufacturer’s instructions. The following primers were used: IL-2 forward, 5′-TCC TGA GCA GGA TGG AGA AAT-3′; IL-2 reverse, 5′-GGC AGT CCT TGT TTG AA-3′; IL-10 forward, 5′-ACA GCC GGG AAG AAT ACA AC-3′; IL-10 reverse, 5′-CAG CTG CTT TGT TTG AA-3′; IL-17 forward, 5′-AGG TCT GCA ATG CGT GTC TG-3′; IL-17 reverse, 5′-TCT GGA GAA GAT GTC ACC-3′; TGF-β forward, 5′-AGG AGA GTC CAG CAG TAC T-3′; TGF-β reverse, 5′-GAA GCC CCT GTT CGT CT-3′; 5′-TTA CTA GGC AGA TGG CCA CA-3′; 5′-TTA CTA GGC AGA TGG CCA CA-3′. The hypoxanthine-guanine phosphoribosyltransferase reverse, 5′-AA TCT GAA CCT TAC ACC TG-3′; and hypoxanthine-guanine phosphoribosyltransferase gene was amplified and served as endogenous control. PCR was performed using an optimal condition. One microliter first-strand cDNA product was amplified with platinum Taq polymerase (Invitrogen) and gene-specific primer pairs. Each sample was assayed in triplicate, and experiments were repeated twice. The relative expression was calculated by plotting the threshold cycle and average relative expression was determined by the comparative method (2−ΔΔthreshold cycle).

Cytokine ELISA

ELISA kits for the detection of IL-2, IL-10, IL-17, and IFN-γ were purchased from eBioscience. Standard procedures were followed to detect release of cytokines in culture supernatants in a variety of settings (detail in figure legends to each experiment).

Preparation of mononuclear cells from the CNS, intracellular cytokine staining, and flow cytometry

On days 17–20 after EAE induction, mice were sacrificed and perfused with PBS through the left heart ventricle to eliminate contaminating blood cells in the CNS. The CNS mononuclear cells were then prepared, as we have described (28). For intracellular cytokine staining, the CNS-infiltrating cells from mice were stimulated in vitro with PMA (50 ng/ml) and ionomycin (1 μM) for 5 h. GolgiStop (BD Biosciences) was added (1:1500) during the last 2 h of incubation. The cells were stained for the cell-surface marker CD4, followed by a standard intracellular cytokine staining for IFN-γ, IL-10, and IL-17. The following Abs were used: anti-CD4 (clone GK1.5; BD Biosciences), anti-IL-10 PE (JES5-16E3; BD Biosciences), anti−IFN-γ-allophycocyanin (XMG1.2; BD Biosciences), anti−IL-17 PE (TC11-18H10; BD Biosciences), and rat IgG1 isotype control PE or allophycocyanin (BD Biosciences). Cells were analyzed on an FACSCalibur flow cytometer (BD Biosciences), and respective isotype controls were obtained from BD Pharmingen. Cells were analyzed on an FACSCalibur flow cytometer (BD Biosciences).

Purification of CD4+CD25+ and CD4+CD25− T cells from 2D2 TCR-transgenic mice or C57BL6 mice

CD4+ T cells were purified from 2D2 TCR-transgenic mice or C57BL6 mice by negative selection. Briefly, spleen and lymph node cells from donor mice were incubated with a mixture of mAbs (anti-CD4 mAb TIB210, anti-Flr mAb 2A4, and anti-CD11c mAb N418). After removing the unbound Abs, the cells were incubated with anti-rat IgG-coated magnetic beads (Dynal Biotech). A magnet was used to remove the Ab-bound cells. The remaining cells were CD4+ T cells. To isolate CD4+CD25+ and CD4+CD25− T cells, the purified CD4+ T cells were further stained with anti-CD25–PE Ab (7D4; BD Biosciences) followed by separation of CD4+CD25+ and CD4+CD25− T cells using anti-PE MACS bead technology. In some experiments, the purified CD4+ T cells were stained with anti-CD4–FITC, and anti-CD25–PE, CD4+CD25−, and CD4+CD25+ cells were subsequently separated by high-speed sorting using a flow cytometer.

Lymphocyte proliferation and Treg-suppressed proliferation assay

For lymphocyte proliferation assay, splenocytes from EBI3−/− or WT mice with EAE, draining lymph node cells from EBI3−/− or WT mice immunized with MOG35–55/CFA, or splenocytes from 2D2EBI3−/− or 2D2EBI3+/+ mice were cultured in Click’s EHAA medium (Invitrogen) in the presence of graded concentrations of MOG35–55 for 60 h; 1 μCi/ml [3H]-Tritium (ICN Pharmaceuticals) was then pulsed into the cultures, and incorporation of [3H]-Tritium was measured in a liquid scintillation β plate counter 12 h later. For Treg-suppressed proliferation assay, 1 × 107/ml purified CD4+CD25− T cells from EBI3−/− mice were cocultured with graded numbers of CD4+CD25− Treg cells from WT or EBI3−/− mice in the presence of irradiated splenocytes (2 × 105/ml) from EBI3−/− Rag1−/− mice and 0.1 μg/ml anti-CD3 mAb (2C11). After 48 h, 1 μCi/ml [3H]-Tritium was pulsed into the cultures, and incorporation of [3H]-Tritium was measured in a liquid scintillation β plate counter 12 h later.

Statistics

Mann–Whitney U test was used for comparison of EAEE clinical scores and histology scores at a given time point. We used Wilcoxon signed-rank test to compare EAEE scores in the time-course experiments. The Student t test was used for all other comparisons. In all cases, the α level was set at p < 0.05.
Results

EAE development is marginally enhanced in EBI3⁻/⁻ mice

To understand the role of EBI3-formed cytokines in EAE development, we induced EAE in EBI3⁻/⁻ and WT mice using MOG35–55 emulsified in CFA with two doses of pertussis toxin. As shown in Fig. 1A, EBI3⁻/⁻ mice were susceptible to EAE induction and had similar disease onset compared with WT mice. However, in WT mice, disease peaked at about day 19 and persisted thereafter; EBI3⁻/⁻ mice reached to peak disease earlier (at about day 17) and had significantly higher disease scores than WT mice between days 14 and 16. However, after day 20, EAE scores were similar between both groups of mice. In five independent experiments, we consistently observed increased mean maximal scores (Table I) and mean accumulating scores (Table I) in EBI3⁻/⁻ mice compared with WT mice. However, mean day of onset and mean incidences of EAE did not differ between the two groups of mice.

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** (A) EAE development in EBI3⁻/⁻ and WT mice. To induce EAE, C57BL/6, and EBI3⁻/⁻ mice were immunized with MOG35–55 as described in Materials and Methods. A representative experiment out of five experiments is shown. *p < 0.05, **p < 0.01 by Mann–Whitney U test. (B) Representative histology of spinal cords from an EBI3⁻/⁻ and a WT mouse. Scale bars, 100 μm. (C) Inflammation scores of the spinal cords from mice with EAE. Seventeen days after EAE inductions, groups of EBI3⁻/⁻ (n = 5) and WT mice (n = 5) were sacrificed, and spinal cords were processed for H&E staining. Each spinal cord was evaluated and scored as described in Materials and Methods. **p < 0.01 by Mann–Whitney U test. (D) CNS-infiltrating cells in mice with EAE. Seventeen days after EAE inductions, groups of EBI3⁻/⁻ (n = 5) and WT mice (n = 5) were sacrificed, and spinal cords were removed. Mononuclear cells (MNC) were isolated, counted, and stained for CD4 as described in Materials and Methods. *p < 0.05 by Student t test.

### Table I. Summary of EAE development in five independent experiments

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Mice</th>
<th>Incidence</th>
<th>Mean Accumulating Score</th>
<th>Mean Maximal Score</th>
<th>Mean Day of Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WT</td>
<td>7/10</td>
<td>22.1 ± 11</td>
<td>1.85 ± 1.42</td>
<td>11.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>EBI3⁻/⁻</td>
<td>10/10</td>
<td>26.1 ± 7</td>
<td>2.45 ± 0.87</td>
<td>12.43 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>4/4</td>
<td>22.2 ± 6.1</td>
<td>2.1 ± 0.93</td>
<td>13.75 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>EBI3⁻/⁻</td>
<td>5/5</td>
<td>27.7 ± 9.4</td>
<td>3.1 ± 0.88*</td>
<td>13.6 ± 1.36</td>
</tr>
<tr>
<td>3</td>
<td>WT</td>
<td>5/5</td>
<td>17.2 ± 2.2</td>
<td>1.6 ± 0.32</td>
<td>14.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>EBI3⁻/⁻</td>
<td>6/6</td>
<td>17 ± 3.3</td>
<td>2 ± 0.5</td>
<td>14.8 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>WT</td>
<td>6/7</td>
<td>19.6 ± 10.6</td>
<td>2.92 ± 0.9</td>
<td>16.1 ± 4.16</td>
</tr>
<tr>
<td></td>
<td>EBI3⁻/⁻</td>
<td>5/5</td>
<td>29.1 ± 4.6</td>
<td>3.6 ± 0.16*</td>
<td>13 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>5/5</td>
<td>20.6 ± 7.2</td>
<td>1.4 ± 0.36</td>
<td>13.2 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>EBI3⁻/⁻</td>
<td>4/4</td>
<td>29.2 ± 11.8</td>
<td>1.6 ± 0.6</td>
<td>12 ± 0</td>
</tr>
</tbody>
</table>

Data shown are mean ± SD. Mean maximal score is the average of maximal score of each mouse in each experiment. Mean accumulating score is the average of accumulating scores of each mouse. The daily EAE scores of each mouse were added together for a period of 25–40 d to obtain the accumulating score for each mouse. Day of EAE onset was considered as when the first sign of EAE appears in each mouse. In experiments 3 and 5, reduced pertussis toxin (100 ng/mouse) was used for the EAE induction.

* *p < 0.05 by Mann–Whitney U test.
mice. Examination of histology in spinal cords revealed more inflammatory lesions in the white matter of EBI3−/− mice than in WT mice on day 17 (Fig. 1B, 1C). In addition, more CNS-infiltrating mononuclear cells were harvested from the brains and spinal cords of EBI3−/− mice with EAE on day 17 than in WT mice (Fig. 1D). However, the number of CNS-infiltrating CD4+ T cells did not differ significantly between the two groups on day 17 postimmunization (p.i.).

Increased Th17 responses in EBI3−/− mice

To understand the phenotype of EAE in EBI3−/− mice, we investigated the immune responses in the CNS of mice with EAE. Increased expression of IFN-γ and IL-17 genes (Fig. 2A) and increased numbers of IFN-γ and IL-17-producing cells (Fig. 2B, 2C) were detected in the spinal cords of EBI3−/− mice at day 17 p.i. Expression of IL-10 gene and number of IL-10–producing CD4+ T cells (Tr1) were low in the spinal cords of mice with EAE on day 17 p.i.; no significant differences were detected between EBI3−/− and WT mice (Fig. 2A-C). The expression of cytokine genes including IFN-γ and IL-17 in spinal cords diminished during EAE recovery (day 29); however, IL-10 gene expression was still higher in spinal cords of EBI3−/− mice (Fig. 2D). We also detected increased IL-10 gene expression in the spinal cords of EBI3−/− mice (Fig. 2D).

To determine the impact of EBI3 deficiency on the priming and differentiation of myelin Ag-specific T cells in the peripheral lymphoid organs, we immunized EBI3−/− and WT mice with MOG35–55/CFA. Ten days later, draining lymph node cells were evaluated by lymphocyte proliferation assay. As shown in Fig. 3A, lymph node cells from EBI3−/− mice had a stronger proliferation in response to MOG35–55 compared with WT lymph node cells. Similarly, when examining recall proliferative responses of splenocytes from mice with EAE (day 29 p.i.), we found that splenocytes from EBI3−/− mice underwent significantly higher

**FIGURE 2.** T cell responses in the CNS of WT and EBI3−/− mice. (A) qPCR was used to detect the expression of cytokine genes in spinal cords of mice with EAE (day 17 after EAE induction). Expression of cytokine genes in a normal spinal cord of a WT mouse was set as 1. Average and SD of five mice per group are shown. Data shown represent two experiments with similar results. **p < 0.01 by Student t test.** (B) Production of cytokines by CNS-infiltrating CD4+ T cells. MNCs were prepared from spinal cords of mice with EAE (day 17 after EAE induction). Cells were first stained for CD4 followed by intracellular cytokine staining as described in Materials and Methods. Data shown are representative of three experiments with similar results. (C) Quantification of CD4+ T cell subsets in the spinal cords of mice with EAE. MNCs were prepared from each spinal cord of mouse with EAE (day 17 after EAE induction). Cells were counted and stained for CD4 followed by intracellular cytokine staining. Numbers of IL-17–producing CD4+ T cells (Th17), IFN-γ–producing CD4+ T cells (Th1), and IL-10–producing CD4+ T cells (Tr1) were calculated, and data are expressed as mean + SD (n = 5 for each group). **p < 0.05 by Student t test.** (D) Cytokine gene expression in the CNS of mice with EAE (day 29 after EAE induction). Total RNA was prepared from the spinal cords of mice (n = 3 in each group). Data represent two experiments with similar results. *p < 0.05, **p < 0.01 by Student t test.
proliferation (Fig. 3B). We examined the expression of cytokine genes in MOG peptide-restimulated lymph node cells and found that expression of IFN-γ, IL-17, and IL-2 genes were significantly upregulated in cells from EBI3−/− mice (Fig. 3C). We measured cytokines released in the culture supernatants and found that MOG35–55-stimulated lymph node cells from EBI3−/− mice produced more IL-17 and IL-2 than lymph node cells from WT mice. The production of IL-10 and IFN-γ did not differ significantly (Fig. 3D). Because IL-2 production was low in the culture supernatant after 4 d of cell culture, we directly measured IL-2 production in CD4+ T cells from unstimulated lymph nodes by intracellular cytokine staining. As shown in Fig. 3E, higher numbers of CD4+IL-2+ T cells were detected in the lymph nodes from EBI3−/− mice than in WT mice.

Enhanced Th17 response and EAE development in EBI3-deficient 2D2 mice

To further validate the observations in EBI3−/− mice, we bred 2D2 TCR-transgenic mice with EBI3−/− mice and generated EBI3-deficient 2D2 mice (2D2/EBI3−/− mice). Although the frequencies of 2D2 T cells (Vα3.2+Vβ11+) in spleens of 2D2/EBI3−/− mice were similar to that of 2D2/EBI3+/+ mice (not shown), the splenocytes of 2D2/EBI3−/− mice had stronger proliferative response to MOG peptide stimulation (Fig. 4A). After 5 d stimulation with MOG35–55, quantitative PCR (qPCR) analysis of expression for cytokine genes revealed that the most dramatic alteration was in IFN-γ expression in MOG35–55-activated splenocytes by EBI3−/− mice, whereas production of IL-10 and IFN-γ did not significantly differ (Fig. 4C). Finally, although 2D2/EBI3−/− mice rarely develop spontaneous EAE, similar to their WT counterparts, they were more sensitive to induced EAE (Fig. 4D). Upon induction, 2D2/EBI3−/− mice had earlier disease onset and rapidly reached peak disease compared with 2D2/EBI3+/+ mice. Thus, EBI3 deficiency enhances both Th17 responses and EAE development in 2D2 TCR-transgenic mice. To test if EBI3-deficient T cells have superior capacity to cause EAE compared with WT T cells, we purified CD4+CD25− T cells from 2D2/EBI3−/− and 2D2/EBI3+/+ mice and injected 1×106 purified T cells into each Rag1−/− mice i.v. followed by induction of EAE.

Enhanced suppressive functions of CD4+CD25+ Treg cells in EBI3−/− mice

EBI3 was also demonstrated to coexpress with IL-12p35 (2) to form IL-35 in Foxp3+ Treg cells and contributed to the suppressive activity of Treg cells (4). To understand if the enhanced EAE development is due to reduced Treg functions, we investigated the impact of EBI3 deficiency on Treg numbers and functions in the EAE model. As shown in Fig. 6A and 6B, similar numbers of CD4+Foxp3+ Treg cells were found in the thymi of EBI3−/− and WT mice. Interestingly, significantly increased numbers of Treg cells were found in the spleens of EBI3−/− mice. To test if EBI3 deficiency affects the functions Treg cells, we sorted CD4+CD25+ Treg cells from EBI3−/− and WT mice and compared their suppressive activities to the proliferation of EBI3-deficient CD4+CD25− T cells in response to low-dose anti-CD3. The experiment was done in the presence of irradiated splenocytes from EBI3−/− Rag1−/− mice. As shown in Fig. 6C, EBI3-deficient Treg cells had more potent inhibitory effects compared with WT Treg cells. We compared the expression of an array of cytokine genes between EBI3-deficient and WT Treg cells and found similar expression of inhibitory cytokine genes such as IL-10 and TGF-B1 (Fig. 6D).

To determine if EBI3 deficiency affects Treg function during EAE development, we performed a series of adoptive transfer experiments. First, we isolated Treg cells from EBI3−/− and WT mice and adoptively transferred them into EBI3−/−Rag1−/− mice. CD4+CD25+ EBI3−/− T cells were used as effectors. The recipient mice were then immunized for EAE development. We found that mice receiving WT Treg cells developed similar disease as mice receiving no Treg cells. In mice receiving EBI3−/− Treg cells, disease in EBI3−/− recipients was significantly delayed compared with EBI3−/− recipients receiving WT Treg cells. Thus, these experiments reveal that EBI3 deficiency enhances both Th17 responses and EAE development in EBI3−/− mice.
cells, diminished EAE development was observed (Fig. 7A). In a second series of experiments, we injected CD4+CD25- 2D2 T cells from 2D2/EBI3-/− mice into EBI3-/− Rag1-/− mice. The recipient mice also received Treg cells from EBI3-/− or WT mice followed by EAE induction. We also found that mice receiving EBI3-deficient Treg cells developed less severe EAE (Fig. 7B). Finally, we compared Treg cells from 2D2/EBI3-/− mice to Treg cells from 2D2/EBI3+/+ mice for their ability to inhibit EAE development caused by EBI3-deficient CD4+CD25- 2D2 T cells. Surprisingly, CD4+CD25- T cells from 2D2/EBI3+/+ mice did not inhibit, but rather dramatically enhanced, EAE development (Fig. 7C). In contrast, CD4+CD25- T cells from 2D2/EBI3-/− mice significantly inhibited EAE development (Fig. 7C). Thus, EBI3-deficient Treg cells had more potent capacity to inhibit EAE development in vivo.

Discussion

Studies using IL-27Ra−/− mice (12) and IL-27 systemic injection (17, 29) have revealed an inhibitory role of IL-27 in EAE development. Recently, IL-27p28−/− mice have also been shown to have enhanced EAE (23). However, the role of the other component of IL-27, the EBI3 subunit, in EAE development has not been studied. To investigate the contribution of this cytokine subunit, we compared the immune responses and susceptibility of EBI3−/− versus WT mice and EBI3-deficient versus EBI3-sufficient 2D2 TCR-transgenic mice to MOG peptide-induced EAE. We found that EBI3−/− and 2D2/EBI3−/− mice had marginally enhanced EAE development compared with their WT counterparts. The enhanced EAE development in EBI3−/− mice was associated with increased T cell IL-2 production, enhanced T cell proliferation, and Th17 and Th1 differentiation. In addition, we have also found that in the peripheral lymphoid organs of EBI3−/− mice, numbers of CD4+Foxp3+ Treg cells are increased. More strikingly, the suppressive function of CD4+ Foxp3+ Treg cells from EBI3−/− mice is more potent than Treg cells in WT mice. Enhanced Treg responses in EBI3−/− mice may explain why the EAE development is only modestly enhanced compared with WT mice, despite the potent Th17 response induction.

In the primed peripheral lymphoid organs and in the CNS of EBI3−/− and 2D2/EBI3−/− mice, increased Th17 response was consistently observed. IL-27 has a known suppressive effect on Th17 differentiation through IL-27−induced activation of STAT1 and STAT3 (12, 18, 30). Thus, in EBI3−/− mice, the increased IL-17 response is consistent with enhanced Th17 responses consequent to IL-27 deficiency. We also observed enhanced Th1 response in EBI3−/− mice during EAE development. This effect was evident in the CNS during EAE; however, in the peripheral lymphoid organs, the effect was less consistent. The reason could be the smaller difference or the fact that peripheral lymphoid cells were cultured for a long time. IL-27−induced T cell production of
IL-10 has been thought to be an important mechanism in suppressing EAE development (16, 31); however, in EBI3−/− mice with EAE, no decreased IL-10 production was observed. In some cases, especially in the CNS, increased IL-10 expression is observed. Thus, EBI3 deficiency does not seem to globally affect IL-10 production. Because IL-10 has multiple sources in vivo, this observation does not rule out the possibility that IL-10 production by some subsets of T cells are affected in EBI3−/− mice. Another notable observation in this study is that EBI3−/− T cells had increased IL-2 production and proliferation. Enhanced IL-2 production by T cells from EBI3−/− mice is consistent with the known anti-IL-2 effect of IL-27 (32–34) and could explain the stronger proliferation of EBI3−/− T cells.

We have also observed increased numbers of CD4+Foxp3+ Treg cells in the peripheral lymphoid organs in EBI3−/− mice. Because the numbers of Treg cells in the thymi of EBI3−/− and WT mice were similar, it is likely that EBI3 deficiency only affects converted Treg cells. Consistent with this observation, IL-27 has been shown to antagonize TGF-β-driven Treg cell conversion through restricting the expression of Foxp3, CD25, and CTLA-4 (CD152) in vitro (35). A recent study (36) showed that lack of IL-27 signaling (T cell deficient for IL-27Rα−/−) resulted in the rapid conversion of conventional T cells into CD4+Foxp3+ Treg cells, which profoundly blocked colitis development in a T cell adoptive transfer-induced colitis model, in which Treg cells are critical for controlling disease development. In another study (32), IL-27 transgenic mice were found to develop spontaneous autoimmunity, with greatly reduced CD4+Foxp3+ Treg cells, and the effect could be largely attributed to lack of IL-2 production in IL-27-transgenic mice. Indeed, we also found that EBI3−/− T cells produced more IL-2, which likely contributed to the expansion of Treg cells in EBI3-deficient mice. Thus, increased numbers of Treg cells in EBI3−/− mice are consistent with the effects of IL-27 deficiency.

Our adoptive transfer experiments suggest that the disease-enhancing effect of EBI3-deficiency is not T cell intrinsic, but attributed to EBI3 deficiency in the non-T cell compartment. Given that IL-27 is mainly produced in non-T compartments (5), whereas IL-35 can be produced by Treg cells in the T cell compartment (4), this observation does not rule out the possibility that IL-10 production by T cells from EBI3−/− mice cannot be explained by diminished Treg cell functions due to IL-35 deficiency. In fact, we have found that Treg cells from EBI3−/− mice have a more potent inhibitory effect on T cell proliferation and T cell-mediated CNS autoimmunity. Under our experimental condition, we found that Treg cells from WT B6 mice or 2D2 mice did not display substantial inhibitory roles for EAE development, but rather enhanced autoimmunity was observed in mice receiving Treg cells from 2D2 mice. In contrast, Treg cells from EBI3−/− and 2D2’EBI3−/− mice were consistently inhibitory. Because IL-10 and TGF-β1 gene expression are similar in Treg cells from EBI3−/− versus WT mice, it will be interesting to determine what other factors contribute to enhanced suppressive functions of EBI3-deficient Treg cells.

Taken together, our data suggest that EBI3 inhibits Th17, Th1, IL-2, and Treg responses and suppresses EAE development. Although these observations are consistent with the known functions of IL-27, the IL-35 contribution to the suppressive functions of Treg cells is not evident in this model. Increased Treg responses in EBI3−/− mice may explain why the EAE development is only modestly enhanced compared with WT mice, despite enhanced Th17 responses are induced.

Disclosures
The authors have no financial conflicts of interest.
References


