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CUTTING EDGE

Cutting Edge: Attenuated Experimental Autoimmune Encephalomyelitis in Eta-1/Osteopontin-Deficient Mice[1]

Marianne Jansson, Vily Panoutsakopoulou, Jessica Baker, Ludger Klein, and Harvey Cantor[2]

Recent studies indicate that early T lymphocyte activation 1 (Eta-1), also known as osteopontin, is a cytokine contributing to the development of Th1 immunity. In the present report, the role of Eta-1 in experimental autoimmune encephalomyelitis (EAE), a disease associated with Th1 immunity, was examined by analysis of disease progression in Eta-1-deficient (Eta-$1^{-/-}$) mice. Although incidence and onset of peptide-induced EAE were found to be similar in Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice, Eta-$1^{-/-}$ mice displayed significantly lower mean maximal clinical score and faster recovery without spontaneous relapses. Accordingly, decreased inflammatory infiltration and demyelination were observed in the spinal cords of Eta-$1^{-/-}$ mice. Furthermore, in comparison to Eta-$1^{+/+}$, Eta-$1^{-/-}$ $CD4^+$ T cells had reduced expression of IFN-γ and TNF-α upon ex vivo restimulation. Taken together, these results suggest that Eta-1 may sustain autoimmune responses by assisting in maintenance of Th1 immunity during EAE. ***The Journal of Immunology*****, 2002, 168: 2096–2099.**

Production of certain cytokines by professional APC is an early essential step of Th cell polarization during the development of immune responses. Thus, production of IL-12 by dendritic cells triggers Th1 cell development, while early IL-10 production can reverse this development and may skew cells to become Th2 (1). Th1 and Th2 responses exhibit not only cytokine profile differences (2) but also differences in their functional properties, resulting in distinct impacts on immunity against pathogens and self. Studies in murine models demonstrate that Th1 polarization is associated with protective immune responses against several intracellular pathogens, but also linked to autoimmune diseases (3).

Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, MA 02115

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[2] Address correspondence and reprint requests to Dr. Harvey Cantor, Dana-Farber Cancer Institute, 44 Binney Street, Boston MA 02115. E-mail address: Harvey_Cantor@dfci.harvard.edu

Experimental evidence suggest that early T lymphocyte activation 1 (Eta-1),[3] also known as osteopontin (Opn), is a pleiotropic cytokine critical for the generation of Th1 immunity. Eta-1 is expressed by T cells early during bacterial infections (4) and was elevated in granulomatous responses (5), typically thought of as type 1 immune responses. In addition, enhanced expression of Eta-1 has been linked to resistance against certain intracellular microorganisms (4). Recently, Eta-1 was observed to be crucial for the induction of Th1-linked delayed-type hypersensitivity reaction to herpes simplex virus challenge (6). Importantly, stimulation with Eta-1 resulted in up-regulation of IL-12 expression by macrophages, whereas IL-10 expression was down-regulated (6), indicating that Eta-1 may act to determine the relative production of IL-12 and IL-10 by APC.

The balance of IL-10 and IL-12 has been reported to play a delicate role in experimental autoimmune encephalomyelitis (EAE) (7), the murine model for multiple sclerosis (MS). Production of IL-12 is essential for the generation of autoreactive EAE-inducing Th1 cells, whereas IL-10 production antagonizes the disease-promoting effects of IL-12 (7). In a recent study, modulation of the IL-10/IL-12 cytokine circuit by IL-12 decrease following IFN-β administration inhibited development of epitope spreading and EAE progression (8).

In the present study, we demonstrate that mice deficient in Eta-1 (Eta-$1^{-/-}$) production develop significantly milder EAE in comparison to wild-type controls. Furthermore, Eta-$1^{-/-}$ mice exhibit no relapses and $CD4^+$ T cells from these mice express reduced levels of Th1 cytokines. These results suggest that Eta-1 may intensify autoimmune responses during EAE development by facilitating the skewing of Th cells toward type 1 immunity.

Materials and Methods

Mice

C57BL/6 × 129 Eta-1/Opn knockout (Eta-$1^{-/-}$) mice (6, 9) were backcrossed to C57BL/6 for six generations. Wild-type (Eta-$1^{+/+}$) littermate control mice were used for comparison in all experiments.

Induction of EAE

EAE was induced in mice by immunization with a 12-mer synthetic peptide (New England Peptide, Fitchburg, MA) representing aa 172–183 (PVYIYFNTWTTC) of proteolipid protein, $PLP_{172–183}$ (10). One hundred fifty micrograms of $PLP_{172–183}$ peptide and 300 μg of killed *Mycobacterium tuberculosis* (Difco, Detroit, MI) in CFA (Sigma-Aldrich, St. Louis, MO) were injected s.c. by means of three injections over the flanks. In

[3] Abbreviations used in this paper: Eta-1, early T lymphocyte activation 1; Opn, osteopontin; EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein.

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addition, 400 ng of pertussis toxin (List Biological Laboratories, Campbell CA) was injected i.p. on days 0 and 2. Mice were monitored daily and assessed for clinical signs of disease in a blinded fashion according to the following criteria: 0, normal mouse without signs of disease; 1, limp tail; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; and 5, moribund state or death due to EAE. Mean clinical scores at separate days and mean maximal scores were calculated by adding scores of individual mice and dividing with number of mice in each group, also including mice not developing signs of EAE. Average day of onset was calculated by adding the first day of clinical signs of individual mice and dividing with number of mice in the group. Day of onset for mice that did not develop EAE was intentionally considered to be 1 day after completion of experiment.

Histology

Spinal cords were removed and fixed in 10% Formalin. Paraffin-embedded sections were stained with H&E or Luxol Fast Blue for visualization of inflammatory infiltrates and demyelination.

Cell cultures and cytokine determination

Draining lymph nodes and spleens were obtained from $PLP_{172–183}$-immunized mice. Single-cell suspension was obtained and cells were suspended in labeling buffer (2% FBS in PBS) at a concentration of 10^7 cells/ml and incubated with 10 μg/ml purified anti-CD8 and anti-B220 and 5 μg/ml purified anti-CD11b and anti-GR-1 (all from BD PharMingen, San Diego, CA) for 30 min at 4°C. Cells were washed three times in labeling buffer and then resuspended at a concentration of 10^7 cells/ml in labeling buffer. To this cell suspension, 50 μl/ml washed Dynabeads M-450 Sheep anti-Rat IgG (Dynal, Lake Success, NY) was added, and the cells were again incubated for 30 min at 4°C. The bead-negative fraction of cells was collected using a magnet. $CD4^+$ cells were enumerated and resuspended in complete medium (RPMI 1640 medium supplemented with 10% FBS (Sigma-Aldrich), L-glutamine, 2-ME, and antibiotics). Briefly, 1.5×10^5 cells were plated in triplicate wells in 96-well microtiter culture plates (Costar, Corning, NY) along with 3×10^4 spleen-derived $CD11c^+$ dendritic cells, purified using anti-CD11c-conjugated MACS microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer. $PLP_{172–183}$ peptide was added to a final concentration of 10 or 50 μg/ml, while medium was added to control cultures. In parallel, $CD4^+$ cells obtained from naive mice were cultured according to the same protocol with the exception that stimulation instead was provided by plate-bound anti-CD3 complex Ab (BD PharMingen) coated at a concentration of either 2.5 or 5.0 μg/ml. After 96-h cell culture supernatant was harvested and frozen at −80°C. Concentrations of IFN-γ, IL-2, IL-4, IL-10, and TNF-α were analyzed using cytokine capture ELISA (OPTEIA; BD PharMingen).

Semiquantitative RT-PCR for cytokine mRNA detection

Spinal cords were extruded by flushing the vertebral canal with PBS and then rinsed in PBS. RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) according to instructions by the manufacturer. cDNA was reverse transcribed using oligo(dT) primer and semiquantitative PCR amplification of cytokine IFN-γ and TNF-α, and housekeeping β-actin mRNA was done using the following primers: IFN-γ, antisense 5′-ACA CTG CAT CTT GGC TTT GC-3′, sense 5′-CGA CTC CTT TTC CGC TTC CT-3′; TNF-α, antisense 5′-GTA TGA GAT AGC AAA TCG-3′, sense 5′-TTC TGT CTA CTG AAC TTC-3′; β-actin: antisense, 5′-TAA AAC GCA GCT CAG TAA-3′; and sense, 5′-TGG AAT CCT GTG GCA TCC-3′. Ratios of cytokine PCR products to housekeeping gene β-actin PCR products for each sample were obtained using densitometry.

Statistical analysis

Statistical differences between groups with respect to disease score at specific days, mean maximal score, number of days with paralyzing disease, and number of inflammatory foci in spinal cord were evaluated using a nonparametric analysis Mann-Whitney *U* test.

Results

Considering that Eta-$1^{-/-}$ mice displayed impaired type 1 immune responses as well as marked resistance to development of herpes stromal keratitis, a Th1-linked autoimmune disease (6), we tested the role of Eta-1 in the development of EAE. Thus, EAE was induced in Eta-$1^{-/-}$ and Eta-$1^{+/+}$ littermates by s.c. injections of a PLP-derived encephalitogenic peptide ($PLP_{172–183}$) in CFA. Although disease onset and incidence were similar among Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice (Fig. 1 and Table I), the severity was substantially reduced in Eta-$1^{-/-}$, and the mean maximal score was significantly lower when compared with the Eta-$1^{+/+}$ score (Fig. 1 and Table I). In addition, Eta-$1^{-/-}$ mice exhibited improved recovery from EAE by sustaining fewer days of paralysis and lower mortality (Fig. 2). Importantly, although many of the Eta-$1^{+/+}$ mice experienced spontaneous relapses, none of the Eta-$1^{-/-}$ mice did (Fig. 2). Thus, peptide-induced EAE in Eta-$1^{-/-}$ mice appeared to be attenuated compared with disease in their Eta-$1^{+/+}$ littermates.

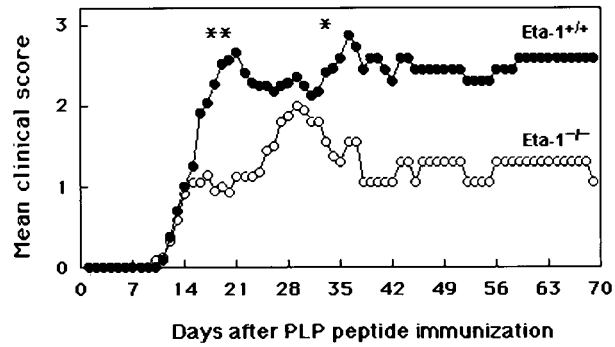

FIGURE 1. Eta-1-deficient mice develop attenuated EAE. Development of EAE was assessed for 35 days after immunization with $PLP_{172–183}$ in Eta-$1^{-/-}$ ($n = 22$) and Eta-$1^{+/+}$ ($n = 23$) littermate mice. From these mice, 11 (Eta-$1^{-/-}$, $n = 4$; Eta-$1^{+/+}$, $n = 7$) were monitored for a period of 70 days. Disease was scored as described in *Materials and Methods*. Depicted are mean clinical scores for Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice at indicated days. *, $p < 0.05$; **, $p < 0.01$.

Histology of spinal cords was performed to examine whether differences in clinical symptoms of EAE between Eta-$1^{-/-}$ and Eta-$1^{+/+}$ were reflected by inflammatory infiltration and demyelination in the CNS. As detected by H&E and Luxol Fast Blue stainings, spinal cord sections from Eta-$1^{-/-}$ mice exhibited significantly lower inflammatory infiltrates as well as demyelinated foci compared with cord sections from wild-type mice (Fig. 3).

To test the cytokine profile, $CD4^+$ T cells obtained from draining lymph nodes and spleen at the onset of disease as well as during recovery (i.e., 3 and 5 wk after immunization) were restimulated ex vivo with $PLP_{172–183}$. $CD4^+$ cells obtained from Eta-$1^{-/-}$ mice during EAE onset and during the recovery secreted significantly less IFN-γ and TNF-α in comparison to cells from Eta-$1^{+/+}$, whereas IL-2 levels were found to be similar among the groups. Proliferation upon PLP-specific stimulation was similar for Eta-$1^{-/-}$ and Eta-$1^{+/+}$ CD4 cells (data not shown). Expression of IL-4 and IL-10 was undetectable in cultures of cells from both groups of mice (data not shown). However, significantly elevated IL-10 was detected in parallel cultures of anti-CD3-stimulated $CD4^+$ T cells obtained from naive Eta-$1^{-/-}$ mice compared with

Table I. *EAE clinical disease in Eta-$1^{-/-}$ and wild-type littermate mice*[a]

	Eta-$1^{+/+}$	Eta-$1^{-/-}$
Incidence	23/23 (100%)	18/22 (82%)
Day of onset	15.1 ± 0.7	19.2 ± 1.6
Maximal score	3.7 ± 0.2	2.6 ± 0.4*

[a] EAE clinical disease monitored during a 35-day follow-up. Incidence was calculated as number and percentage of mice that developed any clinical signs of EAE. For scoring criteria, see *Materials and Methods* and the legend to Fig. 1. The average day of EAE onset and mean maximal score ± SEM.
*, $p < 0.05$.

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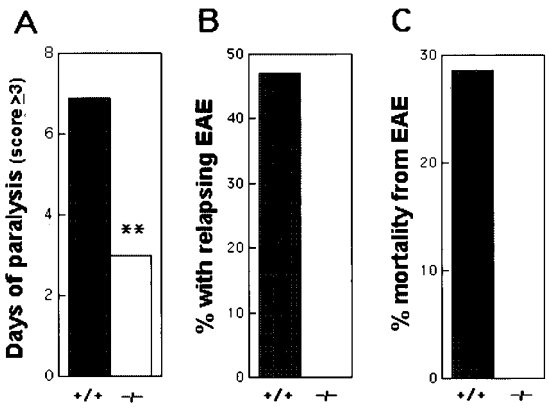

FIGURE 2. Eta-1-deficient mice displayed milder EAE disease with fewer days of paralysis, lack of spontaneous relapsing disease, and decreased mortality. Mice (Eta-$1^{-/-}$, $n = 22$; Eta-$1^{+/+}$, $n = 23$) were scored for 35 days after $PLP_{172–183}$ immunization. *A*, Average number of days mice experienced paralyzing disease corresponding to clinical score 3 or higher (assessed as described in *Materials and Methods*). *B*, Percentage of mice with history of paralysis (scores ≥ 3) that had spontaneous relapses to paralysis (scores ≥ 3). *C*, Mortality illustrates percentage of mice euthanized due to moribund state during a 70-day monitoring experiment. **, $p < 0.01$.

cultures of anti-CD3-stimulated $CD4^+$ T cells from Eta-$1^{+/+}$ littermates (Fig. 4*B*). In the same cultures the overall cytokine profile of Eta-$1^{-/-}$ vs Eta-$1^{+/+}$ $CD4^+$ T cells reflected a Th2 vs a Th1 phenotype, respectively (data not shown).

Furthermore, mRNA levels of IFN-γ and TNF-α from spinal cords of Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice were assessed. Similar to the T cell profile, Eta-$1^{-/-}$ mice displayed reduced levels of IFN-γ and TNF-α mRNA at the site of inflammation compared with wild-type littermates (Fig. 5). Hence, the cytokine profile of $CD4^+$ cells as well as the cytokine mRNA levels in the spinal cord suggest that Eta-$1^{-/-}$ mice exhibit reduced Th1 immunity, which may explain development of milder EAE in these mice.

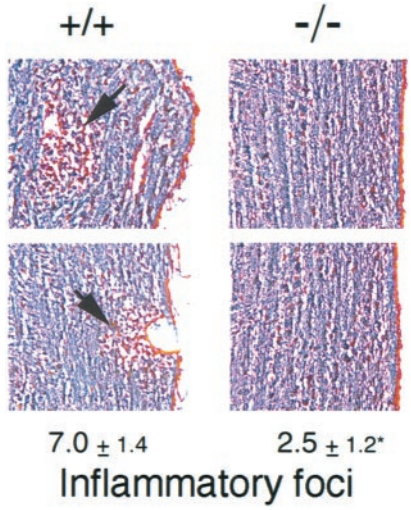

FIGURE 3. Decreased inflammation and demyelination in spinal cords of Eta-1-deficient mice. Representative histology of spinal cord longitudinal sections obtained 3 wk after EAE induction. Inflammatory infiltration and demyelination were visualized by Luxol Fast Blue and hematoxylin. Average number of inflammatory foci/ocular field ± SEM in spinal cords of Eta-$1^{-/-}$ ($n = 4$) and Eta-$1^{+/+}$ ($n = 4$) mice as detected in H&E-stained sections. *, $p < 0.05$

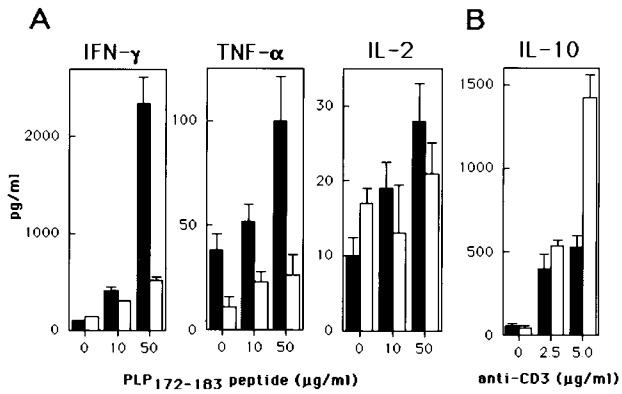

FIGURE 4. Cytokines from $CD4^+$ T cell cultures of Eta-$1^{+/+}$ (■) and Eta-$1^{-/-}$ (□) mice. Cytokine expression in $CD4^+$ T cells derived from EAE-induced mice and ex vivo restimulated with 10 and 50 μg/ml $PLP_{172–183}$ peptide (*A*) and $CD4^+$ T cells obtained from naive mice stimulated with 2.5 and 5.0 μg/ml plate-bound anti-CD3 (*B*). Cell culture supernatants were collected after 96 h and analyzed for content of IFN-γ, IL-2, IL-10, and TNF-α using capture ELISA. Results are depicted as the mean cytokine expression for each group of mice ± SEM.

Discussion

In the present study, we demonstrate that Eta-1 expression has a significant role in sustaining autoimmune destruction as reflected by attenuated EAE development in Eta-1-deficient mice. Eta-$1^{-/-}$ mice displayed reduced mean maximal score, fewer days of paralyzing disease, and no spontaneous relapses. As a result, clinical disease in EAE-induced Eta-$1^{-/-}$ mice was a considerably milder form compared with the lethal and paralytic disease observed in Eta-$1^{+/+}$ littermates. We have previously demonstrated that Eta-$1^{-/-}$ mice are resistant to the development of another autoimmune disease, herpes stromal keratitis (6), pointing to a general role of Eta-1 in Th1-mediated autoimmune destruction.

Certain downstream cytokines play a critical role in the initiation, propagation, and regulation of autoimmune responses. Upon ex vivo PLP peptide restimulation of $CD4^+$ T cells and in analysis of spinal cord mRNA we determined the cytokine profile in Eta-$1^{-/-}$ mice to be skewed, as judged by reduced levels of IFN-γ and

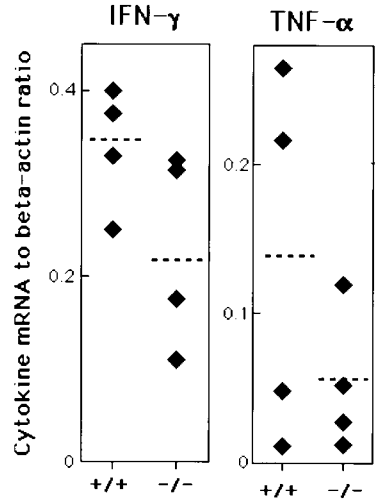

FIGURE 5. Cytokine mRNA expression in the CNS of EAE-induced Eta-$1^{+/+}$ and Eta-$1^{-/-}$ mice. Levels of spinal cord cytokine IFN-γ and TNF-α mRNA obtained 3 wk after EAE induction (by semiquantitative RT-PCR as described in *Materials and Methods*). Dotted lines represent mean ratios.

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TNF-α. Th1 cytokines have generally been considered to be encephalitogenic (11), although conflicting results with respect to EAE development in different cytokine-deficient mice point to complex regulation and function of individual cytokines (12–14). Nonetheless, TNF-α can provoke inflammatory responses (15) and transgenic expression of IFN-γ and TNF-α in the CNS accelerates development of demyelinating disease (16, 17). Conversely, several studies indicate that skewing toward Th2 immunity is protective or alters the outcome of EAE (18).

The Th2 cytokine IL-10 appears to play a regulatory role during development of EAE (19). In our experiments, IL-10 production upon ex vivo PLP peptide restimulation of $CD4^+$ T cells was undetectable, possibly due to the low prevalence of PLP-specific IL-10-producing T cells. Nevertheless, in parallel cultures, we observed that anti-CD3-stimulated $CD4^+$ T cells from naive Eta-$1^{-/-}$ mice expressed elevated levels of IL-10 compared with $CD4^+$ T cells from Eta-$1^{+/+}$ littermates. Interestingly, IL-10 produced by Ag-nonspecific $CD4^+$ T cells has been reported to antagonize encephalitogenic cytokine responses in EAE development (7) and, in the absence of Eta-1, immunoregulatory IL-10-producing cells may play a more dominant role by assisting toward early Th2 skewing. Alternatively, low production of IFN-γ and TNF-α could account for blunted disease expression in Eta-$1^{-/-}$ mice.

EAE, a model for human MS, is thought to primarily be a T cell-mediated autoimmune disease. Eta-1 is expressed by both T cells and macrophages but it is not clear to what extent either source of Eta-1 contributes to the development of autoimmune disease. Since transfer of purified $CD4^+$ T cells (10^7) from naive Eta-$1^{+/+}$ but not Eta-$1^{-/-}$ donors into syngeneic RAG2$^{-/-}$ recipients induces significant EAE after immunization with PLP peptide (data not shown), T cell expression of Eta-1 may be critical for the development of EAE.

Eta-1 also initiates migration of macrophages and dendritic cells to sites of inflammation (20–22, 23) and may contribute in this way to the diminished inflammatory infiltration, decreased demyelination, and reduced Th1 cytokine mRNA in spinal cords of Eta-$1^{-/-}$ mice. Although the role of Eta-1-dependent chemotaxis, macrophage activation and type 1 cytokine expression to EAE remains to be elucidated, the importance of this cytokine to relapsing, progressive and lethal disease is clear. Since these disease elements represent the cardinal clinical features of MS, our findings highlight the importance of studies that correlate human Eta-1/Opn expression with radiographic and clinical signs of remission, relapse and disease progression in MS patients.

While our study was under review, experiments indicating the importance of Eta-1 to rodent EAE and human MS were published by another group (24). In agreement with our findings, Chabas et al. (24) reported nonprogressive EAE in Eta-1-deficient mice as well as decreased production of Th1 cytokines in response to myelin-derived peptide (in this case myelin oligodendrocyte glycoprotein (MOG) 35–55). In addition, their study noted up-regulation of Eta-1 in cDNA libraries obtained from patients with MS as compared with control libraries. Overall, the two independent studies of EAE induction using two distinct myelin-derived peptides ($PLP_{172–183}$ vs MOG 35–55) provide strong support for a critical role of Eta-1 in demyelinating autoimmune disease.

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