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# Increased levels of bioactive IL-16 correlate with disease activity during relapsing experimental autoimmune encephalomyelitis (EAE)

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**Abstract**

Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T-cell mediated disease, which resembles immunopathology of multiple sclerosis (MS). Interleukin (IL)-16 is a CD4+ cell-specific chemoattractant cytokine. In CD4+ T cells, production of bioactive IL-16 from constitutive pro-IL-16 requires cleavage by active caspase-3. We reported reversal of established relapsing disease by IL-16 neutralization. To better understand role(s) of IL-16 in regulation of relapsing EAE, we comparatively analyzed levels of IL-16, active caspase-3 and CD4 in mice with severe relapsing-remitting [(B6 × SJL) F1], and low-relapsing (B6), disease. Elevated levels of IL-16 along with an increase in active-caspase-3 and CD4 levels correlated with stages of clinically active disease in both strains. CNS levels of bioactive IL-16 were notably higher in F1 compared to B6 mice at all stages, being most prominent during relapse. Similar patterns of regulation for IL-16 and active caspase-3 were observed in peripheral lymphoid organs, and in T cells isolated from lymph nodes following T-cell activation in vitro. IL-16 was co-immunoprecipitated with CD4 from CNS of relapsing mice. Our data suggest that caspase-3 mediated production of IL-16 by infiltrating CD4+ T cells, contributes to ongoing neuroinflammation by chemoattraction of additional waves of CD4+ T cells.


*Keywords:* Relapsing autoimmune encephalomyelitis; Interleukin-16; CD4+ T cells; Caspase-3

## 1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune CD4+ Th1 mediated disease. EAE serves as a model for human autoimmune demyelinating disease multiple sclerosis (MS) [1,2]. By producing and locally secreting Th1-type cytokines, such as interleukin-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, encephalitogenic CD4+ T cells induce activation of neighboring astrocytes and microglia, which then in turn produce more inflammatory cytokines and chemokines [3–5]. In addition to cytokine production and stimulation of glial cells, infiltrating T cells directly contribute to local chemokine accumulation [6–8]. Production and local accumulation of chemokines within the CNS compartment has been shown to play a critical role in regulation of mononuclear cell trafficking through the blood–brain barrier (BBB) and induction of CNS inflammation [9]. Findings of elevated levels of several CC and CXC chemokines in CNS in correlation with stages of clinically active disease suggested their role in regulation of inflammatory cell trafficking into CNS [10,11]. This hypothesis was further supported by later experiments, showing complete or partial resistance to EAE induction in either chemokine or chemokine-specific receptor deficient mice [12–14].

We have recently demonstrated that another chemoattractant factor, a cytokine interleukin-16 (IL-16), has been associated with the pathology of relapsing-remitting EAE [15]. As opposed to the majority of CC and CXC chemokines implicated in the immunopathology of EAE, which do not discriminate between cell phenotypes, IL-16 is a CD4+ T-cell-specific chemoattractant cytokine. IL-16 is a more effective chemoattractant for Th1 than for Th2 CD4+ T cells [16]. Cytokine IL-16 has

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been identified as a major source of T-cell chemotactic activity and was originally named leukocyte chemoattractant factor (LCF) [17]. In addition to chemoattraction of CD4+ cells, IL-16 exerts proinflammatory and immunomodulatory properties, which include regulation of CD4+ T-cell activation, and regulation of chemokine induced chemoattraction [18].

The precursor molecule, pro-IL-16 (80 kDa) is constitutively produced in T lymphocytes. Activation and release of bioactive IL-16 (17 kDa) is distinctly regulated among T-cell subsets. In CD4+ T cells, T-cell receptor (TCR) mediated or cytokine-induced T-cell activation, leads to the enzymatic cleavage of pro-IL-16 by activated caspase-3 and release of active C-terminal portion of IL-16 (14–17 kDa), while secretion of IL-16 from CD8+ T cells is caspase-3 independent [18]. It has been suggested that C-terminal cleavage of pro-IL-16 is regulated by phosphorylation of pro-IL-16 on Ser144, which engages Erk1/2 kinase activity, while the secretion of bioactive IL-16 is regulated by MAP kinase [19]. While in quiescent CD4+ T cells only pro-IL-16 (80 kDa) can be detected, upon cell activation, an intermediate product of cleavage (50–60 kDa) and active IL-16 (14–17 kDa) are readily observed. Secreted IL-16 multimerizes into homotetramers composed of 14–17 kDa chains, necessary for the binding to CD4 receptor and subsequent signaling. Bioactive IL-16 induces chemoattraction of CD4+ T cells by binding to CD4 receptor and signaling that involves $p56^{lck}$, protein kinase C, with the requirement for SH2/SH3 recruitment domains, which suggest involvement of other intracellular signaling proteins [20]. While the secreted C-terminal domain of IL-16 achieves its pleiotropic effects on CD4+ cells through CD4-initiated signaling pathways, the residual N-terminal domain translocate to the nucleus, where it induces $G_0/G_1$ cell cycle arrest. Nuclear translocation of N-terminal portion of IL-16 is enabled by the CcN motif, which contains nuclear localization sequence (NLS), protein kinase CK2 substrate site and a cdc2 kinase substrate site [21].

Increased local production of IL-16 has been reported in other CD4+ Th1 mediated autoimmune diseases, such as rheumatoid arthritis and Graves' disease [22,23]. In CNS of (B6 × SJL) F1 mice with relapsing-remitting EAE, we observed IL-16 immunoreactivity confined to CD4+, CD8+ T cells and B cells. Our data suggest that IL-16 play an important role in regulation of relapsing EAE in (B6 × SJL) F1 ($H\text{-}2^{b/s}$) mice, as anti-IL-16 therapy improved clinical and histopathology of relapsing disease [15].

In the present study, we examined and compared mechanisms of IL-16 regulation between relapsing-remitting and non/low-relapsing EAE mice.

## 2. Materials and methods

### 2.1. Mice and induction of EAE

EAE was induced in 8–10-week-old C57BL/6 (B6) ($H\text{-}2^{b}$) and (B6 × SJL) F1 ($H\text{-}2^{b/s}$) female mice (Jackson Laboratories, Bar Harbor, ME) by immunization with $MOG_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK), 99% pure by HPLC, (Caltech, Pasadena, CA), as previously described [8]. Mice were observed daily for clinical symptoms of EAE. The clinical grade was scored as follows: 0.5, partial loss of tail tonicity; 1, complete loss of tail tonicity; 2, flaccid tail and abnormal gait; 3, hind leg paralysis; 4, hind leg paralysis with hind body paresis; 5, hind and foreleg paralysis, 6, moribund.

### 2.2. Tissue sampling

Mice were killed at different stages of relapsing-remitting disease: acute, remission, relapse and chronic. Approximately 3–4 mice from each strain were analyzed at each stage of disease. Mice were anesthetized, perfused through the heart with 10 ml of ice-cold phosphate-buffered saline (PBS), and spinal cord, brain, spleen and regional lymph nodes were dissected. From each dissected tissue, 1–2-mm blocks were cut and embedded in Tissue Compound (Sigma, St. Louis, MO). The remaining tissues were snap-frozen for protein isolation. Frozen blocks and tissue samples were kept at −70 °C until analyzed.

### 2.3. Antibodies

For immunostaining, Western blot and immunoprecipitation, primary antibodies against: human IL-16 unconjugated and R-Phycoerythrin (PE) conjugated (clone 14.1) (1:200), and mouse CD-4 (rat IgG2a,κ—clone RM4-5) (BD Pharmingen, San Diego, CA); human/mouse active Caspase-3 (1:200), (R&D Systems); p85 fragment of polyADP-ribose polymerase (PARP) (Promega, Madison, WI), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz), were used. Isotype matched control antibody used for immunoprecipitation as a control for CD4-specific antibody was purified rat IgG2a, κ (clone R35-95, BD Pharmingen).

### 2.4. Western blot

Protein was isolated from fresh frozen spinal cord, spleen and lymph node tissues using TRIzol reagent (Gibco BRL, Grand Island, NY) according to the procedure recommended by the manufacturer. Equal amounts of protein (approximately 20 μg/lane) from each sample were loaded per lane for Western blot analysis. Samples were loaded at nonreducing conditions onto NuPage Novex Tris-Acetate gels (Invitrogen, Carlsbad, CA), resolved by electrophoresis. Electrophoresed proteins were then transferred from the gel onto nitrocellulose membrane. Membrane was cut into 3–4 strips, each containing proteins of different molecular weight. Each membrane strip was probed separately with the appropriate primary antibody overnight at 4 °C, washed three times with 0.1% Tween 20–Tris-buffered saline, and than incubated with peroxidase conjugated secondary antibody. The membrane bound peroxidase activity was detected by using ECL Plus Western blotting detection kits (Amersham, Arlington Heights, IL). Chemiluminescent images were captured and analyzed by a Kodak Digital Science Image Station 440CF. All blots were studied

within the linear range of exposure. In each sample levels of IL-16, active caspase-3 and CD4 were normalized by corresponding levels of GAPDH.

### 2.5. Immunoprecipitation

CD4 protein was immunoprecipitated from total proteins isolated from spinal cord of EAE mice using a Seize Primary Immunoprecipitation kit (Pierce, Rockford, IL), following the manufacturer's procedure. Immunoprecipitation with control antibody was done similarly in a separate experiment. CD4 and control immunoprecipitates were then subjected to Western blot analysis.

### 2.6. Immunostaining and confocal microscopy

Similarly prepared 6-μm frozen sections were used to analyze phenotypes of infiltrating cells by immunofluorescence, following a routine procedure [8]. Briefly, sections were air-dried, acetone-fixed, treated with 10% normal donkey serum for 10 min, followed by overnight incubation with relevant primary antibody in a moist chamber at +4 °C. The slides were then washed and incubated with secondary fluorochrome-labeled antibodies for 30 min. DAPI staining was performed for 5 min after immunostaining was completed. After washing, the slides were mounted in Gelmount (Biomeda, Foster City, CA) and analyzed by light and fluorescent microscopy. Images were captured on a Nikon Eclipse 600 epifluorescent microscope with a Princeton Instruments Micromax 5 MHz cooled CCD camera.

### 2.7. In vitro T-cell stimulation

From anesthetized (B6 × SJL) F1 (3 relapsing and 2 with chronic disease) and B6 (2 relapsing) mice, spleens were dissected and cells were prepared for in vitro experiments. Splenic and lymph node isolated T cells ($5 \times 10^5$/well) were stimulated with 25 μg/ml of $MOG_{35-55}$ in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 1% normal mouse serum. Cells were cultured in 96-well flat-bottom plates for 72 h and incubated at 37 °C in humidified air containing 5% $CO_2$. Following in vitro activation, approximately $5 \times 10^6$ cells (12 wells) were treated with Brefeldin A (10 μg/ml) (Sigma), prior to collection and protein isolation. Proteins from stimulated T cells were then analyzed by Western blot. From control (B6 × SJL) F1 mice, splenic T cells were similarly isolated and stimulated with PHA (10 μg/ml) for 72 h in vitro. Proteins were isolated from cells at the end of culture period, and subjected to Western blot analysis.

### 2.8. Statistical analysis

The significance of differences between groups was calculated by Student's *t*-test or paired-test as appropriate. The level of statistical significance was set at 95%.

## 3. Results

### 3.1. Increased CNS levels of bioactive IL-16 during clinically active disease correspond to elevation of active-caspase-3 and CD4+ T-cell infiltration

The levels of bioactive IL-16 (17 kDa) in spinal cord from relapsing-remitting (B6 × SJL) F1, and low/relapsing (B6) mice are shown in Fig. 1. Compared to control levels, which were indistinguishable between the two strains of mice, we found markedly higher upregulation of bioactive IL-16, during stages of active disease, in F1 than in B6 mice. Levels of IL-16 were significantly elevated during acute, relapsing and chronic stages of the disease. At each stage of disease, comparison was made between F1 and B6 mice with similar disease severity scores (differences not grater than one grade on a 0–6 scale). Levels of bioactive IL-16 were markedly higher in mice with acute EAE compared to control levels. Bioactive IL-16 peaked at relapsing, slightly decreased at chronic, but still remained significantly higher compared to controls in (B6 × SJL) F1 mice. B6 mice with chronic disease were not examined because the majority of these mice recovered either following disease onset or relapse, similar to previous data [8]. In low-relapsing, B6 mice, levels of bioactive IL-16 were increased but not significantly different between acute and relapsing disease (Fig. 1a,b). Although differences in levels of bioactive IL-16 were significant at acute ($p < 0.05$), strikingly different levels of bioactive IL-16 were observed during relapsing disease between F1 and B6 mice. Relapsing F1 mice had approximately fourfold higher levels of bioactive IL-16 in CNS compared to relapsing B6 mice ($p < 0.001$). During remission, levels of bioactive IL-16 returned close to control values in both strains of mice. In parallel with the increase in levels of bioactive IL-16, an elevation of active caspase-3 in spinal cord of mice with relapsing EAE was observed. Upregulation of active-caspase-3 followed trend similar to bioactive IL-16 throughout the course of relapsing EAE. Differences in the levels of active caspase-3 between the F1 and B6 mice were consistent with those found for bioactive IL-16 (Fig. 1a,c). Relative levels of bioactive IL-16 were approximately three- to fourfold higher compared to relative levels of active caspase-3, during acute and relapsing EAE (Fig. 1b,c). Elevation of active caspase-3 was not followed by elevation of cleaved poly(ADP-ribose) polymerase (PARP) (not shown), suggesting an apoptosis-unrelated role for active caspase-3 in CNS of EAE mice. Active caspase-3 is important for the enzymatic cleavage of bioactive IL-16 (17 kDa) from pro-IL16 (80 kDa) in activated CD4+ T cells.

Previously, we observed that infiltrating CD4+ T cells contained IL-16 in spinal cord of (B6 × SJL) F1 EAE mice [15]. Our data from semiquantitative analysis of immunostained spinal cord sections suggested more extensive infiltration by CD4+ T cells during the acute and especially relapsing disease in (B6 × SJL) F1 then B6 mice [8]. In this experiment we measured levels of CD4 from total proteins isolated from spinal cord by Western blot (Fig. 2). Regulation of CD4 levels paralleled those of bioactive IL-16 and active

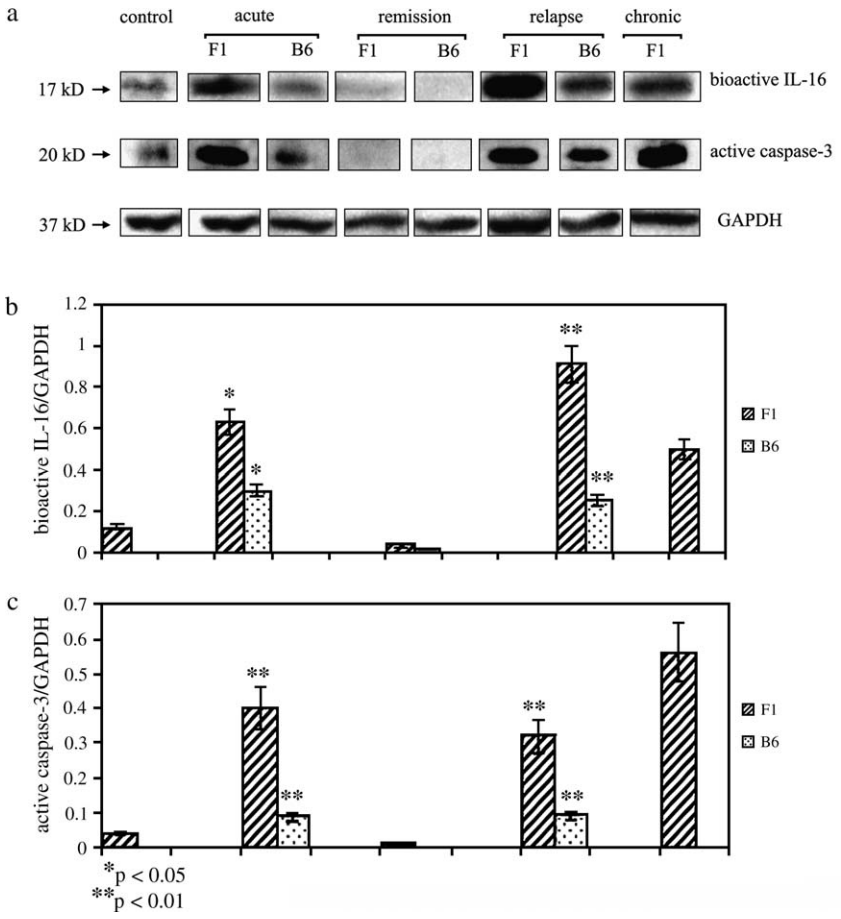


Fig. 1. Similar patterns of regulation between bioactive IL-16 (17 kDa) and active caspase-3 are observed in CNS of (B6 × SJL) F1 and B6 mice throughout the course of relapsing disease. (a) Levels of bioactive IL-16 and active caspase-3 from total proteins isolated from spinal cord of EAE mice were analyzed by Western blot. Compared to low constitutive expression, marked upregulation in levels of both proteins was observed during acute, relapsing and chronic stages of disease in both strains of mice. (b) Production of bioactive IL-16 was consistently higher in F1 compared to B6 mice. (c) Levels of active caspase-3, similarly to bioactive IL-16, were markedly higher in F1 compared to B6 mice. Approximately 20 μg of total protein was loaded per lane. From each stage of the disease of each mouse strain total of 3–4 mice were analyzed. Representative blot shows: acute-F1 (12 dpi, grade 4.5), B6 (20 dpi, grade 5); remission-F1 (28 dpi, grade 1), B6 (23 dpi, grade 0.5); relapse-F1 (68 dpi, grade 4, 3rd relapse), B6 (23 dpi, grade 4, 1st relapse); and chronic-F1 (70 dpi, grade 3); B6 (30 dpi, grade 4). Data for CD4, IL-16 (80 and 55 kDa) from these samples are shown in Figs. 2, 6 and 7.

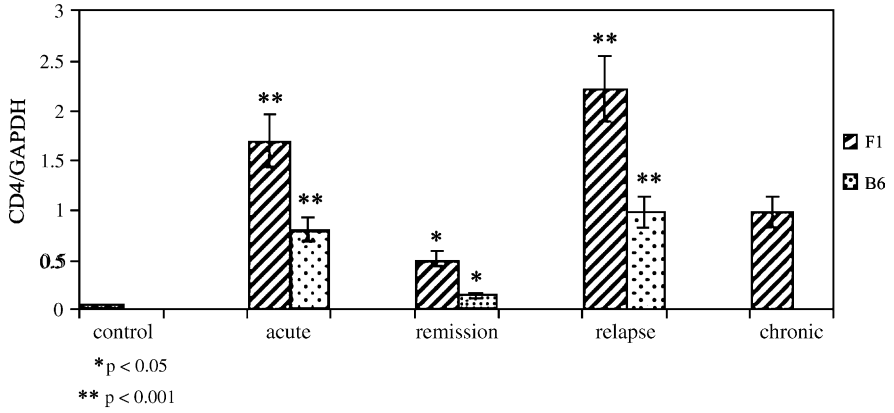


Fig. 2. Levels of CD4 protein in CNS of (B6 × SJL) F1 and B6 mice were measured by Western blot. Similar patterns of regulation between levels of CD4 and production of bioactive IL-16 and active caspase-3 (Fig. 1) were observed. Markedly higher levels of CD4 were found in F1 then in B6 mice.

caspase-3 in CNS of relapsing mice. Levels of CD4 were higher in (B6 × SJL) F1 than in B6 mice, which was consistent with our previous report. We than preceded to investigate whether infiltrating CD4+ T cells produce bioactive IL-16 in the CNS of EAE mice. Because of the lack of antibody that would distinguish between the pro-IL-16, which is constitutively present in CD4+ T cells, and bioactive IL-16, which is cleaved from pro-molecule by active caspase-3 upon cell activation, we analyzed spinal cord sections by two-color immunostaining and confocal microscopy. The colocalization of IL-16 with active caspase-3 in infiltrating cells in spinal cord of relapsing EAE mouse is shown in Fig. 3. Active caspse-3 was observed in most IL-16-containing infiltrating mononuclear cells (Fig. 3, arrows) but also in neighboring cells within the CNS. While the majority of IL-16 immunoreactive infiltrating cells contained active caspase-3, only a few cells without apparent colocalization between IL-16 and active caspase-3 were observed (Fig. 3, arrowheads).

### *3.2. Splenic and lymph node T cells from EAE mice produce IL-16 in vitro*

Overall, data from analysis of IL-16, active caspase-3 and CD4 regulation, and colocalization in CNS of relapsing-remitting and low-relapsing strains of mice suggested that bioactive IL-16 could be produced locally by infiltrating CD4+ T cells. Moreover, our results implied that its production could be important for regulation of acute and especially relapsing and chronic stages of disease. To explore the further potential of CD4+ T cells to produce bioactive IL-16 in EAE, we analyzed peripheral lymphoid organs, spleen and inguinal lymph nodes. We expected to find evidence for activation of IL-16 in regional lymph nodes and spleen of EAE mice, as a result of CD4+ T-cell activation by immunization with $MOG_{35-55}$ in complete Freund's adjuvant. Of especial interest to us was to investigate whether activation of IL-16 occurs at later stages of disease, relapse and chronic disease. High levels of bioactive IL-16 (17 kDa) and IL-16 (55 kDa) were found in inguinal lymph nodes and spleen of both strains of mice with either relapsing or chronic disease. Similarly as observed in CNS, levels of active caspase-3 corresponded to levels of bioactive IL-16 in peripheral lymphoid organs (Fig. 4). Distinctly from CNS, in the periphery marked differences between F1 and B6 strains in levels of bioactive IL-16 and active caspase-3 were not observed.

### *3.3. Levels of IL-16 in T cells following either polyclonal activation, or restimulation with cognate antigen in vitro*

In addition to analyzing IL-16 protein in intact peripheral lymphoid organs, we isolated T cells from lymph nodes and

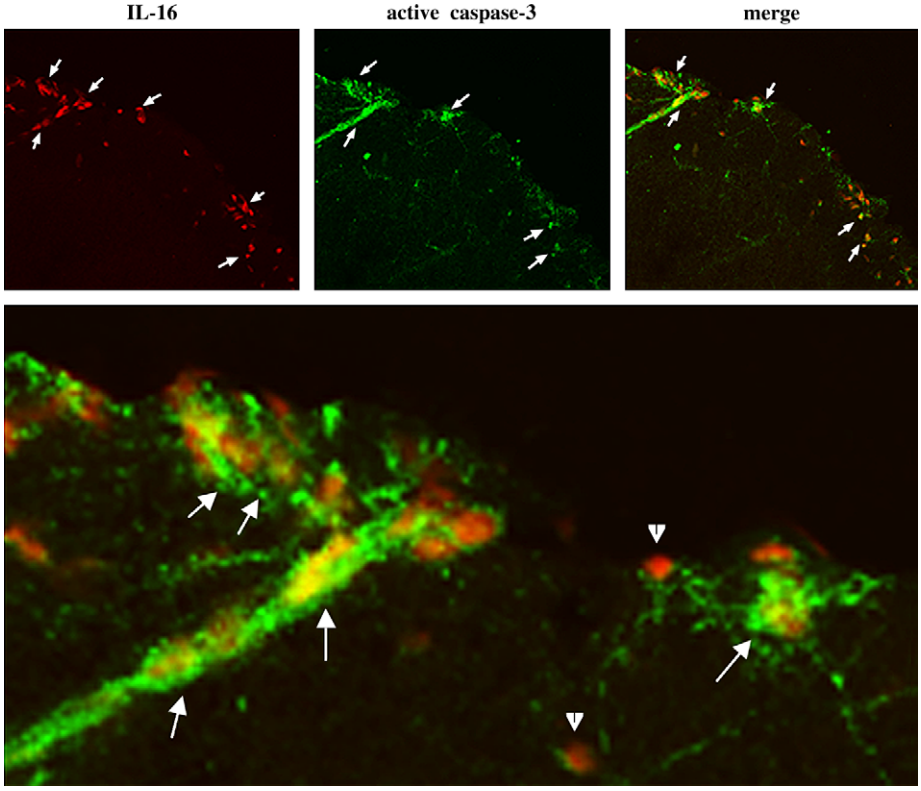


Fig. 3. Colocalization between IL-16 and active-caspase-3 in infiltrating mononuclear cells in spinal cord was analyzed by confocal microscopy on two-color immunostained spinal cord frozen sections. A representative image shows dorsal column of lumbar spinal cord from F1 mouse at fourth EAE relapse, 83 dpi, with clinical grade of 2. Note in upper panel, IL-16+ mononuclear cells in submeningeal spaces and along the penetrating blood vessel (at arrows), active caspase-3 (arrows) and their colocalization (merge, arrows). Detail of merge image is shown in lower panel. Colocalized IL-16 and active caspse-3 were observed in most of IL-16+ infiltrating cells (arrows). Active caspase-3 was also observed in the neighboring cells, which did not contain IL-16. Few IL-16+ cells did not show active capse-3 immunostaining (arrowheads). Two-color immunostaining × 40 and × 200.

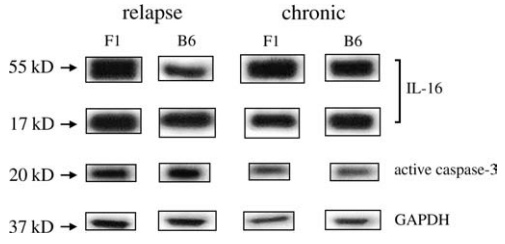


Fig. 4. Levels of IL-16 (17 and 55 kDa) and active caspase-3 in spleen and lymph node of relapsing mice were analyzed by Western blot. Note marked upregulation of IL-16 (55 kDa) and bioactive IL-16 (17 kDa) during relapsing and chronic stages. Active caspase-3 was elevated correspondingly. Representative samples from 3–4 similar experiments show: relapse (F1: 36 dpi, grade 3; B6: 21 dpi, grade 2); chronic (F1: 70 dpi, grade 4; B6: 25 dpi, grade 1).

spleens from mice immunized with $MOG_{35-55}$, and restimulated cells with antigen in vitro. Control cells isolated from spleen of non-immunized mice and polyclonally activated naïve splenocytes were analyzed, and IL-16 activation was compared (Fig. 5). In control splenocytes, IL-16 was not observed at appreciable levels. Following 72 h activation with PHA, both bands indicating activation of IL-16, the 55 kDa and 17 kDa, were observed. Similarly, 72 h restimulation with $MOG_{35-55}$ of either splenic or lymph node isolated T cells from immunized mice resulted in activation of IL-16. Active caspase-3 was not observed at times optimal for bioactive IL-16 (17 kDa) and IL-16 (55 kDa) production (not shown). It is likely that cleavage of caspase-3, its intracellular accumulation and later degradation of the active caspase-3 fragment is regulated differently than cleavage of bioactive IL-16.

### *3.4. Levels of IL-16 (55 kDa) are higher during active stages of disease in both strains of mice*

We also found elevated levels of IL-16 (55 kDa), which is believed to be an intermediate product of pro-IL-16 cleavage.

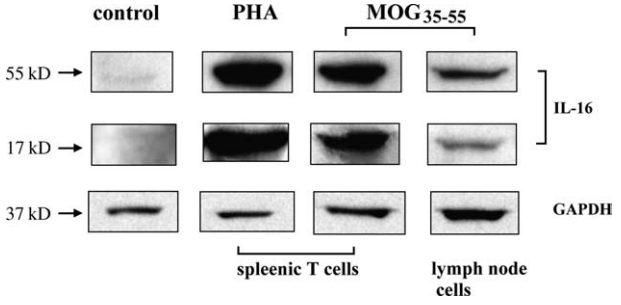


Fig. 5. Production of bioactive IL-16 (55 and 17 kDa) was observed in activated T cells, isolated from mice immunized with $MOG_{35-55}$. Ten days after immunization isolated T cells were in vitro restimulated with $MOG_{35-55}$ (50 μg/ml) for 72 h. In a different experiment, T cells isolated from naïve control mice were activated with PHA (10 μg/ml) for 72 h. For either restimulation with $MOG_{35-55}$ or polyclonal activation, approximately $5 \times 10^6$ cells were stimulated/well in a 96-well plate. Following stimulation cells were collected, and total proteins were isolated and analyzed by Western blot. Representative blot from two similar experiments shows production of IL-16 17 kDa, and 55 kDa, following T-cell activation, compared to control unstimulated T cells isolated from spleen of naïve mice.

In spinal cord of mice with EAE, IL-16 (55 kDa) was the most prominent compared to pro-IL-16 (80 kDa) (Figs. 6 and 7) and bioactive IL-16 (17 kDa) (Fig. 1). Similar temporal regulation of all three forms of IL-16 in CNS of EAE mice was observed. Throughout the course of relapsing EAE in both strains of mice, IL-16 proteins of 80, 55 and 17 kDa were upregulated during clinically active disease (acute, relapse and chronic). At each examined stage of the disease, F1 and B6 mice with similar day post immunization (dpi) clinical grade and duration of symptoms were compared. Generally, B6 mice had delayed onset of EAE compared to F1 mice (15 vs. 9), and lower mean severity scores at onset (3+- vs. 4+-), similar to findings we reported previously [8]. In order to assure fair comparison between two strains of mice, which distinctly regulate their immune responses to $MOG_{35-55}$, the priority was given to similarities in clinical scores, which reflected severity of disease, rather than the day of onset. Mice with severity scores different more than one grade on the scale of 1–6 were not compared. At acute and relapsing stages a correlation between levels of IL-16 and severity was not observed.

Temporal regulation of the levels of pro-IL-16 in CNS of EAE mice followed similar patterns as observed for activated IL-16 55 kDa fragment and bioactive IL-16 (17 kDa). Increased levels of pro-IL-16 were observed in acute, relapsing and chronic disease stages. Distinct to levels of activated fragments (55 and 17 kDa), which were highly elevated at acute and relapsing disease, levels of pro-IL-16 were markedly increased during relapsing and chronic compared to acute disease. Strain differences in levels of pro-IL-16 were observed and were consistent with those described for IL-16 55 and 17 kDa (see Fig. 1). Overall, (B6 × SJL) F1 strain contained more pro-IL-16, and produced more activated 55 kDa and bioactive (17 kDa) IL-16 throughout the course of relapsing disease compared to B6 strain.

### *3.5. IL-16 co-immunoprecipitates with CD4*

Our data suggest that infiltrating CD4+ T cells could produce bioactive IL-16 and subsequently release it locally. The importance of such production is linked to the major biological function of bioactive IL-16, which is CD4+ T-cell chemoattraction. IL-16 binds to CD4 molecule in a ligand-receptor specific fashion. IL-16 initiated signaling through CD induces specific chemotaxis of CD4+ T cells. By co-immunoprecipitation experiments, we investigated whether IL-16 and CD4 are functionally related in the CNS of EAE mice. Co-immunoprecipitation of CD4 and IL-16 is shown in Fig. 8. Total proteins were isolated from spinal cord of a (B6 × SJL) F1 mouse at third relapse, 68 dpi, with clinical severity score of 4. In two separate experiments, immunoprecipitation was done with anti-CD4 and an isotype-matched control antibody. Immunoprecipitated proteins were resolved by electrophoresis and blotted onto the membrane. Membranes were probed with anti-IL-16 antibody, then stripped and probed with anti-CD4 antibody. A strong anti-IL-16 specific band corresponding to 17 kDa fragments, a less intense band corresponding to 50 kDa fragment and a faint band of approximately 62 kDa

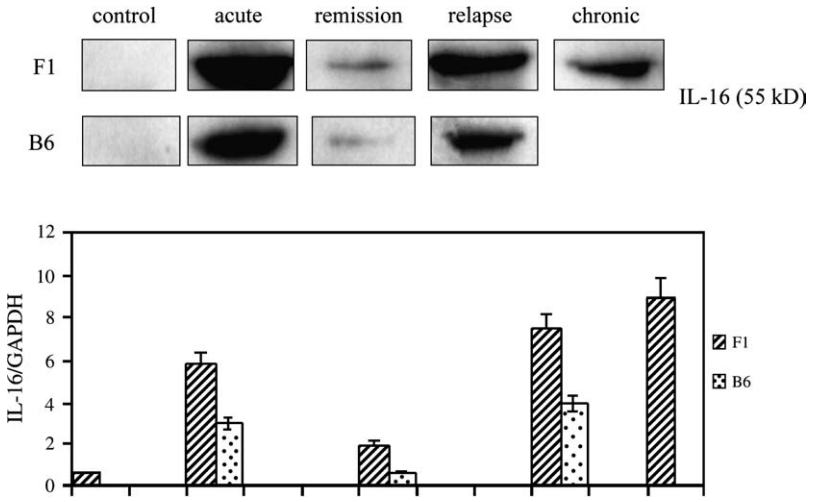


Fig. 6. Regulation of IL-16 (55 kDa) in spinal cord of F1 and B6 mice, with relapsing disease was similar to those of bioactive IL-16 (Fig. 1) and pro-IL-16 (Fig. 7). Elevated levels of IL-16 (55 kDa) during acute, relapse and chronic disease, compared to control were observed in both relapsing-remitting (F1) and low-relapsing (B6) mice. Upregulation of IL-16 was markedly higher in F1 compared to B6 mice, with similar disease.

were observed in CD4 immunoprecipitated proteins. No IL-16 or CD4 specific reactivity was evident in immunoprecipitates with the control antibody. Anti-CD4 antibody confirmed presence of CD4 of approximately 50 kDa in CD4 immunoprecipitates.

## 4. Discussion

Previous work from our laboratory has suggested an important role of IL-16 in regulation of relapsing disease in (B6 × SJL) F1 mice [15]. To better understand the role of IL-16 in orchestrating inflammation of the central nervous system (CNS), we extended our previous observations and sought answers to several questions relevant for the regulation of bioactive IL-16 in relapsing EAE. To test our hypothesis that IL-16 has a role in regulation of relapsing disease, we examined and compared regulation of IL-16 between two genetically distinct strains of mice, (B6 × SJL) F1 and the parental B6. Following immunization with $MOG_{35-55}$ (B6 × SJL) F1 mice develop severe relapsing-remitting while B6 mice develop low-relapsing disease. In addition these two strains exhibit distinct histopathology of CNS lesions and distinct regulation of their T-cell responses to $MOG_{35-55}$ [8]. Our data show major differences in levels of IL-16 produced in CNS between these two strains of mice. In CNS of F1 mice, which consistently develop severe, relapsing-remitting disease, levels of bioactive IL-16 were markedly higher compared to the low-relapsing B6 strain. Elevation of bioactive IL-16 during acute, relapse and chronic, and its clearance from CNS during remission, may suggest its participation in mechanisms of inflammation, which accompany stages of clinically manifested disease. Markedly higher levels of bioactive IL-16 during relapse as compared to acute and its persistence in chronic disease further underscored the possibility that IL-16-mediated mechanisms might be of greater importance in the regulation of chronic inflammation in CNS, in the relapsing-remitting F1 strain. Throughout the course of relapsing-remitting EAE, temporal regulation of IL-16 very much resembles that reported for several chemokines, which are critically involved in regulation of CNS inflammation, such as MCP-1, MIP-1α, MIP-1β, and others [24]. While the role of chemokines in the orchestration of the initial CNS

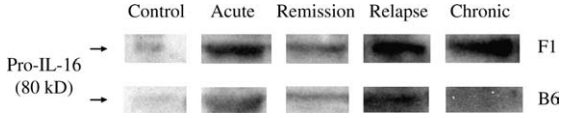


Fig. 7. Levels of pro-IL-16 (80 kDa) in CNS of EAE mice were analyzed by Western blot. Note upregulation of pro-IL-16 during acute, relapsing and chronic EAE, similarly as observed for IL-16 (55 kDa) (Fig. 6), and bioactive IL-16 (17 kDa) (Fig. 1).

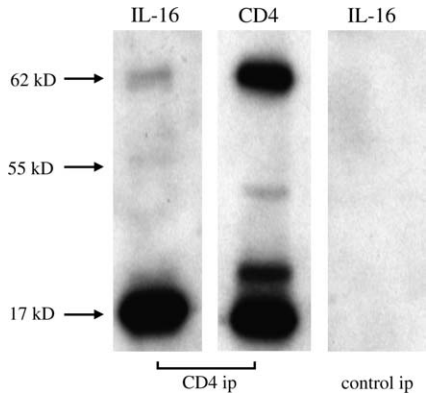


Fig. 8. IL-16 co-immunoprecipitated with CD4 in CNS from mice with relapsing disease. CD4 was immunoprecipitated using an anti-CD4 specific antibody, from total proteins isolated from spinal cord and subjected to Western blotting using anti-IL-16 antibody. The membrane was then stripped and reblotted with anti-CD4 antibody. In a separate, similarly performed experiment, immunoprecipitation was done with control antibody, which was isotype-matched with anti-CD4 specific antibody. IL-16 specific immunoreactivity was not observed with control immunoprecipitates. Blot shows results from spinal cord of (B6 × SJL) F1 mouse, at third relapse, 68 dpi, with clinical grade of 4.

inflammation and onset of disease has been clearly established, our data imply that bioactive IL-16 may be more important in regulating relapsing and chronic stages of disease.

Bioactive IL-16 (17 kDa) represents secreted C-terminal portion of a larger precursor molecule, pro-IL-16 (80 kDa). By specifically binding and signaling through CD4, bioactive IL-16 induces chemoattraction of CD4+ cells. The minimal C-terminal peptide RRKS (corresponding to $Arg^{106}$ to $Ser^{109}$) was shown to be critical for mediating chemoattracting activity of mature IL-16 [25]. Active caspase-3 is essential for the cleavage of bioactive IL-16 in activated CD4+ T cells [18]. To investigate whether CD4+ infiltrating T cells may locally produce IL-16, we proceeded to determine levels and regulation of active caspase-3 in CNS. We found that both active caspase-3 and CD4 levels are regulated similarly to bioactive IL-16 in CNS of relapsing EAE mice. Similar correlations between levels of bioactive-IL-16 and active capsase-3 have been demonstrated in thyrocytes, which also require caspase-3 enzymatic cleavage of pro-IL-16 in order to produce and secrete IL-16 [26]. Similarities in patterns of regulation between active caspase-3, CD4 and bioactive IL-16 raised the possibility that active caspase-3 may be contained in CD4+ infiltrating cells, where it may cleave pro-IL-16. Previously, we described CD4+ IL-16+cells within inflammatory infiltrates in F1 relapsing mice [15]. Previously and currently used IL-16 specific antibody does not recognize a distinction between pro-IL-16 and bioactive IL-16. Therefore, it was necessary to investigate whether active caspase-3 and IL-16 immunoreactivity colocalize. We show in Fig. 2 colocalization between IL-16 and active caspase-3 in mononuclear infiltrating cells, further supporting the importance of infiltrating CD4+ T cells as a local source of bioactive IL-16 in F1 mice. Active caspase-3 immunoreactivity was not exclusive for IL-16 containing mononuclear cells, but was also observed in some neighboring IL-16$^-$ cells. This finding corresponds to observations from other EAE studies reporting the presence of active caspase-3 in oligodendrocytes, which suggested other potentially important roles, such as apoptosis of oligodendrocytes [27]. Finding significantly higher levels of CD4 protein in CNS of mice with severe relapsing-remitting disease (F1), compared to low-relapsing B6 mice, was consistent with our previously reported differences in relative numbers of infiltrating CD4+ T cells [8]. Although our data support the role of CD4+ T cells in producing bioactive IL-16 in CNS of EAE mice, we expect that some other cell phenotypes could also elaborate and locally release IL-16. We have shown IL-16-immunoreactive B220+ B cells and CD8+ T cells but not Mac-3+ macrophage/microglia in CNS of F1 relapsing mice [15]. Presence of IL-16 has been reported in microglia in one rat EAE model, although it was not made clear whether this was pro-IL-16 or bioactive IL-16, and if so mechanisms of its production have not been addressed [28]. Potential of other infiltrating and resident CNS cells to produce IL-16 in (B6 × SJL) F1 EAE mice is currently being investigated in our laboratory (Skundric, unpublished data). In this paper we show data relevant for production of IL-16 by CD4+ T cells, because these cells represent the major infiltrating phenotype in relapsing F1 mice differently than in B6, where macrophage infiltration predominates. The importance of CD4+ T-cell-derived IL-16 for relapsing disease is further supported by recent findings from several laboratories that reactivation of CD4+ T cells within the CNS compartment rather than in the periphery represents one of the crucial mechanisms of relapse regulation in mice [29].

Despite the site of reactivation in both B6 and SJL strains of mice, autoreactive and other non-specifically activated CD4+ T cells recirculate between CNS and peripheral lymphoid organs throughout the course of relapsing-remitting disease [30,31]. To advance our understanding of IL-16 production by activated T cells, not only in CNS but also in peripheral lymphoid organs, we analyzed IL-16 and active caspase-3 regulation in inguinal lymph nodes and spleens from mice with relapsing EAE. In peripheral lymphoid organs of mice with relapsing and chronic EAE, we detected elevated levels of processed IL-16 (55 kDa), bioactive IL-16 (17 kDa), and active caspase-3. This data suggest that activated lymphocytes, including CD4+ T cells, serve as sources of bioactive IL-16. Similar to our findings, the presence of processed forms of IL-16 ranging from 32 kDa to 78 kDa have been reported in activated CD4+ T cells in vitro, and in vivo at the site of delayed-type hypersensitivity reaction [32,33]. To investigate further whether CD4+ T cells activated in either non-specific or antigen-specific manner produce bioactive IL-16 in a caspase-3 specific manner, we isolated T cells from regional lymph nodes of (B6 × SJL) F1 mice immunized with $MOG_{35-55}$. We detected production of IL-16 (17 and 55 kDa) and elevation of active caspase-3, following either polyclonal activation or restimulation with cognate antigen in vitro.

Another important question that we addressed was whether biological function of elevated bioactive IL-16 in CNS of mice with relapsing EAE was to provide a chemoattractant signal for CD4+ T cells. To examine whether IL-16 functionally relates to CD4 in CNS, we performed co-immunoprecipitation experiments. Data from those experiments revealed that IL-16 (17 kDa, 50–60 kDa) co-immunoprecipitated with CD4, which suggested a functional relationship between IL-16 and CD4 in CNS. Our data are consistent with recently published studies showing an association between the cleaved C-terminus and intracellular N-terminus of IL-16 with CD4 co-receptor or with kinases activated through TCR/CD4 signaling events [19,20]. This observed relationship between IL-16 (17 kDa) and CD4 in CNS of mice with EAE further supports the role of bioactive IL-16 in CD4+ T-cell chemoattraction.

Overall, we demonstrate an elevated production of bioactive IL-16, which corresponds to increased activation of caspase-3 and massive infiltration by CD4+ T cells in CNS of (B6 × SJL) F1 mice with severe relapsing disease. We show similar patterns of regulation for IL-16 and active caspase-3 in peripheral lymphoid organs of relapsing EAE mice, and in T cells isolated from lymph nodes following T-cell activation in vitro. Finally, we provide evidence of a functional relationship between CD4 and IL-16 proteins in CNS, suggesting that one of the important roles of locally produced IL-16 may be in chemoattraction of additional waves of CD4+ T cells. We propose that infiltrating CD4+ T cells serve as an important source

of bioactive IL-16 in mice with relapsing disease. In turn, elevation of intratechal bioactive IL-16 bears relevance for perpetuation and worsening of disease by providing a specific chemoattractant signal for additional waves of CD4+ T cells.

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