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Thiamine Deficiency Promotes T Cell Infiltration in Experimental Autoimmune Encephalomyelitis: The Involvement of CCL2

Zhe Ji,* Zhiqin Fan,* Ying Zhang,† Ronghuan Yu, † Haihua Yang, † Chenghua Zhou,* Jia Luo, ‡ and Zun-Ji Ke*§

Multiple sclerosis (MS) is a complex multifactorial disease that results from the interplay between environmental factors and a susceptible genetic background. Experimental autoimmune encephalomyelitis (EAE) has been widely used to investigate the mechanisms underlying MS pathogenesis. Chemokines, such as CCL2, are involved in the development of EAE. We have previously shown that thiamine deficiency (TD) induced CCL2 in neurons. We hypothesized that TD may affect the pathogenesis of EAE. In this study, EAE was induced in C57BL/6J mice by the injection of myelin oligodendroglial glycoprotein (MOG) peptides 35–55 with or without TD. TD aggravated the development of EAE, which was indicated by clinical scores and pathologic alterations in the spinal cord. TD also accelerated the development of EAE in an adoptive transfer EAE model. TD caused microglial activation and a drastic increase (up 140%) in leukocyte infiltration in the spinal cord of the EAE mice; specifically, TD increased Th1 and Th17 cells. TD upregulated the expression of CCL2 and its receptor CCR2 in the spinal cord of EAE mice. Cells in peripheral lymph node and spleen isolated from MOG-primed TD mice showed much stronger proliferative responses to MOG. CCL2 stimulated the proliferation and migration of T lymphocytes in vitro. Our results suggested that TD exacerbated the development of EAE through activating CCL2 and inducing pathologic inflammation. The Journal of Immunology, 2014, 193: 2157–2167.
receptors. Chemokines play a major role in the immune response because they regulate the migration and activation of leukocytes. CCL2 was first identified as a potent chemotaxon for monocytes in response to proinflammatory stimuli. CCL2 regulates the migration and activation of monocytes, T cells, NK cells, and basophils. CCL2 binds solely to CCR2, a seven-transmembrane-spanning protein that is functionally linked to downstream signaling pathways through heterotrimetric G proteins. In addition to being a receptor for CCL2, CCR2 serves as the receptor for four other ligands: MCP-2, -3, -4, and -5. CCL2 and CCR2 have been implied in EAE (30–36). For example, anti-CCL2 Ab blocks relapses of adoptive transfer EAE in SJL mice (37). Mice that lack CCR2 fail to develop EAE after active immunization and are resistant to induction of EAE by the adoptive transfer of primed T cells from syngeneic wild-type mice (29, 38).

Thiamine is a cofactor of key enzymes in glucose metabolism (39). Thiamine deficiency (TD) causes mild impairment of oxidative metabolism and induces Wernicke–Korsakoff syndrome in humans. TD causes regionally selective neuronal death, mitochondrial dysfunction, energy shortage, and chronic oxidative stress in the brains of humans and animals (40, 41). TD in animals has been used to model some neurodegenerative diseases (41). Subclinical TD is common in the elderly population (42). However, it is unclear whether the status of thiamine will affect MS and EAE. Our previous studies demonstrated that TD increased CCL2 expression in the CNS (41). In this study, we demonstrated that TD enhanced the EAE severity by activating T cell reaction and increasing CCL2 expression in the spinal cord.

Materials and Methods

Peptides and Abs

Rat MOG35–55 peptides were obtained from Biosynth International (Naperville, IL) and purified by HPLC, and the purity of the peptide was >95%. The sequence of MOG35–55 was MEVGWYRSPFSRVHLYRNGK. Purified hamster anti-mouse CD3ε, FITC rat anti-mouse CCL2, allophycocyanin rat anti-mouse CD8a, allophycocyanin rat anti-mouse IL-4, and PE rat anti-mouse IL-17A Abs were purchased from BD Pharmingen (Basel, Switzerland), goat anti-mouse ionized calcium-binding adaptor molecule-1 (IBA1) Ab was obtained from Abcam (Cambridge, U.K.), rat anti-mouse CCL4, rabbit anti-mouse CCL2 Ab, and CCL2 (rat recombinant) were purchased from AbD Serotec (Raleigh, NC). FITC rat anti-mouse IFN-γ Ab was purchased from eBioscience (San Diego, CA); rabbit anti-mouse CCR2 Ab was purchased from Abcam (Cambridge, U.K.); 2-(1-benzyl-indazol-3-yl) methoxy-2-propyl propionic acid (bindarit) was synthesized by and obtained from Angelini (Angelini Research Center, Pomezia, Italy).

Animal models

Active immunization model of EAE. C57BL/6J mice were obtained from Shanghai Laboratory Animal Center (Shanghai, China). The procedure for animal surgery was performed in accordance with the Guidelines of Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Induction of EAE was performed as previously described (43). In brief, mice were s.c. injected at two sites with 100 μg rat MOG peptides 35–55 (MEVGWYRSPFSRVHYLRNGK; >95% purity; Bio-Synthesis, Rockland, Rockland, ME) according to manufacturer's instruction. The method for bindarit treatment in animals has been previously described (44). The method for bindarit treatment in animals has been previously described (44). In brief, bindarit was prepared as a suspension in DMSO at a concentration of 40 mg/ml. Then mice were given daily i.p. injection of bindarit (or vehicle DMSO) at 200 mg/kg for 3 consecutive days, beginning 24 h before MOG immunization. This treatment was repeated daily for 3 days. This schedule was designed to minimize trauma associated with daily injections at times of peak neurologic disease and physical compromise.

Immunohistochemistry and immunofluorescence staining

For immunohistochemical (IHC) analysis of spinal cord tissues, mice were euthanized at the peak of EAE by intracardiac perfusion with ice-cold PBS, followed by 4% paraformaldehyde solution, under anesthesia. Spinal cords were rapidly dissected and sectioned at a thickness of 25 μm. The sections were incubated with 0.3% hydrogen peroxide, blocked by the incubation with 10% BSA at 37°C for 1 h, then incubated overnight at 4°C with a primary Ab (rabbit anti-mouse CD45 Ab, 1/1,000; goat anti-mouse IBA1 Ab, 1/1,000). The sections were then incubated with appropriate biotinylated secondary Abs at 37°C for 1 h and treated with diaminobenzidine. All Abs were diluted in 1% BSA in PBS. Negative controls were performed by the incubation of preimmune IgG. After detecting inflammation with antibodies, the sections were stained with H&E.

For immunocytofluorescence staining, tissue sections or cells from LNs were rinsed in PBS, blocked by incubation with 1% BSA at 37°C for 1 h, and then incubated overnight at 4°C with primary Abs (rabbit anti-CCL2 polyclonal Ab, 1/200; rat anti-mouse CD4 Ab, 1/50; rat anti-mouse CD8a, 1/50). The sections were incubated with appropriate FITC secondary Abs at 37°C for 1 h. The bright-field images were taken on a BX51 Olympus microscope (Olympus Corporation, Tokyo, Japan); immunofluorescence images were recorded using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY). For quantification, five sections from each mouse were used for cell counting. Cells were counted using ImageJ (National Institutes of Health) in a designated area. Data represent mean ± SD of five mice for each group.

T cell proliferation

To examine the proliferation of T cells, we isolated LNs and spleen from MOG35–55-immunized mice and cultured T cells in a 96-well plate (1 × 10^5/well) in the presence of MOG35–55 (0, 0.8, 4, 20, and 100 μg/ml), CCL2 (20 μg/ml), or Con A (10 μg/ml; Sigma-Aldrich). Cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Life Technologies), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin/streptomycin, and 2 × 10^-5 M 2-ME (Life Technologies) for 72 h. Cell proliferation was determined using an AMR PLUS kit (Lonza Rockland, Rockland, ME) according to manufacturer's instruction. The absorbance was analyzed with a luminometer (Bio-Tek, Atlanta, GA).

Flow cytometry

T cells (1 × 10^6/ml) obtained from LNs were washed and resuspended in PBS. Cells were stained for surface markers with specific primary Abs
appropriate FITC-conjugated secondary Abs in FACS buffer at 4 ºC for 40 min. Cells were washed twice and resuspended in the 200–400 µl PBS for flow cytometry analysis as previously described (49, 50). The cell sorting was performed with a FACSCalibur (BD Biosciences, San Diego, CA) equipped with CellQuest software (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA). For intracellular staining, cells were maintained in a 6-well plate (2 × 10^6/well) and treated with MOG35–55 (20 µg/ml) for 72 h. An inhibitor of protein transport brefeldin A (1:10 dilution; BD Biosciences, San Jose, CA) was added to the cultures during the last 4–5 h; then cells were collected, fixed, and permeabilized using Cytofix/Cytoperm (BD Biosciences, San Jose, CA), followed by the staining using fluorescence-labeled rat anti-mouse Abs directed against IFN-γ, IL-4, or IL-17. Cells were then analyzed with FACSCalibur. Isotype-matched Abs were used as controls. For the CCL2 binding experiments, draining LN cells were incubated with IgG, CCL2, or CCL2 plus anti-CCL2 Ab for 60 min. The cells were then incubated with appropriate FITC Abs and analyzed with FACSCalibur.

Transwell migration assay

Chemotaxis assays were performed with purified LN T cells using uncoated 8-µm transwell filters (Corning Costar, Corning, NY) as previously described (51). In brief, T cells (2 × 10^5/ml) suspended in RPMI 1640 containing 1% FBS were added to the upper chamber; CCL2 (5 µg/ml) as a T cell chemokine was added to the lower chamber. Cells were allowed to migrate for 4 h at 37 ºC in 5% CO2 atmosphere. The transwell filters were collected and fixed with methanol for 10 min, then stained with Diff-Quick (Baxter Diagnostics, Deerfield, IL) for 20 min. The migrated T cells were counted under a microscope at ×400 magnification (Axiovert 200M; Carl Zeiss). Data are expressed as mean ± SD for duplicate filters from four independent experiments.

Statistical analysis

Statistical analysis was assessed by ANOVA followed by Student–Newman–Keuls analyses. An unpaired t test was used for the analysis of quantitative data of cell counting. Data were presented as means ± SD. A difference in which p < 0.05 was considered statistically significant.

Results

TD exacerbates EAE in active immunization model

We investigated the effects of TD on EAE using an active immunization model of EAE. The EAE mice developed a monophasic disease characterized by ascending paralysis 10–18 d after immunization, and EAE mice with TD showed markedly more severe neurologic dysfunction. Table I summarizes the neurobehavioral features of MOG35–55–induced EAE mice with or without TD. In our EAE mouse model (MOG injection), 11 of 16 (69%) mice experienced development of fatal EAE, whereas in EAE plus TD model, 13 of 16 (81%) mice experienced development of fatal EAE (Table I). The onset of EAE was earlier in the EAE plus TD model (13 d) compared with the EAE mouse model (16 d). The neurologic signs of EAE in mice were quantified and expressed as clinical scores and presented in Fig. 1A.

![FIGURE 1. Effect of TD on the progression of EAE. EAE was induced in C57BL/6 mice by injection of MOG35–55 with or without TD as described in Materials and Methods. The clinical score (A) and body weight (B) was determined in these mice as described in Materials and Methods. Data were presented as mean ± SEM; *p < 0.05, compared with EAE mice; n = 20 for each group. (C) The infiltration of inflammatory cells in the spinal cord was detected by H&E staining on the peak of EAE. Scale bar, 50 µm. (D) The number of hematoxylin+ cells in the spinal cord was quantified using ImageJ software in a designated area. Data were presented as mean ± SD; ***p < 0.001, compared with EAE mice; n = 5 for each group.](http://www.jimmunol.org/Downloadedfrom)
TD alone revealed neurologic alterations, such as clumsy gait or poor righting ability after 25 d. The clinical score for the EAE model peaked at day 20 and then slightly declined; however, in the EAE plus TD model, the clinical score was significantly higher and kept increasing after day 20 (Fig. 1A). In addition, in the EAE plus TD model, mice had a more severe loss of body weight (Fig. 1B).

We next performed histopathologic analysis on spinal cords of EAE mice with or without TD. Inflammatory cell infiltration in lumbosacral enlargement was examined by H&E staining (Fig. 1C), and the infiltrated cells were quantified (Fig. 1D). Compared with controls, the number of infiltrated cells increased by 283 and 613% in EAE mice and EAE plus TD mice, respectively (Fig 1D).

**FIGURE 2.** Effect of TD on microglial activation in EAE mice. EAE in C57BL/6 mice was induced by injection of MOG35–55 with or without TD. The activation of microglia was measured by IHC staining with an Ab directed against CD45 (A) or Iba1 (C) on day 20 after MOG35–55 injection. Scale bars, 50 μm. The number of CD45+ cells (B) and active microglia (D) were determined. Data were presented as mean ± SD; **p < 0.01, ***p < 0.001, compared with EAE mice; n = 5 for each group.
To determine the identity of inflammatory cells, we performed IHC analysis of CD45 and Iba1 on the spinal cord (Fig. 2). CD45 is a marker for the mononuclear cells from the circulation, whereas Iba1 is a marker for microglia in the CNS. In the EAE plus TD model, there were much more CD45+ cells (139% increase) compared with the EAE model. EAE activated microglia in the spinal cord; the active microglia displayed a larger cell body and thicker processes. TD significantly increased the number of active microglia in EAE mice (Fig. 2C, 2D). We further examined the infiltrated lymphocytes in the spinal cord by IHC of CD4 and CD8. CD4+ and CD8+ cells were only observed in EAE mice, and there were no CD4+ and CD8+ T lymphocytes in the spinal cord of wild-type and TD mice. TD drastically increased CD4+ and CD8+ cells in EAE mice (Fig. 3). We have also performed FACS to test the proportion of CD4 and CD8 cells obtained from spleen and peripheral LN (PLN) in different group mice on the onset and peak of EAE. The results showed there were no obvious differences between different groups (Supplementary Fig. 1).

**TD potentiates Ag-stimulated proliferation of T lymphocytes**

We hypothesized that TD increased the proliferation of T lymphocytes in response to the Ag. To test this hypothesis, we examined the Ag-stimulated proliferation of T lymphocytes isolated from the PLN and spleen of control, TD mice, and EAE with or without TD mice on the onset and peak of EAE. Although other cells may be present, T lymphocytes are major cells in the PLN. MOG did not stimulate the proliferation of lymphocytes isolated from control and TD mice, but significantly increased the proliferation of lymphocytes isolated from EAE mice (Fig. 4). More importantly, the lymphocytes isolated from EAE mice plus TD mice displayed a much stronger and concentration-dependent response to MOG-mediated proliferation.
TD aggravates EAE in adoptive transfer model

We hypothesized that the Ag-specific T cells isolated from EAE plus TD would cause more severe EAE. To test this hypothesis, we transferred encephalitogenic cells from mice treated with MOG with or without TD for 10 d to recipient mice that were irradiated sublethally. The encephalitogenic cells isolated from MOG-treated mice caused EAE, which was shown by the clinical score and the loss of body weight, whereas the encephalitogenic cells isolated from MOG plus TD–treated mice induced more severe EAE in recipient mice, which was indicated by higher clinical scores and greater loss of body weight (Fig. 5A). Next, we transferred encephalitogenic cells isolated from MOG-treated mice to recipient mice that were treated with or without TD. In the recipient mice that were treated with TD, the encephalitogenic cells caused a quicker and more severe development of EAE, as determined by the clinical scores (Fig. 5B). Consistently, the encephalitogenic cells induced greater body weight loss in the recipient mice that were treated with TD (Fig. 5B).

CD4+ T cells have been classified into distinct subsets, namely, Th1 (characterized by the production of IFN-γ), Th2 (characterized by the production of IL 4), and Th17 (characterized by the production of IL 17). IFN-γ–producing Th1 cells and IL-17–producing Th17 cells have been implicated in EAE induction. We examined the relative populations of Th1, Th2, and Th17 cells in the LN using flow cytometry after 10 d of induction. There was a significant increase in the IFN-γ–producing cells (23% more) and IL-17–producing cells (56% more) in the EAE plus TD model compared with the EAE only model (Fig. 5B). Consistently, the encephalitogenic cells induced greater body weight loss in the recipient mice that were treated with TD (Fig. 5B).

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CCL2 is involved in TD-induced exacerbation of EAE

We have demonstrated that the expression of CCL2 was selectively induced by TD in the brain (52). We hypothesized that CCL2 may mediate TD-induced exacerbation of EAE. To test this hypothesis, we examined the expression of CCL2 in the spinal cord with IHC. Both MOG and TD treatment increased the number of cells expressing CCL2, but MOG plus TD further increased CCL2+ cells in comparison with MOG or TD-treated group alone (Fig. 6A, 6B). To confirm that CCL2 can regulate T lymphocyte infiltration, we first determined whether CCL2 can directly bind to T cells. We treated LN T cells with IgG, CCL2, or CCL2 plus anti-CCL2 Ab for 60 min, and then determined the amount of CCL2 bound to T cells by flow cytometry. Anti-CCL2 Ab blocked the binding of CCL2 to T cells, indicating that CCL2 specifically bound to T cells (Fig. 6C, 6D). More importantly, CCL2 increased the proliferation and migration of T lymphocytes in vitro (Fig. 6E, 6F).

To confirm the involvement of CCL2, we treated mice with bindarit, an inhibitor of CCL2 synthesis. Bindarit inhibited the expression of CCL2 in the spinal cord (Supplemental Fig. 2C, 2D). Bindarit had therapeutic effects on mice of EAE plus TD, lowering the clinical scores and alleviating the loss of body weight (Supplemental Fig. 2A, 2B). Mice that suffered EAE plus TD had a slower progression and a maximum mean clinical score of 2.5. Bindarit-treated mice with EAE plus TD had a slower progression and a maximum mean clinical score of 1.5.

TD increases the expression of CCR2 in T lymphocytes

CCR2 is a chemokine receptor that responds predominantly to CCL2. We examined the expression of CCR2 in lymphocytes isolated from LNs by flow cytometry. The expression of CCR2 on lymphocytes was confirmed by isolated T cells from LN (Supplemental Fig. 3). Approximately 30% of lymphocytes expressed CCR2, and CCR2 was observed in both CD4+ and CD8+ cells (data not shown). We next examined the effect of MOG and TD on CCR2 expression in CD4+ and CD8+ T lymphocytes in the spinal cord. MOG significantly increased the number of CCR2+ CD4+ or CD8+ T lymphocytes in the spinal cord and TD potentiated the effect of MOG (Figs. 7, 8).

Discussion

MS is considered a T lymphocyte–mediated CNS autoimmune disease that results from the interplay between environmental...
factors and a susceptible genetic background (15, 53, 54). Epidemiologic studies show that environmental influences, such as diet, play an important role in the development of MS (5). Vitamin deficiency has been implicated in the pathogenesis of MS. For example, there is a correlation between vitamin D deficiency and MS (7, 8). Vitamin D supplements have been considered a potential therapeutic strategy for MS patients (9, 10). MS and vitamin B12 deficiency share some common inflammatory, neurodegenerative, and pathophysiologic characteristics, and a decreased level of vitamin B12 has been demonstrated in MS patients (55, 56). The supplementation with vitamin B12 improved clinical outcomes in MS patients (13). In an animal model of MS, both nicotinic acid and fumaric acid esters have been shown to improve neurologic function, and this effect was accompanied by a significant reduction in inflammatory infiltrates (57). Fumaric esters have recently been evaluated in phase III trials for MS (58–60), and nicotinic acid has been shown to have neuroprotective and anti-inflammatory effects during acute ischemic stroke (61).

Thiamine (vitamin B1) deficiency produces a mild impairment of oxidative metabolism, and TD in animals has been used to model the diminished metabolism and reduced activity of the thiamine-dependent mitochondrial enzymes that occur in the brain of aging-related neurodegenerative disorders (40, 62). In this study, we show that TD exacerbates EAE, which is a widely used model to study the pathogenesis of MS (63). Two models of EAE were used in our study: an active immunization model that was achieved by s.c. injection of myelin Ag (MOG) and an adoptive transfer model that transferred purified MOG-specific T cells obtained from EAE mice into naive mice. In both active and adoptive transfer EAE models, TD drastically increases the progression of EAE, which is indicated by a significantly worse clinical score and a more severe loss of body weight (Figs. 1, 5). The mice of TD alone showed neurologic alterations, such as clumsy gait or poor righting ability after 25 d.

The clinical severity of EAE is directly associated with T cell activation (64). We show that TD increases the number of infiltrated cells and active microglia in the spinal cord. We further demonstrate that TD increases the number of infiltrated cells and active microglia in the spinal cord. These results indicated that TD exacerbates the development of EAE by inducing T activation. Neuroantigen-specific CD4+ T cells can initiate and sustain neuroinflammation and pathology in EAE (15). Depending on the major cytokines produced, CD4+ T cells have been classified into distinct subsets, namely, Th1, Th2, and Th17. Recently, many studies have been undertaken to identify the T cell subsets that are involved in tissue-specific autoimmune diseases. IFN-γ-expressing Th1 cells were initially considered to be the effector CD4+ T cell subset that induced EAE. Later, Th17 cells were also reported to cause more severe EAE; animal models that were deficient in IL-17A or the
IL-17RA receptor were more resistant to EAE (65–67). We demonstrate that TD significantly increases the number of CD4+ cells and specifically the number of Th1 and Th17 cells, but not Th2 cells (Figs. 3, 5). These results are consistent with the role of these T cells in EAE. TD also increases the number of CD8+ T cells (Fig. 3). The role of CD8+ T cells in EAE is unclear, although some studies indicate a potential pathogenic action for CNS-targeted CD8+ T cells (68, 69).

A critical event in the pathogenesis of EAE is the entry of both Ag-specific T lymphocytes and Ag-nonspecific mononuclear cells into the CNS. Chemokines are a key mediator that regulates the transmigration of T cells and monocytes across the blood–brain barrier (70). CCL2, one of the first chemokines to be characterized, regulates the activity of monocytes, dendritic cells, and NK cells, and plays an important role in innate immunity (35); it is also associated with pathologic inflammation (71). CCR2 is a CC chemokine receptor that responds predominantly to CCL2. In murine EAE, the expression of CCL2 mRNA in the brain and spinal cord was upregulated and may mediate the onset of EAE (31, 32). We have previously demonstrated that neuronal CCL2 plays an important role in TD-induced microglia recruitment/activation and neurodegeneration in the brain (52). We show in this study that TD increases the expression of CCL2 and CCR2 in spinal cord of EAE in mice (Figs. 6–8). In humans, CCR2 is expressed in monocytes and CD4+ T cells in the circulation (72). Our results show that CCR2 is expressed in both CD4+ and CD8+ T cells.
T cells in C57BL6 mice. Our data indicate that CCL2 can directly activate T cells as it binds to T cells and promotes the proliferation and migration of T cells in vitro (Fig. 6). Bindarit, a CCL2 synthesis inhibitor, suppresses the expression of CCL2 in the spinal cord and has therapeutic effects on EAE plus TD mice (Supplemental Fig. 2). These indicate the involvement of CCL2. Taken together, our results suggest that TD upregulates CCL2 and CCR2, and through its interaction with CCR2, CCL2 enhances the proliferation and recruitment of encephalitogenic T cells into the spinal cord, which causes more pathologic inflammation. The mechanisms underlying TD-induced CCL2/CCR2 upregulation are currently unknown. TD causes oxidative stress. Oxidative stress is detrimental to neurons and may promote inflammatory activities and the production/secretion of CCL2. Although a direct regulation cannot be ruled out, it is more likely that TD modulates CCL2 expression through an indirect mechanism, such as oxidative stress. Regardless of the mechanisms, CCL2/CCR2 signaling plays an important role in TD-potentiated EAE.

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Disclosures
The authors have no financial conflicts of interest.

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