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Microglial activation is a major contributor to neurologic dysfunction in thiamine deficiency

Dongmei Wang, Alan S. Hazell *

Department of Medicine, University of Montreal, Montreal, Quebec, Canada

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ABSTRACT

In Wernicke's encephalopathy and thiamine deficiency (TD), the cause of this brain disorder, development of inflammation is an important aspect of the disease process. How this pathological mechanism relates to the neurologic impairment associated with TD, however, remains unclear. A key feature of the inflammatory process is the activation of microglia. In the present study, we evaluated the role of microglial activation in the pathophysiology of TD by examining the relationship between levels of CD11b/c and CD68, two proteins associated with microglial activation, and neurological dysfunction under conditions of TD. Rats with TD showed large increases in expression of both CD11b/c and CD68 in the vulnerable thalamus and inferior colliculus, with no change in mRNA levels in the relatively non-vulnerable frontal cortex. These alterations in CD11b/c and CD68 expression were reflected in dramatic upregulation of both proteins by immunoblotting and immunohistochemical methods. Co-treatment of rats with TD and the anti-inflammatory drug minocycline prevented microglial activation, and onset of neurological changes, including loss of righting reflex, was delayed by approximately 39 h, compared to animals with TD alone. In addition, co-treatment of rats with TD and *N*-acetylcysteine prevented the increase in CD11b/c and CD68, but did not alter the onset of neurological impairment. These results suggest that microglial activation plays a role in the development of neurological impairment in TD and possibly Wernicke's encephalopathy, and that while development of oxidative stress may be involved in microglial activation, the basis of this neurologic dysfunction is likely to be multifactorial in nature.

1. Introduction

Selective cerebral vulnerability is a major consequence of Wernicke's encephalopathy (WE), the neurological component of the Wernicke-Korsakoff syndrome, a neuropsychiatric disorder characterized by changes in behaviour that include a striking memory loss, oculomotor disturbances, and ataxia [1]. The underlying cause of WE is thiamine deficiency (TD), in which focal areas of the brain exhibit symmetrical hemorrhagic and ischemic-like lesions, occuring most frequently in diencephalic regions such as the thalamus and mammillary bodies, but which also extend caudally through midbrain structures such as the inferior colliculus and other periventricular brainstem areas that include the vestibular nuclei and inferior olivary complex in particular [2].

Cerebral inflammation is now recognized as a key component of several neurological diseases including stroke, multiple sclerosis and Alzheimer's disease, along with other conditions such as brain trauma. During the 1960s, alterations in glial cell morphology in TD including evidence of swelling and the appearance of phagocytic vacuoles [3,4] were first reported. These findings are consistent with pathological changes that can be attributable to the presence of an inflammatory process. More recently, further evidence has been described in support of this mechanism in TD; increased microglial reactivity, an indication of inflammation, is an early cellular response, while production of pro-inflammatory cytokines in both vulnerable and non-vulnerable regions of brain have been reported [5–7].

Previous studies have identified activated microglia as a major feature of TD [8], in which levels of CD11b/c (OX-42) and CD68 (ED-1), localised predominantly in these cells in brain are increased, occuring at early stages of the disorder, prior to development of neuronal cell death, and localized to areas of focal cerebral vulnerability, suggesting this cellular response may contribute to neurological dysfunction and subsequent lesion formation. In addition, increased levels of pro-inflammatory cytokines, such as interleukins IL-1beta, IL-6 and tumor necrosis factor-alpha have been reported in the brains of TD animals [7]. Development of oxidative stress is also known to be an early event in TD and which can produce functional impairment in vulnerable areas of the brain [9–12]. In the present study, we have examined the expression of CD11b/c and CD68 in vulnerable and non-vulnerable brain regions in TD, and the effects of the anti-inflammatory drug minocycline

* Corresponding author. Address: NeuroRescue Laboratory, Hôpital Saint-Luc (CHUM), 1058 St-Denis, Montreal, Quebec, Canada H2X 3J4. Fax: +1 514 412 7734.
E-mail address: alan.stewart.hazell@umontreal.ca (A.S. Hazell).

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on neurological dysfunction, along with the well-established antioxidant *N*-acetylcysteine (NAC).

2. Materials and methods

2.1. Materials

Pyrithiamine hydrobromide, minocycline hydrochloride, protease inhibitor cocktail, 3,3′-diaminobenzidine (DAB), and mouse monoclonal antiserum against β-actin were purchased from Sigma Chemical Company (St. Louis, MO, USA.). Monoclonal antisera against CD68 and CD11b/c were purchased from AbD Serotec (Raleigh, NC, USA) and Cederlane Laboratories Ltd (Burlington, ON, Canada), respectively. Biotinylated and HRP-conjugated donkey anti-mouse IgG secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). NAC (“Parvolex”) was purchased from Bioniche Pharma (Toronto, ON, Canada). Streptavidin-horseradish peroxidase (HRP) conjugate was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Polyvinylidene difluoride (PVDF) membranes and broad-range protein markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Enhanced chemiluminescence (ECL) kits were purchased from New England Nuclear (Boston, MA, USA.) and X-OMAT autoradiography film was purchased from Kodak (Ile des Soeurs, Quebec, Canada). All other materials and chemicals were purchased from Amersham Canada Ltd (Oakville, Ontario, Canada).

2.2. Model of TD

All animal procedures were conducted in accordance with guidelines set out by the Canadian Council on Animal Care and were approved by the University of Montreal Animal Ethics Committee. Male Sprague–Dawley rats (225 g) were fed a TD diet (Ralston Purina Inc., Richmond, IN, USA) and injected daily with pyrithiamine (0.5 mg/kg body weight, i.p.; Sigma Chemical Co., St. Louis, MO, USA), a thiamine antagonist that inhibits the enzyme thiamine diphosphate phosphokinase. Symptoms of TD including rotational and backward movements, ataxia, opisthotonus, nystagmus, loss of righting reflex (LRR), and convulsions were assessed daily. Rats were killed by decapitation within 6 h of the appearance of LRR at the symptomatic stage [13]. None of the rats exhibited obvious seizures at this stage. Rats placed on a TD diet (limited in quantity to that consumed by their TD counterparts) and injected daily with thiamine (100 μg in 0.2 mL saline, i.p.), served as pair-fed controls (PFC).

TD animals co-treated with minocycline received an initial dose of minocycline (90 mg/kg, i.p.) in sterile saline followed by daily injections (45 mg/kg, i.p.) every 12 h [14], commencing at day 0 of TD, with the vehicle control group receiving equivalent injections of saline only. TD rats co-treated with NAC were administered the drug (163 mg/kg body weight, i.p.) daily [15], commencing at day 0 of TD.

2.3. Real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from rat brains (frontal cortex, posterior medial thalamus and inferior colliculus) using the Trizol reagent (Invitrogen Canada Inc., Burlington, ON, Canada), according to the manufacturer’s instructions. Expression levels were assessed by real-time PCR in which cDNA was synthesized using a thermoscript RT-PCR system (Invitrogen). One microgram of RNA was reverse transcripted in a RotorGene 3000t Real time DNA detection system (Corbett Life Science, Sydney, Australia) using the QuantiTect SYBRGreen I PCR kit (Qiagen, Valencia, CA, USA). The PCR program used was 95 °C for 15 min, followed by 35 (β-actin) or 45 cycles (CD11b/c and CD68) (94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s). Oligonucleotide primer sequences were designed using the Primer 3 software [16] based on the following GenBank accession numbers: NM_012711 (CD11b/c), NM_001031638 (CD68), and NM_031144 (β-actin). The specificity of the oligonucleotide primers was verified using the program BLASTN from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The forward and reverse primers used were: CD11b/c (OX-42, Integrin alphaM), 5′-CATCACCGTGAGTTCCACAC-3′ and 5′-GAGAACTGGTTCTGGCTTGC-3′ CD68 (ED-1), 5′-CTGTTGCGGA AATACAAGCA-3′ and 5’-GGCAGCAAGAGAGATTGGTC-3′ and β-actin, 5′-GTCGTACCACTGGCATTGTG-3′ and 5′-CTCTCAGCTGTGGTGG TGAA-3′. Expression levels were normalized to the housekeeping gene beta-actin. A relative quantification was performed by comparing the threshold cycle values of samples with serially diluted standards.

2.4. Immunoblotting studies

At the appropriate time, animals (all groups, $n = 6$) were sacrificed and the brains removed, followed by dissection of the posterior medial thalamus, inferior colliculus, and frontal cortex on dry ice. The tissue was stored at -80 °C until ready for study. The dissected tissue was homogenized in buffer containing 50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate (pH 8.0) and a protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO, USA), and centrifuged at 10,000 *g* for 10 min, 4 °C. Protein content of all samples was determined by the method of Lowry et al. [17] using bovine serum albumin (BSA) as the standard. Sample buffer was added to aliquots of the tissue (30 μg) and the samples boiled for 5 min. Aliquots were subjected to (SDS)-polyacrylamide gel electrophoresis

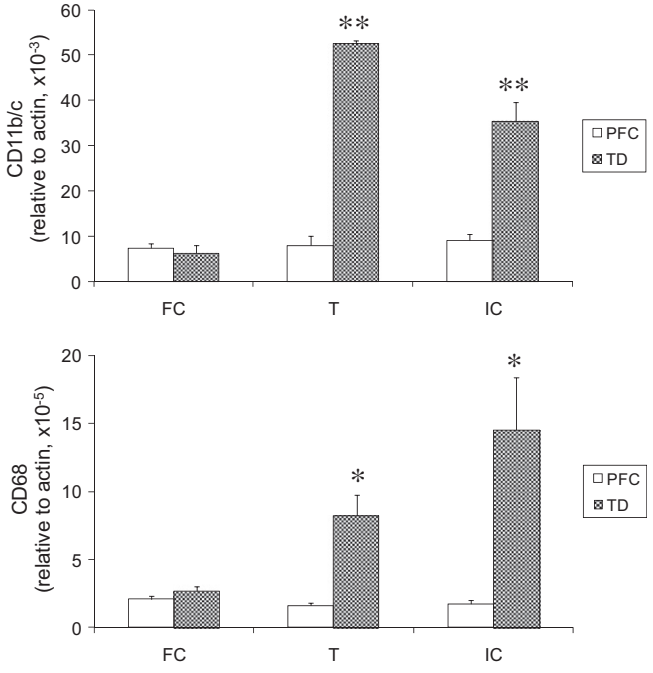

Fig. 1. Expression of CD11b/c and CD68 mRNA in the brains of rats with TD. Relative levels of CD11b/c and CD68 mRNA are shown in the non-vulnerable frontal cortex (FC), medial thalamus (T) and inferior colliculus (IC) of PFC and TD rats at the loss of righting reflex stage. TD resulted in increased expression of CD11b/c and CD68 in the vulnerable thalamus and inferior colliculus compared to control animals. The non-vulnerable frontal cortex was unaffected. Data represent mean ± SEM in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group (Student’s *t*-test).

(8% polyacrylamide) and the proteins subsequently transferred to PVDF membranes by wet transfer at 20 V over 24 h. The transfer buffer consisted of 48 mM Tris (pH 8.3), 39 mM glycine, 0.037% SDS, and 20% methanol. Membranes were subsequently incubated in blocking buffer (10 mM Tris, 100 mM NaCl, 5% nonfat dried milk, and 0.1% Tween-20) followed by incubations with monoclonal antisera directed against CD68 (1:1,000), CD11b/c (1:1,000), or β-actin (1,20,000). Reblocking was followed by incubation with HRP-coupled anti-mouse IgG (1:10,000) secondary antiserum. Each incubation step was of 1 h duration following which blots were washed several times with buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20). For the detection of specific antibody binding, the membranes were treated in accordance with the ECL-kit instructions and apposed to photosensitive X-OMAT film. Signal intensities were subsequently measured by densitometry using Adobe Photoshop (Toronto, ON, Canada). Linearity of the relationship between optical density and protein concentration was verified using appropriate standard curves.

2.5. Immunohistochemistry

Rats (all groups, $n = 4$) were deeply anesthetized with pentobarbital (60 mg/kg) and perfused transcardially as described previously [13]. Brains were removed and post-fixed overnight in

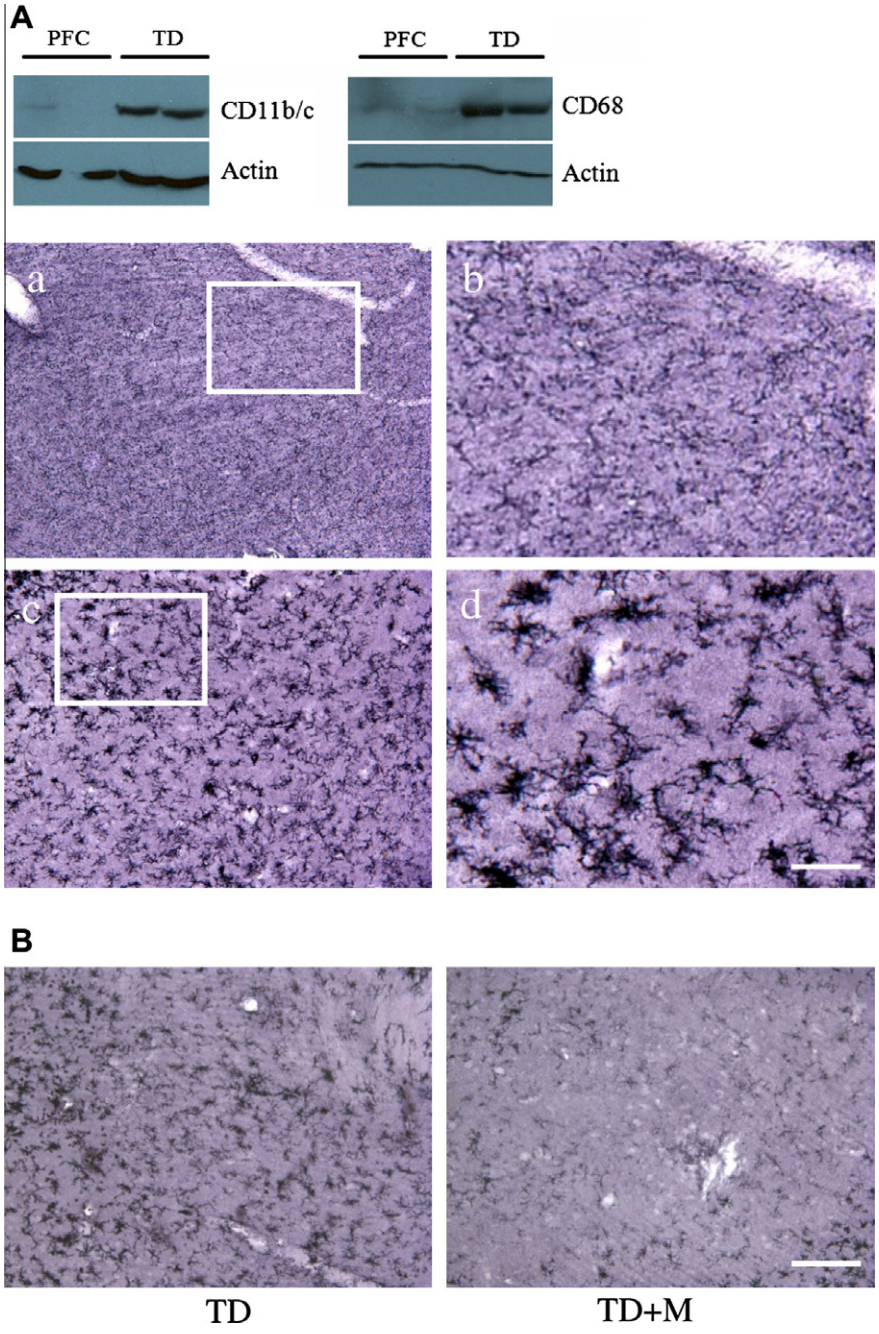

Fig. 2. Microglial activation in the brains of rats with TD. (A) Representative immunoblots of CD11b/c and CD68 are displayed for the medial thalamus of pair-fed control (PFC) and TD rats in which both microglial markers are dramatically increased in TD animals. Representative photomicrographs show CD11b/c immunoreactivity in the medial thalamus from PFC (a, b) and TD (c, d) rats at the loss of righting reflex stage. Mild constitutive staining of CD11b/c is present in control animals and is dramatically increased in TD. Panels b) and d) show content of inset boxes displayed in (a) and (c). Bar: (a) and (c), 150 μm; (b) and (d), 75 μm. (B) Effect of minocycline on microglial activation in TD. Photomicrographs of representative sections of brain show CD11b/c immunostaining in TD and TD + minocycline (TD + M) treated rats at the level of the posterior thalamus. Co-treatment with minocycline prevented the upregulation of CD11b/c in TD animals. Bar: 150 μm.

neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, and 1.5% methanol, pH.7.0. Coronal sections of 40-μm thickness were cut at the level of medial posterior thalamus, inferior colliculus, and frontal cortex using a vibrotome according to the rat brain atlas of Paxinos and Watson [18]. Immunohistochemistry was performed according to Hazell and colleagues [13]. Briefly, brain sections were then incubated for 10 min in phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were washed in PBS (3 × 10 min), blocked for 20 min in 0.5% Triton X-100 and 1% donkey serum, and incubated with 0.5% Triton X-100, along with 1% donkey serum and primary mouse antisera directed against CD11b/c (1:200) or CD68 (1:200) at 4 °C overnight. Sections were then washed (3 × 10 min) and incubated in PBS with 0.5% Triton X-100 containing biotinylated donkey anti-mouse IgG (1:100). Sections were then incubated for 1 h in streptavidin-HRP conjugate (1:100) followed by washing (3 × 10 min), and then incubation with DAB (0.05%) in PBS containing 25 mg/ml nickel ammonium sulfate for signal enhancement and in the presence of H_2O_2 (0.03%) for 2–10 min. Sections were then mounted on polylysine-coated slides (Thermo Fisher Scientific, Ottawa, ON, Canada), dehydrated in graded alcohols, cleared in xylene, and coverslipped with Permount. Negative controls consisted of omission of primary antibody, resulting in loss of immunoreactivity. Microglial cell numbers were counted using Image-Pro Plus (V6.2) software (Media Cybernetics Inc., Bethesda, MD, USA) in four adjacent boxes (0.06 mm^2 each) at a magnification of 400× using an Olympus BX51 microscope and attached Spot RT digital camera.

3. Results

Treatment of rats with TD resulted in a loss of righting reflexes after 13–14 days, preceded by development of ataxia, opisthotonus, nystagmus, and piloerection. Fig. 1 shows the results of qRT-PCR analysis, in which TD treatment resulted in a 6-fold increase in the level of expression of CD11b/c in the medial thalamus ($p < 0.01$), and a 3-fold increase in the inferior colliculus compared to PFC rats ($p < 0.01$). This effect of TD, however, was not observed in the frontal cortex, where CD11b/c expression was unaffected. Examination of CD68 expression in the medial thalamus showed a 5-fold increase in levels ($p < 0.05$) while in the inferior colliculus, expression of this gene was raised 8-fold compared to PFC values ($p < 0.05$) (Fig. 1). In the frontal cortex, CD68 expression was unchanged relative to control animals.

Immunoblotting analysis indicated that CD11b/c and CD68 protein levels were both increased in the medial thalamus by over 30-fold, relative to PFC animals ($p < 0.01$) (Fig. 2A). Brain sections from PFC rats showed mild constitutive immunoreactivity for CD11b/c in the medial thalamus, while in TD animals CD11b/c staining was considerably enhanced in this brain region, consistent with the immunoblotting findings (Fig. 2A). In addition, while CD68 immunoreactivity was not detectable in the thalamus or inferior colliculus of PFC rats, TD animals showed dramatic induction of this protein in these brain regions (not shown).

When TD rats were co-treated with minocycline, CD11b/c immunoreactivity was considerably reduced in the medial thalamus to almost basal levels (Fig. 2B). Examination of the effect of minocycline on neurological dysfunction in TD rats indicated that while TD alone resulted in development of ataxia and opisthotonus, culminating in LRR at 13.7 ± 0.3 days following commencement of treatment, co-treatment with minocycline delayed the onset of these changes and, in particular, this latter stage by approximately 39 h (15.0 ± 0.4 days, $p < 0.01$) (Fig. 3). In addition, co-treatment of TD rats with NAC completely prevented the increase in CD11b/c due to TD alone ($p < 0.05$), while induction of CD68 was partially blocked by NAC administration ($p < 0.05$) (Fig. 4). However, neurologic impairment was unaffected temporally in TD animals treated with this drug.

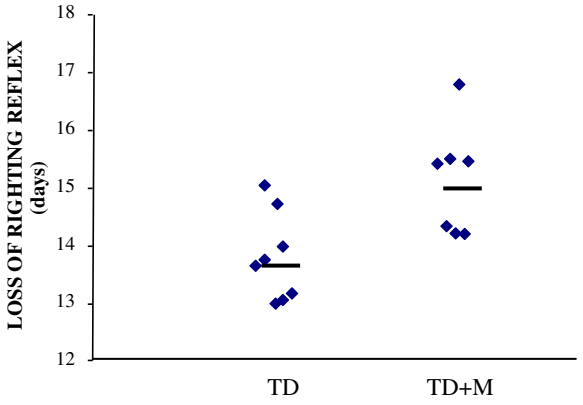

Fig. 3. Effect of minocycline on neurological dysfunction in TD. Rats co-treated with TD and minocycline (TD + M) showed a delay in the loss of righting reflexes compared to rats treated with TD alone (Mann–Whitney *U*-test).

4. Discussion

Microglial activation is a key feature of a wide range of neurological disorders that include neurodegenerative diseases [19] as well as traumatic brain injury [20] and stroke [21]. These cells are known to play a major role in the regulation of inflammatory processes in the brain, and produce cytokines and high levels of NADPH oxidase as well as NO synthase, along with large amounts of superoxide anion and NO in response to pro-inflammatory stimuli [22] and [23]. In the present study, we have demonstrated that TD results in large increases in CD68 and CD11b/c at the mRNA and protein level, not only in the vulnerable thalamus as previously reported by other groups [8] but also in other brain regions known to be damaged in this disorder such as the inferior colliculus, with sparing of the relatively non-vulnerable cerebral cortex. Such findings indicate that microglial activation is a consequence of TD and is linked to focal vulnerability in this disorder.

In a recent microarray study, we were able to demonstrate that in the thalamus and inferior colliculus during TD, inflammatory genes represent the largest functional group of transcripts upregulated [6]. These include the pro-inflammatory cytokines (IL-6, IL-18, TNF-α, AIF1 and osteopontin), chemokines (MCP-1, MIP-1α, MIP-1β and Gro1), interferons (IFNs) and IFN-inducible proteins, indicating these two brain regions exhibit a large inflammatory response in TD. This is consistent with other studies that have reported cytokine production in vulnerable areas of the brain in TD [5–7]. Several transcription factors known to control inflammatory gene expression (Egr-1, c-EBP-β, c-EBP-δ, CPBP and Klf-4) were also upregulated following TD. Of these, Egr-1 and c-EBP-β may play an important role in starting the inflammatory cascades following oxidative impairment. Levels of these various inflammatory-related gene products in different brain regions may be an important factor(s) in the determination of selective vulnerability in TD.

In the present report, treatment of TD rats with minocycline led to a significant delay in the onset of LRR of approximately 39 h. This represents to our knowledge, the first example of a drug with the ability to slow the development of neurological impairment in this brain disorder. Minocycline also blocked the increase in CD11b/c due to TD, suggesting that the delay in onset of LRR may be at least partly due to its anti-inflammatory effects. Interestingly, treatment with the antioxidant NAC considerably reduced the increase in both CD11b/c and CD68 due to TD. Previous studies

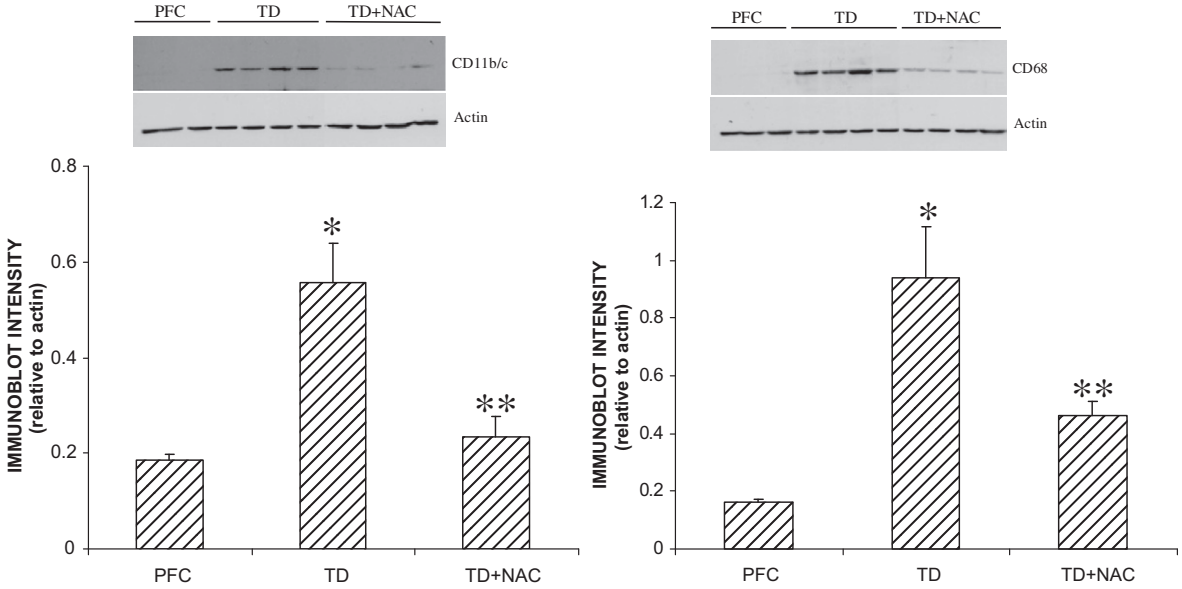

Fig. 4. Effect of antioxidant treatment on microglial activation. Results show representative blots of CD11b/c, CD68, and β-actin in the medial thalamus of pair-fed controls (PFC), TD rats, and TD rats co-treated with NAC. Immunoblot analysis indicates that NAC prevented upregulation of both microglial proteins in TD animals. Data represent mean ± SEM in each group. $^{*}p < 0.05$ compared with PFC group, $^{**}p < 0.05$ compared with TD group (one-way ANOVA with *post hoc* Dunnett's test for multiple comparisons).

have suggested that inflammation represents the earliest response to developing TD, occurring as early as day 8 of TD [8]. However, since NAC was able to reduce the extent of microglial activation, this indicates that part of the inflammatory response in vulnerable brain regions in TD is likely due to oxidative stress, and that this may occur prior to the onset of inflammation. Earlier studies have involved the use of antioxidant treatment in TD in which elements of excitotoxicity, including glutamate transporter downregulation and alterations in complexin levels were blocked concomitant with increased neuronal survival [12] and [24], supporting the premise that oxidative stress plays an important role in the pathogenesis of TD-induced brain damage. On the other hand, onset of neurologic impairment was unaffected by NAC treatment, suggesting a more complex, multifactorial basis in TD.

Although minocycline has well-established anti-inflammatory properties, it should also be noted that mechanisms of neuroprotection associated with this drug are broad and remain to be fully elucidated. For example, although minocycline has been shown to suppress microglial activation and cytokines in injured brain [25,26], it can also downregulate apoptosis and protect against oxidative stress [27,28]. In addition to oxidative stress, apoptosis is known to occur under conditions of TD [29–31]. Thus, it cannot be excluded that these other effects of minocycline may also be involved to some degree in the ability of this drug to delay the onset of neurological deficits in TD.

In conclusion, the present results show that microglial activation is a response to TD that occurs in focal regions of vulnerability in the affected brain, and which is linked to neurological dysfunction. Prevention of the increase in CD11b/c and CD68 associated with activation of microglia results in a considerable delay in the onset of LRR, suggesting this glial response plays an important role in the development of neurological changes. In addition, development of oxidative stress may occur in advance of the inflammatory process, an important issue for our understanding of the sequelae of events that underlie the pathophysiology of this disorder.

Acknowledgments

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