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Source / Izvornik: **Neuroimmunomodulation, 2013, 20, 152 - 163**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.1159/000346546

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:184:893650

Original Paper

NeuroImmunoModulation

Neuroimmunomodulation 2013;20:152–163
DOI: 10.1159/000346546

Received: August 2, 2012
Accepted after revision: December 17, 2012
Published online: March 13, 2013

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Key Words
Albino Oxford and Dark Agouti rats · Experimental autoimmune encephalomyelitis · Metallothioneins I/II · Zinc · Copper

Abstract
Objectives: Compared to the Dark Agouti (DA), the Albino Oxford (AO) rat strain exhibits lower susceptibility to the induction of experimental autoimmune encephalomyelitis (EAE). Here, we investigated the potential contribution of the heavy metal-binding proteins metallothioneins (MTs) I/II to these effects. ***Methods:*** Rats were immunized with bovine brain homogenate emulsified in complete Freund's adjuvant or only with complete Freund's adjuvant. The expression patterns of MTs mRNA and proteins and tissue concentrations of Zn^{2+} and Cu^{2+} were estimated in the brain and in the liver on days 7 and 12 after immunization, by real-time PCR, immunohistochemistry and inductively coupled plasma spectrometry, respectively. Additionally, the hepatic transforming growth factor beta and nuclear factor kappa B immunoreactivities were tested. ***Results:*** Clinical signs of EAE were not induced in AO rats, but they upregulated the expression of MT I/II proteins in the brain (hippocampus and cerebellum) and in the liver, similarly as DA rats. The transcriptional activation of MT-I occurred, however, only in DA rats, which accumulated also more zinc in the brain and in the liver. In contrast, intact AO rats had greater hepatic MT-I mRNA immunoreactivity and more Cu^{2+} in the hippocampus. Besides, in immunized AO rats a high upregulation of transforming growth factor beta and nuclear factor kappa B immunoreactivities was found in several hepatic structures (vascular endothelium, Kupffer cells and hepatocytes). ***Conclusions:*** Our data show that AO and DA rats differ in constitutive and inductive MT-I gene expression in the brain and in the liver, as well as in the hepatic cytokine profile, suggesting that these mechanisms may contribute to the discrepancy in the susceptibility to EAE.

Introduction

Metallothioneins (MTs) belong to a highly conserved family of low molecular weight, cysteine-rich proteins [1–6]. As zinc- and copper-binding proteins they are involved in several physiological processes, such as cell differentiation, proliferation and apoptosis, which are ubiquitous in eukaryotes and are expressed particularly in fast growing tissues [7–9]. Besides, MT I+II isoforms participate in several pathophysiological states, since tissue injuries, exposure to heavy metals and other stressful condi-

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tions upregulate the transcription of MTs by the glucocorticoid-responsive elements (GREs), by the antioxidant (or electrophile) response element, by the elements activated by STAT-3 (signal transducers and activators of transcription) proteins through cytokine signaling, as well as by metal response elements, showing that MTs are multifunctional proteins that participate in a variety of cellular functions [1, 6, 10, 11].

The protective effects of MTs within the mammalian CNS have been repeatedly emphasized [1, 5, 12–14]. Thus, MT-I and MT-II immunostaining dramatically increases in Alzheimer disease [4], as well as in brain lesions during multiple sclerosis [15] and experimental autoimmune encephalomyelitis (EAE) [16], where they prevent the oxidative stress and demyelination and reduce the axonal damage [17], participating in the clinical recovery of EAE [18, 19]. Confirming these data, recently we showed that a chronic relapsing form of EAE (CR-EAE) induced in Dark Agouti (DA) rats was characterized by dynamic changes in the expression of MT I+II and by a marked dysbalance in metal ion homeostasis in the brain and in the liver [20]. Moreover, we found MT upregulation and labile zinc movements even in the presymptomatic phase of CR-EAE, showing that these changes might predict the development of EAE [Jakovac et al., in press].

Trying to provide some information about the genetic background of these important neuroprotective mechanisms, in this study we extended our analysis to the early induction phase of EAE in DA and Albino Oxford (AO) rats, which have different susceptibility to EAE. The data showed that constitutive and induced MT I+II gene expression in EAE-resistant and EAE-prone rats is different both in the organs that were damaged by the autoimmune attack (hippocampus and cerebellum), as well as in the liver, pointing to the high involvement of the central and peripheral MTs-related mechanisms in the induction of EAE. Besides, since only in AO rats, immunized with encephalitogen, the upregulation of transforming growth factor beta (TGF-β) immunoreactivity was found in the liver, the data imply that a hepatic immunosuppressive environment may contribute to the induction of resistance toward EAE.

Materials and Methods

Experimental Animals

For the experiments, we used male DA and AO rats, aged 2–3 months. They were bred and maintained according to the guide for Institutional Animal Care and used with approval of the local ethical committee.

EAE Induction

Immunization was performed by bovine brain white matter homogenate (BBH) emulsion in complete Freund's adjuvant (CFA; Sigma, St. Louis, Mo., USA), as previously described [20]. Each animal received 2 × 0.1 ml of emulsion, which was injected subcutaneously, in each hind footpad. The control group was injected with the same dose of CFA. Animals were sacrificed on day 7 after immunization (before the appearance of any clinical symptoms of EAE) and on day 12 (during the appearance of clinical symptoms of EAE in DA rats). The severity of disease was assessed clinically according to the following criteria: 0 = no symptoms; 1 = flaccid paralysis of tail; 2 = hind legs paresis; 3 = hind legs paralysis with incontinence, and 4 = death of animal.

Tissue Preparation for Paraffin Slices

Tissue samples of the brain and the liver were rapidly removed from 9 rats in each time interval and fixed in 10% buffered formalin solution during 24 h. The tissue was then embedded in paraffin wax and sections were cut at 4 μm using HM 340E microtome (Microtom, Germany). Heat-induced epitope retrieval was done prior to the staining procedure by heating tissue slides in boiled citrate buffer of pH 6.0 for 4 times, 5 min each, using a microwave steamer.

Immunohistochemistry

Immunohistochemical studies were performed by Dako EnVision+ System, peroxidase (DAB) kit, on tissue sections embedded in paraffin wax, according to the manufacturer's instructions (Dako Corporation, USA). The MTs I+II were identified by the mouse monoclonal anti-MT I+II antibody, as previously described [20]. Briefly, after washing, mouse monoclonal anti-MT I+II (clone E9; Dako Cytomation, USA; diluted at 1:50) antibodies were added to tissue samples and incubated overnight at 4°C in a humid environment, followed by 45 min incubation with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin-containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreaction product was visualized by adding substrate-chromogen (DAB) solution. Tissues were counterstained with hematoxylin, dehydrated through graded ethanols, mounted using Entelan (Sigma-Aldrich, Germany) and examined with Olympus BX51 microscope (Olympus, Tokyo, Japan). The same protocol was used for the visualization of T lymphocytes, TGF-β and nuclear factor kappa B (NFκB) immunoreactivity after the application of anti-CD3, anti-TGF-β and anti-NFκB p65 monoclonal antibodies, respectively, at the dilutions of 1:100 or 1:1,000, respectively (Abcam Inc; Cambridge, Mass., USA). The specificity of the reaction was confirmed by substitution of specific antibodies with mouse irrelevant IgG1 kappa immunoglobulins, used under the same conditions and dilutions as the primary antibodies.

Immunohistochemical Staining Quantification

Immunohistochemical staining quantification was performed using Cell F v3.1 software (Olympus Soft Imaging Solutions). Captured images were subjected to intensity separation. They were subsequently inverted, resulting in grey scale images with different intensity ranges, depending on the strength of immunohistochemical signals. Regions of interest were set up to cover the cytoplasm of immunopositive cells to measure grey intensity. Twenty regions of interest per field (400×) were analyzed in ten fields per micro-

scopic slide of tissue samples, obtained from BBH+CFA and CFA-treated AO and DA rats.

Tissue Preparation for RNA Isolation

Tissue for RNA isolation was obtained from intact rats and rats immunized with BBH+CFA, sacrificed by exsanguinations on days 7 and 12 after immunization. The brain was dissected out from the skull and placed into ice-cold phosphate-buffered saline in a Petri dish. The cerebellum and the hippocampus were carefully isolated after a coronal cut behind the inferior colliculi and removal of diencephalon from each hemisphere. The liver was rapidly removed and rinsed in the ice-cold phosphate-buffered saline to remove any surface blood. All tissue samples were snap-frozen in liquid nitrogen and stored at –80°C.

RNA Extraction, Reverse Transcription and Real-Time PCR Analysis

Total RNA was extracted from frozen tissues, using Trizol Reagent (Invitrogen, USA), according to the protocol provided by the manufacturer (1 ml of Trizol per 0.1 mg of tissue). RNA was assayed by ultraviolet spectrophotometric measurements at a wavelength of 260 nm, and its purity was estimated by the ratio of A260/A280. Total RNA (5 μg) was treated with turbo DNA-free reagent (Ambion; Applied Biosystems, USA) to remove contaminating genomic DNA, followed by reverse transcription (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, USA), according to the manufacturer's instructions. The selected cDNA transcripts were amplified using TaqMan probes specific for MT-1a (Rn00821759_g, TaqMan® Gene Expression Assay; Applied Biosystems, USA). For normalization, endogenous control 18S (Hs03003631_g1, TaqMan® Gene Expression Assay; Applied Biosystems, USA) was used.

Real-time PCR was performed using a TaqMan® Universal PCR Master Mix (Applied Biosystems, USA), according to the manufacturer's procedure. Each reaction was performed in a 25-μl reaction mixture containing 5 μg of cDNA with a 7300 Real-Time PCR System (Applied Biosystems). The reaction mixture was denatured for one cycle of 10 min at 95°C and incubated for 40 cycles (denaturing for 15 s at 95°C and annealing and extending for 1 min at 60°C). The amplification data were analyzed with a 7300 Real-Time PCR System software version 1.3. Values were expressed as relative quantities (RQ) normalized to the endogenous control gene 18S. Analyses were carried out in tissue samples consisting of a pool of 3 rats, and the experiments were repeated 3 times.

Tissue Sample Preparation for Mineralization and Inductively Coupled Plasma Spectrometry

Liquid tissue samples of brain, hippocampus, cerebellum and liver (n = 5) were introduced into the apparatus by pneumatic nebulization. Metal ions measurements were performed using a Philips PU 7000 ICP spectrometer, by the ASTM D 19756–91 method (power 1 kW, coolant 12 liter/min, nebulizer 38 psi), at a fixed wavelength of 213.856 nm for zinc and 324.754 nm for copper.

Statistical Analysis

Data were expressed as mean ± SE. Differences between groups were assessed by Friedman's one-way analysis of variance, Mann-Whitney U test (clinical scores), and by two-tailed Student's t test for unpaired samples (RNA analysis and metal tissue concentrations). The level of significance was set at $p < 0.05$.

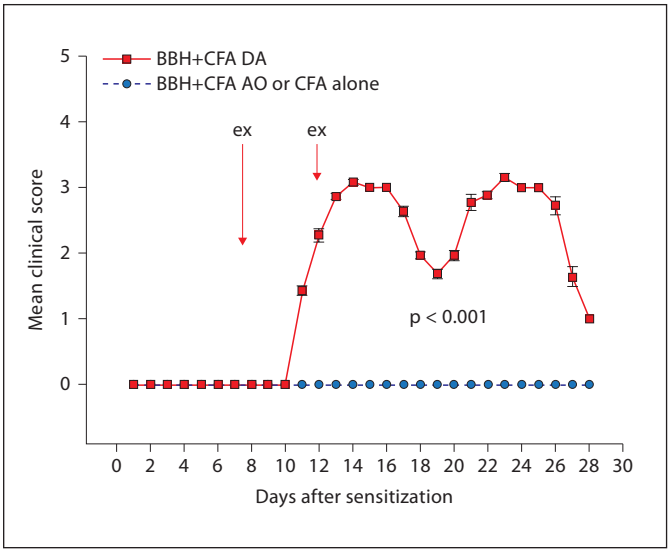

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Fig. 1. Clinical score of DA and AO rats (n = 18) with CR-EAE induced by injection of BBH+CFA. The control group rats (n = 18) were injected with CFA alone. Arrows point to the time when tissue samples for the detection of MT I/II immunoreactivities were taken (i.e. in intact rats and on days 7 and 12 after treatment with BBH+CFA or CFA). Data are mean ± SE.

Results

Clinical Course of EAE in DA and AO Rats

DA and AO rats were immunized with BBH+CFA, but only genetically susceptible DA rats (n = 18) developed a typical chronic relapsing form of disease, characterized by two peaks of clinical symptoms (on the 12th and the 22nd postimmunization day), as we previously described [20]. In contrast, AO rats (n = 18), similarly as the control rats treated by CFA (n = 18), did not develop any clinical symptoms of EAE (fig. 1; $p < 0.001$).

Expression of MT I+II Proteins, mRNA and Tissue Metals of EAE-Resistant and EAE-Prone Rats

To elucidate the potential role of MTs in the development of resistance to EAE, the AO and DA rats were immunized with BBH+CFA or with CFA and sacrificed on days 7 and 12 after immunization, i.e. before and after the appearance of clinical symptoms in the DA rat strain. The expression patterns of MTs I/II proteins, MT-I mRNA and tissue content of metals (zinc and copper) were determined in tissue samples of the whole brain, in the isolated hippocampus and cerebellum, as well as in the liver. The data were also compared with the corresponding findings of the tissues obtained from intact AO and DA rats.

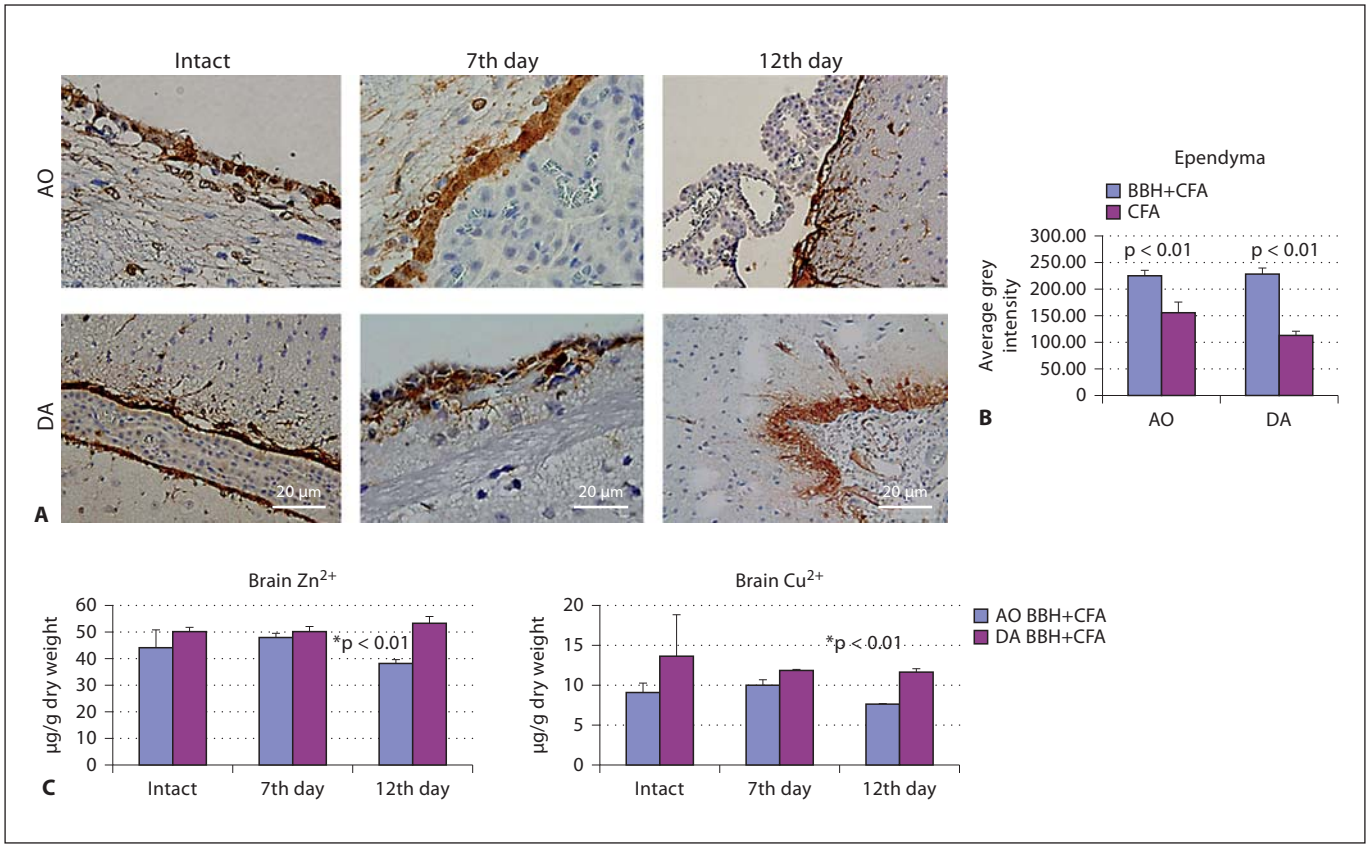

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Fig. 2. A Expression of MT I+II proteins in the subventricular area of AO and DA rats immunized with BBH+CFA. Analyzes were done on paraffin-embedded sections of tissue obtained at the preclinical phase of EAE and at the time of first attack in EAE-prone rats (days 7 and 12, respectively). The results are representative findings of 3 rats. **B** Immunohistochemical, cell-based staining quantification of MT I/II expression in the ependyma found in analogous paraffin-embedded sections obtained from experimental (BBH+CFA) and control (CFA-treated) AO and DA rats on the 12th postinjection day. The corresponding average grey distribution of MTs expression was calculated from twenty regions of interest (magnification 400× per field) in ten fields per microscopic slides of tissue samples. Results are mean ± SE. **C** Zinc and copper content in the homogenate of the whole brain in AO and DA rats immunized with BBH+CFA. Results are mean ± SE (n = 5). Statistical significance between AO and DA rats is indicated by p values with an asterisk.

Whole Brain

In both rat strains, after immunization with BBH+CFA, upregulation of MT I/II proteins was found in subarachnoid regions on glial cells and on choroid plexus epithelial cells (fig. 2A). Changes were visible already on the 7th postinjection day. Immunohistochemical staining quantification analysis also showed that they were of the similar intensity in both strains, but significantly greater that those found in CFA-treated rats (fig. 2B; $p < 0.01$). However, measurement of the essential metals in the homogenate of the whole brain revealed that DA rats accumulated more Zn^{2+} and Cu^{2+} than AO rats during the EAE attack (fig. 2C; $p < 0.01$).

Hippocampus

To detect the regional distribution of MTs in the brain, the expression patterns of MT I+II proteins, MT-I gene and tissue metals were also compared in the tissues of the isolated hippocampus and the cerebellum of DA and AO rats. The data revealed that in both strains, the MT I/II proteins were upregulated in the subgranular layer of the dentate gyrus. Changes were greater than those in CFA-treated rats (fig. 3B; $p < 0.01$), and on the 12th postinjection day, there was a higher expression in DA rats immunized with BBH+CFA (fig. 3B; $p < 0.05$). Moreover, DA rats reacted with a higher expression of MT I mRNA to immunization, pointing to the difference at the transcriptional level (fig. 3C; $p < 0.001$). Besides, metal tissue anal-

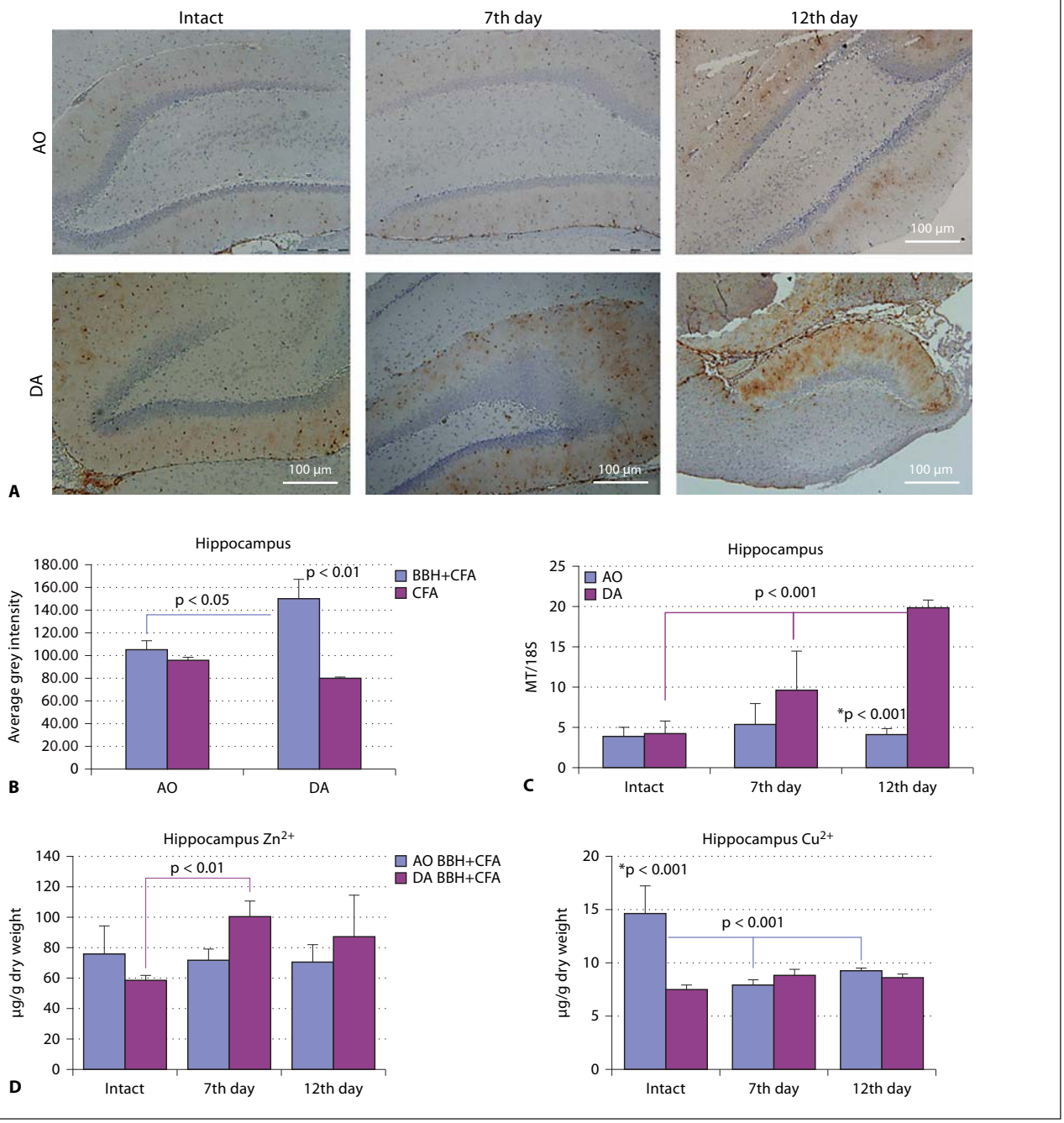

Fig. 3. A Expression of MT I+II proteins in the hippocampus of AO and DA rats immunized with BBH+CFA. Analyzes were done on paraffin-embedded sections of tissue obtained at the preclinical phase of EAE and at the time of first attack in EAE-prone rats (days 7 and 12, respectively). The results are representative findings of 3 rats. **B** Immunohistochemical, cell-based staining quantification of MT I/II expression in the hippocampus found in analogous paraffin-embedded sections obtained from experimental (BBH+CFA) and control (CFA-treated) AO and DA rats on the 12th postinjection day. The corresponding average grey distribution of MTs expression was calculated from twenty regions of interest (magnification 400× per field) in ten fields per microscopic slides of tissue samples. Results are mean ± SE. **C** Expression of MT-I mRNA in the hippocampus of AO and DA rats after immunization with BBH+CFA. Results are mean ± SE of RQ of MT-I mRNA normalized to the endogenous control gene 18S. Analyses were made in tissue samples consisting of a pool of 3 rats for each time point during the course of EAE in three separate experiments. **D** Zinc and copper content in the hippocampus of AO and DA rats immunized with BBH+CFA. Results are mean ± SE (n = 5). Statistical significance between AO and DA rats is indicated by p values with an asterisk.

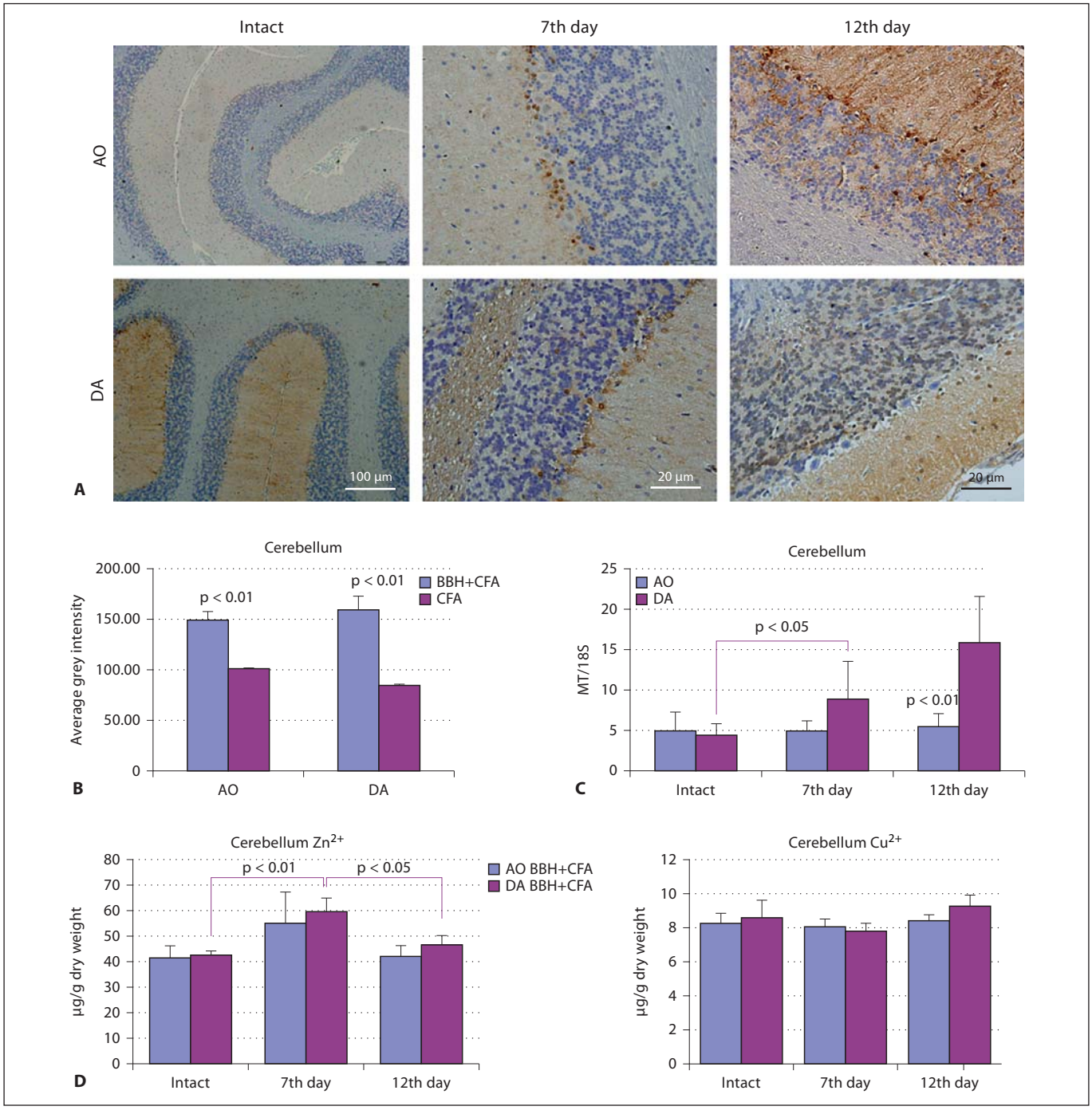

Fig. 4. A Expression of MT I+II proteins in the cerebellum of AO and DA rats immunized with BBH+CFA. Analyzes were done on paraffin-embedded sections of tissue obtained at the preclinical phase of EAE and at the time of first attack in EAE-prone rats (days 7 and 12, respectively). The results are representative findings of 3 rats. **B** Immunohistochemical, cell-based staining quantification of MT I/II expression in the cerebellum found in analogous paraffin-embedded sections obtained from experimental (BBH+CFA) and control (CFA-treated) AO and DA rats on the 12th postinjection day. The corresponding average grey distribution of MTs expression was calculated from twenty regions of interest (magnification 400× per field) in ten fields per microscopic slides of tissue samples. Results are mean ± SE. **C** Expression of MT I mRNA in the cerebellum of AO and DA rats immunized with BBH+CFA. Results are mean ± SE of RQ of MT-I mRNA normalized to the endogenous control gene 18S. Analyses were made in tissue samples consisting of a pool of 3 rats for each time point during the course of EAE in three separate experiments. **D** Zinc and copper content in the cerebellum of AO and DA rats immunized with BBH+CFA. Results are mean ± SE (n = 5).

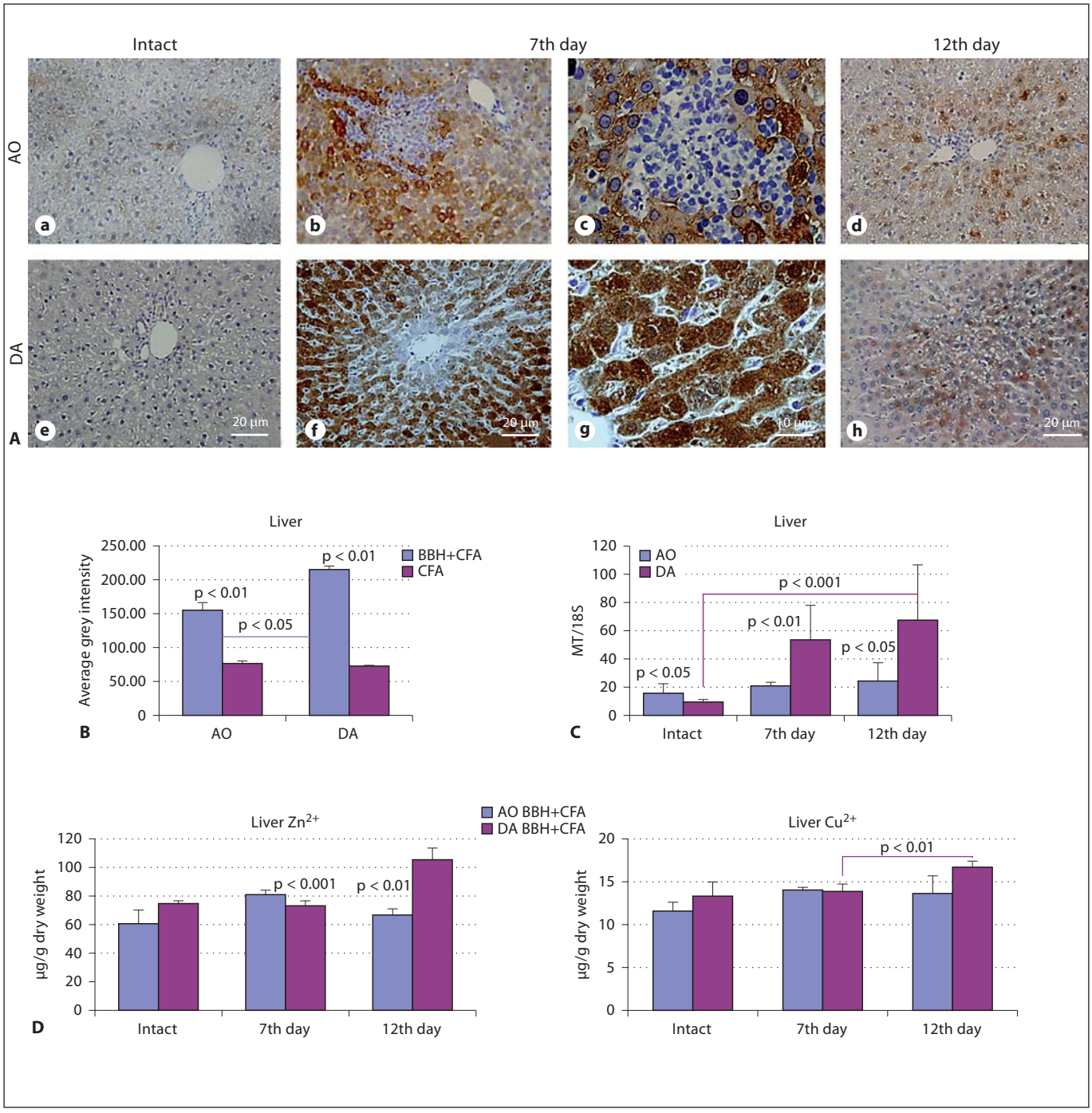

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Fig. 5. A Expression of MT I+II proteins in the liver of AO and DA rats immunized with BBH+CFA. Analyzes were done on paraffin-embedded sections of tissue obtained at the preclinical phase of EAE and at the time of first attack in EAE-prone rats (days 7 and 12, respectively). The results are representative findings of 3 rats. **B** Immunohistochemical, cell-based staining quantification of MT I/II expression in the liver found in analogous paraffin-embedded sections obtained from experimental (BBH+CFA) and control (CFA-treated) AO and DA rats on the 12th postinjection day. The corresponding average grey distribution of MTs expression was calculated from twenty regions of interest (magnification 400× per field) in ten fields per microscopic slides of tissue samples. Results are mean ± SE. **C** Expression of MT I mRNA in the liver of AO and DA rats immunized with BBH+CFA. Results are mean ± SE of RQ of MT-I mRNA normalized to the endogenous control gene 18S. Analyses were made in tissue samples consisting of a pool of 3 rats for each time point during the course of EAE in three separate experiments. **D** Zinc and copper content in the liver of AO and DA rats immunized with BBH+CFA. Results are mean ± SE (n = 5).

ysis showed that in the hippocampal area, DA rats initially accumulated more zinc than AO rats (fig. 3D; $p < 0.01$). In contrast, intact AO rats had significantly greater levels of copper than DA rats (fig. 3D; $p < 0.001$).

Cerebellum

Similar patterns of MT I/II proteins and MT I gene expressions were found in the cerebellum. Thus, in both rat strains a high MT I/II immunoreactivity was present in the granular and molecular layers of the cerebellum (fig. 4A). Changes were greater than those in CFA-treated rats (fig. 4B). However, similarly as in the hippocampus, MT-I mRNA increased only in DA rats (fig. 4C), which exhibited also a greater accumulation of zinc after immunization (fig. 5D).

Expression of MT-I Gene, MT I+II Proteins and Tissue Metals in the Liver of EAE-Resistant and EAE-Prone Rats

Owing to our previous findings that EAE-prone DA rats exhibit a high upregulation of MT I/II protein expression during attacks of CR-EAE particularly in the liver [20], herein we compared also the hepatic pattern of their expression in AO and DA rats. The data showed that DA rats reacted to immunization with BBH+CFA by an earlier and higher cytoplasmic upregulation of MT I/II immunoreactivity in hepatocytes (fig. 5Af, g). The effect was also confirmed by immunohistochemical staining quantification analysis, which confirmed that upregulation of MTs in rats immunized with BBH+CFA was greater than that induced by CFA (fig. 5B). Furthermore, DA rats showed a greater hepatic MT-I gene transcription on days 7 and 12 after immunization (fig. 5C; $p < 0.05$), as well as a higher hepatic accumulation of zinc than AO rats on the 12th postinjection day (fig. 5D; $p < 0.01$). In contrast, the constitutive MT-I mRNA expression in AO rats was found to be greater than that in DA rats (fig. 5C; $p < 0.05$). Besides, on the 7th postinjection day, multiple cellular infiltrates surrounded by MT I/II positive hepatocytes were found in the liver of AO rats (fig. 5Ab, c).

TGF-β and NFκB Immunoreactivity in the Liver of EAE-Resistant and EAE-Prone Rats

Preliminary phenotypic analysis revealed that cellular infiltrates in the liver of AO rats contained some CD3+ lymphocytes (fig. 5Ab). Furthermore, a high TGF-β1 immunoreactivity was found on sinusoidal epithelial cells (fig. 5Ad), as well as on vascular endothelial cells (fig. 5Ae) and on multiple hepatocytes (fig. 5Af). Moreover, only in AO rats, a high nuclear and cytoplasmic NFκB immunoreactivity was found on multiple cells in infiltrates and in large hepatocytes around these infiltrates, respectively (fig. 6h). In addition, in AO rats a greater number of NFκB-positive Kupffer cells than in DA rats was found (fig. 6i vs. g).

Discussion

EAE is widely used as an animal model for multiple sclerosis, a human demyelinating inflammatory disease. It arises from a break in tolerance toward self-antigens and is characterized by the development of myelin-reactive Th1 and Th17 lymphocytes, macrophages and several bystander cells, which in susceptible strains of animals infiltrate the brain and develop the typical remitting relapsing course of the disease [21–24]. The outcome, however, depends on the interplay between the pathogenic and regulatory T cell populations, as well as on several local and systemic inflammatory and anti-inflammatory mechanisms that affect the development of immunogenic and tolerogenic lymphatic subsets and regulate the susceptibility of target organs or target tissue structures to the autoimmune attack. The pathogenesis is multifactorial and dependent on the presence of genes that determine both the immune functions and the target organ susceptibility, as well as on the additional modulatory factors, such as the interrelationship between the neuroendocrine and the immune system and the environmental factors that may affect the structure of tissues damaged by autoimmune attack [25–27].

In this context, it has been repeatedly shown that AO and DA rats significantly differ in their reactivity to immunization of brain or spinal homogenate emulsified in CFA. Thus, although the AO rats exhibit mild neuroinflammation even without the presence of clinical symptoms [28, 29], the DA rats usually accumulate more CD4+ T cells and CD11b+ cells in brain and spinal cord lesions and secrete more IL-2, IFN-γ and IL-17 [28, 30]. Besides, DA rats react to immunization with a greater initial influx of macrophages and dendritic cells in the CNS than AO rats [31], which, however, enhance the chemokine expression in microvessels and in target tissues damaged by autoimmune processes [32].

Moreover, recently it was reported that DA and AO rats might differ also in the production of TH-1 and TH-17-inducing cytokines and in the secretion of IL-6 from draining lymph node cells (DLNC) [30]. Supporting these findings, we showed herein that after immunization with encephalitogen, DA and AO rats differ in some aspects of

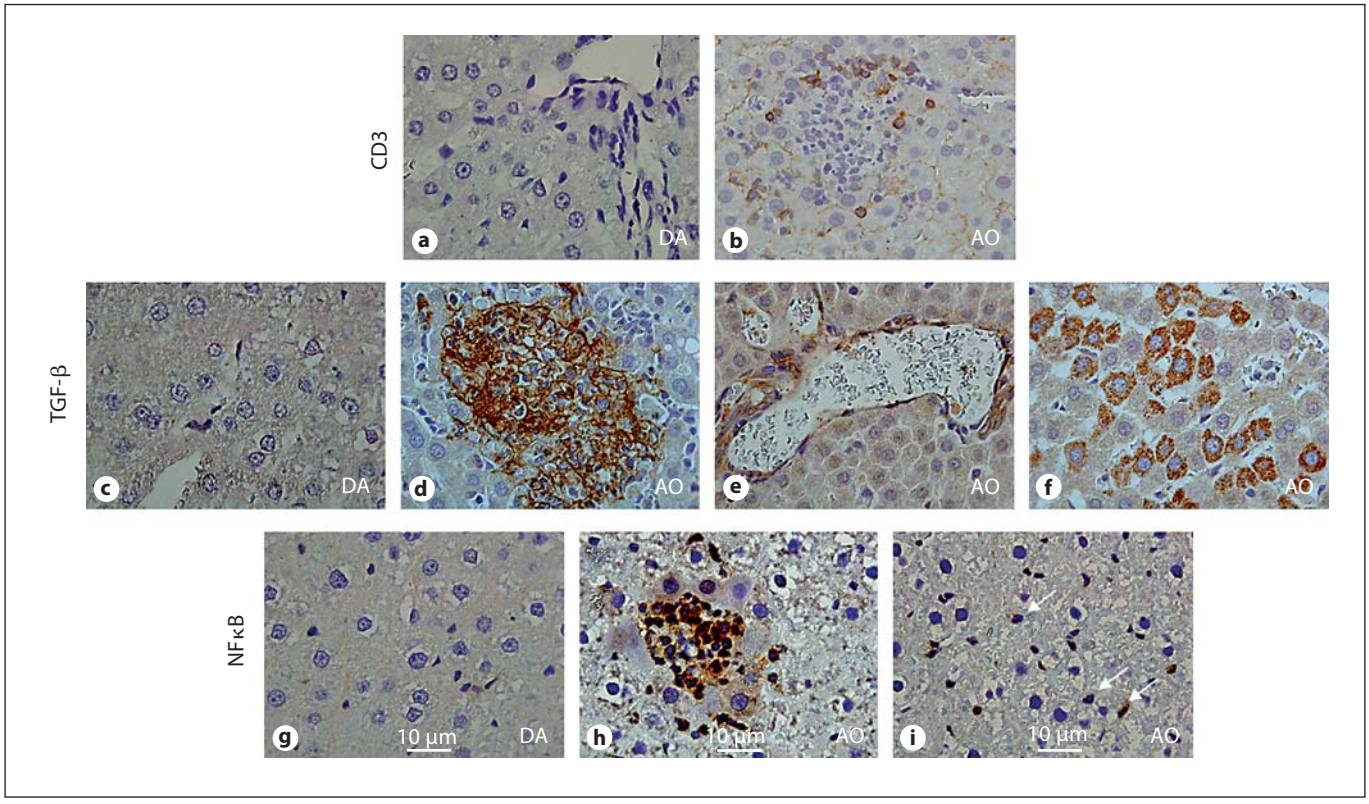

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Fig. 6. Immunohistochemical staining of T lymphocytes, TGF-β and NFκB in the liver specimen of DA (**a**, **c**, **g**) and AO rats (**b**, **d–f**, **h**, **i**), after the application of anti-CD3, anti-TGF-β and anti-NFκB p65 monoclonal antibodies. Tissue samples were taken on the 7th day after immunization with BBH+CFA. The results are representative findings of 3 rats.

MTs-related events. Thus, although they similarly upregulated the MT I/II proteins in the subventricular area and in the cerebellum, they exhibited a different pattern of MT-I gene expression in the hippocampus, in the cerebellum and in the liver and had a different capacity to maintain the trace metal homeostasis in these organs. Since these changes were greater in EAE-prone DA rats, the data imply that the transcription of MTs correlated with the intensity of autoimmune damage. However, the findings of specific TGF-β-related changes in the liver permit us also to speculate that the hepatic microenvironment contributed to the induction of resistance to EAE in AO rats.

In both strains, the changes in the MT-I/II proteins were present particularly in cells that form the blood-brain and the blood-cerebrospinal fluid barrier and provide stimulatory signals for T cells invasion (endothelial cells, ventricular subependyma, microglia/macrophages and astroglial cells around the ventricle walls). High expression was also found on glial cells in the subgranular layer of the hippocampal dentate gyrus and on cells in the granular and molecular layers of the cerebellum, implying that in these areas MTs were involved in suppression of neuroinflammation, which occurred even in AO rats. The tissue metal dysregulation was, however, noticed only in EAE-prone DA rats, which accumulated zinc particularly in the hippocampus and cerebellum, implying that the greater oxidative or nitrative stress in these rats led to the release of zinc from the MT-I/II, due to oxidation of thiols responsible for coordinate binding of zinc [10]. Besides emphasizing the link between the intensity of inflammation and the expression of the MT-I gene, we showed herein that the MT-I mRNA increased only in DA rats, both in these target organs (fig. 3C, 4C) and in the liver (fig. 5C). In the latter, the transcription of MTs might be viewed as part of an acute phase response [2, 6], which contributed also to the sequestration of zinc in DA rats during the attack of disease (fig. 5D).

The mechanisms need to be elucidated, but generally the hypothesis is in agreement with current knowledge showing that MT I+II isoforms may be expressed on several endothelial, epithelial and astroglial cells and transcriptionally rapidly induced by metals that affect the metal response elements by the metal-responsive transcription factor-1 or the antioxidant response element, STAT-3 and GREs, in response to redox status, cytokine signaling and stressful conditions, respectively [6, 11]. The issue is covered by numerous excellent reviews [1, 10, 33–35] and discussed in our previous reports showing that the expression of MT I/II proteins in DA rats correlated with the severity of clinical symptoms during the CR-EAE, as well as with the intensity of zinc and copper dysregulation in the brain, spinal cord and liver [20].

Herein, we show that dysbalances of zinc homeostasis in the brain and in the liver probably contributed also to the greater transcriptional activation of the MT-1 gene in EAE-prone DA rats, pointing to the participation of MTF-1, which is a sensor of zinc concentration in the cytoplasm [2, 6, 11]. However, the influences of proinflammatory cytokines, such as TNFα, IL-1a, IL-1β and IL-6, and greater intensity of stress cannot be ruled out. Regarding the latter, it should be also emphasized that the recovery of animals from EAE [36] and from other types of autoimmune diseases [37] is highly dependent on the activation of the cytokine hypothalamic-pituitary-adrenal axis feedback circuit, which increases the levels of endogenous glucocorticoids (GCs), as the immunosuppressive end-product of this pathway [reviewed in 38–40]. Noteworthy, some EAE-susceptible rat strains display genetic deficiency in this axis that reduces the basal levels of corticosterones and secretion of corticotropin-releasing hormone during the attack of disease [21, 41–43]. Our data, however, imply that the secretion of GCs and GRE-induced MT-I gene transcription in DA rats was not defective, since the significant upregulation of MT-I mRNA was observed in the hippocampus and the cerebellum (fig. 3C, 4C), i.e. in the brain areas that are usually involved in the response to stress [44]. However, it should be emphasized that GCs in the brain often exert different – even opposed – effects depending on the concentration of GCs and duration of exposure, on the type of implicated cells in affected brain areas (microglia, astroglia or neurons), as well as on the type of receptors for GCs (mineraloreceptors vs. glucoreceptors) [reviewed in 39].

Additionally, our data underline that in the pathogenesis of EAE, the liver plays a marked role (fig. 5, 6), where the GCs and proinflammatory cytokines commonly exert the effects on the transcription of genes involved in the metabolic axes and in maintenance of hepatic immunologic homeostasis [reviewed in 40, 45]. Moreover, during the acute phase reaction, MT I/II and cytokines participate in the creation of the specific hepatic cytokine microenvironment that governs the local balance between tolerance and immunity [46, 47]. In this context, we showed herein that the transcription of MT-I mRNA was markedly upregulated in the liver of EAE-prone DA rats (fig. 5C), but that AO rats had a greater basal level of MT-I mRNA (fig. 5C) and increased TGF-β immunoreactivity in several hepatic structures (vascular endothelium, liver sinusoidal endothelial cells, Kupffer cells and hepatocytes (fig. 6), suggesting that the hepatic cytokine profile contributed to the activation of processes that restrain of the autoimmune process in the AO rat strain. This hypothesis remains to be proved, but it is in agreement with current knowledge showing that TGF-β and its combination with IL-6 regulate the differentiation of naive T cells into the regulatory T cells or into the autoaggressive Th17 cells [48–50]. Since, TGF-β in the absence of IL-6 promotes the appearance of regulatory T cells, it can be speculated that hepatic TGF-β contributed to the induction of resistance to EAE in AO rats. This hypothesis seems to be highly supported by data of Markovic et al. [30] who showed that AO rats do not express any detectable level of IL-6 mRNA in DLNC during the induction phase of EAE, in contrast to DA rats, which produced this cytokine. Critical influences of TGF-β and IL-6 for the induction of Treg cells or pathogenic Th17 cells were confirmed also in murine myelin oligodendrocyte glycoprotein [51] and in proteolipid protein-induced EAE [52]. Besides, it was shown that IL-6 might completely inhibit the generation of Treg cells induced by TGF-β [51], as well as that the administration of TGF-β1 in vivo might improve the clinical course of EAE, similarly as polyphenolic phytochemical curcumin, which after the downregulation of the expression of IL-6, IL-21, ROR t signaling and inhibition of STAT3 phosphorylation inhibited the differentiation and development of Th17 cells [53]. It should be also emphasized that TGF-β signaling might affect the processes of unique antigen presentation by local adipocyte progenitor cell populations in the liver, such as dendritic cells, Kupffer cells, and liver sinusoidal endothelial cells that result in induction of the antigen-specific peripheral tolerance rather than in induction of T cell immunity [47, 54, 55]. Our data showing the greater activation of NFκB in Kupffer cells of AO rats seem to point in this direction (fig. 6h vs. i). The high impact of the liver and hepatic microenvironment on the prevention of EAE was recently reviewed by Hoffman and Herzog [56], who emphasized

that the introduction of antigens into the liver may be used as a preventative or therapeutic intervention for autoimmune disease in the brain, particularly owing to the induction of TGF-β-dependent generation of myelin basic protein-specific CD4+CD25+Foxp3+ Tregs after the expression of myelin basic protein in the liver [56, 57].

Our data also point to the high TGF-β immunoreactivity in hepatocytes (fig. 6) and to the high MT I/II protein expression in hepatocytes, located around the foci of lymphoid cells (fig. 4C), implying that signals from liver parenchymal cells might contribute to the induction of T cell tolerance to encephalitogen in AO rats. These data, however, need further explanation, owing to the complex relationship between various cells and factors that participate in the mechanisms, such as immune ignorance, clonal deletion or immune deviation, which in the liver might induce tolerance against the encephalitogen [47].

In conclusion, we showed herein that the expression of MTs, particularly at the transcriptional level, correlates with the susceptibility of DA and AO rats to the induction of EAE and with the concomitant tissue metal dysregulation, confirming that MTs participate in housekeeping processes, such as protection against ROS, regulation of the catalytic activity of numerous metalloenzymes and stabilization of the conformation of zinc-dependent protein domains, such as zinc fingers, zinc clusters and RING fingers that are commonly found in transcriptional regulatory proteins [1, 2, 6, 19, 58]. Differences were visible in the brain, hippocampus and cerebellum, as well as in the liver. Besides showing that EAE-resistant AO rats, early after sensibilization with encephalitogen, markedly upregulated the TGF-β in nonparenchymal and parenchymal cells in the liver, we point to the possibility that the hepatic immunosuppressive environment contributes to the induction of tolerance against EAE in rats.

Acknowledgements

This work was supported by grants from the Croatian Ministry of Science (projects 62-06213411337 and 62-0621341-0061).

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