Journal of Neuroimmunology 298 (2016) 32–41

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Microglia response in retina and optic nerve in chronic experimental autoimmune encephalomyelitis

Lioba Horstmann [a], Sandra Kuehn [a], Xiomara Pedreiturria [b], Kathrin Haak [b], Christiane Pfarrer [c], H. Burkhard Dick [a], Ingo Kleiter [b,1], Stephanie C. Joachim [a,*,1]

[a] Experimental Eye Research Institute, University Eye Hospital, Ruhr-University Bochum, In der Schornau 23-25, 44892 Bochum, Germany
[b] Department of Neurology, St. Josef-Hospital, Ruhr-University Bochum, Bochum, Germany
[c] Department of Anatomy, University of Veterinary Medicine Hannover, Germany

ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form 7 June 2016
Accepted 22 June 2016

Keywords:
EAE
MOG
Multiple sclerosis
Microglia
Demyelination
Retinal ganglion cells

ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is a common rodent model for multiple sclerosis (MS). Yet, the long-term consequences for retina and optic nerve (ON) are unknown. C57BL/6 mice were immunized with an encephalitogenic peptide ($MOG_{35–55}$) and the controls received the carriers or PBS. Clinical symptoms started at day 8, peaked at day 14, and were prevalent until day 60. They correlated with infiltration and demyelination of the ON. In MOG-immunized animals more microglia cells in the ONs and retinas were detected at day 60. Additionally, retinal ganglion cell (RGC) loss was combined with an increased macroglia response. At this late stage, an increased number of microglia was associated with axonal damage in the ON and in the retina with RGC loss. Whether glial activation contributes to repair mechanisms or adversely affects the number of RGCs is currently unclear.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) with a wide variety of clinical signs (Compston and Coles, 2008). During MS, inflammatory demyelinating lesions develop throughout the CNS, including the optic nerves (ON). A common initial presentation of MS is optic neuritis, leading to impaired vision (Ransohoff et al., 2015). This vision defect is often, but not always reversible. Most MS patients have a persistent reduction of vision-related quality of life 5 to 8 years after an optic neuritis (Cole et al., 2000). The disease usually starts before the age of 40 and more females, three per one male, are affected (Compston and Coles, 2008). Progressive accumulation of disability, including the visual system, affects the majority of patients during the course of MS. The most common visual symptoms include decrease in visual acuity and contrast sensitivity (Balcer and Frohman, 2010), defects in binocular vision, visual field abnormalities (Nakajima et al., 2010), reduced colour vision (Villoslada et al., 2012), blurred vision and diplopia. There is an association between MS and some ocular inflammatory diseases such as uveitis (Biousse et al., 1999, Le Scanff et al., 2008). The clinical manifestation of relapses is variable and results from immune cell migration across the blood-brain-barrier during attacks. Typically lesions are due to demyelination in the white matter, known as plaques (Friese et al., 2014). The relationship between inflammation, demyelination and later neurodegenerative changes, particularly in the visual system, is still not well-known.

The animal model of MS, experimental autoimmune encephalomyelitis (EAE), is a powerful tool to study disease pathogenesis and therapeutic interventions (Gold et al., 2006). With regard to the visual tract, axonal damage and loss of neurons are found in EAE animals (Guan et al., 2006). During acute EAE, the ON shows an influx of inflammatory cells and a strong demyelination (Shindler et al., 2008). Both, in MS and the EAE model, the ON and the retina are affected by inflammation (Talla et al., 2013). The main inflammatory cells are microglia and T-cells. Th1 and Th17 cells are crucial pathologic T-cell subtypes in MS and the EAE animal model (Fletcher et al., 2010). We have previously shown, that the clinical score of acute $MOG_{35–55}$-induced EAE correlates with the severity of inflammation and demyelination in the ON (Horstmann et al., 2013). In eyes of EAE animals with acute optic neuritis the retinal ganglion cells (RGCs) are significantly reduced (Shindler et al., 2006).

Abbreviations: CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; LFB, luxol fast blue; GFAP, glial fibrillary acid protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; ON, optic nerve; PKCα, protein kinase Cα,; PTx, pertussis toxin; RGC, retinal ganglion cells.

* Corresponding author at: Experimental Eye Research, University Eye Hospital, Ruhr-University Bochum, In der Schornau 23-25, 44892 Bochum, Germany.
E-mail address: stephanie.joachim@rub.de (S.C. Joachim).

[1] These authors contributed equally and should therefore be regarded as equivalent senior authors.

http://dx.doi.org/10.1016/j.jneuroim.2016.06.008
0165-5728/

2. Material and methods

2.1. Animals

All experiments that involved animals were performed in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and approved by the animal care committee of North Rhine-Westphalia, Germany. C57BL/6 mice (Charles River) were bred in our facility and housed under environmentally controlled conditions with free access to food and water ad libitum in the absence of pathogens.

2.2. Induction and evaluation of EAE

To induce EAE, 7 to 10-week-old C57BL/6 mice were immunized subcutaneously with 100 mg/kg MOG_{35-55} peptide (provided by Charité, Berlin) in complete Freund's adjuvant (CFA) (Thermo Fisher, Waltham, USA) containing 200 μg *mycobacterium tuberculosis H37Ra* (BD Difco, USA) (MOG; n = 7). In addition, mice received 200 ng pertussis toxin (PTx; Merck Millipore, Darmstadt, Germany) intraperitoneally on days 0 and 2. Two control groups were included in this study, one was injected with the same doses of CFA and PTx as the MOG group (CFA/PTx; n = 4), while the other one received only PBS (PBS; n = 3) (Kleiter et al., 2010). Experimental groups were matched for age and sex.

Clinical assessment of EAE was performed daily, according to the following criteria: 0 = no disease, 0.5 = partial tail paralysis, 1 = tail paralysis or waddling gait, 1.5 = partial tail paralysis and waddling gait, 2 = tail paralysis and waddling gait, 2.5 = partial limb paralysis, 3 = paralysis of one limb, 3.5 = paralysis of one limb and partial paralysis of another, 4 = paralysis of two limbs, 4.5 = moribund state and 5 = death (Kleiter et al., 2010). Also, the cumulative EAE score for each group was calculated (Kleiter et al., 2007).

60 days after MOG-immunization mice were sacrificed and perfused with 4% paraformaldehyde (Sigma Aldrich, Munich, Germany). Brain, eyes and ONs were removed and post-fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany), drained in sucrose (VWR, Langenfeld, Germany), embedded in Tissue Tec (Thermo Scientific, Cheshire, UK) and frozen at −80 °C.

2.3. Histopathological evaluation of optic nerves and brain

5 μm thick longitudinal ON sections were stained with hematoxylin and eosin (H&E, Merck, Darmstadt, Germany) and luxol fast blue (LFB, RAL Diagnostics, Martillac Cedex, France). All slides were dehydrated in ethanol, followed by incubation in xylene (Merck, Darmstadt, Germany), before mounting with Eukitt (VWR, Langenfeld, Germany). 3 pictures (dorsal, middle and periphery) per ON section (6 sections/ON) were taken with a microscope (Axio Imager M1, Zeiss, Oberkochen, Germany) at a 400× magnification. Additionally, the left cerebrum and cerebellum were sagittally cut in 20 μm slices and stained with H&E as previously described. Pictures of all slices of one hemisphere were taken with a microscope (Olympus BX51, Hamburg, Germany) at a 20× magnification.

All photos were evaluated using ImageJ (Version 1.44p; NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij) in a masked fashion. The inflammatory cell infiltration of the ON was assessed by a four-point scale: 0 = no infiltration, 1 = mild cellular infiltration, 2 = moderate infiltration, 3 = severe infiltration and 4 = massive infiltration (Horstmann et al., 2013, Shindler et al., 2012). LFB-stained ON sections were graded as follows: 0 = no demyelination, 1 = moderate demyelination and 2 = severe demyelination (Horstmann et al., 2013, Shindler et al., 2006). The CNS inflammatory index was quantitatively assessed by measurement of the area of inflammatory infiltrates divided by the total area of the cerebrum or cerebellum on the respective section. Additionally, the total number of meningeal/cortical lesions per section was counted.

2.4. Immunohistochemistry of optic nerves and retina

Longitudinal ON (5 μm, 1 nerve/animal) and retinal cross-sections (10 μm, 1 retina/animal) were prepared for immunohistochemistry as follows. Sections were dehydrated for 30 min and then rehydrated for 10 min in PBS (Santa Cruz, Dallas, USA). The sections were blocked in 10% appropriate serum in 0.1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) plus PBS. 6 sections per tissue were used for each staining. In the ON, macroglia cells were investigated with GFAP antibody and microglia cells were stained with Iba1 (Table 1). In the retina, RGCs were labeled with Brn-3a, a neuronal transcription factor (Table 1). Macroglia cells (mainly astrocytes) were investigated with GFAP antibody and Müller glia via vimentin. Additionally, microglia cells were stained with Iba1. PKCα was used to label bipolar cells. All slides were counterstained with DAPI as nuclear marker. In general, four photos were taken from each retinal section, two from the periphery and two from the central part. Three photos were taken from dorsal, middle and peripheral part of each ON section with an ApoTome.2 microscope (Zeiss, Oberkochen, Germany) in a 400× magnification. All photos were masked and analyzed using ImageJ. To measure expression of GFAP and vimentin, an ImageJ macro was used for area analysis (Horstmann et al., 2013). First, each image was transformed into a greyscale. After background subtraction (20 pixel), the lower and upper threshold was set (GFAP: lower threshold = 8.68, upper threshold = 260; vimentin: lower threshold = 4.03, upper threshold = 90). Then, the labeled area was measured via ImageJ. Regarding all other stainings, labeled cells were counted. The $Iba1^+$ microglia in the retina were distinguished into resting and active, based on their shape and they were only counted in the ganglion cell layer (GCL) and inner plexiform layer (IPL). While ramified microglia represent a resting/inactive state, microglia with a round/amoeboid formation are considered highly active (Gramlich et al., 2011b).

2.5. Statistical analyses

Data are presented as mean ± SD. A p-value < 0.05 was considered to be statistically significant. The three groups were compared by ANOVA, followed by Tukey post-hoc test (Statistica; V12; Tulsa, Ok, USA). The correlation between demyelination and infiltration was significant with a correlation coefficient (r^2) near 1.0.

3. Results

3.1. Clinical course of chronic EAE

Mice developed clinical signs of EAE starting at day eight after MOG_{35-55} peptide immunization. The average score ranged between 2 and 2.5 and peaked at day 14 (MOG: 2.6 ± 0.4; CFA/PTx: 0.0 ± 0.0, p < 0.001; PBS: 0.0 ± 0.0, p < 0.001; Fig. 1A). After 20 days, a partial remission of the disease was observed and most animals showed only tail paralysis and waddling gait. However, animals deteriorated again in the further course. A cumulative EAE score of 114.5 ± 24.2 was noted in the MOG-immunized group compared to 0.0 ± 0.0 in the CFA/PTx- and the PBS-treated group (p < 0.001). Additionally, nine days after MOG-immunization differences in weight were noted. In accordance with EAE severity, the lowest weight was measured at day 11 (MOG: 87.0 ± 8.5%; CFA/PTx: 120.0 ± 7.9%; PBS: 111.0 ± 3.9%, Fig. 1B). The weight changes within each group remained comparable from day 31 (MOG: 113.0 ± 8.2%; CFA/PTx: 130.0 ± 9.1%; PBS: 123.0 ± 1.9%, Fig. 1B) on up to day 60 (MOG: 113.0 ± 14.5%; CFA/PTx: 129.0 ± 6.1%; PBS: 122.0 ± 5.4%, Fig. 1B).

3.2. Brain inflammation

The inflammatory index in the cerebrum was significantly higher in MOG-immunized animals (0.2 ± 0.08) compared to both control