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Reduced cuprizone-induced cerebellar demyelination in mice with astrocyte-targeted production of IL-6 is associated with chronically activated, but less responsive microglia

Filip Petković[a,b,*], Iain L. Campbell[c], Berta Gonzalez[a], Bernardo Castellano[a]

[a] Department of Cell Biology, Physiology and Immunology, Institute of Neuroscience, Autonomous University of Barcelona, Bellaterra 08193, Spain
[b] Department of Immunology, Institute for Biological Research "Sinisa Stankovic", 11000 Belgrade, Serbia
[c] School of Molecular Bioscience, University of Sydney, Sydney, NSW 2006, Australia

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ABSTRACT

Background: Cerebellar pathology is a frequent feature of multiple sclerosis (MS), a demyelinating and neuroinflammatory disease of the central nervous system (CNS). Interleukin (IL)-6 is a multifunctional cytokine with a potential role in MS. Here we studied cuprizone-induced cerebellar pathology in transgenic mice with astrocyte-targeted production of IL-6 (GFAP-IL6), specifically focusing on demyelination, oligodendrocyte depletion and microglial cell response.

Results: Over the course of cuprizone treatment, when compared with WT mice, GFAP-IL6Tg showed a reduced demyelination in the deep lateral cerebellar nuclei (LCN). The oligodendrocyte numbers in the LCN were comparable between WT and GFAP-IL6Tg mice after 4–6 weeks of cuprizone treatment, however after the chronic cuprizone treatment (12 weeks) we detected higher numbers of oligodendrocytes in GFAP-IL6Tg mice. Contrary to strong cuprizone-induced microglial activation in the LCN of WT mice, GFAP-IL6Tg mice had minimal cuprizone-induced microglial changes, despite an already existing reactive microgliosis in control GFAP-IL6Tg not present in control WT mice.

Conclusions: Our results show that chronic transgenic production of IL-6 reduced cuprizone-induced cerebellar demyelination and induced a specific activation state of the resident microglia population ($Iba1^+$, $CD11b^+$, $MHCII^+$, $CD68^-$), likely rendering them less responsive to subsequent injury signals.

1. Introduction

Multiple sclerosis (MS) is a demyelinating and inflammatory disease affecting various regions of the central nervous system (CNS), including the cerebellum (Weier et al., 2015). In the most widely used MS model, experimental autoimmune encephalomyelitis (EAE), neuropathological manifestations are largely restricted to the spinal cord, therefore a vast majority of EAE studies have focused exclusively on this part of the CNS. On the other hand, cuprizone-induced demyelination model provides robust, as well as timely and anatomically predictable demyelination of various brain regions, including cerebellum (Kipp et al., 2009; Groebe et al., 2009). In spite of extensive cerebellar neuroinflammation and demyelination in the cuprizone model, very few studies have focused on this part of the CNS. The time course of cerebellar demyelination, oligodendrocyte (OL) loss, microgliosis and astrogliosis in the cuprizone model have been described in detail by Skripuletz et al. (2010). Interleukin (IL)-6 is a cytokine with potential, yet not clarified, role in MS (reviewed in Petković and Castellano, 2016). We have recently reported that cuprizone-fed transgenic mice with astrocyte-targeted production of IL-6 (GFAP-IL6Tg) showed reduced demyelination, reduced axonal damage, and lower microglial and astroglial activation in the corpus callosum (Petković et al., 2016). Taking into account that the cerebellum is the region of the brain where the highest production of IL-6 occurs in these transgenic mice (Quintana et al., 2009), in this report we expanded our study investigating the effect of localized and chronic production of IL-6 on cuprizone-induced cerebellar neuropathology, with special emphasis on IL-6-induced microglial phenotype alterations.

Abbreviations: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; Iba, ionized calcium-binding adapter molecule; IL, interleukin; LCN, lateral cerebellar nucleus; MBP, myelin basic protein; MHC, major histocompatibility complex; MS, multiple sclerosis; OL, oligodendrocyte; pMϕ, perivascular macrophages; WT, wild type

* Corresponding author at: Unitat d'Histologia, Torre M5, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.
E-mail addresses: petkovicfilip@yahoo.com (F. Petković), iain.campbell@sydney.edu.au (I.L. Campbell), berta.gonzalez@uab.cat (B. Gonzalez), bernardo.castellano@uab.cat (B. Castellano).

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2. Material and methods

In this study male GFAP-IL6Tg mice and wild type (WT) C57BL/6 litter mates of 8–12 weeks of age were used. Mice were maintained with food and water *ad libitum* in a 12 h light/dark cycle during the experiments. All experimental work was conducted according to Spanish regulations (Ley 32/2007, Real Decreto 1201/2005, Ley 9/2003, y Real Decreto 178/2004) in agreement with European Union directives (86/609/CEE, 91/628/CEE i 92/65/CEE) and was approved by the Ethical Committee of the Autonomous University of Barcelona, Spain. In order to induce demyelination, WT and GFAP-IL6Tg mice were fed with 0.2% (w/w) cuprizone (bis–cyclohexanone-oxaldihydrazone, Sigma-Aldrich) mixed into standard powdered ground chow for 4, 6 or 12 weeks (n = 4–8/genotype and time point). Control groups of WT and GFAP-IL6Tg mice (n = 4–5/genotype) were fed with standard chow. After 4, 6 or 12 weeks of cuprizone treatment, mice were intracardially perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4), brains were isolated and processed for paraffin embedding. Paraffin sections, 10 μm thick, obtained using a Leitz 1512 microtome were used for immunohistochemical detection of myelin basic protein (MBP), Olig2, ionized calcium-binding adapter molecule (Iba1) and CD68. Briefly, activity of endogenous peroxidase was blocked by 5 min incubation with 2% H_2O_2 in 70% methanol. Sections were then incubated for 1 h in blocking buffer solution (BB; 0.05 M Tris-buffered saline (TBS), pH 7.4, containing 10% fetal calf serum, 3% bovine serum albumin (BSA) and 0.1% Triton X-100), which was followed by incubation with primary antibodies (overnight at 4 °C and 1 h at room temperature) (Table 1) diluted in BB. As negative controls, sections incubated in BB without primary antibody were used. After washing with TBS + 0.1% Triton X-100, sections were incubated for 1 h at room temperature with the appropriate secondary antibodies (anti-rat biotinylated; 1:500; BA-4001; Vector Laboratories, Inc.; Burlingame, CA or anti-rabbit biotinylated; 1:500; BA-1000; Vector Laboratories, Inc.; Burlingame, CA) diluted in BB. After 1 h in streptavidin-peroxidase (1:500; SA-5004; Vector Laboratories, Inc.; Burlingame, CA), the reaction was visualized by incubating the sections with DAB (DAB kit SK-4100; Vector Laboratories, Inc.; Burlingame, CA) following the manufacturer's instructions. Sections immunostained for CD68 were counterstained with toluidine blue for nuclei visualization. Sections were air dried, dehydrated in graded alcohols and after xylene treatment coverslipped with DPX. In addition, groups of control and cuprizone-fed (4 weeks) WT and GFAP-IL6Tg mice were processed for cryostat sectioning, as previously described (Petković et al., 2016). Free-floating, 30 μm thick, cryostat sections were used for immunohistochemical detection of CD11b and major histocompatibility complex (MHC) II. Firstly, activity of endogenous peroxidase was blocked by 10 min incubation with 2% H_2O_2 in 70% methanol. Sections were then incubated for 1 h in blocking buffer solution (BB; 0.05 M Tris-buffered saline (TBS), pH 7.4, containing 10% fetal calf serum, 3% bovine serum albumin (BSA) and 1% Triton X-100), which was followed by overnight incubation at 4 °C and 1 h at room temperature incubation with the primary antibody (Table 1) diluted in BB. After washing with TBS + 1% Triton X-100, sections were incubated for 1 h at room temperature with conjugated secondary antibody (anti-rat biotinylated; 1:500; BA-4001; Vector Laboratories, Inc.; Burlingame) diluted in BB and subsequently with streptavidin-peroxidase for 1 h. The reaction was visualized using DAB and sections immunostained for MHCII were counterstained with toluidine blue for nuclei visualization. Stained sections were analyzed and photographed with a DXM 1200F Nikon digital camera mounted on a Nikon Eclipse 80i brightfield microscope. Densitometric analysis was performed using analySIS software and final values (arbitrary units) were calculated by multiplying the percentage of the stained area by the mean intensity of the staining, as previously described (Almolda et al., 2014). The number of $Olig2^+$ cells was determined using the "Automatic nuclei counter (ITCN)" plug-in from NIH ImageJ software (Wayne Rasband, National Institutes of Health, USA), as previously described (Petković et al., 2016). Statistical analysis was performed using GraphPad Prism Software. All results are presented as mean + standard error of the mean. For the comparison between WT and GFAP-IL6Tg mice, non-parametric Mann-Whitney *U* test was performed. Significant differences were indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

3. Results and discussion

Our results showed that cuprizone-induced cerebellar pathological changes in WT mice were largely restricted to deep cerebellar nuclei, which is in agreement with previous reports (Groebe et al., 2009; Skripuletz et al., 2010). Regarding the cerebellar cortex and the white matter of the cerebellar lobes, our findings in WT mice, in concordance with previous observations by others (Groebe et al., 2009), did not show significant demyelination after chronic (12 week) cuprizone treatment, although mild demyelination has been reported by some (Skripuletz et al., 2010). Our findings showed that all three deep cerebellar nuclei were strongly demyelinated, however, for the purpose of clarity, we focused on the lateral cerebellar nuclei (LCN) (Fig. 1a). Thus, in WT mice, LCN were extensively demyelinated after 6 weeks of cuprizone treatment (Fig. 1A, E), and progressed to almost complete demyelination after 12 weeks of treatment (Fig. 1G), but we did not detect demyelination in the cerebellar cortex (Fig. 1J, L). In cuprizone-treated GFAP-IL6Tg mice, demyelination of the LCN was significantly lower after 6 and 12 weeks of cuprizone treatment, compared with WT mice (Fig. 1F, H, I). In concordance with previous observations (Campbell and Powell, 1996), we found myelin alterations in the granule cell layer of the cerebellar cortex and subjacent white matter in control GFAP-IL6Tg mice (Fig. 1K). However, remarkably, we did not detect any further increase in myelin degeneration in cuprizone-fed GFAP-IL6Tg mice, even after chronic treatment (Fig. 1M). Additionally, our findings showed a loss of $Olig2^+$ oligodendrocytes (OL) in the LCN of both WT and GFAP-IL6Tg mice fed with cuprizone (Fig. 1O–V). Quantitative analysis showed that the number of OL was similar in both WT and GFAP-IL6Tg mice, except after 12 weeks of treatment, when OL number was almost 60% higher in GFAP-IL6Tg mice (Fig. 1W). In general, these observations demonstrated that cuprizone-induced neurohistopathological changes were significantly less severe in GFAP-IL6Tg, than in WT mice.

In agreement with other studies (Groebe et al., 2009; Krauthausen et al., 2014), our observations showed that cuprizone treatment in WT mice induced a strong activation of microglia in the LCN. Microglia are most likely the main effector cells in the CNS, and have been suggested

Table 1
Primary Abs used for immunohistochemistry.

Ab	Host	Directed against	Dilution	Ag retrieval	Supplier cat. number
MBP	Rat	Myelin basic protein	1:1000	None	Abcam (ab7349)
Iba1	Rabbit	Ionized calcium-binding adaptor molecule	1:1000	None	Wako (019-19741)
Olig2	Rabbit	Oligodendrocyte transcription factor 2	1:2000	Citrate	Millipore (AB9610)
CD68	Rat	Phagolysosomal protein	1:500	None	BioRad (MCA1957)
CD11b	Rat	CR3 complement receptor subunit	1:1000	None	Serotec (MCA74G)
MHCII	Rat hybridoma	Major histocompatibility complex II	1:25	None	ATCC (TIB-120)