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# Immunohistochemical localization of galectin-3 in the brain with Theiler's murine encephalomyelitis virus (DA strain) infection

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**Abstract :** Galectin-3 is a β-galactoside-binding lectin that plays a role in neuroinflammation through cell migration, proliferation, and apoptosis. In the present study, regulation of galectin-3 was examined in the brain of mice infected with the Daniel strain of Theiler's murine encephalomyelitis virus (TMEV) at days 7 and 81 post-infection by immunohistochemistry. Immunohistochemistry revealed that galectin-3 was mainly localized in ionized calcium-binding adapter 1-positive macrophages/activated microglia, but not in Iba-1-positive ramified microglia. Galectin-3 was also weakly detected in some astrocytes in the same encephalitic lesions, but not in neurons and oligodendrocytes. Collectively, the present findings suggest that galectin-3, mainly produced by activated microglia/macrophages, may be involved in the pathogenesis of virus induced acute inflammation in the early stage as well as the chronic demyelinating lesions in Daniel strain of TMEV induced demyelination model.

**Keywords :** encephalomyelitis, galectin-3, macrophages, microglia, Theiler's murine encephalomyelitis virus

## Introduction

Galectin-3 is a β-galactoside-binding animal lectin that is found in a variety of tissues including the immune system in normal mice [9] and is associated with several biological functions including cell adhesion, signaling, proliferation, differentiation, and apoptosis [7], depending on its localization. Intracellular galectin-3 plays a role in cell survival, whereas extracellular galectin-3 mediates cell migration and apoptosis. Besides the biological role of galectin-3 in normal tissues, it plays pleitrophic roles in the course of inflammation through the facilitation of cell migration and activation [1, 22, 23]. Although some studies have shown that galectin-3 is not expressed in normal central nervous system (CNS) tissues [18], more recent work has reported galectin-3 expression by ependymal cells and astrocytes from the subventricular zone in normal uninjured mice [3], a process in which ependymal cilia establish chemotactic gradients and astrocytes form glial tubes, which combine to aid neuroblast migration [3]. Galectin-3 has also been shown to drive oligodendrocyte differentiation [17]. Upon injury, galectin-3 is upregulated in macrophages/activated microglia, but not in ramified microglia in brains infected with Junin virus [6], in the case of the spinal cord after peripheral infection with herpesvirus [10] or following experimental autoimmune encephalomyelitis (EAE) induction [19]. A recent review suggested that galectin-3 is exclusively expressed in macrophages and activated microglia capable of phagocytosis of myelin debris, but not in ramified microglia that do not phagocytose degenerated myelin [20].

Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease (TMEV-IDD) is used as a model of chronic primary multiple sclerosis [5]. It has been suggested that in TMEV-IDD, axonal injury precedes demyelination, and the lesion develops from the axons to the myelin [13, 21]. Subsequently, the initial axonal damage might result in the release of neuroantigens that induce an autoimmune response against myelin antigens, finally attacking the myelin by autoimmune $CD4^+$ T cells [15, 21]. During the course of TMEV infection, acute encephalitis was preceded, preferentially around ventricles [11], and then followed by chronic demyelination [21]. However, the early response in the brain remains to be further examined in relation to the expression of galectin-3, which is a pro-inflammatory mediator.

The aim of this study was to examine galectin-3 expression in CNS tissues infected with Daniel's (DA) strain of TMEV in the acute phase at day 7 post-infection and in the chronic phase at day 81 post-infection to elucidate the possible involvement of galectin-3 in the development of TMEV induced pathology.

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## Materials and Methods

Female SJL/J mice were purchased from Harlan Europe and maintained in our in-house colony [Instituto Cajal, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain) according to the protocols of our institution. Animals were housed in cages with filter tops in a laminar flow hood and maintained on food and water *ad lib.* in a 12-h dark–light cycle. Five to six-week-old mice were inoculated intracerebrally in the right cerebral hemisphere with $10^6$ plaque-forming units (PFU) of Daniel's (DA) TMEV strain, which was generously donated by Dr. Moses Rodriguez (Mayo Clinic, USA), in 30 μL Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, as previously described [2, 4]. Handling of animals was performed in compliance with the guidelines of animal care set by the European Union (86/609/EEC) and Spanish regulations (BOE67/8509-12; BOE 1201/2005) on the use and care of laboratory animals and was approved by the local Animal Care and Ethics Committee of the CSIC.

Animal tissue was processed as previously described [14]. Briefly, mice were perfused transcardially with saline. Brains ($n$ = 5/group) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), washed in 0.1 M PB, cryoprotected with 15% and later 30% sucrose solution in 0.1 M PB, frozen at –80°C, and processed for OCT embedding. Pieces of brains were routinely processed for paraffin embedding and used for histopathological examination. Paraffin sections (5 μm thick) were routinely stained with hematoxylin and eosin and examined under a light microscope.

The following primary antibodies were used. Rat anti-galectin-3 monoclonal antibody was purified by affinity chromatography from the supernatant of hybridoma cells (clone TIB-166, M3/38.1.2.8. HL.2; American Type Culture Collection, USA) and used at a concentration of 1 μg/mL for immunohistochemistry. This antibody has been used to detect galectin-3 in various animal tissues, including mouse tissues [9]. Rabbit anti-ionized calcium binding adapter 1 (Iba-1) (Wako Chemical Pure Industry, Japan) and mouse anti-glial fibrillary acidic protein (GFAP) (Sigma, Spain) were used for the identification of microglia and astrocytes, respectively. For the identification of neurons, mouse anti-neuron specific nuclear protein (NeuN) (Millipore, USA) was used. For oligodendrocyte labeling, mouse monoclonal anti-adenomatus polyposis coli (APC) [Cat. no. OP80; CalBiochem (EMD Millpore), USA] was used. The secondary antibodies were Alexa Fluor 488-conjugated goat anti-rat secondary antibody, Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody, and Alexa Fluor 594-conjugated goat anti-mouse secondary antibody. All secondary antibodies were purchased from Molecular Probes (USA). All other reagents were obtained from standard suppliers.

Free-floating coronal brain sections (30 μm thick) were processed as described previously [14]. Briefly, free-floating sections were washed with 0.1 M PB three times for 10 min, permeabilized with PBT (PB with 0.2% Triton X-100) three times for 10 min, and then blocked with 5% normal goat serum (Vector Laboratories, UK) in PBT for 1 h at room temperature. The sections were then incubated overnight at 4°C with a primary antibody cocktail of rat anti-galectin-3 primary antibody (diluted 1 : 1,000) and either rabbit anti-Iba-1 antibody (diluted 1 : 1,000), mouse anti-GFAP antibody (diluted 1 : 1,000), mouse anti-NeuN (diluted 1 : 1,000), or anti-APC (diluted 1 : 1,000). The following day, the sections were rinsed with PBT and incubated for 1 h with a cocktail of Alexa Fluor 488-conjugated goat anti-rat IgG and either Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG. After three washings with PBS for 10 min, sections were incubated with 4',6-diamidino-2-phenylindole (DAPI) (diluted 1 : 1000) (Molecular Probes) in PBS for 30 min to visualize nuclear morphology. Then, sections were rinsed with PBS three times for 10 min and mounted. In all cases, specificity of immunofluorescence staining was confirmed by omitting the primary antibody. The sections were examined by confocal microscopy (Leica TCS SP5; Leica Microsystems, Germany).

## Results

Vehicle-injected mouse brains (Fig. 1A, C) showed no

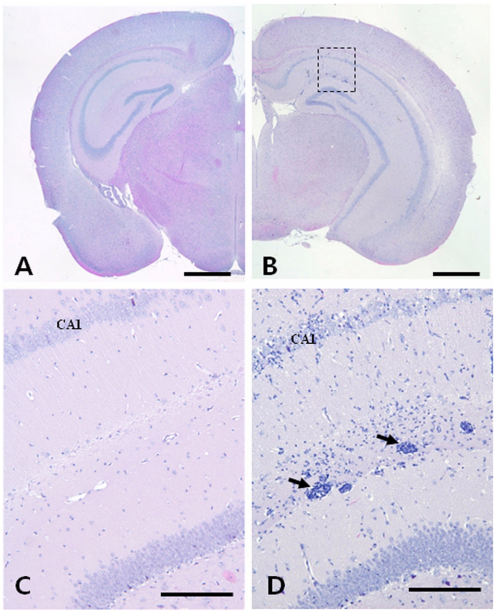


**Fig. 1.** Histological findings of vehicle-treated (A and C) and TMEV-infected (B and D) brains at day 7 post-infection. At low magnification, no histological abnormalities were seen in the vehicle-treated brain (A and C), whereas the brain of TMEV-infected mice showed infiltration of inflammatory cells throughout the brain, including the hippocampus (B and D). (C) and (D) show higher magnification of the hippocampus seen in (A) and (B). (D) is a higher magnification of the inset in (B) and shows severe encephalitis with perivascular cuffing (arrows). Scale bars = 1 mm (A and B). Scale bars = 200 μm (C and D).

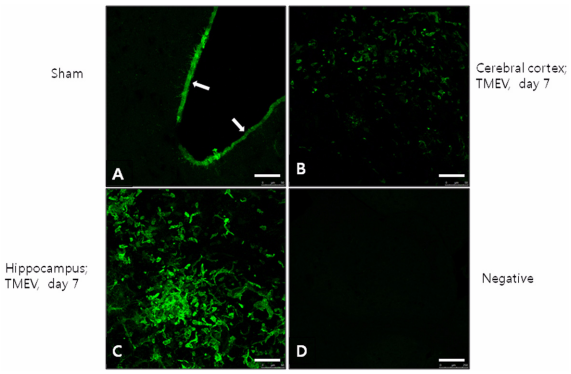


**Fig. 2.** Representative confocal images of galectin-3 in the brain of sham-infected (A) and TMEV-infected (B and C) mice. Galectin-3 was localized exclusively in ependymal cells in the third ventricle of sham-infected mice (A). In the brain with TMEV infection, galectin-3 was detected in the cerebral cortex (B) and hippocampus (C). (D) Negative control. Scale bars = 50 μm (A~D), 250 μm (D).

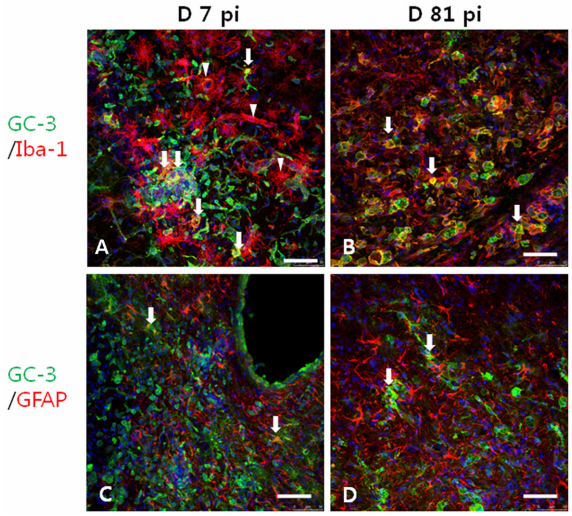


**Fig. 3.** Confocal merged images of galectin-3 (A~D, green) and either Iba-1 (A and B) or GFAP (C and D) in TMEV-infected brain at day 7 (A and C) and day 81 (B and D) post-infection. At day 7, galectin-3 (A, green) was occasionally colocalized with Iba-1-positive cells (A, arrows), whereas ramified Iba-1-positive microglia were devoid of galectin-3 in peripheral lesions (A, arrowheads). The majority of galectin-3-positive cells were colocalized with Iba-1 at day 81 post-infection (B, yellow, arrows). Galectin-3 (C and D, green) was occasionally colocalized in GFAP-positive cells (C and D, red), but the number of colocalized cells was a few (C and D, yellow, arrows) in the brain at both day 7 (C) and day 81 (D) post-infection. Scale bars = 50 μm (A~D).

inflammatory lesions. In TMEV-infected brains at day 7 post-inoculation (Fig. 1B, D), random infiltration of inflammatory cells was found throughout the brain tissues, including the cerebral cortex, hippocampus (Fig. 1D, arrows), striatum, and pons. Perivascular cuffings were also found in the brain parenchyma near the ventricles, including the lateral, third and fourth ventricles. The general features of neuropathology were similar to those in previous studies [11].

In sham-infected control brain, galectin-3 (Fig. 2A) immunostaining was observed in some ependymal cells (Fig. 2A, arrows) and lining ventricles, as shown previously [3], but not in neurons and glial cells. In TMEV-infected brains at day 7 post-infection (Fig. 2B and C), galectin-3-positive cells were randomly localized in various brain regions including the cerebral cortex (Fig. 2B) and especially the hippocampus (Fig. 2C). To study the cell phenotype of galectin-3 labeling in both the acute (7 days post-infection) and chronic phase (81 days post-infection) of TMEV-IDD, double immunofluorescence was carried out using cell-specific markers. At day 7 post-infection, galectin-3-positive cells were observed in the brain parenchyma (Fig. 3A, green), showing that galectin-3 was colocalized in a few Iba-1-positive cells with rounded morphology (Fig. 3A, yellow, arrows), whereas ramified Iba-1-positive microglial cells were not positive for galectin-3 (Fig. 3A, red, arrowheads). In the chronic lesion at day 81, the majority of cells expressing galectin-3 (Fig. 3B, green) were Iba-1-positive activated microglia/macrophages with rounded morphology (Fig. 3B, red) (Fig. 3B, yellow color with merged image, arrows), but galectin-3 never colocalized with Iba-1-positive cells with ramified morphology. Our data suggest that galectin-3 is only expressed in activated microglia/macrophages. Double immunofluorescence of galectin-3 (Fig. 3C and D, green) and GFAP (Fig. 3C and D, red) showed that a few astrocytes were positive for galectin-3 in acute (day 7 post-infection) (Fig. 3C, arrows) and chronic (day 81 post-infection) (Fig. 3D, arrows) TMEV infection. However, galectin-3 immunostaining was not observed in either NeuN-positive neurons or APC-positive oligodendrocytes (data not shown).

## Discussion

This is a first study to show that galectin-3 is upregulated in the brain of TMEV-infected mice. Histopathological changes induced by TMEV show infiltration of inflammatory cells in the form of perivascular cuffing throughout the brain lesions, including in the cerebral cortex and hippocampus, as shown in previous studies [11, 21].

Galectin-3 seems to function as a pro-inflammatory mediator in that its deficiency reduces EAE severity [8], and CNS inflammation accompanies an increase of galectin-3 [6, 16, 24]. However, a neuroprotective role for galectin-3 is suggested by the observation that galectin-3 knockout mice had defective microglial proliferation and a significant increase in ischemic lesions in a model of ischemic brain injury [12].

As for the cell type of galectin-3 expression in the CNS, there is general agreement that macrophages/activated microglia are the main cell phenotypes for galectin-3 expression during CNS inflammation with Junin virus infection [6], herpesvirus infection [24], ischemic injury [12], and EAE [19, 20]. Even though the expression of galectin-3 is very low in other glial cells, astrocytes and ependymal cells have been

suggested as another candidate for galectin-3 expression in the Junin-virus-infected mouse brain [6] and normal CNS development [3], respectively.

In the present study, we confirmed that galectin-3 was mainly found in macrophages/activated microglia, but not in ramified Iba-1-positive microglia, in acute (day 7 post-infection) and chronic (day 81 post-infection) TMEV infection. Here, we also found that a few astrocytes were able to express galectin-3 in TMEV-infected mice.

Galectin-3 expression in microglia is associated with phagocytosis of degenerating myelin *in vitro* and *in vivo* in EAE, but not in other conditions such as CNS Wallerian degeneration [20]. Additionally, galectin-3 is required for microglia activation and proliferation in brain ischemic injury [12].

Taken together, these findings lead us to postulate that galectin-3 mainly originates from macrophages/activated microglial cells, rather than ramified microglia, in acute as well as in chronic TMEV infection. It is postulated that galectin-3 facilitates migration of inflammatory cells and microglial proliferation in TMEV-infected brain, but further studies are necessary to demonstrate the functions of galectin-3 in TMEV-IDD.

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