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Inflammatory responses in Multiple Sclerosis normal-appearing white matter and in non-immune mediated neurological conditions with wallerian axonal degeneration: a comparative study.

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Abstract

Inflammatory-like changes in the white matter (WM) are commonly observed in conditions of axonal degeneration by different etiologies. This study is a systematic comparison of the principal features of the inflammatory-like changes in the WM in different pathological conditions characterized by axonal damage/degeneration, focusing in particular on Multiple Sclerosis (MS) normal-appearing white matter (NAWM) compared to non immune-mediated disorders. The study was performed on sections of NAWM from 15 MS cases, 11 cases of non immune-mediated disorders with wallerian axonal degeneration (stroke, trauma, amyotrophic lateral sclerosis), 3 cases of viral encephalitis, 6 control cases.

Common features of the inflammatory-like changes observed in all of the conditions of WM pathology were diffuse endothelial expression of VCAM-1, microglial activation with expression of M2 markers, increased expression of sphingosine receptors. Inflammation in MS NAWM was characterized, compared to non immune-mediated conditions, by higher VCAM-1 expression, higher density of perivascular lymphocytes, focal perivascular inflammation with microglial expression of M1 markers, ongoing acute axonal damage correlating with VCAM-1 expression but not with microglia activation.

Inflammatory changes in MS NAWM share all the main features observed in the WM in non immune-mediated conditions with wallerian axonal degeneration (with differences to a large extent more quantitative than qualitative), but with superimposition of disease-specific perivascular inflammation and ongoing acute axonal damage.

Keywords: Multiple Sclerosis (MS), normal-appearing white matter (NAWM), microglia, axon, inflammation, VCAM-1, sphingosine, S1P1, wallerian degeneration.

Background

A complex relationship exists between axonal damage and inflammation in the CNS white matter (WM). Inflammation can induce axonal dysfunction and damage, through mechanisms well characterized in immune-mediated disorders, for example as observed in acute Multiple Sclerosis (MS) demyelinating lesions [Smith KJ and McDonald WI, 2003]. However, also axonal damage/degeneration due to any other pathological condition determines secondary inflammatory-like changes in the WM, through yet not fully known damage signals [Hussain RH et al, 2014; Russo M and McGavern DB, 2015; Burda JE and Sofroniew MV, 2014; Amor et al, 2014]. These inflammatory changes can be observed in experimental models of CNS lesions and can be found in the WM in any non-immune mediated neurological disorder with wallerian axonal degeneration [Griffin et al, 1992; Raivich, 1998; Graves et al, 2004; Griffith et al, 2010; Palin et al, 2008]. They are known to differ both quantitatively and qualitatively from “real” brain inflammation that is observed in immune-mediated or infectious neurological disorders [Estes ML and Mc Allister AK, 2014; Graeber MB, 2014]. However, systematic studies evaluating differences and similarities between different conditions of WM pathology are lacking.

Widespread inflammatory changes in the normal-appearing white matter (NAWM), distant from focal demyelinating lesions, are a distinctive feature of MS [Kutzelnigg A et al, 2005; Filippi M and Rocca MA, 2005; Zeiss T et al, 2008; Frischer JM et al, 2009]. Several studies have described the pathological changes in MS NAWM, but little data is available on the specificity of such findings and on comparisons with other conditions of WM pathology from other causes. Widespread wallerian axonal degeneration is known to occur in MS NAWM [Evangelou et al, 2000; Casanova et al, 2003; Dzedzic et al, 2010].

The aim of this study was to perform a systematic comparison of the principal features of the inflammatory-like changes observed in the WM, in several pathological conditions characterized by axonal damage/degeneration. Another aim was also to add to current data the notion of what is actually specific to MS NAWM and what are instead more aspecific features of WM undergoing axonal damage.

The NAWM in MS was compared with the WM in non immune-mediated neurological conditions with axonal degeneration (NINCs) (such as wallerian degeneration in stroke or traumatic brain injury, or neurodegeneration of corticospinal tracts in ALS), and with the WM in infectious CNS disorders (with inflammation as an appropriate response to CNS infection, such as in viral encephalitis). We specifically addressed microglial activation, markers of M1/M2 macrophage differentiation, expression of vascular adhesion molecules, markers of lymphocytes and dendritic cells, expression of effectors of tissue damage, expression of sphingosine receptor and markers of acute axonal damage.

Materials and Methods

This study was performed on autoptic formalin-fixed, paraffin-embedded brains, assessing the following pathological conditions:

- MS NAWM, distant from focal demyelinating lesions (14 MS cases; 6 relapsing-remitting (RR) MS, 7 secondary progressive (SP) MS, 1 hyperacute MS)
- pure wallerian degeneration (pontine and spinal corticospinal tracts degeneration after a middle cerebral artery ischemic stroke: 1 case)
- wallerian degeneration and focal axonal damage (diffuse WM injury after brain trauma resulting in vegetative state: 4 cases)
- neurodegenerative disorders with axonal degeneration (corticospinal tracts degeneration in amyotrophic lateral sclerosis (ALS): 6 cases)
- infectious CNS disorders, with WM inflammation as an appropriate response to CNS infection (HSV-1 viral encephalitis in immunocompetent host: 3 cases)

Samples from 6 brains of patients without neurological disorders were used as controls.

For each case, tissue blocks were selected including the corticospinal tracts in the cervical spinal cord and in the pons; the lateral portion of the temporal lobe was selected for the viral encephalitis cases. In MS brains only NAWM areas were selected, defined by absence of demyelination (normal Luxol staining and MBP immunostaining), at least 1 cm distant from focal demyelinating lesions.

WM areas in diffuse traumatic brain injury were selected excluding areas of focal necrosis or haemorrhage. In viral encephalitis cases, temporal lobe subcortical WM was selected excluding areas of focal necrosis or haemorrhage.

Tissue was obtained from the archives of the University of Turin and the University of Genoa. Post-mortem interval was less than 36 hours in all cases and controls.

Mean age of death in MS cases was 50.2 years (range 27-66 years); mean duration of disease course was 15.1 years (range 6 months-30 years). Mean age of death was 59.4 years (range 52-65 years) in control cases, 57.2 years (range 42-72 years) in the cases of non immune-mediated neurological conditions with axonal degeneration. Disease course in MS cases (RR or SP MS) was retrospectively defined from hospital records. The clinical features of all cases are summarized in Supplementary Table 1.

Histology and immunohistochemistry

Consecutive 5 μm sections were obtained. Standard hematoxylin/eosin, Luxol and Bodian stainings were performed for each section. Immunohistochemistry was performed with the antibodies listed in Supplementary Table 2 (HLA-DR, CD68, CD163, mannose receptor, iNOS, MMP-9, MPO, CD40, CD3, CD20, CD138, CD83, CD86, fibrinogen, IgG, VCAM-1, ICAM-1, S1P1, S1P3, APP, MBP). After deparaffinization, sections were treated with 3% H_2O_2 for 10 minutes and then processed for antigen retrieval; details on antigen retrieval are listed in Table 1. The sections were incubated with 10% normal serum for 30 minutes; they were later incubated overnight with the primary antibodies. After washing with TBS, the sections were incubated at room temperature for 30 minutes with the Envision complex (Dako, Glostrup, Denmark). Peroxidase labeling was visualized with 10% 3,3-diaminobenzidine. Sections were counterstained with hematoxylin. Double immunohistochemistry was performed as needed to confirm colocalization of antigens. Immunohistochemistry was performed as described above. First, peroxidase labeling for the first antibody was visualized with 10% 3,3-diaminobenzidine, then immunohistochemistry for the second antibody was performed after blocking with normal serum, and peroxidase labeling was visualized with VIP red (Vector Laboratories, Burlingame, USA).

Image acquisition

The sections were examined using either a Zeiss Axiophot microscope or a Zeiss Axio Imager.A1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were acquired using either a Nikon Digital Sight DS-DM camera (Nikon Corporation, Tokyo, Japan) or a Zeiss AxioCam MRc5 camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Evaluation of immunohistochemistry, statistical analysis

The percentage of vessels showing VCAM-1 endothelial immunostaining was assessed in 100X microscopic fields in at least 40 fields for each tissue block; all blood vessels were identified on a morphological basis in high magnification (100X) microscopic fields, by two different pathologists (M.V. and P.C.)

The number of HLA-DR+ cells, CD3+ lymphocytes, CD20+ lymphocytes, CD163+ cells was assessed in 40X microscopic fields in at least 40 fields for each tissue block, and expressed as number of elements/ mm^2 .

The number of APP+ axonal spheroids was assessed in 100X microscopic fields in at least 40 fields for each tissue block, and expressed as number of elements/ mm^2 . Axonal loss, deformation and thickening in MS NAWM were assessed on a visual basis in Bodian-stained sections.

Immunostaining for S1P1, mannose receptor, CD138, CD40, iNOS, MMP-9, CD83, CD86,

fibrinogen, IgG was assessed by two different observers, using a semi-quantitative scoring system (-, +, ++, +++), in 20X microscopic fields in at least 40 fields for each tissue block.

Differences in the percentage of VCAM-1+ vessels, and in the number of HLA-DR+ cells, CD3+ lymphocytes, CD20+ lymphocytes, CD163+ cells, APP+ axonal spheroids between MS NAWM, WM in NINCs and control WM was assessed using the Mann-Whitney U test.

Correlations between the percentage of VCAM-1+ vessels, and the number of HLA-DR+ cells, CD3+ lymphocytes, CD20+ lymphocytes, CD163+ cells, APP+ axonal spheroids, and between the above and clinical parameters (age, MS disease duration, MS disease course), were evaluated using the Spearman's Rank Correlation Test.

Results

Vascular adhesion molecules expression, blood-brain barrier disruption

- Findings in MS

In MS NAWM, endothelial immunostaining for the vascular adhesion molecule VCAM-1 was detected on most vessels (mean percentage of VCAM-1 positive vessels in MS NAWM $68.62\% \pm 8.63$) (Table 1, Figure 1A, 1B). Fibrinogen and IgG immunostaining was detected only inside blood vessels and not in the adjacent parenchyma (Figure 2A, Table 1). IgG immunostaining was however detected on some axons in MS NAWM.

The percentage of VCAM-1 positive vessels was significantly higher in progressive MS than in RRMS cases (mean percentage of VCAM-1 positive vessels RRMS $63.14\% \pm 7.36$; mean percentage of VCAM-1 positive vessels progressive MS $73.42\% \pm 6.80$; $p = 0.015$) (Figure 7A). A significant correlation was found, in MS NAWM, between the percentage of VCAM-1+ vessels and the number of CD3+ T-lymphocytes (Rho 0.800; $p < 0.0001$) (Figure 8A). A significant correlation was also found between the percentage of VCAM-1+ vessels and the number of APP+ axonal spheroids (Rho 0.658; $p = 0.008$) (Figure 8B). The percentage of VCAM-1+ vessels also correlated with age (Rho 0.640; $p = 0.010$) and disease duration (Rho 0.759; $p = 0.003$) (Figure 8C).

- Findings in NINCs

In the WM in NINCs, VCAM-1 endothelial immunostaining was detected on vessels (Figure 1C, 1D), but to a significantly lower extent than in MS NAWM (mean percentage of VCAM-1 positive vessels $39.77\% \pm 13.16$; $p < 0.0001$) (Table 1, Figure 7B). Fibrinogen and IgG immunostaining was detected only inside blood vessels.

- Findings in controls

In the WM in control brains no VCAM-1 endothelial immunostaining was observed (Table 2, Figure 1E). Fibrinogen and IgG immunostaining was detected only inside blood vessels.

- Findings in viral encephalitis

In viral encephalitis WM, intense VCAM-1 immunoreactivity was observed on most blood vessels, and frequent fibrinogen and IgG immunostaining in the parenchyma was detected, suggesting overt blood-brain barrier disruption.

Endothelial immunostaining for the vascular adhesion molecule ICAM-1 was only very rarely detected in MS NAWM, and never observed in the WM in NINCs or in controls (while it was quite common in viral encephalitis).

Lymphocytes, plasmacells, dendritic cells

- Findings in MS

In MS NAWM, a few perivascular lymphocytes were observed: mainly CD3+ T-lymphocytes (Figure 1F), uncommon CD20+ B-lymphocytes (Figure 2B). CD138+ plasmacells were very rare. Rare CD3+ T-lymphocytes were sometimes detected also in the parenchyma close to blood vessels. Mean density of CD3+ T-lymphocytes in MS NAWM was $2.54 \text{ cells/mm}^2 \pm 0.76$, while mean density of CD20+ B-lymphocytes was $0.33 \text{ cells/mm}^2 \pm 0.20$ (Table 2). The number of CD3+ T-lymphocytes in MS NAWM correlated with disease duration (Rho 0.725; $p = 0.005$).

A few CD83+ mature dendritic cells were detected in MS NAWM, in the perivascular space and in the parenchyma close to blood vessels (Table 1, Figure 2C).

- Findings in NINCs

In the WM in NINCs, very few perivascular CD3+ T-lymphocytes were observed (Figure 1G), almost ten times less frequent than in MS NAWM (mean density of CD3+ T-lymphocytes $0.29 \text{ cells/mm}^2 \pm 0.39$; $p < 0.0001$) (Table 1, Figure 7C); no CD3+ T-lymphocytes were found in the parenchyma. No CD20 or CD138 stained cells were found.

No CD83+ mature dendritic cells were found in the WM in NINCs (Table 1).

- Findings in controls

In control WM no perivascular or parenchymal CD3, CD20, CD138 stained cells were observed (Table 1). No CD83+ mature dendritic cells were found in the WM in controls (Table 1).

- Findings in viral encephalitis

In viral encephalitis WM, strong and diffuse lymphocyte infiltration was observed. Perivascular cuffs of CD3+ T-lymphocytes and CD20+ B-lymphocytes were observed close to many blood vessels; CD3+ T-lymphocytes were often found also in the parenchyma (Table 1, Figure 1H).

Perivascular and parenchymal CD83+ dendritic cells were found in viral encephalitis.

Microglia/macrophages phenotype

Microglia activation

- Findings in MS

Intense HLA-DR immunostaining on microglia was detected in the NAWM in all MS brains. Mean density of HLA-DR positive cells in MS NAWM was $56.85 \text{ cells/mm}^2 \pm 32.93$ (Table 1, Figure 3A, 3B). Microglia nodules were frequently detected in focal areas of more intense NAWM inflammation (Figure 3C). Perivascular macrophages were common in MS NAWM; these also showed HLA-DR immunostaining.

A correlation was found between density of HLA-DR positive cells in MS NAWM and age (Rho 0.533; $p = 0.033$), disease duration (Rho 0.626; $p = 0.022$), percentage of VCAM-1+ vessels (Rho 0.744; $p = 0.008$).

- Findings in NINCs

Intense HLA-DR immunostaining was observed on microglia in the WM also in NINCs (Figure 3D, 3E), although to a slightly lower extent than in MS NAWM (mean $41.70 \text{ cells/mm}^2 \pm 22.79$; $p = 0.188$) (Table 1, Figure 7D). Microglial nodules were few or absent.

- Findings in controls

In control WM, HLA-DR immunostaining was observed only in rare cells inside blood vessels, probably circulating leukocytes, while no appreciable immunostaining was found in the brain parenchyma (Table 1).

- Findings in viral encephalitis

In viral encephalitis WM, diffuse activation of microglia was observed, expressing intense HLA-DR immunoreactivity; microglia nodules were common (Table 1, Figure 3F).

Effectors of tissue damage

- Findings in MS

In MS NAWM, no iNOS or MMP-9 immunostaining was found, except in focal areas close to some blood vessels, associated with a higher density of activated microglia, in which weak iNOS and even weaker MMP-9 immunostaining was observed (Table 1, Figure 4A). MPO immunostaining in MS NAWM was detected only on occasional intravascular cells, probably circulating leukocytes (Table 1).

-Findings in NINCs and controls

In the WM in NINCs or in controls, no iNOS, MMP-9 or MPO immunostaining was detected in the parenchyma; MPO immunostaining was detected on rare intravascular cells (Table 1).

-Findings in viral encephalitis

In viral encephalitis WM, iNOS and MMP-9 immunoreactivity was diffusely observed on microglia and macrophages (Table 1, Figure 4B).

Markers of M1/M2 differentiation

- Findings in MS

Expression of costimulatory molecules CD40 and CD86 was observed only in focal areas close to some blood vessels in MS NAWM (Table 1, Figure 2E, 2D).

Expression of CD163, a marker highly expressed in alternatively activated M2 macrophages [Moestrup SK and Møller HJ, 2004], was observed on perivascular leukocytes in MS NAWM, as well as on some cells in the parenchyma (Table 1, Figure 4D).

- Findings in NINCs

In the WM in NINCs, CD40 immunostaining was almost absent (Table 1), except for some very weak expression in ALS corticospinal tracts. No CD86 immunostaining was observed.

Expression of CD163 in the WM in NINCs followed the same pattern observed in MS NAWM, with presence of both perivascular and some parenchymal CD163+ leukocytes (Table 1, Figure 4E). The mean number of CD163+ cells was significantly higher in MS NAWM ($25.16 \text{ cells/mm}^2 \pm 26.44$) and in the WM in NINCs ($30.40 \text{ cells/mm}^2 \pm 28.86$), if compared to control WM ($5.41 \text{ cells/mm}^2 \pm 6.44$) ($p < 0.0001$). It did not differ however between MS NAWM and WM in NINCs ($p = \text{n.s.}$) (Table 1, Figure 7D).

- Findings in controls

No CD40 or CD86 immunostaining was found in control WM.

CD163 expression was observed only on perivascular cells in control WM (Table 1, Figure 4F).

- Findings in viral encephalitis

In viral encephalitis WM, diffuse intense CD40 immunostaining was observed (Table 1, Figure 2F); CD86 positive cells were also common.

A very high number of CD163+ cells were observed in the parenchyma and in perivascular cuffs in viral encephalitis WM (Table 1, Figure 4G).

Expression of mannose receptor, a marker of CNS perivascular macrophages (perivascular macrophages expressing the mannose receptor are known to be the only constitutively phagocytic cells in the normal CNS) [Galea I et al, 2005], was observed only on perivascular macrophages. No appreciable differences were found in mannose receptor expression between MS NAWM, WM in NINCs and control WM (Table 1, Figure 4C).

Sphingosine receptors expression

- Findings in MS

Diffuse S1P1 expression was detected on glial cells in MS NAWM (Table 1, Figure 5A). S1P3 expression was detected mainly on hypertrophic reactive astrocytes in the WM neighbouring demyelinating lesions; weak expression of S1P3 was found on glial cells in MS NAWM.

- Findings in NINCs

Diffuse expression of S1P1 on glial cells was observed also in the WM in NINCs (Table 1, Figure 5B), with an extent comparable to MS NAWM. Weak expression of S1P3 was found on glial cells in the WM in NINCs.

- Findings in controls

In control WM, S1P1 expression on glial cells was weak and detectable only on a low number of cells (Table 1, Figure 5C).

- Findings in viral encephalitis

Diffuse intense S1P1 and S1P3 expression was observed on glial cells in viral encephalitis WM.

Axonal pathology

Presence of thickened and grossly distorted axons was a common feature in MS NAWM.

Uncommon APP+ axonal spheroids, indicating acute axonal damage, were detected in the NAWM in most MS cases (mean density of APP+ axonal spheroids $7.11/\text{mm}^2 \pm 5.67$) (Figure 5D).

No APP+ axonal spheroids were detected in the WM in NINCs (with the exception of the cases of diffuse traumatic brain injury) or in control WM.

Discussion

This is a systematic study of comparison of different patterns of WM inflammatory changes by different etiologies, focusing in particular on MS NAWM, in comparison with non immune-mediated neurological conditions (NINCs) with inflammatory changes in the WM secondary to wallerian axonal degeneration, and with infectious neurological disorders. This also to add to current data the notion of what is actually specific to MS NAWM and what are instead aspecific features of WM pathology shared with NINCs.

In our series, diffuse inflammatory changes and ongoing axonal damage are always observed in the NAWM in MS brains, with extent increasing along with age and duration of disease, also in progressive MS cases with very long disease duration. Several features observed in MS NAWM appear however to be not specific, being found in the WM (although to a relatively lower extent)

also in conditions of axonal degeneration in non primarily inflammatory neurological disorders, and are probably part of a general aspecific response to WM damage.

Common features of the aspecific inflammatory-like changes observed in WM pathology by different etiologies were endothelial expression of vascular adhesion molecules (VCAM-1), recruitment of T-lymphocytes, sphingosine receptor upregulation, diffuse activation of microglia with increased expression of M2 macrophages markers. The aspecific inflammatory-like changes observed in the WM in any condition of focal damage or in wallerian degeneration are probably instrumental for the removal of myelin and axonal debris, and for the presentation of antigens to T-cells for routine immune surveillance; reparative and neuroprotective roles are also hypothesized [Lowenstein, 2002; Griffiths et al, 2010; Griffin et al, 1992; Ousman SS and Kuber P, 2012; Estes ML and Mc Allister AK, 2014; Graeber MB, 2014; Amor et al, 2014].

Inflammation in MS NAWM was characterized by higher expression of vascular adhesion molecule VCAM-1 and by far greater number of T-lymphocytes, and furthermore by presence of functional APCs (mature dendritic cells). Focal switching of microglia close to blood vessels to a pro-inflammatory M1 phenotype, with expression of effectors of tissue damage, was observed in MS NAWM only. These features probably represent the superimposition of disease-specific immune responses on the aspecific inflammatory-like response to tissue damage.

Upregulation of VCAM-1 on endothelia, together with microglial activation, appears to be a prominent feature both in MS NAWM and in the WM in NINCs (although significantly higher in MS NAWM). In presence of CNS tissue damage, endothelial expression of VCAM-1 probably promotes selective entry of T-lymphocytes in the CNS [Engelhardt B and Ransohoff RM, 2005] for routine immune surveillance [Lowenstein PR, 2002; Engelhardt B and Ransohoff RM, 2012]. VCAM-1 endothelial expression does not appear alone to be sufficient for the persistence of T-lymphocytes in the WM: this is in fact not observed in the WM in NINCs, despite the presence of widespread endothelial VCAM-1 immunoreactivity. Presence of perivascular functional APCs (mature dendritic cells) is another distinctive feature observed in MS NAWM only; these may allow effective presentation of antigens to infiltrating T-cells, contributing to the persistence of T-lymphocytes in MS NAWM [Plumb et al, 2003; Serafini et al, 2006; Windhagen et al, 1995; Greter et al, 2005; McMahon et al, 2006]. VCAM-1 expression in MS NAWM significantly correlates with age and disease duration, and is higher in SPMS than in RRMS.

Diffuse microglial activation with HLA-DR upregulation is found in MS NAWM and in the WM in NINCs. The phenotype of microglia does not however suggest a full activation with a pro-inflammatory phenotype such as observed in viral encephalitis. Both in MS NAWM and in the WM in NINCs, microglia does not generally express effectors of tissue damage (iNOS, MPO, MMP-9). Likewise, markers of M1 proinflammatory macrophages (such as CD40 and CD86) are not usually expressed in MS NAWM or in NINCs. Conversely, the expression of a marker of M2 alternatively-activated macrophages such as CD163 is increased both in MS NAWM and in NINCs if compared to controls. In MS NAWM, however, the expression of M1 macrophage markers and of effectors of tissue damage (iNOS, MMP-9) can be observed in focal areas close to some blood vessels, associated with a higher local density of activated microglia. This perivascular inflammation observed only in MS NAWM might represent the superimposition of disease-specific immune responses. Overall, activated microglia in MS NAWM might not play a major role in furthering axonal damage [Melief et al, 2013], except in such focal perivascular areas in which it appears to be shifted to a pro-inflammatory phenotype. Accordingly, in MS NAWM acute axonal damage does not correlate with the extent of diffuse microglial activation, but rather with the expression of VCAM-1 on endothelia. While the overall number of axons showing ongoing signs of acute damage in MS NAWM appears to be very low, it might become important in the long term in a chronic disease such as MS.

Sphingosine receptors expression on glial cells is increased equally both in MS NAWM and in the WM in NINCs, likely as another non disease-specific response to tissue damage. Upregulation of

S1P1 and S1P3 has also been observed in experimental models of CNS injury [Van Doorn R et al, 2010; Wei et al, 2011; Li C et al, 2012; Norimatsu et al 2012].

In conclusion, aspecific inflammatory-like changes (endothelial VCAM-1 expression, recruitment of T-lymphocytes, sphingosine receptors upregulation, activation of microglia with increased expression of M2 markers) are shared between several conditions of WM pathology by different etiologies, including also MS NAWM. All these probably play a role in the routine immune surveillance of the CNS. MS therapies interfering with these mechanisms, such as natalizumab (targeting VCAM-1/VLA-4 interaction) or fingolimod (targeting sphingosine receptors), are in fact associated with a risk of CNS infection by opportunistic viruses (i.e. JC virus) [D'Amico E et al, 2016]. Inflammatory changes in MS NAWM share all the main features observed in the WM in non immune-mediated conditions with wallerian axonal degeneration, with differences to a large extent more quantitative than qualitative. In MS NAWM, in addition, disease-specific immune responses appear to be superimposed on the aspecific inflammatory-like response to tissue damage, and may be responsible of detrimental perivascular inflammation and additional acute axonal damage.

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Figure legends:

Figure 1

- A: VCAM-1+ endothelial immunostaining in MS corpus callosum NAWM (20X). Scale bar 50 μ m.
B: VCAM-1+ endothelial immunostaining in MS spinal cord (corticospinal tracts) NAWM (20X). Scale bar 50 μ m.
C: VCAM-1+ endothelial immunostaining in ALS spinal cord (corticospinal tracts) WM (20X). Scale bar 50 μ m.
D: VCAM-1+ endothelial immunostaining in ALS spinal cord (corticospinal tracts) WM (40X). Scale bar 25 μ m.
E: VCAM-1, control WM (20X). Scale bar 50 μ m.
F: perivascular CD3+ T-lymphocytes in MS corpus callosum NAWM (20X). Scale bar 50 μ m.
G: rare isolated perivascular CD3+ T-lymphocyte in ALS spinal cord (corticospinal tracts) WM (20X). Scale bar 50 μ m.
H: thick perivascular and parenchymal CD3+ T-lymphocytes infiltrates in viral encephalitis subcortical WM (10X). Scale bar 100 μ m.

Figure 2

- A: absent fibrinogen leakage outside blood vessels in MS corpus callosum NAWM (10X). Scale bar 100 μ m.
B: perivascular CD20+ B-lymphocytes, MS subcortical NAWM (20X). Scale bar 50 μ m.
C: perivascular CD83 immunostaining, MS corpus callosum NAWM (20X). Scale bar 50 μ m.
D: perivascular CD86 immunostaining, MS corpus callosum NAWM (40X). Scale bar 25 μ m.
E: CD40+ cells in MS corpus callosum NAWM (20X). Scale bar 50 μ m.
F: CD40+ cells in viral encephalitis subcortical WM (20X). Scale bar 50 μ m.

Figure 3

- A: HLA-DR. MS spinal cord NAWM (2.5X). Scale bar 400 μ m.
B: HLA-DR. ALS spinal cord WM (2.5X). Scale bar 400 μ m.
C: HLA-DR. MS corpus callosum NAWM (20X). Scale bar 50 μ m.
D: HLA-DR. Diffuse traumatic brain injury subcortical WM (20X). Scale bar 50 μ m.
E: HLA-DR. Microglia nodule in MS NAWM (40X). Scale bar 25 μ m.
F: HLA-DR. Viral encephalitis subcortical WM (10X). Scale bar 100 μ m.

Figure 4

- A: localized weak iNOS immunostaining in MS corpus callosum NAWM (40X). Scale bar 25 μ m.
B: diffuse iNOS immunostaining in viral encephalitis subcortical WM (10X). Scale bar 100 μ m.
C: Mannose receptor positive perivascular macrophages in MS corpus callosum NAWM (20X). Scale bar 50 μ m.
D: CD163+ leukocytes in MS corpus callosum NAWM (20X). Scale bar 50 μ m.
E: CD163+ leukocytes in corticospinal tracts degeneration after middle cerebral artery ischemic stroke (pons corticospinal tracts) (20X). Scale bar 50 μ m.
F: perivascular CD163+ leukocyte in control spinal cord WM (20X). Scale bar 50 μ m.
G: CD163+ leukocytes in viral encephalitis subcortical WM (20X). Scale bar 50 μ m.

Figure 5

- A: S1P1 glial immunostaining in MS pons corticospinal tracts NAWM (20X). Scale bar 50 μ m.
B: S1P1 glial immunostaining in ALS pons corticospinal tracts WM (20X). Scale bar 50 μ m.
C: S1P1 glial immunostaining in control pons corticospinal tracts WM (20X). Scale bar 50 μ m.
D: APP+ axonal transection spheroids in MS subcortical NAWM (20X). Scale bar 50 μ m.

Figure 7

- A: % of VCAM-1+ vessels in RRMS NAWM vs progressive MS NAWM
- B: % of VCAM-1+ vessels in control WM, MS NAWM and WM in NINCs
- C: Density of CD3+ T-lymphocytes in control WM, MS NAWM and WM in NINCs (cells/mm²)
- D: Density of HLA-DR+ cells in control WM, MS NAWM and WM in NINCs (cells/mm²)
- E: Density of CD163+ cells in control WM, MS NAWM and WM in NINCs (cells/mm²)

Figure 8

- A: Correlation between % of VCAM-1+ vessels and density of CD3+ T-lymphocytes (cells/mm²) in MS NAWM
- B: Correlation between % of VCAM-1+ vessels and density of APP+ axonal spheroids (/mm²) in MS NAWM
- C: Correlation between % of VCAM-1+ vessels in MS NAWM and disease duration (data on

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