In multiple sclerosis lesions macrophages display markers for both classically and alternatively activated phenotype
Chapter 8

ABSTRACT:

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by demyelinating lesions associated with inflammatory infiltrates. Macrophages play a prominent role, with diverging effects possibly associated with different states of activation. The two extreme states of activation are: classically activated (CA/M1), pro-inflammatory, macrophages and alternatively activated (AA/M2), growth promoting, macrophages. Neuropathological reports indicate that these different subtypes are present in MS lesions, however, no systematic exploration of the macrophage phenotype in MS lesions has been performed. The aim of this study is to determine the phenotype of the macrophages present in MS lesions.

Based on literature, we selected a panel of markers for the different activation states. FACS analysis of human macrophages stimulated in vitro with LPS and interferon-gamma (IFN-γ) to induce CA macrophages, showed that Fc-gamma-receptor (FcγRI) and CD40 were significantly upregulated on CA macrophages. Of the AA phenotype markers, only the mannose receptor (MR) was significantly upregulated after stimulation of human macrophages in vitro with interleukin (IL)-4 and -10. Expression of inducible nitric oxide synthase (iNOS), CD68, FcyRII, CD86, factor XIIa (FXIIa), CD163 and E-cadherin was not significantly different between CA and AA macrophages after in vitro stimulation. Macrophages in active and chronic active MS lesions expressed both CA and AA markers, with a higher expression of CA markers. Microglia in the surrounding normal appearing white matter (NAWM) expressed CA markers. The AA markers were predominantly expressed by perivascular macrophages (PVM).

These findings indicate that in MS lesions macrophages posses a CA phenotype based on the markers investigated.
Markers for CA and AA macrophages in MS lesions

INTRODUCTION:

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of the central nervous system (CNS). It is the most common cause of neurological disability among young adults, affecting approximately one in 1000 individuals in Europe and Northern America.1 Major pathological hallmarks of MS are multiple demyelinated lesions, which are associated with perivascular infiltrates, astrogliosis, axonal damage and axonal loss.2,4

Macrophages are present in high numbers in MS lesions and are correlated with lesion activity.5 In fact, their presence is used as a marker for lesion activity.2,6,7 Upon activation macrophages secrete a plethora of mediators, such as pro-inflammatory cytokines, nitric oxide (NO), reactive oxygen species (ROS) and glutamate, which are able to induce damage to oligodendrocytes and neurons.8-13 A decrease in the expression of CD200 and CD47, macrophage inhibitory molecules, was found in MS lesions indicating that release of inhibition of macrophages could play a role in MS.14 Injection of clodronate liposomes, which eliminates infiltrating macrophages, suppressed both axonal damage and the clinical signs of experimental autoimmune encephalomyelitis (EAE) 15,16, an animal model of MS. Macrophages are associated with axonal damage that in MS lesions.17-20 Altogether, these reports point to a role for macrophages in tissue damage in MS lesions.

However, the role that macrophages play is more complex and not only detrimental, since they also exert beneficial effects during MS. Phagocytosis of myelin debris by macrophages is considered beneficial, since myelin debris is growth inhibiting for both axonal sprouting and remyelination.21-23 In addition, macrophages may be involved in remyelination.24-26 Macrophages are involved in neuronal regeneration and repair27-29 and in addition express neurotrophic factors.30-32

Macrophages are not one homogeneous population and many different activational subpopulations exist.33-35 An increasing body of evidence exists indicating that environmental signals contribute to this heterogeneity by influencing the activational status of macrophages.36-39 Macrophages are subdivided into different activational states with distinct functions, the two most extreme phenotypes are classically activated (CA) and alternatively activated (AA) macrophages.38,39 CA macrophages are generated in vitro by stimulation of macrophages with IFN-γ and LPS. They are considered to be cytotoxic and proinflammatory.38,39 Generation of AA macrophages is not straightforward, since various stimuli, not leading to a CA phenotype, have been designated as inducing alternative activation.39 Therefore, alternatively activated macrophages can display a wide variety of phenotypical and functional characteristics. Mosser et al. introduced 2 categories in AA macrophages: wound healing and regulatory39. Wound healing macrophages are generated using IL-4 or IL-13. They are involved in tissue repair since they secrete extracellular matrix (ECM) molecules and anti-inflammatory cytokines. These macrophages do not produce pro-inflammatory cytokines.38,39 Regulatory macrophages are generated by exposure to IL-10 or glucocorticoids. They are potent inhibitors of inflammation, but they do present antigens to T-cells.38,39
Although the functional differences of the various activation states of the macrophages have been well characterized, evaluation of marker expression has been more challenging, since not one marker is exclusively expressed on either phenotype. Furthermore, marker expression differs between species. For CA macrophages, the production of NO is a reliable marker for mouse CA macrophages, while in human macrophages NO production is not. A panel of putative markers defining the phenotypes of CA and AA macrophages has been described in literature (Table 1). The most consistent and oldest marker for the AA phenotype is mannose receptor (MR). Next to MR, the markers CD163, factor XIIIa (FXIIIa) and E-cadherin are preferentially expressed by the AA phenotype. Recently CD40 was established as a marker for the CA phenotype. Furthermore, FcγRI and II, CD86 and inducible nitric oxide synthase (iNOS) are upregulated on CA macrophages.

In MS lesions macrophages might play different roles depending on their activational phenotype. Several reports indicate that differently activated macrophages are present in MS lesions. Foamy macrophages in MS lesions express anti-inflammatory cytokines without pro-inflammatory cytokines, indicating an alternative activation. Indications that CA macrophages are present in MS lesions are supported by the expression of iNOS, FcγR and CD40 by macrophages in MS lesions. Untill now, the distribution of AA and CA markers on macrophages in MS lesions has not been studied systematically. In this study the expression of a panel of markers for CA and AA macrophages was investigated in MS lesions. These markers were tested on their differential expression on CA and AA macrophages in vitro using FACS analysis. The results indicate that the CA phenotype is dominant in active and chronic active MS lesions.

**MATERIALS AND METHODS:**

**Autopsy material**

Human brain tissue was obtained at autopsy from one case without neurological complications (control) and from nine MS patients. Patient characteristics are listed in Table 2. The rapid (short post-mortem delay) autopsy regimen of the Netherlands Brain Bank in Amsterdam (coordinator dr. I. Huitinga) was used to acquire the samples, with the approval of the Medical Ethical Committee of the VU University Medical Center. Tissue samples from subcortical white matter or corpus callosum were obtained from control cases. For MS tissue, the clinical diagnosis
was confirmed neuropathologically by Prof P. van der Valk and Dr. W. Kamphorst (department of Pathology, VU medical center). Tissue samples from MS cases were obtained after ex vivo MRI scanning of the autopsy brain slices, as described by de Groot et al. 7. Brain tissue samples were snap-frozen and stored in liquid nitrogen.

Classification of lesion staging was based on standard histopathological stainings for inflammatory cells (anti-major histocompatibility complex (MHC) class II) and myelin (anti-proteolipid protein (PLP)) as described before 44,45. Based on these findings five lesions sampled in this study were classified as active with myelin loss and abundant perivascular and parenchymal macrophages. Four lesions were identified as chronic active with demyelinated areas containing fewer MHC class II-positive cells and a hypercellular lesion rim.

<table>
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<tr>
<th>Number</th>
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<th>Sex</th>
<th>Disease duration</th>
<th>PMD (hrs:min)</th>
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<td>F</td>
<td>Pneumonia</td>
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<tr>
<td>MS lesions</td>
<td>5</td>
<td>50 ± 9.5</td>
<td>3 M, 2 F</td>
<td>22 ± 14</td>
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<tr>
<td>Active</td>
<td>4</td>
<td>60.5 ± 26.7</td>
<td>3 M, 1 F</td>
<td>23 ± 3</td>
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</table>

Table 2: Details of autopsy brain tissue

**Human macrophages**

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donoruffy coats (Sanquin Blood Bank, Amsterdam, The Netherlands) using Ficoll (Lymphoprep™, Axis-Shield, Oslo, Norway) density gradient. Afterwards, monocytes were isolated by Percoll (GE Healthcare, Uppsala, Sweden) density centrifugation, resulting in >80% pure monocytes. Monocytes were cultured in 6-wells plates (Greiner Bio-One; Alphen a/d Rijn, the Netherlands) at a concentration of 2x10^6 cells/ml in macrophage medium (RPMI (Invitrogen, Breda, the Netherlands), supplemented with 5% (v/v) human AB serum (Sanquin Blood Bank), and 1% (v/v) penicillin-streptomycin-glutamine (PSG) (Invitrogen)), at 37°C, 5% CO₂. Monocytes differentiated into macrophages in the course of 5-7 days of culturing. Before each experiment macrophages were washed to remove non-adherent cells, resulting in >95% pure macrophages cultures.

The CA phenotype was generated using 5x10³ U/ml recombinant human IFN-γ (U-Cytech, Utrecht, the Netherlands) and 10 ng/ml Escherichia coli LPS (026:B6; Sigma-Aldrich, Zwijndrecht, the Netherlands) for 48 h. To induce the AA phenotype macrophages were exposed to 10 ng/ml human IL-4 and IL-10 (ImmunoTools; Friesoythe, Germany) for 48 h.

**FACS analysis**

For FACS analysis, macrophages were washed twice with PBS and incubated with the first antibody (for list see Table 3) for 1 h. After washing, cells were incubated with the fluorescently labeled secondary antibody for 1 h. The macrophages were analysed using flow cytometry (FACS Calibur, Becton Dickinson, Erembodegem,
Belgium) combined with Cellquest Pro software (Becton Dickinson). As a negative control, omission of the primary antibody was included.

<table>
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<tr>
<th>Antigen</th>
<th>animal</th>
<th>Dilution</th>
<th>2nd Antibody</th>
<th>dilution</th>
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<td>1:400</td>
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<td></td>
<td></td>
<td>Goat-anti-mouse biotinylated</td>
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<td>NOS2 (Santa Cruz; Huissen, the Netherlands)</td>
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<td>Goat-anti-mouse 488</td>
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<td></td>
<td></td>
<td>Goat-anti-mouse bio</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Goat-anti-mouse bio</td>
<td></td>
</tr>
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<td>FcγRII (Serotec; Oxford, UK)</td>
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<td>Goat-anti-mouse 488</td>
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</tr>
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<td>Goat-anti-rat bio</td>
<td></td>
</tr>
<tr>
<td>FXIIIa (abcam; Uithoorn, the Netherlands)</td>
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Table 3: Primary antibodies and dilutions used in FACS analysis and immunohistochemical stainings.

The FACS analysis data are expressed as mean fluorescent intensity (MFI). The data from 3 separate experiments performed in duplicate were averaged and expressed as mean ± SEM. In order to obtain a better insight into the relative upregulation of the different markers on CA versus AA macrophages the results were expressed relative to control macrophages. The data were analysed using a one-way ANOVA with Bonferroni correction in SPSS (15.0.0, Chicago, USA). A p-value smaller than 0.05 was considered significant.

**Immunohistochemistry**

Frozen sections of MS lesions and normal control brain tissue (mounted on superfrost glass slides) were air dried for 10 min, after which they were incubated with acetone for 10 min. Sections were rehydrated by washing in phosphate buffered saline (PBS) and pre-incubated with 10% normal goat serum in PBS. Subsequently, the sections were washed three times and incubated with the first antibody overnight at 4°C. The antibodies were diluted in PBS containing 1% bovine serum albumin (BSA; Boehringer-Mannheim, Mannheim, Germany). The antibodies used are listed in Table 3.
Markers for CA and AA macrophages in MS lesions

After washing the sections were incubated with biotinylated secondary antibody for one h at room temperature. The sections were incubated for another h with a streptavidin-biotin-HRP complex (strep-ABComplex HRP 1:200; DAKO, Vector), after washing. Sections were washed, incubated with 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich) and counterstained with haematoxylin (Sigma-Aldrich). Finally the sections were dehydrated and embedded using Entellan (Sigma-Aldrich). Omission of the primary antibody was used as a negative control. Images were taken on a Nikon E800 (Amstelveen, the Netherlands). Images were processed using Adobe Photoshop 6.0 (San Jose, USA).

RESULTS

Expression of CA and AA markers on macrophages generated in vitro

In order to assess the phenotype of CA and AA human macrophages generated in vitro we analysed the expression of a panel of markers based on literature (Table 2) using flow cytometry. Macrophages were obtained from 3 different donors. For the CA macrophages we observed that CD40 and FcγRI were upregulated significantly compared to AA macrophages. CD68 and iNOS appeared to be slightly but consistently upregulated on CA macrophages compared to AA macrophages, while CD86 and FcγRII did not appear different between CA and AA macrophages. B) For the AA markers only MR was upregulated on AA macrophages. For CD163, KLF2 and E-cadherin no difference was observed. For FXIII a slight upregulation was observed for AA macrophages compared to CA macrophages. C) Per-}

expression of markers on CA and AA macrophages stimulated in vitro, with IFN-γ and LPS or IL-4 and IL-10 respectively. The data are expressed as the mean of 3 separate experiments with 3 different donors. A) Expression of CA macrophage markers. CD40 and FcγRI were highly upregulated on CA macrophages. *indicates p<=0.05.
(1.5 and 2.3 fold respectively) in all experiments compared to AA macrophages (Figure 1A and C). Both CD68 and iNOS were slightly but consistently upregulated in all experiments (Figure 1A). The expression of CD86 and FcγRII did not differ between CA and AA macrophages. Only MR was significantly upregulated, 2.5

Figure 2: Expression of markers for the CA and AA phenotype in control brain. Sections from white matter of control brain were stained using immunohistochemistry with DAB as a chromogen. Arrows point to PVM and arrowheads to microglia. CD68 was clearly visible on PVM and microglia. CD40 also showed a very clear microglial staining and PVM. FcγRII stained microglia and PVM. FcγRII showed nice microglia. CD86 was mainly present on PVM and to a lower extent on microglia. The iNOS staining was very weak, however some microglia and PVM were visible. The AA marker MR was highly expressed on PVM. Microglial staining was lower compared to PVM. CD163 was very highly expressed on PVM and some microglia. FXIIIa was highly expressed on PVM and microglia. Finally E-cadherin staining was very light, but visible on PVM and microglia.
Markers for CA and AA macrophages in MS lesions

fold, in AA macrophages compared to CA macrophages (Figure 1B and C). The expression of CD163, FXIIIa and E-cadherin did not differ between CA and AA macrophages.

Expression of CA and AA macrophage markers in normal brain

Markers for both CA and AA macrophages were expressed in control white matter (see Figure 2). First the markers for the CA phenotype will be discussed. CD68, which is present in the lysosomes of macrophages, was present in control white matter. It was strongly expressed in all microglia and perivascular macrophages (PVM) (Figure 2). CD40 was expressed highly by all microglia in the parenchyma and the PVM (Figure 2). FcyRI was expressed mainly in microglia (Figure 2). Also some positive PVM could be observed, but this appeared to be less frequent compared to CD68. FcyRII staining pattern was similar to FcyRI, being mainly present on microglia and PVM. Staining intensity of FcyRII was lower compared to FcyRI. CD86, a co-stimulatory molecule, had a very light staining pattern and again both microglia and PVM were stained. The expression of iNOS was very low in the control white matter, with some microglia and some PVM staining weakly positive.

Staining for the AA markers was also observed in the control brain. The AA marker, MR was highly expressed by PVM (Figure 2). Some microglia expressed MR, but they were stained weakly. CD163 was predominantly expressed in PVM, although some microglia could also be observed expressing CD163 highly. E-cadherin was weakly expressed in the control white matter. The endothelium was stained, as were both microglia and PVM. The staining pattern for FXIIIa also showed expression in blood vessels, with clear PVM. In the parenchyma FXIIIa microglia stained positive.

Expression of CA and AA macrophage markers in MS lesions

In active MS lesions and the rim of chronic active MS lesions numerous macrophages are present. The expression of markers for the CA phenotype was upregulated compared to the normal appearing white matter (NAWM) and control (Table 4). The expression of CA markers was similar in active and chronic active lesions. In Figure 3 expression of markers of the CA phenotype are depicted. Staining for markers of the CA phenotype was increased compared to the NAWM. The CA markers, except iNOS, were highly expressed by macrophages present in the lesion. In the NAWM, the staining intensity of CD40 on microglia was increased compared to the control brain. In MS lesions CD40 was highly expressed. Near the rim of the lesion the transition of “resting”, highly branched and weakly CD40 positive microglia into activated, ameboid microglia highly positive for CD40 was visible. In the lesion both PVM and foamy macrophages highly expressed CD40. The staining pattern was typical for membrane staining, clearly showing the cell processes of the macrophages and microglial cells. FcyRI was highly expressed on macrophages and microglial cells at the lesion border. Foamy macrophages and PVM clearly expressed high levels of FcyRI. FcyRII showed a similar staining pattern as observed for FcyRI, with high expression on macrophages at the lesion border, foamy macrophages and PVM. All macrophages in the rim of the lesion
were strongly positive for CD68, showing a granular, intracellular staining pattern. In addition, PVM showed strong staining for CD68. The CD86 expression was visible on foamy macrophages, PVM and some hypertrophic astrocytes. CD86 expression was also present at the lesion border. Only weak expression of iNOS was observed in foamy macrophages. The macrophages at the rim of the lesion were negative for iNOS.

<table>
<thead>
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<th>Lesion</th>
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</table>

Table 4: Semi-quantification marker expression in MS lesions. Scales range from - to +++.

The expression level of MR, the AA marker, was slightly increased in MS lesions compared to the NAWM (Table 4). The expression of MR was similar in active and chronic active lesions. MR expression was mainly present on PVM (Figure 4). A subpopulation of foamy macrophages appeared to be slightly MR positive. E-cadherin was only lightly expressed in the lesion area and did not appear to be upregulated. Some foamy macrophages and PVM were positive for E-cadherin. FXIIIa expression was increased in the lesion. The macrophages at the lesion border expressed this marker and PVM expressed FXIIIa highly. Foamy macrophages and some hypertrophic astrocytes also were positive for FXIIIa.
Markers for CA and AA macrophages in MS lesions

Figure 3: Expression of markers for the CA phenotype in an active lesion. Images were taken at the rim of the lesion. Using the CA markers CD68, CD40, FcγRI and FcγRII the staining intensity of the macrophages in present in the lesions was high. The PVM and foamy macrophages expressed these markers highly. CD86 staining was weak. The PVM and some foamy macrophages expressed CD86. The CA marker iNOS was expressed at a low level. Both PVM and some foamy macrophages were weakly stained. visible.

DISCUSSION:

The aim of this study was to investigate the activational phenotype of the macrophages present in MS lesions. Outside the CNS, diverging effects of CA and AA macrophages have been well established, with CA macrophages being cytotoxic and AA macrophages being considered growth promoting, wound healing and anti-inflammatory. In the CNS, macrophages and microglial cells, the resident macrophages of the brain, have diverging effects on neuronal integrity, but no reports are available on their activational phenotype. Based on literature we selected a panel of markers differently expressed by human CA and AA macrophages and we determined the phenotype of the macrophages and...
microglia present in and around MS lesions.

By FACS analysis of *in vitro* stimulated human macrophages we established the most discriminating markers for each of the different activational phenotypes. For the generation of the CA phenotype, macrophages were stimulated *in vitro* with IFN-γ and LPS for 48 h. Expression of iNOS and CD68 was slightly, but consistently increased in CA macrophages. The extent of upregulation of these markers was too modest to be useful in discriminating between CA and AA phenotypes in tissues using immunohistochemistry. Both FcγRI and CD40 were significantly upregulated after stimulation with IFN-γ and LPS compared to AA and control macrophages. CD40 has only been recently established as a potential marker for the CA phenotype. In our experiments, CD40 appeared to be a reliable marker to discriminate between CA and control or AA macrophages *in vitro*. The expression of CD40 was significantly and consistently upregulated on CA macrophages. Interestingly a recent report suggests that CD40 signaling is even instrumental in the polarization
Markers for CA and AA macrophages in MS lesions

of macrophages towards an CA phenotype in mice, since stimulation of CD40 increased expression of proinflammatory cytokines, iNOS and CCL2 while abrogation of CD40 signaling via tumor necrosis factor receptor-associated factor 6 induces IL-10 expression.

For the in vitro induction of the AA phenotype, we stimulated the macrophages with IL-4 and IL-10. Of all the markers tested for AA macrophages only MR was upregulated using this protocol. The other markers, CD163, FXIIIa and E-cadherin, were not discriminative. This confirms that the state of alternative activation is not represented by one phenotype or one stimulation protocol, but a wide variety of stimuli induces a wide variety of phenotypical and functional differences in macrophages.

The subdivision of macrophages in M1 versus M2 or CA and AA may be useful, but bares the risk of oversimplification and does not reflect the full degree of macrophage plasticity. Furthermore, since no marker was found to be exclusive for either phenotype the expression of one marker is not sufficient for the characterization of a specific phenotype. Therefore, a panel of markers should always be used, as well as a precise description of the in vitro stimulation protocol.

In MS lesions, both active and the rim of chronic active, numerous macrophages are present. Expression of markers specific for macrophages are therefore increased in the lesion compared to the NAWM. We observed that the markers for the CA phenotype, CD68, CD40 and FcγRI, were highly expressed on macrophages in MS lesions. CD86 and FcγRII were also expressed on macrophages in MS lesions, although to a lower extent. Hardly any cells positive for iNOS were found. CD40 was expressed on activated microglia and macrophages in the lesion rim. Together with the in vitro data, our findings show that CD40 is a suitable marker for classical activation in MS lesions.

The expression of the AA markers MR and CD163 was increased, mainly in perivascular macrophages, as was described previously. Foamy macrophages only weakly expressed MR and CD163. E-cadherin was very lightly expressed on macrophages in MS lesions. All these data indicate that only a few macrophages in and around MS lesions have an AA phenotype. The PVM and foamy macrophages express both, CA and AA, categories of markers. Expression of markers was similar between active and chronic active lesions.

The fact that the CA phenotype is more prominent also in chronic active MS, indicates that there is no massive transition to an anti-inflammatory AA phenotype in chronic lesions. Some macrophages in MS lesions, in particular PVM and foamy macrophages, express markers of both AA and CA macrophages. In another neurodegenerative disorder, Alzheimer’s disease, microglial cells were found to upregulate mRNA levels for both markers for the CA and AA phenotype, indicating a dual state of activation expressing functional characteristics of both CA and AA macrophages.

In MS brain material we did not observe upregulation of AA markers on microglial cells, but expression of the CA marker CD40 was increased in the NAWM.

The finding of the persistence of the CA phenotype is in line with the finding that during spinal cord injury the CA/M1 phenotype seemed to persist, while induction
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of the AA/M2 phenotype was transient \(^{56}\). Kigerl et al. hypothesized that this could be the reason for the hampered functional repair, since AA macrophages have been described to be beneficial during repair. Similarly, the persistence of the CA phenotype in MS lesions may contribute to the remyelination failure and the slow burning neurodegeneration \(^{5,57}\), due to the secretion of cytotoxic substances such as glutamate, iNOS and proinflammatory cytokines \(^{38,39}\).

**REFERENCE LIST**

Markers for CA and AA macrophages in MS lesions


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