On the role of macrophages, microglia and the extracellular matrix in remyelination

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Chapter 4

ECM molecules prevent the generation of OPC differentiation-supporting macrophages and microglia in the absence of appropriate anti-inflammatory signals

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Abstract
Demyelination and incomplete remyelination are hallmarks of multiple sclerosis (MS). Although oligodendrocyte progenitor cells (OPCs) are present in most lesions, they ultimately fail to differentiate into mature myelinating oligodendrocytes. Aberrant expression of extracellular matrix (ECM) molecules in MS lesions, such as chondroitin sulphate proteoglycans (CSPGs) and (aggregated) fibronectin, directly contribute to the failure of OPC differentiation. Here, we examined whether the ECM in MS lesions might interfere with the generation of classically- and/or alternatively-activated bone marrow-derived macrophages (BMDMs) and microglia, and whether this affects OPC maturation. Our findings indicate that dimeric plasma fibronectin (pFn) coatings reduced the expression of the anti-inflammatory marker arginase-1 in alternatively-(IL-4) activated BMDMs and microglia. In contrast, aggregated Fn (aFn) induced pro-inflammatory features in alternatively-(IL-4)-activated BMDMs and microglia, including increased iNOS expression and a tendency to release TNFα. CSPGs hardly interfered with the generation of classically-(IFNγ+LPS) and alternatively-(IL-4)-activated cells. Secreted factors by BMDMs and microglia that were grown on aFn, pFn and/or CSPG coatings, inhibited OPC differentiation, which is not evident upon BMDM and microglia IL-4 co-stimulation. In addition, analysis of proMMP-7 levels, an enzyme involved in ECM remodelling, revealed that although aFn coatings reduced the release of proMMP7 expression in IL-4-activated BMDMs, the levels were still sufficient to efficiently degrade aFn in vitro. Hence, while ECM coatings as such may interfere with the generation of remyelination supporting BMDMs and microglia, reduce alternatively-activated features (pFn) or introduce pro-inflammatory features (aFn) in IL-4-activated BMDMs and microglia, alternative activation suffices to generate OPC differentiation-supporting BMDMs and microglia.
Introduction

Multiple sclerosis (MS) is a chronic demyelinating and disabling disease of the central nervous system. In the majority of patients, inadequate remyelination, i.e., the generation of new myelin membranes that restores saltatory conduction and prevents axon degeneration, contributes to the progressive deterioration of the disease (Compston & Coles, 2008; Franklin & ffrench-Constant, 2008; Irvine & Blakemore, 2008). Although oligodendrocyte progenitor cells (OPCs) responsible for remyelination, are present in most lesions, they ultimately fail to differentiate into mature myelinating oligodendrocytes, thereby frustrating remyelination (Lucchinetti et al., 1999; Chang et al., 2002; Kuhlmann et al., 2008). In fact, an unfavorable molecular and cellular signaling environment in (chronic) MS lesions, including dysregulated levels of growth factors and extracellular matrix (ECM) proteins, as well as a dysregulated activation of infiltrating macrophages and resident microglia, collectively contribute to perturbed OPC maturation and hence a failure of remyelination (Chamberlain et al., 2016; Franklin & ffrench-Constant, 2008; Olsen & Akirav, 2015).

Dynamic remodelling of the ECM, involving transient expression and/or degradation, is an effective mechanism to regulate glia cell behaviour, including OPC differentiation upon CNS injury. For example, dimeric fibronectin, chondroitin sulphate proteoglycans (CSPGs) and laminin are readily expressed or upregulated, following toxin-induced demyelination or induction of EAE, an animal model for MS (Lau et al., 2012; Stoffels et al., 2013; Back et al., 2005). Whereas upon experimentally induced demyelination in animal models these ECM molecules are cleared and only transiently expressed, their persistent and aberrant expression is a salient feature of MS. Specifically, (aggregates of) fibronectin, high molecular weight (HMW) hyaluronan, and CSPGs are persistently present in MS lesions and each of these ECM proteins prevents OPC maturation, thereby contributing to
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remyelination failure (Sobel & Ahmed 2001; Back et al., 2005; Sloane et al., 2010; Lau et al., 2012; Stoffels et al., 2013). While astrocytes synthesize most ECM proteins (Wiese et al., 2012; Jones & Bouvier 2014), microglia and macrophages are important cellular players in the degradation of ECM proteins (Brown et al., 2009; Lu et al., 2011; Valentin et al., 2009). Microglia and macrophages are major sources of matrix metalloproteinase (MMPs), which have been implicated in extracellular ECM degradation (Lu et al., 2011). Our previous data indicate an upregulation of MMP-7, which is implicated in fibronectin and CSPG degradation (chapter 2, Lu et al., 2011) during early remyelination in the lysolecithin demyelination-remyelination model (chapter 2). Also in the cuprizone demyelination-remyelination model, several MMPs are significantly upregulated just prior to the onset of remyelination, supporting a role of MMPs in dynamic ECM remodelling upon demyelination (Ulrich et al., 2006; Škuljec et al., 2011). Accordingly, MMPs are implicated in the pathogenesis of MS (Anthony et al., 1997; Cossins et al., 1997; Lindberg et al., 2001), and the inability to clear ECM proteins in MS lesions may be attributed to a perturbed expression and/or malfunctioning of these proteolytic enzymes.

Important cellular constituents of MS lesions are macrophages, which either arise from resident microglia or from infiltrated monocytes/macrophages that enter the CNS as a consequence of a disrupted blood-brain barrier. Using the microglia specific marker TMEM119, it has been estimated that approximately 45% of the macrophage-like cells in active MS lesions are derived from resident microglia, while their number decreases when the lesion further develops (Zrzavy et al., 2017). Microglia and macrophages respond rapidly to micro-environmental changes, and as such they express the appropriate receptors for binding to ECM proteins (Beachley et al., 2015; Milner & Campbell, 2003; Asea et al., 2002), implying that ECM proteins may regulate activation of these cells. Therefore, the abnormal ECM environment in MS lesions may not only influence the behaviour of OPCs, but also that of resident...
microglia and infiltrated macrophages. Models of microglia and macrophages activation are often simplified in classically- or alternatively-activated microglia and macrophages. Classically-activated cells are considered as pro-inflammatory, showing enhanced antigen presentation, secretion of pro-inflammatory cytokines, and release of nitric oxide (NO), causing a perturbation of OPC differentiation (Miron et al., 2013; Miron & Franklin, 2014; Lloyd & Miron, 2016; Miron, 2017). Alternatively-activated or pro-regenerative microglia and macrophages produce factors that promote OPC differentiation (e.g. IGF-1, activin-A), MMP-7, and likely also other MMPs (chapter 2; Nagorsen et al., 2005; Lively & Schlichter, 2013). Alternatively-activated microglia and macrophages are imperative for remyelination to proceed (Lloyd & Miron, 2016; Miron, 2017; Miron & Franklin, 2014). In active MS lesions, an intermediate state between pro-inflammatory activation and anti-inflammatory activation is observed (Vogel et al., 2013), indicating that a full shift from classically-activated to pro-regenerative microglia and macrophage is prevented.

We hypothesize that the abnormal ECM environment in MS lesions may, next to directly deregulate OPC behaviour, also indirectly affect OPC differentiation by interfering with the appropriate activation of microglia and macrophages. To this end, we examined in the current study whether the observed prolonged expression in MS lesions of Fn, either in its dimeric or aggregated form, and CSPGs may 1) interfere with the generation of alternatively-activated microglia and/or macrophage phenotype, 2) lead to an alteration in secreted factors that either promote or impair OPC differentiation and/or 3) reduce the expression of MMP7. With regard to the latter, we previously demonstrated its ability to degrade remyelination-inhibiting Fn aggregates (chapter 2), presumably due to its reduced expression in MS lesions (chapter 2; Anthony et al., 1997; Cossins et al., 1997; Lindberg et al., 2001). We show that dimeric plasma fibronectin
(pFn) reduced arginase-1 expression, an anti-inflammatory feature of alternatively-activated macrophages and microglia, whereas aFn induced iNOS expression, a pro-inflammatory feature, in alternatively-activated macrophages and microglia, and a decrease the secretion of proMMP-7. However, the remaining MMP levels were sufficient to degrade aFn in vitro, while aFn-matrix-exposed alternatively-IL-4-activated macrophages and microglia still promote OPC differentiation, in contrast to secreted factors derived from aFn-matrix-exposed unstimulated cells. Hence, these findings suggest that the ECM interferes with the microglia/macrophages phenotype, but that promoting an anti-inflammatory microenvironment in MS lesions likely suffices for OPC differentiation to proceed.

Materials and Methods

Primary cell cultures

Primary glia cells were obtained from forebrains of 1-2 day-old Wistar rats (Harlan and Charles River), as described (Bsibsi et al., 2012). Briefly, mechanical and enzymatic (papain) digestion was used to obtain a single cell suspension. Cells were cultured on poly-L-lysine (PLL, 5 µg/ml, Sigma Aldrich, St. Louis, MO)-coated tissue culture flasks (Nalge Nunc, Naperville, IL) until a tight astrocyte monolayer was formed on which OPCs and microglia adhere.

Microglia. To obtain microglia, the tissue culture flasks were shaken on an orbital shaker at 150 rpm for 1 hour. The medium was centrifuged for 5 min at 150 g. Cell pellets were resuspended in microglia culture medium (DMEM [Life Technologies, Paisley, UK]; 10% fibronectin-free FBS [see below]; 1% antibiotics [Life technologies,] and 1% glutamin [Life technologies]). Microglia were cultured in 10-cm dishes with rat recombinant macrophage colony-stimulating factor (M-CSF, 10 ng/ml, Peprotech, Rocky Hill, NJ) for 3-5 days at a density of 2.0 × 10^6 cells per dish (Corning, Lowell, MA).
OPCs. OPCs were obtained by shaken the tissue flask overnight on an orbital shaker at 240 rpm and further purified via differential adhesion as described previously (Maier et al., 2005, Bsibsi et al., 2012). Isolated OPCs were plated on 13-mm coverslips in a 24 wells plate at a density of $3.0 \times 10^4$ cells per well and cultured for 2 days in SATO medium (Maier et al., 2005) supplemented with the growth factors FGF-2 (10 ng/ml, Peprotech, London, UK) and PDGF-AA (10 ng/ml, Peprotech, London, UK). OPC differentiation was initiated by growth factor withdrawal and culturing in SATO medium supplemented with 0.5% FCS or SATO with conditioned medium obtained from treated BMDMs or microglia in a 1:1 ratio for 3 days.

Bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDMs) were isolated from of upper limbs and hind legs of P0-P2 Wistar rats as described previously (chapter 2). Briefly, skin and muscle were removed from limbs and legs. The marrow cavity was opened by sharp scalpels, and the bone marrow from femora and tibiae was flushed by a syringe and 25-gauge needle with BMDM medium (Roswell Park Memorial Institute (RPMI)-1640 medium [Life Technologies], 10% fibronectin-free FBS [see below], 1% sodium pyruvate [Life Technologies], 1% antibiotics [Life Technologies]). The suspension was centrifuged for 5 min at 150 x g, and cells were resuspended in BMDM medium and plated in 10-cm dishes at a density of $2.5 \times 10^6$ cells per dish and cultured with rat M-CSF (10 ng/ml, Peprotech, Rocky Hill, NJ) for 5-7 days.

Fibronectin-free serum
A gelatin sepharose 4B column (GE healthcare) was used to bind plasma fibronectin present in FBS, according to manufacturer’s instructions (O'Keefe, et al., 1984). Briefly, FBS was passed though the column, and the fibronectin-free flow through was collected and filtered (0.2 μm, Whatman, GE
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healthcare life sciences, Freiburg, Germany) and store aliquots at -20 °C until further use. The absence of fibronectin in FBS was confirmed by Western blot.

Generation of fibronectin aggregates

Astrocytes. To remove remaining microglia and OPC isolation, the tissue culture flasks were shaken on an orbital shaker for another 24 hours at 240 rpm overnight. The astrocyte monolayer was trypsinized once, cultured in T162 flasks and at approximately 90% confluency trypsinized and used to generate fibronectin aggregates.

Deoxycholate (DOC)-insoluble aggregated fibronectin was derived from primary rat astroglial matrices as described previously (Stoffels et al., 2013). Briefly, the tissue culture flasks with the remaining astrocyte monolayer after the removal of microglia ad OPCs were shaken on an orbital shaker for 24 hours at 240 rpm overnight. The astrocyte monolayer was trypsinized once, cultured in T162 flasks and at approximately 90% confluency trypsinized. To generate fibronectin aggregates, the astrocytes were plated on 10-cm dishes at a density of 1.0 × 10^6 cells per dish for 1 hour, and treated with the toll-like receptor 3 agonist poly(I:C) (50 μg/mL, GE Healthcare, Germany) for 48 hours to induce fibronectin aggregation (Stoffels et al., 2013). Cells were removed by water-lysis for 2 hours at 37 °C. The remaining fibronectin aggregate containing astroglial deposits were scraped in ice-cold DOC buffer (2 % deoxycholate [Sigma Aldrich] in 20mM Tris-HCl supplemented with complete Mini Protease inhibitor cocktail [Roche, Mannheim, Germany], pH 8.0). After at least 30 min on ice, the suspension was centrifuged at 16,300 g for 30 min at room temperature. The fibronectin aggregate-containing pellet was washed three times in PBS, followed by resuspension in PBS with a syringe and 25-gauge needle. The level and the proper separation of fibronectin aggregates from fibronectin dimers was routinely checked by Western blot.
Treatment of microglia and bone marrow-derived macrophages

Tissue culture plastic was uncoated or pre-coated with CSPGs (0.5 µg per well in 6-well-plate; 0.05 µg per well in an 8-well chamber slide; Merck, Temecula, CA), plasma Fn (50 µg per well in 6-well-plate; 5 µg per well in a 8-well chamber slide, Sigma Aldrich) or aggregated fibronectin (50 µg per well in 6-well-plate; 5 µg per well in 8-well chamber slide) overnight at 4 °C overnight. Gently scraped and centrifuged microglia and macrophages were plated at a density of 1.0 × 10^6 cells per well in a 6-well plate (1 m) and 5×10^4 cell per well in a 8-well chamber slide (300 µl). Microglia and BMDMs were subsequently untreated or treated with rat recombinant IFNγ (400 ng/ml, Peprotech) and lipopolysaccharide (LPS, 200 ng/ml, Sigma), to induce the classically-activated phenotype or rat recombinant IL-4 (40 ng/ml, Peprotech) to promote the alternatively-activated phenotype for 6 hours (RT-qPCR) or 48 hours (Western blot). To generate conditioned medium and to avoid direct effects of IFNγ, LPS or IL-4 on OPCs, BMDMs and microglia plated on the indicated coatings were treated for 24 hours, after which the medium was changed to SATO+1%FCS. Medium was collected after 24 hours, i.e., 48 hours after plating.

Fn degradation assays

Fibronectin aggregates (5 µg, see above) were incubated with 35 µl non-conditioned medium or conditioned medium in the presence of the general MMP-activator, 4-aminophenylmercuric acetate (APMA, 2 mM, Sigma Aldrich) in 50 µl MMP reaction buffer (50 mM Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, 0.05% Brij-35, pH 7.5) and incubated at 37°C for 72 hours. Of note, conditioned medium was made with plasma fibronectin-free serum. The reaction was terminated by adding non-reducing SDS sample buffer and heating at 95°C for 10 min. The extent of degradation was analysed by Western blotting.
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**TNFα ELISA**
The level of rat TNFα microglia/macrophage conditioned-medium was determined by a commercial ELISA kit using TNFα as standard (R&D systems, catalog # DY 008), according to manufacturer’s instructions.

**Immunocytochemistry**
Cells were fixed with 2% paraformaldehyde (PFA) in medium for 5 min followed by 4% PFA in PBS for 20 min at room temperature. Cells were permealized in ice-cold methanol for 5 min (MBP) or with 0.1% Triton X-100 for 20 min (iNOS), after which cells were blocked with 1% normal goat serum (NGS) for 30 min at room temperature to prevent nonspecific binding. Cells were incubated with primary antibodies (table 1) for 2 hours (MBP) at 4 °C overnight (iNOS), followed by a 1 hour incubation with the appropriated TRITC- (MBP, 1:50, Jackson ImmunoResearch, West Grove, PA) or Alexa 546 (iNOS, Life technologies)-conjugated antibodies. Cell nuclei were stained with DAPI (1 µg/ml, Sigma). Cells were covered with DAKO mounting medium and analyzed with a conventional immunofluorescence microscope (Olympus AX70 or Leica DMI 6000 B). In each experiment, at least 250 cells were scored and the percentage of MBP-positive cells or iNOS- positive cells of total DAPI-stained cells was calculated.

**Western blot analysis**
Cells were detached from plates by scraping in PBS, following by centrifugation at 9,200 x g for 5 min. Cell were lysed by sonication in TNE buffer (50 mM Tris-HCl, 150 mM M NaCl, and 5 mM EDTA, pH 7.5) for 10 seconds on ice. A Bio-Rad DC Protein Assay (Bio-Rad Laboratories, CA) was used to measure total protein concentration, using BSA as standard. Proteins in lysate (50 µg) or medium (40 µl) were denatured at 95°C for 5 min within SDS reducing loading buffer, separated by a 12.5% SDS-PAGE gel, and transferred
to PVDF (Immobilon-FL) using the wet blotting system at 500 mA for 1 hour in cold transfer buffer. Membranes were blocked in Odyssey blocking buffer for 30 min (1:1 with PBS; Li-Cor Biosciences, Lincoln, NE), and incubated overnight at 4°C with the primary antibodies (table 1). Membranes were washed three times with PBS containing 0.5% Tween-20 (PBST), and incubated with appropriate IRDye®-conjugated secondary antibodies (1:3000; Li-Cor Biosciences) for 1-2 hours at room temperature. Finally, membranes were washed three times with PBST, proteins were visualized with the Odyssey Infrared Imaging System (Li-Cor Biosciences) and intensities analyzed with Scion image software.

Table 1: Primary antibodies used during WB and ICC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution WB</th>
<th>Dilution ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-actin (mAb)</td>
<td>Sigma</td>
<td>1:1000</td>
<td>n.a.</td>
</tr>
<tr>
<td>anti-arginase-1 (mAb)</td>
<td>BD Biosciences</td>
<td>1:250</td>
<td>n.a.</td>
</tr>
<tr>
<td>anti-EIIIA-fibronectin (3E2, mAb)</td>
<td>Sigma</td>
<td>1:500</td>
<td>n.a.</td>
</tr>
<tr>
<td>anti-fibronectin (pAb)</td>
<td>Millipore</td>
<td>1:1000</td>
<td>n.a.</td>
</tr>
<tr>
<td>anti-MBP (mAb)</td>
<td>Serotec</td>
<td>n.a.</td>
<td>1:250</td>
</tr>
<tr>
<td>anti-MMP7 (pAb)</td>
<td>GeneTex and Bioworld</td>
<td>1:1000</td>
<td>n.a.</td>
</tr>
<tr>
<td>anti-iNOS (mAb)</td>
<td>BD Biosciences</td>
<td>1:500</td>
<td>1:250</td>
</tr>
</tbody>
</table>

n.a.: not applicable; mAb: monoclonal antibody; pAb: polyclonal antibody; WB: Western blot; ICC: immunocytochemistry

Real-time, quantitative polymerase chain reaction reaction (real-time qPCR)

Total RNA was extracted from cells with the RNeasy Micro Kit (Qiagen, Hamburg, German), according to the manufacturer’s instructions. Total RNA (1 μg) reverse transcribed by oligo (dT)12-18 (500 μg/ml), 10 mM dNTP Mix, 0.1 M dithiothreitol (DTT), 5× first strand buffer and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (all from Invitrogen, Venlo, Netherlands). Real-time qPCR was performed by an Applied Biosystems 7900HT Real-Time PCR System. For each reaction, 10 ng cDNA, 10 pM primers (table 2) and Absolute SYBR Green Rox Mix (Thermo Scientific, Landsmeer, the Netherlands) were mixed. Gene expression was calculated by the \(2^{-\Delta\Delta ct}\) method (Livak & Schmittgen, 2001) and GADPH and HMBS were used as housekeeping gene.
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Table 2: Primer pair set sequences used during RT-qPCR

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2 (Rn)</td>
<td>GCTTCTGTCTGAGCAAAG</td>
<td>CAGGGTCTGAGAGTGTC</td>
<td>90</td>
</tr>
<tr>
<td>MMP3 (Rn)</td>
<td>GGCGGGGAGAAGTCTGTCT</td>
<td>AGACGGGCAATAAGGAGA</td>
<td>100</td>
</tr>
<tr>
<td>MMP7 (Rn)</td>
<td>CGGAGATGCTCATTGAGCA</td>
<td>CATGAGTGCCAACAAACAG</td>
<td>83</td>
</tr>
<tr>
<td>MMP8 (Rn)</td>
<td>TGGAGACATTCCTTGAGGACT</td>
<td>AGGTTGGACAGGGTGCTGT</td>
<td>155</td>
</tr>
<tr>
<td>MMP9 (Rn)</td>
<td>TGTATGGTGCTGCTCCTAAAC</td>
<td>GTGGGACACATACTGCAGG</td>
<td>91</td>
</tr>
<tr>
<td>MMP11 (Rn)</td>
<td>GGCAACTGTGAAAGGGAGCAG</td>
<td>AAGTTGTCCCATGCAAGTA</td>
<td>148</td>
</tr>
<tr>
<td>MMP12 (Rn)</td>
<td>TGGTACCTTAGGCCCATGCTT</td>
<td>AGGAACAGTTTTGTGCCCTTG</td>
<td>108</td>
</tr>
<tr>
<td>MMP13 (Rn)</td>
<td>GCCAGAATCTCCCAACCA</td>
<td>CCCAGCACTGTAGCCTT</td>
<td>176</td>
</tr>
<tr>
<td>MMP14 (Rn)</td>
<td>AATAAGTACATACGCTCCATG</td>
<td>GAGACTGACAGGATTCTTC</td>
<td>91</td>
</tr>
<tr>
<td>ADAMTS4 (Rn)</td>
<td>GCCGGATTCATCACTGACTT</td>
<td>GCGGTACAGCATAGTCTCT</td>
<td>117</td>
</tr>
<tr>
<td>TIMP1 (Rn)</td>
<td>GGGTCCCTGCTGCATAATGCTA</td>
<td>ATGGCTGACAGGAAACAC</td>
<td>99</td>
</tr>
<tr>
<td>TIMP2 (Rn)</td>
<td>TGGACCTGGGAGAAAGAAGAG</td>
<td>TCCCCGGGCAACATAAGTC</td>
<td>97</td>
</tr>
<tr>
<td>TIMP3 (Rn)</td>
<td>CCCACACAGCTACATGCTA</td>
<td>GCTCTTCTCCACCACCTTG</td>
<td>167</td>
</tr>
<tr>
<td>TIMP4 (Rn)</td>
<td>TGCCAAATTACCCATCCCTGCTA</td>
<td>ATAGAGCTTCCGCTCCACGA</td>
<td>93</td>
</tr>
<tr>
<td>TNFα</td>
<td>ATGGGCAGTGTACTTATCTACTC</td>
<td>GTATGAAATGGCAAAATCGC</td>
<td>101</td>
</tr>
<tr>
<td>HBMS (Rn)</td>
<td>CCGAGCCAAGCACCAGGAT</td>
<td>CTCCCTCAGG TGCTCAGA</td>
<td>107</td>
</tr>
<tr>
<td>GAPDH (Rn)</td>
<td>CATCAAGAAGGTTGGAAGC</td>
<td>ACCACCTGTGCTGTA</td>
<td>204</td>
</tr>
</tbody>
</table>

Rn, rattus norvegicus; RT-qPCR: real-time quantitative analysis

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments. When values of two means were compared, statistical significance was calculated by a paired Student’s t-test. When absolute values of more than two means were compared, statistical significance with control was calculated by a one-way ANOVA followed by a Dunnett’s Multiple Comparison test. Statistical analysis was performed with a one sample t-test when relative values of the conditions were calculated by setting the control as 1 in each independent condition. Statistical differences were calculated using GraphPad Prism software (version 5.03). In all cases, p <0.05 was considered significant.
Results
Fibronectin aggregates induce iNOS expression, while plasma fibronectin reduces arginase-1 expression in IL-4-activated bone marrow-derived macrophages and microglia

As a natural response to demyelination in MS lesions, astrocytes deposit among others the ECM proteins CSPGs and cellular Fn. In addition, pFn enters the lesioned area via the disrupted blood-brain barrier (Sobel & Mitchell, 1989; van Horssen et al., 2005; Satoh et al., 2009). While their expression is transient upon experimentally induced demyelination, in MS lesions CSPGs and Fn are not cleared and their presence persists (Sobel & Mitchell, 1989; Sobel & Ahmed, 2001; van Horssen et al., 2005; Stoffels et al, 2013). In fact, the chronic inflammatory nature of MS lesions and the perturbed expression of MMPs, including MMP-7, induce the formation of Fn aggregates (aFn), which consist of both cellular and pFn. To assess whether the persistent ECM environment in MS lesions affects the phenotype of infiltrated macrophages, we cultured bone marrow-derived macrophages (BMDMs) on CSPG, pFn or aFn coatings, and stimulated the macrophages with IFNγ+LPS, to skew the cells to the classically-activated phenotype, or with IL-4 that on uncoated dishes drives the cells to alternatively-activated macrophages. Indeed, as shown in figure 1 (white bars), when BMDMs were cultured on uncoated dishes, exposure to IFNγ+LPS induced the expression of the pro-inflammatory marker iNOS (Fig. 1B,E,H, white bars), while the expression of arginase-1, a marker for alternatively-activated macrophages, was increased upon IL-4 treatment compared to control, i.e., unstimulated BMDMs (Fig. 1C,F,I, white bars). CSPG coatings hardly if at all affected the expression of iNOS and arginase-1 in control, IFNγ+LPS- and IL-4-stimulated BMDMs (Fig. 1A-C, white vs black bars). On pFn coatings, the arginase-1 levels were significantly reduced in IL-4-activated BMDMs, compared to those in IL-4-activated BMDMs that were grown on uncoated dishes, although arginase-1 expression was still
increased, compared to control (Fig. 1D,F, white vs black bars). pFn coatings hardly affected iNOS expression in control, IFNγ+LPS- and IL-4-stimulated BMDMs (Fig. 1D,E, white vs black bars). In contrast, aFn coatings alone increased arginase-1 expression in both unstimulated and in IL-4-activated BMDMs (Fig. 1G,I, white vs black bars), indicating that aFn potentiated the effect of IL-4. In addition, aFn coatings induced iNOS expression in unstimulated BMDMs (Fig. 1G,H, white vs black bars), which is in line with our previous observations (chapter 2). Strikingly, the effect of aFn coatings on iNOS expression was also apparent in IL-4-activated BMDMs (Fig. 1G,H, white vs black bars), suggesting that an aFn environment triggers pro-inflammatory phenotype features in IL-4-activated BMDMs. Similar findings were observed with microglia (Fig. 2). Thus, pFn coatings, while ineffective as such, markedly reduced arginase-1 levels in IL-4-activated microglia, while aFn coatings increased arginase-1 levels in IL-4-activated microglia, compared to IL-4-activated microglia grown on uncoated dishes (Fig. 2D,F, white vs black bars). In contrast to BMDMs, CSPG coatings also tend to decrease arginase-1 levels in IL-4-activated microglia (Fig. 2A,C, white vs black bars). In contrast to BMDMs, the expression of iNOS is slightly reduced in IFNγ+LPS-activated microglia compared to IFNγ+LPS-activated microglia cultured on uncoated dishes, while the expression is still increased compared to unstimulated control microglia (Fig. 2D,E, white vs black bars). In line with the findings in BMDMs, aFn coatings induced iNOS expression both in unstimulated microglia and IL-4-activated microglia (Fig. 2D,E). Hence, CSPG coatings were seemingly ineffective, while pFn and aFn coatings differentially modulated IL-4-activated BMDMs and microglia. pFn coatings disturbed expression of arginase in IL4-activated BMDMs and microglia, while aFn induced iNOS expression in these cells. To further examine the potential disturbing effect of aFn coatings on alternative activation of BMDMs and microglia, i.e., by promoting pro-inflammatory features, we next examined the levels of TNFα.
Fig 1. Plasma fibronectin and aggregated fibronectin differentially interfere with classically- and alternatively-activated bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDMs) were left unstimulated (ctrl), cultured on chondroitin sulphate proteoglycans (CSPGs, A-C), plasma fibronectin (pFn, D-F) or fibronectin aggregates (aFn, G-I), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively-activated BMDMs, respectively. Then, the expression of iNOS (A,B,D,E,G,H) as a marker for classically-activated BMDMs, and arginase-1 expression (A,C,D,F,G,I) indicative for alternative polarization, were analyzed by Western blotting (45-50 µg). Actin served as a loading control. Representative blots are shown in A, D and G; quantitative analysis in B, C, E, F, H, and I. Note that pFn coatings decrease arginase-1 expression (F, n=12) and aFn coatings promote iNOS expression (H, n=8) in alternatively-(IL-4)-activated BMDMs, while CSPG coatings hardly interfere with BMDM polarization (B,C, n=4). In addition, aFn coatings induce iNOS (H, n=10) and arginase-1 (I, n=11) expression in unstimulated BMDMs, while CSPG and pFn coatings hardly interfere with iNOS and arginase-1 expression in unstimulated BMDMs. Bars represent mean values of each condition relative to control cells (set at 1 for each independent experiment). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when compared to control (* p<0.05, ** p<0.01). A paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (# p < 0.05).
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Fig 2. Plasma fibronectin and aggregated fibronectin differentially interfere with classically- and alternatively-activated microglia. Microglia were left unstimulated (ctrl), cultured on chondroitin sulphate proteoglycans (CSPGs, A-C), plasma fibronectin (pFn, D-F) or fibronectin aggregates (aFn, G-I), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively activated macrophages, respectively. Then, the expression of iNOS (A,B,D,E,G,H), as a marker for classically-activated microglia, and arginase-1 expression (A,C,D,F,G,I) indicative for alternative polarization, were analyzed by Western blotting (45-50 µg). Actin serves as a loading control. Representative blots are shown in A, D and G; quantitative analysis in B, C, E, F, H, and I. Note that CSPG coatings slightly reduce arginase-1 expression (C, n=5, p=0.06). pFn coatings markedly decrease arginase-1 expression (F, n=9), and aFn coatings slightly promote iNOS (H, n=7) and arginase-1 (I, n=9) expression in alternatively (IL-4)-activated microglia. In addition, aFn (H, n=6), and to a lesser extent pFn (E, n=3) coatings induce iNOS expression in unstimulated microglia, while CSPG coatings hardly interfere with iNOS expression in unstimulated microglia (B, n=5). Bars represent mean values of each condition relative to control cells (set at 1 for each independent experiment). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when compared to control (* p< 0.05, ** p<0.01). A paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (# p< 0.05, ## p<0.01).

Fibronectin aggregates modulate the secretion of TNFα in IL-4-activated bone marrow-derived macrophages

Upon pro-inflammatory activation, BMDMs and microglia readily enhance the mRNA expression of TNFα (Martinez & Gordon, 2014). Also in our experimental setting, TNFα mRNA levels in BMDMs and microglia, cultured
on uncoated dishes, were prominently increased upon exposure to IFNγ+LPS, but not IL-4, compared to unstimulated BMDMs and microglia (Fig. 3A,B and Fig. 4A,B, respectively, white bars). Remarkably, both pFn and aFn coatings reduced the TNFα mRNA levels in IFNγ+LPS-activated BMDMs (Fig. 3A,B, white vs black bars) and microglia (Fig. 4A,B, white vs black bars), while the levels were still higher than in unstimulated control BMDMs and microglia. The TNFα mRNA levels of control and IL-4-activated BMDMs, grown on aFn coatings, but not pFn coatings, tend to increase, compared to control and IL-4 activated BMDMs, respectively, that were grown on uncoated dishes (Fig. 3B, white vs black bars). This increase in TNFα mRNA levels on aFn coatings was hardly observed in microglia (Fig. 4B, white vs black bars). Analysis of TNFα levels in BMDM-conditioned medium confirmed the slight, but reproducible, increase in control and IL-4-activated BMDMs on aFn coatings (Fig. 3D). This effect appears to be aFn- and BMDM-specific, as on pFn coatings the release of TNFα is similar to BMDMs grown on uncoated dishes (Fig. 3C, white vs black bars), while microglia, cultured on aFn and pFn coatings, were seemingly ineffective in enhancing TNFα levels (Fig. 4C,D, white vs black bars). Notably, the marked decrease in TNFα mRNA levels in IFNγ+LPS-activated BMDMs and microglia on pFn and aFn coatings, was not reflected by a decrease in TNFα levels in conditioned medium (Figs. 3C,D and 4C,D respectively, white vs black bars). Therefore, aFn coatings, but not pFn coatings, tend to slightly increase TNFα mRNA expression as well as secretion of TNFα in IL-4-activated BMDMs, but not microglia. Hence, aFn coatings induced pro-inflammatory features in anti-inflammatory IL-4-activated BMDMs, which may disturb their anti-inflammatory properties. As TNFα levels on aFn coatings were only slightly enhanced in conditioned medium, i.e., not to the extent as observed in IFNγ+LPS-activated BMDMs, we next examined whether aFn conveys pro-inflammatory features to all cells.
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Fig 3. Aggregated fibronectin increases TNFα levels and the number of iNOS+ cells in alternatively-activated bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDMs) were left unstimulated (ctrl), cultured on plasma fibronectin (pFn, A,C) or fibronectin aggregates (aFn, B,D), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively-activated BMDMs, respectively. Then, TNFα mRNA expression levels were analyzed using quantitative real-time PCR (A,B; 6 h; A, n=3-4; B, n=3-4; against HMBS (shown) and GAPDH (not shown, but yielding comparable findings), TNFα levels were measured in conditioned medium using ELISA (C,D; 24 h; C, n=3; D, n=6; 200 µl) and percentage of iNOS-positive (red) cells of total DAPI-stained (blue) cells was determined using immunocytochemistry (E,F; 48 h; n=6). Representative images are shown in E. Note that aFn coatings tend to increase TNFα mRNA expression (B, p=0.08) and release (D, p=0.13), while in addition an increase in the percentage of iNOS-positive cells (E,F) in control and alternatively- (IL-4) activated BMDMs is observed. Remarkably, both pFn (A) and aFn (B) coatings decrease TNFα mRNA expression in IFNγ+LPS-activated BMDMs, which is not reflected by a decrease in TNFα release (C and D, respectively). Bars represent mean expression levels versus control (A,B, normalized control set at 1 for each independent experiment, horizontal line), mean absolute TNFα levels (C,D) or mean percentage of iNOS-positive cells (F). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when relative levels are compared to control (A,B; * p<0.05), a one-way ANOVA when absolute values are compared to control (C,D,F, Dunnett’s Multiple Comparison test, *** p<0.001), and a paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (A-D,F, # p<0.05, ### p<0.001). Scale bar is 25 µm.
Fibronectin aggregates induce iNOS expression in a subset of bone marrow-derived macrophages and microglia

Given the increase in iNOS expression on aFn coatings in control and IL-4-activated BMDMs and microglia (Figs. 1 and 2), we performed iNOS immunocytochemistry to examine the number of cells that express this pro-inflammatory marker. The number of iNOS-expressing BMDMs grown on aFn coatings was 10-fold higher than in control BMDMs (Fig. 3E,F, 34.8±2.1 vs 3.5±0.4). Similarly, a 6-fold increase was observed in the number of iNOS expressing cells in IL-4-activated BMDMs on aFn coatings, compared to control IL-4-activated BMDMs (Fig. 3E,F, 34.8±2.1 vs 5.5±0.6). These findings are consistent with the increased iNOS expression shown on Western blot (Fig. 1G,H). However, the number of iNOS-positive cells in IL-4-activated and aFN-exposed BMDMs was 2-fold less than in IFNγ+LPS-stimulated BMDMs (Fig. 3E,F, 34.7±4.5 vs 70.7±5.1, respectively), indicating that iNOS was expressed in only a subset of IL-4-activated BMDMs grown on aFn coatings. aFn coatings did not interfere with the number of iNOS-positive BMDMs upon IFNγ+LPS stimulation. Similar results were obtained with microglia. As shown in figure 4E,F, the number of iNOS-positive microglia were significantly increased when cultured on aFn-coatings, both for unstimulated (Fig. 3E,F, 29.8±2.81 vs 3.0±0.7) and IL-4-activated cells (Fig. 3E,F, 36.0±2.7 vs 7.8±1.4), while aFn coatings hardly if at all altered the percentage of iNOS-positive cells in IFNγ+LPS-activated BMDMs (Fig. 3E,F, 73.8±4.2 vs 79.0±5.6). As for BMDMs, the number of iNOS-positive cells in aFn-matrix-exposed IL-4-activated microglia was 2-fold less than in IFNγ+LPS-activated microglia (Fig. 3E,F, 36.0±2.7 vs 79.0±5.6). Hence, these findings show that aFn coatings modulate iNOS expression in only a subset of control and IL-4-activated BMDMs and microglia. As differential activation of macrophages and microglia results in the release of distinct soluble factors
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Fig 4. Aggregated fibronectin increases the number of iNOS+ cells in alternatively-activated microglia. Microglia were left unstimulated (ctrl), cultured on plasma fibronectin (pFn, A,C) or fibronectin aggregates (aFn, B,D,F), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively-activated microglia, respectively. Then, TNFα mRNA expression levels were analyzed using quantitative real-time PCR (A,B; 6 h; A, n=3; B, n=3), TNFα levels were measured in conditioned medium using ELISA (C,D; 24 h; C, n=3; D, n=7; 200 µl) and percentage of iNOS-positive (red) cells of total DAPI-stained (blue) cells was determined using immunocytochemistry (F; 48 h; n=5). Representative images are shown in E. Note that both pFn (A, p=0.09) and aFn (B, p=0.09) coatings tend to decrease TNFα mRNA expression in IFNγ+LPS-activated microglia, which is not reflected by a decrease in TNFα release (C and D, respectively). In addition, aFn coatings increase the percentage of iNOS-positive cells (F) in control and alternatively-(IL-4)-activated microglia. Bars represent mean expression levels versus control (A,B, control set at 1 for each independent experiment, horizontal line), mean absolute TNFα levels (C,D) or mean percentage of iNOS-positive cells (F). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when relative levels are compared to control (A,B), a one-way ANOVA when absolute values are compared to control (C,D,F), Dunnett’s Multiple Comparison test, * p<0.05, *** p<0.001), and a paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (## p < 0.01). Scale bar is 25 µm.

(Rawji & Yong, 2013), we next examined whether ECM coatings may alter the content or levels of BMDM and microglia-secreted factors that potentially affect OPC differentiation.
Conditioned medium derived from bone marrow-derived macrophages, grown on CSPG or fibronectin aggregate coatings, inhibits OPC differentiation, which is rescued upon co-treatment with IL-4

OPCs in monoculture readily differentiate into mature oligodendrocytes that elaborate myelin-like membranes, as determined by the expression and localization of MBP, a myelin-specific protein that is imperative for in vivo myelination (Baron & Hoekstra, 2010; Ozgen et al., 2014). To determine whether classically- and alternatively-activated BMDMs, cultured on the MS-relevant ECMs, modulate OPC differentiation via secreted factors, OPCs were cultured with non-conditioned medium (NCM) or conditioned BMDM medium. Importantly, 24 h after activation of BMDMs with IFNγ+LPS or IL-4, fresh medium without stimulators was added, and supernatants were collected 24 h later. In this manner, a direct effect of IFNγ+LPS or IL-4 on OPC differentiation is excluded. As shown in figure 5, 3 days after initiating OPC differentiation, conditioned medium of IFNγ+LPS-activated BMDMs decreased the number of MBP-positive cells compared to NCM-treated OPCs, consistent with previous findings (Wang et al., 2013; Moore et al., 2015; Lloyd & Miron, 2016; Miron, 2017). OPC differentiation was unaffected, following exposure to conditioned medium of IL-4-activated and control BMDMs, compared to NCM-treated OPCs (Fig. 5B-D, white bars). Quantitative analysis of OPC differentiation upon addition of conditioned medium derived from control and IFNγ+LPS-activated BMDMs grown on CSPG (Fig. 5B, black bars), pFn (Fig. 5C, black bars), and aFn (Fig. 5A,D, black bars) coatings, revealed a perturbed differentiation of OPCs compared to NCM-treated OPCs. Moreover, the number of MBP-positive cells was more prominently reduced in the presence of conditioned medium of IFNγ+LPS-activated BMDMs, cultured on aFn coatings than IFNγ+LPS-activated BMDMs cultured on uncoated plastic (Fig 5A,D, white vs black bars). Strikingly, simultaneous activation of BMDMs with IL-4 overcomes the inhibiting effect on early OPC.
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differentiation, as was also observed with conditioned medium of unstimulated BMDMs cultured on CSPGs and aFn coatings (Fig 5B,D, white vs black bars). Hence, while aFn coatings induced pro-inflammatory features in both control and alternatively-IL-4-activated BMDMs, differentiation of OPCs via secreted factors is inhibited upon exposure to control aFn-matrix-exposed BMDM conditioned medium, but not conditioned IL-4-activated aFn-exposed BMDM medium.

Fig 5. Conditioned medium of aggregated fibronectin-exposed control, but not alternatively-(IL-4)-activated bone marrow-derived macrophages inhibit OPC differentiation. Bone marrow-derived macrophages (BMDMs) were left unstimulated (ctrl), cultured on chondroitin sulphate proteoglycans (CSPGs, B, n=3), plasma fibronectin (pFn, C, n=4) or fibronectin aggregates (aFn, A,D, n=4), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively-activated macrophages, respectively. Then, BMDM-conditioned medium was added to oligodendrocyte progenitor cells (OPCs). The number of MBP-positive cells of total DAPI-stained cells was determined 3 days after initiating differentiation. The percentages of MBP-positive cells in cells cultured with non-conditioned medium was 24.0±10.2%. Note that addition of conditioned medium of aFn-matrix-exposed BMDMs inhibits OPC differentiation, which was not evident with conditioned medium of aFn-matrix-exposed IL-4-activated BMDMs (D). Bars represent mean expression levels versus non-conditioned medium which was set at 1 for each independent experiment (horizontal line). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when compared to control (* p<0.05, ** p<0.01, *** p<0.001). A paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (# p < 0.05). Representative images of D are shown in A. Scale bar is 25 μm.
Conditioned medium derived from microglia grown on plasma fibronectin or fibronectin aggregate coatings, inhibits OPC differentiation, which is rescued upon co-treatment with IL-4

Using a similar experimental protocol as applied for BMDMs, the effect of ECM coatings on microglia-derived secreted factors and OPC differentiation was analyzed next. Similar to BMDM-conditioned medium, conditioned medium of IFN+LPS-activated microglia decreased the number of MBP-positive cells, compared to NCM-treated OPCs, while conditioned medium of IL-4-activated microglia had seemingly no effect on OPC differentiation (Fig. 6B-D, white bars). Secreted factors derived from unstimulated microglia cultured on pFn and aFn coatings decreased OPC differentiation by approx. 40 and 60% respectively (Fig. 6C,D). As observed for BMDMs, conditioned medium derived from microglia cultured on pFn and aFn coatings and stimulated with IL-4, rescued the inhibiting effect of conditioned medium of unstimulated microglia on pFn and aFn coatings (Fig. 6C,D, white vs black bars). In contrast, pFn and aFn coatings exaggerated the inhibiting effect of conditioned medium of IFNγ+LPS-activated microglia on OPC differentiation (Fig. 6C,D, white vs black bars). Conditioned medium derived from microglia grown on CSPG coatings, hardly if at all affect the differentiation of OPCs (Fig. 6B, white vs black bars). Hence, these results indicate that the effect of ECM coatings on OPC differentiation was at least in part mediated through secreted factors by activated BMDMs and microglia. On the other hand, factors in conditioned medium of IL-4-activated BMDMs and microglia protected OPCs against ECM-mediated inhibition of differentiation via these cells. Thus, when appropriately co-stimulated, ECM coatings have little effect on microglia and macrophage-mediated regulation of OPC differentiation via secreted factors.
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**Fig 6.** Conditioned medium of aggregated fibronectin-exposed control, but not alternatively-(IL-4)-activated microglia inhibit OPC differentiation. Microglia were left unstimulated (ctrl), cultured on chondroitin sulphate proteoglycans (CSPGs, B, n=4), plasma fibronectin (pFn, C, n=4) or fibronectin aggregates (aFn, A,D, n=4), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively activated macrophages, respectively. Then, microglia-conditioned medium was added to oligodendrocyte progenitor cells (OPCs) and the number of MBP-positive cells of total DAPI-stained cells, as a marker for OPC differentiation, was determined 3 days after initiating differentiation. The percentage of MBP-positive cells in cells cultured with non-conditioned medium was 24.0±10.2%. Note that addition of conditioned medium of pFn and aFn-matrix-exposed microglia inhibits OPC differentiation, which was less evident with conditioned medium of pFn and aFn-matrix exposed IL-4-activated microglia (C and D, respectively). Bars represent mean expression levels versus non-conditioned medium which was set at 1 for each independent experiment (horizontal line). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when compared to control (*p< 0.05, ** p<0.01, *** p<0.001). A paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (# p < 0.05). Representative images of D are shown in A. Scale bar is 25 μm.

Fibronectin aggregates decrease the secretion of proMMP7 by IL-4-activated bone marrow-derived macrophages

Being the main producers of MMPs, macrophages and microglia also indirectly contribute to OPC differentiation, by regulating the timely degradation of ECM molecules. In our previous work, we demonstrated that IL4-activated BMDMs and microglia predominately produce proMMP7 (chapter 2), which is able degrade fibronectin (aggregates) and CSPGs (chapter 2; Lu et al., 2011). To examine, whether ECM molecules regulate the production and/or secretion of
proMMP7, we analyzed (pro)MMP7 expression in BMDM and microglia lysates and conditioned medium. Consistent with our previous findings,

![Figure 7](image_url)

Fig 7. Plasma fibronectin and aggregated fibronectin decrease proMMP-7 expression in alternatively-activated bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDMs) were left unstimulated (ctrl), cultured on chondroitin sulphate proteoglycans (CSPGs, A–C), plasma fibronectin (pFn, D–F) or fibronectin aggregates (aFn, G–I), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively-activated macrophages, respectively. Then, the levels of proMMP-7 in total lysates (A,B,D,E,G,H; cells; 45–50 µg) and conditioned medium (A,C,D,F,G,I; med; 40 µl) were analyzed by Western blotting. Actin served as a loading control. Representative blots are shown in A, D and G; quantitative analysis in B, C, E, F, H, and I. Note that aFn (G,I, n=5), but not CSPG (A,C, n=6) and pFn (D,F, n=6) coatings, decrease the release of proMMP-7 in alternatively-IL-4-activated BMDMs. Bars represent mean values of each condition relative to control cells (set at 1 for each independent experiment). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when compared to control (* p<0.05, ** p<0.01). A paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (# p < 0.05).

exposure to IL-4, but not IFNγ+LPS, increased proMMP7 levels in BMDMs and microglia, compared to the respective unstimulated cells, both in cell lysates (respectively Fig. 7B,E,H and Fig. 8B,E,H, white bars) and conditioned medium (respectively Fig. 7C,F,I and Fig. 8C,F,I,white bars). pFn, and more prominently aFn coatings alone, but not CSPG coatings, tend to increase
proMMP7 expression in both BMDMs [Fig. 7A,B (CSPGs), 7D,E (pFn), 7G,H (aFn), white vs black bars] and microglia [Fig. 8A,B (CSPGs), 8D,E (pFn), 8G,H (aFn), white vs black bars], without a significant effect on the level of secreted proMMP7. Remarkably, the expression of proMMP7 was higher in IL-4-activated microglia cultured on aFn coatings than in microglia that were activated with only IL-4 (Fig. 8G,H, white vs black bars). This finding was not observed in BMDMs (Fig. 7G,H, white vs black bars) and neither occurred in either cell type cultured on CSPG (Figs. 7A,B and 8A,B, white vs black bars) and pFn (Figs. 7D,E and 8D,E, white vs black bars). The increased proMMP7 expression was not reflected by an increase in secreted proMMP7 levels in aFn-exposed IL-4 activated microglia, which were in fact even slightly reduced (Fig. 8G,I, white vs black bars). In contrast, the levels of proMMP7 were significantly reduced in conditioned medium, obtained from IL-4-activated BMDMs grown on aFn coatings compared to IL-4-activated BMDMs alone (Fig. 87G,I), while being still increased compared to unstimulated BMDMs. pFn coatings do not significantly affect proMMP7 levels in BMDM and microglia conditioned medium. CSPG coatings tend to slightly decrease proMMP7-levels in lysates and conditioned medium of IFNγ+LPS-activated microglia (Fig. 8B,C), but not IFNγ+LPS-activated BMDMs (Fig. 7B,C). As the decreased levels of secreted proMMP7 in IL-4-activated BMDMs cultured on aFn may affect ECM remodelling, we next performed aFn degradation assays.
Fig 8. Plasma fibronectin, aggregated fibronectin and CSPGs hardly affect proMMP-7 expression in alternatively-activated microglia. Microglia were left unstimulated (ctrl), cultured on chondroitin sulphate proteoglycans (CSPGs, A-C), plasma fibronectin (pFn, D-F) or fibronectin aggregates (aFn, G-I), or treated with interferon-γ (IFNγ) and lipopolysaccharide (LPS) or interleukin-4 (IL-4), to induce classically- or alternatively activated macrophages, respectively. Then, the levels of proMMP-7 in total lysates (A,B,D,E,G,H; cells; 45-50 µg) and conditioned medium (A,C,D,F,G,I; med, 40 µl) were analyzed by Western blotting. Actin serves as a loading control. Representative blots are shown in A, D and G; quantitative analysis in B, C, E, F, H, and I. Note aFn (G,H, n=6), but not that CSPG (A,B, n=5) and pFn (D,E, n=5) coatings tend to increase proMMP-7 expression, but not its release (G,I, n=7). Bars represent mean values of each condition relative to control cells (set at 1 for each independent experiment). Error bars show the standard error of the mean. Statistical analyses were performed using the one-sample t-test when compared to control (* p< 0.05). A paired student’s t-test was performed to compare the effect of the respective coating at similar treatment, i.e., IFNγ+LPS or IL-4 (# p < 0.05).

Fibronectin aggregates do not interfere with IL-4-activated bone marrow-derived macrophages-mediated degradation of aggregated fibronectin

Previously, we demonstrated that alternatively-IL-4-activated BMDMs, and to a lesser extent microglia, were able to cleave fibronectin aggregates at proper MMP-activating conditions, i.e., upon incubation with the MMP activator APMA, to activate the proMMPs that are present in the conditioned medium
ECM molecules interfere with microglia/macrophage polarisation (Gibbs et al., 1999). The degradation was likely mediated by MMP7, as similar degradation products were obtained with IL-4-activated BMDM conditioned medium, as with recombinant active MMP7 (chapter 2). Incubation with aFn for 72 hours at 37°C and subsequent Western blot analysis, using a polyclonal anti-Fn antibody, showed that at MMP-activation conditions, conditioned medium of IL-4-activated, but not of control and IFNγ+LPS-activated BMDMs, cleaved Fn aggregates into 3 major degradation products with approx. molecular weights of 61, 31 and 21 kDa (Fig. 9A, arrows). The previously noted 9 kDa degradation product, detected with the polyclonal fibronectin antibody, was not visible at the present degradation conditions, suggesting that only partial degradation has occurred. Western blot analysis with an anti-EIIIA fibronectin antibody, i.e., recognizing cellular fibronectin within the aggregates, visualized a major degradation product around 13-14 kDa (Fig. 9B, arrow), consistent with previous findings (Shinde et al., 2008; chapter 2). Therefore, although the proMMP7 levels were reduced in conditioned medium, culturing BMDMs on aFn coatings did not interfere per se with the degradation of Fn aggregates, as similar products were observed with conditioned medium of aFn-exposed IL-4-activated BMDMs and BMDMs that were stimulated with IL-4 only (Fig. 9A,B). However, some additional degradation products with a molecular weight of 96, 54, 47 and 36 kDa were visualised with the polyclonal anti-Fn antibodies, when aFn was incubated with conditioned medium of IL-4-activated BMDMs cultured on aFn coatings (Fig. 9A, arrowheads). These additional degradation products may represent partially cleaved products or, alternatively, may indicate that other MMPs than MMP7 were expressed and potentially secreted upon aFn coatings, giving rise to different degradation products. Therefore, an RT-qPCR analyses was performed to determine which metalloproteinase (MMPs, ADAMTS), capable of degrading fibronectin, and their natural inhibitors, TIMPs, were specifically expressed in aFn-matrix-exposed IL-4-activated BMDMs. As
shown in table 3, upon IL-4 activation of the 9 examined MMPs only MMP7 mRNA is increased upon IL-4 stimulation compared to control BMDMs. No

| Table 3: RT-qPCR analysis of bone marrow-derived macrophages (BMDMs) |
|-------------------------|-----------------|-----------------|
|                         | IL-4            | IL-4+aFn        |
| MMP2                   | 0.77±0.12       | 0.68±0.09       |
| MMP3                   | 0.83±0.32       | 1.00±0.31       |
| MMP7                   | 2.89±0.57       | 2.82±0.78       |
| ADAMST4                | 0.60±0.23       | 0.78±0.20       |
| MMP8                   | 0.27±0.16       | 0.79±0.39       |
| TIMP1                  | 1.07±0.28       | 1.20±0.23       |
| MMP9                   | 0.95±0.19       | 1.22±0.65       |
| TIMP2                  | 1.13±0.09       | 1.10±0.18       |
| MMP11                  | 1.14±0.41       | 1.10±0.29       |
| TIMP3                  | 0.71±0.24       | 0.58±0.11       |
| MMP12                  | 0.97±0.18       | 1.24±0.39       |
| TIMP4                  | 1.21±0.42       | 1.18±0.14       |

All data relative to (untreated) control are shown, which was set to 1 at each independent experiment; Data are expressed as mean±SEM (n=4); Statistical differences were assessed with a paired student’s t-test (not significant); RT-qPCR: real-time quantitative PCR

prominent differences of the examined MMP and TIMP mRNA expression were observed, when plated on uncoated dishes or aFn coatings. Hence, likely the decreased levels of proMMP7, being secreted by IL-4-activated BMDMs, may account for the partial degradation of aggregated Fn. Partial degradation was not evident upon visualisation with the anti-EIIA fibronectin antibody, although the level of the 13-14 kDa product appeared slightly higher on the blot (Fig. 9B). Hence, aFn coatings only marginally interfere with IL-4-activated BMDM-mediated degradation of aggregated fibronectin. Thus,
at least *in vitro*, although the proMMP7 levels of IL-4-activated BMDMs grown on aFn were reduced, they were still sufficient to degrade Fn aggregates.

**Discussion**

Microglia and macrophage activation is often categorized as either classical or alternative (Nagorsen et al., 2005; Martinez & Gordon, 2014; Murray, 2017; Wolf et al., 2017), classical being pro-inflammatory and detrimental, and alternative anti-inflammatory and beneficial for repair. However, microglia and macrophages do not constitute uniform phenotypes, but rather adopt heterogeneous phenotypes, instructed by an interplay of regional cues elicited upon injury. Here, we show that besides being dependent on instructive soluble signals, such as the classical phenotype activators IFN\(\gamma\) and LPS, and the alternative phenotype activator IL-4, the identity of BMDMs and microglia also depends on the nature of the ECM. Thus, dimeric pFn coatings dampened anti-inflammatory features, such as arginase-1 expression in alternatively-IL-4-activated BMDMs and microglia, while aFn coatings promoted pro-inflammatory features, such as iNOS and TNF\(\alpha\) release, in a subset of unstimulated and IL-4-activated BMDMs and microglia. In addition, our findings indicate that this local regulation of the immune response may regulate OPC differentiation. Thus, CSPG- or aFn-matrix-exposed BMDMs and pFn- or aFn-matrix-exposed microglia reduced OPC differentiation via secreted factors, an effect that is counteracted by IL-4 stimulation. Hence, these findings suggest that the transient and spatial presence of dimeric Fn upon demyelination may prevent premature OPC differentiation by reducing anti-inflammatory features of infiltrating macrophages and resident microglia, and by secreting factors that inhibit OPC differentiation. Moreover, the persistent presence of aFn may account for the observed mixed microglia/macroage phenotype in MS lesions, i.e., harboring both pro-inflammatory and anti-inflammatory features that may contribute to the inhibition of OPC differentiation. Thus, from a translational perspective,
offering an anti-inflammatory stimulus to the MS lesions microenvironment may counteract this ECM-induced microglia/macrophage-mediated perturbation of OPC differentiation.

As a response to a demyelinating insult, microglia and macrophages first adopt a classically-activated phenotype, while the alternatively-activated phenotype dominates at the initiation of remyelination (Miron et al., 2013; Miron & Franklin, 2014; Miron, 2017). Upon CNS demyelination, dimeric Fn and CSPGs are readily expressed, while transient components of the ECM play an important role in the timing of remyelination (Lau et al., 2013; Stoffels et al., 2013), not only by direct binding to OPCs via integrin receptors (Milner & ffrench-Constant, 1994; Milner et al., 1996; Blaschuk et al., 2000; Baron et al., 2002), but as shown in the present work, also indirectly by modulating microglia and macrophage phenotypes. Thus, in vitro, coatings of dimeric pFn, and to a lesser extent those of CSPGs modulated phenotype features of alternatively-IL-4-activated BMDMs or microglia, while the ECM proteins seemingly did not interfere with the classically-IFNγ+LPS-activated BMDMs and microglia phenotype. This indicates that these transiently expressed ECM proteins at the onset of demyelination may prevent the premature presence of alternatively-activated microglia and macrophages. Also, in the absence of stimuli, CSPG coatings account for BMDM-, but not microglia-secreted factors that inhibit OPC differentiation, while secreted factors derived from microglia, but not BMDMs, cultured on pFn coatings, reduced OPC differentiation, which is potentiated in classically-IFNγ+LPS-activated microglia. Notably, and in contrast to the present findings with rat microglia, CSPG coatings activate mouse microglia leading to the production of insulin-like growth factor (IGF-1) that promotes OPC maturation and survival, and prevent LPS-activated microglia to release pro-inflammatory cytokine TNFα (Martinez & Gordon, 2014). The relatively high levels of insulin in our non-conditioned medium
may have masked the beneficial effect of IGF-1. On the other hand, CSPG 
counters induced the release of soluble factors in BMDMs that perturbed OPC 
differentiation, indicating that also OPC differentiation inhibitory factors were 
generated by CSPG coatings. The secreted factor(s) in CSPG- and 
pFn-matrix-exposed BMDM and microglia-conditioned medium that prevent(s) 
OPC differentiation, remain(s) to be determined. Interestingly, irrespective of 
the nature of this factor, upon co-stimulation with IL-4, the effect of CSPG and 
pFn coatings on BMDM or microglia-mediated inhibition of OPC 
differentiation was abolished. This is in line with previous findings, which 
showed that IL-4-activated microglia remain committed to their phenotype, 
even when they are pre-exposed to LPS (Stout et al., 2005; Fenn et al., 2014; 
Tanaka et al., 2015). Thus, even when microglia or macrophages are exposed 
to dimeric Fn and CSPGs, upon simultaneous or subsequent exposure to 
sufficient levels of IL-4, an alternatively-activated microglia and macrophage 
phenotype that supports OPC differentiation, is acquired. Potential factors, 
secreted by these alternatively-IL-4-activated BMDMs and microglia, and that 
may account for this rescue, are activin-A, IGF-1, galectin-3, and/or CXCL-12 
(Yu et al., 1996; Abe et al., 2002; Ebert et al., 2002; Wynes & Riches, 2003; 
Mantovani et al., 2004; Butovsky et al., 2005; Novak et al., 2012; Miron et al., 
2013).

While the expression of dimeric Fn and CSPGs is transient at healthy 
conditions, allowing for robust remyelination, their expression persists in 
demyelinated MS lesions, among others due to aberrant expression and 
regulation of enzymes, such as MMPs, that mediate their degradation (Lu et al., 
2011; chapter 2). While CSPGs (aggrecan, neurocan and versican) are mainly 
deposited at the edge of the lesions (Maeda & Sobel, 1996; Sobel & Ahmed, 
2001), dimeric Fn assembles into aggregates (Stoffels et al., 2013). We have 
previously shown that aFn coatings induce pro-inflammatory and 
anti-inflammatory features in unstimulated BMDMs and microglia (chapter 3).
Here, we have extended these findings and demonstrated that αFn coatings promote pro-inflammatory features, likely in a subset of IL-4-activated BMDMs and microglia, while the expression of the anti-inflammatory marker arginase-1 in IL-4-activated BMDMs and microglia, was potentiated on αFn coatings. The distinct and non-overlapping effects of pFn and αFn on IL-4-activated BMDMs and microglia indicate that they likely operate via different mechanisms. Possibly, the fact that Fn aggregates may also act as scaffold for other proteins, including other ECM molecules and heat shock proteins (Hsps), accounts for this difference (Engvall et al., 1978; Mosher 1989; Williams et al., 1994). Similarly, and even more prominently as observed for dimeric pFn and CSPGs, αFn induced BMDMs and microglia to adopt a phenotype that is detrimental to OPC differentiation, whereas co-stimulation with IL-4, in spite of the acquired pro-inflammatory features on αFn coatings, protects against this detrimental effect. Therefore, in MS lesions, resident microglia and infiltrating macrophages may be prevented to adopt an appropriate, alternatively-activated phenotype for remyelination. Indeed, a mixed macrophage/microglia phenotype with classically- and alternatively-activated features has been observed in MS lesions (Vogel et al., 2013; Peferoen et al., 2015), which may thus be caused by sustained activation due to the persistent presence of distinct ECM molecules. The lack of appropriate profiles, or levels of and response to cytokines and chemokines may then prevent them to protect against ECM-modulated macrophages and microglia-mediated inhibition of OPC differentiation.

It should however be kept in mind that pFn, αFn and CSPGs also directly inhibit OPC differentiation (Siebert and Osterhout, 2011; Pendleton, et al., 2013; Stoffels et al., 2013; Baron et al., 2014; Keough, et al., 2015; Qin et al., 2017), implying that their complete degradation is required to allow for OPC differentiation to resume. In MS lesions, ECM remodelling is disturbed, among
ECM molecules interfere with microglia/macrophage polarisation

others, by reduced expression of MMP7 (chapter 2). Alternatively-(IL-4)-activated macrophage and microglia are the main producers of MMP7, while aFn, but not pFn and CSPG coatings, reduce the release of proMMP7, but not the expression levels, by BMDMs. Interestingly, MMP7 degrades aggregated Fn (Lu et al., 2011), and the remaining proMMP7 levels in aFn-matrix-exposed IL-4-activated BMDM suffice to partially degrade aggregated aFn in vitro. Hence, ECM coatings may not interfere with the regulation aFn degradation. However, whether pFn and CSPG coatings modulate the expression and release of other MMPs, thereby (de)regulating their own clearance, remains to be determined. In this respect, MMP2 and MMP9 levels are more abundant in CSPG-matrix-exposed microglia (Rolls et al., 2008), and a potential positive feedback regulation of their degradation has been suggested. Of note, our preliminary data indicate that pFn reduced proMMP9 levels in IL-4-activated BMDMs and microglia (data not shown).

Taken together, our findings suggest that upon CNS demyelination, the timely expression of Fn and CSPGs are important determinants for the transient generation of a macrophage and microglia phenotype that inhibits OPC differentiation. In MS lesions, as a result of non-optimal regulation of ECM degradation, infiltrating macrophages and resident microglia are exposed over a prolonged time interval to this otherwise transient ECM microenvironment, i.e., (aggregated) Fn and CSPGs are still major components of the ECM. Manipulating the phenotype of macrophages and microglia in MS lesions, for example by exposure to IL-4, may be a promising therapeutic strategy to relieve ECM-mediated inhibition of OPC differentiation due to macrophages and microglia. However, given the reported presence of IL-4 in astrocytes and that macrophages in MS lesions harbor the IL-4 receptor (Hulshof et al., 2002; Nair et al., 2008), macrophages and microglia of MS patients may not appropriately respond to IL-4. To this end, the identification of the IL-4-induced BMDMs and microglia secreted factor(s) that overcome
ECM-mediated inhibition of OPC differentiation, is required to device a specific approach for effective treatment of MS.

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References


