On the role of macrophages, microglia and the extracellular matrix in remyelination
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Chapter 6

Summary and Perspectives
Summary

Multiple sclerosis (MS) is a central nervous system disease, displaying typical characteristics like chronic inflammation, demyelination and failure of remyelination (Compston & Coles, 2008; Podbielska et al., 2013; Popescu et al., 2013). Aberrant behavior of CNS resident and infiltrating immune cells and as yet poorly identified environmental factors, contribute to the etiology and pathology of MS (Franklin & ffrench-Constant, 2008; Gregory et al., 2011). For example, several studies have shown that structural perturbation of distinct extracellular matrix (ECM) molecules, such as the aggregation of fibronectin, may occur in MS lesions that prevent the differentiation of oligodendrocyte progenitor cells (OPCs) into myelinating oligodendrocytes (Back et al., 2005; Lau et al., 2012; Stoffels et al., 2013), thereby contributing to remyelination failure of axons in MS lesions. Accordingly, insight into the underlying mechanism(s) of fibronectin aggregation and/or development of means to preclude or eliminate such aggregates, might be useful tools in clarifying the pathology of the disease and the rescue of remyelination in MS, respectively.

In chapter 1, an overview is presented of the role of ECM molecules in (re)myelination, and cellular proteolytic systems that modulate the ECM at regular, physiological conditions. To complete remyelination, transient expression and distinct remodelling of the ECM is required to support microenvironment-dependent remyelination. However, it is apparent that distinct ECM molecules, transiently present upon demyelination at physiological conditions, remain persistently localized in MS lesions. This persistent presence may lead to an inhibition of OPC differentiation to mature oligodendrocytes, the myelinating cells of the central nervous system. As a result, failure of remyelination occurs. Particularly, fibronectin aggregates (aFn) are dominantly present in MS lesions and an obstacle for remyelination. Matrix metalloproteinases (MMPs) are enzymes capable of remodelling ECM components, including fibronectin. The expression of several MMPs is altered.
in MS, which may thus contribute to a failure of remodelling ECM molecules, necessary for remyelination. Therefore, we hypothesized that local application of MMPs, capable of degrading fibronectin aggregates, might serve as a useful tool in efforts to rescue remyelination in MS.

In chapter 2, MMP expression was studied in demyelinating animal models and in MS lesions. Our findings demonstrated that the expression of proMMP3 and MMP7 was increased upon experimentally-induced demyelination during the remyelination phase. The pattern of MMP3 and MMP7 expression was in line with the previously observed increased expression of fibronectin upon toxin-induced demyelination, followed by its removal at remyelination (Stoffels et al., 2013). In vitro, MMP7, but not MMP3, degraded dimeric fibronectin and αFn. However, coatings of degraded fragments of dimeric fibronectin and αFn still perturbed OPC maturation. In active MS lesions, (pro)MMP7 was mainly localized to HLA-DR-positive microglia and macrophages, while proMMP7 expression was downregulated in chronic MS. IL-4-activated microglia and macrophages were identified as a major cellular source of proMMP7, and at proper MMP-activating conditions, αFn is cleaved with conditioned medium, obtained from IL-4-activated microglia and macrophages. Hence, reduced MMP7 levels may contribute to the inability to clear remyelination-impairing fibronectin (i.e., aggregates) in MS lesions.

Complete remyelination requires the support of an alternative, regenerative phenotype of microglia and macrophages (Miron et al., 2013). Also, αFn likely not only directly perturbs OPC differentiation, but may also prevent remyelination in MS lesions by modulating the phenotype of resident microglia and infiltrating macrophages. Therefore, in chapter 3, we investigated whether αFn coatings could modulate microglia and macrophage phenotypes. The data revealed that both dimeric plasma fibronectin and αFn induced microglia and
macrophages to adopt an amoeboid morphology, and stimulated phagocytosis by macrophages. Coatings of aFn, but not plasma fibronectin promote both microglia and macrophages to release nitric oxide, a feature of classical IFNγ-activated microglia and macrophages. In addition, aFn coatings promote macrophages to express arginase-1 and increase arginase activity, similar to regenerative IL-4-activated cells. Strikingly, this effect of aFn was TLR4- and integrin-independent. Proteomic analysis showed that aFn contains other proteins, including Hsp70 and thrombospondin-1, and therefore aFn acted as a scaffold for other signaling proteins. This may explain the distinct effect of plasma fibronectin and aFn on microglia and macrophage activation. Indeed, Hsp70 stimulated macrophages to produce iNOS, and Hsp70 treatment and thrombospondin-1 coatings induced the expression of arginase-1 in macrophages. Together, this study demonstrated that aFn probably caused macrophages, and likely also microglia, to adopt a phenotype that possessed both features of pro-inflammatory and regenerative microglia and macrophages.

Besides aFn, likely other ECM molecules, such chondroitin sulfate proteoglycans (CSPGs), similarly modulate the phenotype of microglia and macrophages. In chapter 4, we therefore studied whether coatings of CSPGs, an ECM that is persistently present at the edge of MS lesions (Sobel & Ahmed, 2001), triggered features of a pro-inflammatory and alternative nature in microglia and macrophage. In addition, we examined whether aFn, plasma fibronectin and CSPG coatings altered pro-inflammatory (IFNγ+LPS) or regenerative (IL-4) activation of microglia and macrophages. Both aFn and plasma fibronectin, but not CSPGs, interfere with alternatively regenerative (IL-4)-activated microglia/macrophages. Coatings of aFn induce iNOS expression, a pro-inflammatory marker, while plasma fibronectin coatings reduce arginase-1 expression, a regenerative marker, in IL-4 activated cells. CSPGs, plasma fibronectin and aFn coatings cause microglia and macrophages
to secrete factors that inhibit OPC differentiation, but this inhibition was rescued by co-treatment with IL-4. As shown in chapter 2, MMP7 cleaves aFn, and proMMP7 is mainly secreted by IL-4 activated macrophages. We observed that although aFn coatings decreased the secretion of proMMP7 by IL-4-activated macrophages, aFn could still be degraded extracellularly by IL-4-activated macrophages at proper MMP activating conditions. Hence, these data indicate that IL-4 activation of aFn-exposed microglia and macrophages not only suffices to generate OPC differentiation-supporting microglia and macrophages, but may also contribute to ECM remodelling (Fig. 1).

To further assess whether IL-4 is sufficient to induce a phenotype of microglia that support OPC differentiation, and therefore remyelination, in the presence of aFn, in chapter 5, the cytokines IL-4 and IFNγ were applied in toxin-demyelinated organotypic forebrain slice cultures (OFSCs). As fibronectin does not aggregate spontaneously upon toxin-induced demyelination (Stoffels et al., 2013), we first had to induce endogenous fibronectin aggregation in OFSCs. We showed that deposited aFn was deposited upon exposure to the TLR3 agonist poly(I:C) after, but not before lysolecithin-induced demyelination. Moreover, remyelination was impaired in poly(I:C)-treated demyelinated OFSCs. Single exposure to IL-4, but not IFNγ, overcomes the impaired remyelination in poly(I:C)-treated, i.e., aFn-containing, demyelinated OFSCs. IL-4 induced a different morphological phenotype of microglia, and increased the number microglia that express the regenerative marker CD206. In addition, proMMP7 was released in the medium upon IL-4 treatment, which may add to degradation of aFn when appropriately activated. However, as shown in oligodendrocyte monocultures, IL-4 may also directly act on oligodendrocytes and increase OPC maturation. Hence, IL-4 may be an interesting tool to overcome aFn-mediated inhibition of OPC maturation, acting on both microglia and oligodendrocytes.
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Fig 1. Graphical abstract of the main findings in this thesis. Fibronectin aggregates (aFn) persistently exist in multiple sclerosis (MS) lesions and inhibit remyelination (box). Matrix metalloproteinase 7 (MMP7) is able to cleave remyelination-imparing aFn. However, proMMP7 expression is reduced in MS lesions, which may prevent aFN degradation. At healthy conditions, IL-4 activated microglia and macrophages secrete proMMP7, which needs to be cleaved by a still unknown factor (?) to become activated and proteolytically cleave aFn (black arrows). In MS lesions, aFn may perturb the proper activation of microglia and macrophages. aFn serves as scaffold for other proteins, including Hsp70 and thrombospondin, and these proteins induced pro-inflammatory and anti-inflammatory features in microglia and macrophages. In addition, the factors secreted by microglia and macrophages, when plated on aFn coatings, inhibit oligodendrocyte differentiation (red arrows). This aFn-mediated inhibition is rescued upon co-treatment with IL-4. Importantly, adequate levels of proMMP7 are still secreted that fragment aFn at proper activating conditions (green arrows).

Perspectives

The work presented in this thesis offers approaches to overcome remyelination failure by fibronectin aggregates (aFn) in multiple sclerosis (MS) lesions that may prevent secondary neurodegeneration and disease progression (Fig.1). A straightforward approach is the removal of aFn by its degradation via matrix
metalloproteinases (MMPs). Fibronectin aggregates are persistently present in MS, among others due to the lack of an appropriate protease to degrade fibronectin, allowing the formation of aFn by astrocytes, triggered by the chronic inflammatory MS lesion environment. In this thesis, we show that MMP7 is able to cleave aFn in vitro (chapter 2). However, whether MMP7 degrades aFn in vivo, and whether this functionally will overcome remyelination failure, remains to be determined. As fibronectin does not aggregate upon toxin-induced demyelination (Stoffels et al., 2013, Espitia Pinzon et al., 2017), this is technically challenging to investigate. The use of organotypic brain slices would be an option, but as shown in chapter 5, the poly(I:C)-induced aFn is only transiently increased, while aFn degradation is rather difficult to analyse. Thus, the development of an experimental animal model that upon demyelination forms aFn and as a consequence shows remyelination failure, i.e., mimicking MS lesions, is essential. A prerequisite for such a model is that the expression and/or activity of MMPs, and MMP7 in particular, are also reduced, thus leading to the persistent presence of fibronectin, and allowing for its aggregation. Indeed, when aFn is injected in cuprizone demyelinated corpus callosum, failure of OPC differentiation is only transient, presumably because of degradation of the (injected) aFn (Qin et al., 2017; chapter 5, Fig. 2). In this regard, it will be of interest to investigate whether aFn is formed in a (conditional) knockout of MMP7, and whether remyelination fails when MMP7 is lacking.

Although MMP7 degrades fibronectin and aFn in vitro, the fibronectin-derived fragments still inhibit OPC differentiation and maturation (chapter 2). Given that the OPCs are plated on coatings of aFn-derived fragments, i.e., are immobilized, it is likely that in vivo, the released fragments are soluble and cleared by microglia and macrophage-mediated phagocytosis, or alternatively further subjected to degradation and susceptible for degradation by other
MMPs or proteases. It is intriguing that the fragments obtained from MMP7-cleaved aFn differ from those obtained from MMP7-cleaved plasma and cellular fibronectin fragments (chapter 2), which indicates that identification of MMP7 cleavage sites in (dimeric) fibronectin and fibronectin aggregates will be helpful to modify MMP7 in such a way that it specifically degrades aFn. This is important as simply applying MMP7 is not desirable, since MMP7 also cleaves other ECM proteins, including laminin that promotes OPC survival and maturation (Buttery & ffrench-Constant 1999; Lu et al., 2001; Rosenberg et al., 2002; Baron et al., 2014), activates harmful cytokines (Gearing et al., 1995; Yamamoto et al., 2014) and is toxic to neurons (Newman et al., 2001). Thus, the direct application of active MMP7 as a therapeutic tool to cleave and/or remodel fibronectin aggregates in MS, will require careful targeting and timing. Of note, an advantage of increasing MMP7 in MS lesions will be that other remyelination-impairing ECM molecules, such as chondroitin sulfate proteoglycans (CSPGs), could also be degraded by MMP7 (Rolls et al., 2008; Lu et al., 2011).

Our findings show that proMMP7 expression levels are reduced in chronic MS lesions where aFn is prominently present, which may be due to the inappropriate activation of lesion-resident cells (chapter 2). Thus, an alternative approach to directly applying MMP7 to remove fibronectin aggregates from MS lesions is to induce cells to locally and transiently increase MMP7 expression. IL4-activated microglia and macrophages are a major source of proMMP7 (chapter 2), but an unresolved issue is how proMMP7, secreted by microglia and macrophages, is activated, given that in vitro, activation by other cellular sources or additional stimuli for microglia and macrophages are required. MMP7 can be activated by plasmin and e.g. MMP3 (Imai et al., 1995; Lu et al., 2011), MMP3 being expressed at high levels in astrocytes upon demyelination (Ulrich et al., 2006; Škuljec et al., 2011) and in MS lesions (Anthony et al., 1997; Cossins et al., 1997; Lindberg et al. 2001).
Interestingly, our very recent preliminary data suggest that HSPB5-treated macrophages release active MMP7, but this needs to be confirmed. Notably, HSPB5 has been shown to trigger a TLR2-mediated protective response in microglia, which produced chemokines and immune-regulatory mediators (Bsibsi et al., 2013; Bsibsi et al., 2014). Although MMP7 does not appear in the top genes that were upregulated, it is possible that HspB5-treated macrophages secrete a factor that may activate proMMP7.

Microglia and macrophages in active MS lesions possess an intermediate state harbouring both pro-inflammatory and anti-inflammatory phenotype markers (Vogel et al., 2013; Peferoen et al, 2015). We show that in active MS lesions, MMP7 is present in the majority of HLA-DR-positive microglia/macrophages, while MMP7 is occasionally present in microglia/macrophages that are positive for either of the two differential phenotype markers CD40 and CD206. To obtain more insight into which microglia/macrophage phenotype produces proMMP7 in active MS lesions, immunohistochemical analysis of MMP7 with more markers for microglia/macrophages, such as the CD68 (related tissue damage) and Iba1 (structural studies), and in situ hybridization to localize MMP7 mRNA, is suggested (Hendrickx et al., 2017). For the latter, this is also worthwhile to analyse in toxin-induced experimental animal models, to identify all cells that produce MMP7 in demyelinated lesions. Finally, using the recently identified microglia-specific markers, e.g., TMEM119 (Bennet et al., 2016), it could be revealed whether resident microglia and/or infiltrating macrophages express MMP7.

An alternative approach to overcome remyelination failure by aggregated fibronectin is a search for means to bypass the inhibitory action of aFn. In a recent study, we demonstrated that GD1a overcomes remyelination failure by aFn by directly acting on OPCs (Qin et al., 2017). In this thesis, we show that
aFn may also indirectly affect OPC maturation by activating microglia and macrophages, as well as by interfering with pre-determined microglia and macrophage phenotypes (chapter 3 and 4). Thus, our findings demonstrate that aFn induces features of pro-inflammatory, classically activated and alternatively regenerative in macrophage in vitro (chapter 3 and 4), while OPC differentiation is perturbed by aFn-exposed microglia and macrophages via secreted factors (chapter 4, Fig. 1). Our preliminary findings suggest that also in vivo aFn may induce differential activated markers in microglia. Upon intralesional injection of aFn in cuprizone-demyelinated corpus callosum at the onset of remyelination, both IL1-β, a classically-activated marker, and CD206-positive microglia, an alternatively-activated marker were visible two days post aFn, but not PBS, injection (Fig. 2). Intriguingly, the number of PLP-mRNA-expressing cells was reduced upon intralesional aFn injection compared to PBS injection, indicating an aFn-mediated delay in OPC maturation (Fig. 2). Strikingly, at 7 days post aFn injection, aFn-injected lesions are virtually similar to PBS-injected lesions, i.e., staining of IL1-β and CD206 was hardly detected, and apparently similar numbers of PLP-mRNA expression cells were noticed. This indicates a transient effect of aFn on microglia and OPC maturation, which may be due to the presence of proteases, such as MMP7, that may cleave aFn (chapter 2). Indeed, MMP7 immunohistochemistry revealed that at the site of aFn injection, MMP7 is upregulated (Fig. 2). This suggests that MMP7, and maybe other proteases, are involved in the degradation of aFn, and corroborates that this process maybe dysregulated in MS lesions, due to the lack of MMP7 expression. This is an interesting topic for further research.

The lack of MMP7 expression in chronic MS lesions may be due to inappropriate activation of microglia and macrophages among others by the ECM environment (chapter 3 and 4). We show here that the ECM environment
Fig 2. Effect of intraloeal injection of aggregated Fn on microglia and OPC differentiation. Animals were fed with cuprizone for 5 weeks (5wks) to allow for demyelination. At day 0, saline (control) or fibronectin aggregates (aFn) were injected into the demyelinated corpus callosum, and analyzed 2 and 7 days after saline or aFn injection. A. Schematic representation of the experimental setup. B. Double immunohistochemistry for the general microglia marker Iba1 (green) and the pro-inflammatory marker IL-1β (red). C. Double immunohistochemistry for the general microglia marker Iba1 (green) and the pro-regenerative marker CD206 (red). D. Double immunohistochemistry for the general microglia marker Iba1 (green) and MMP7 (red). E. In situ hybridization for PLP mRNA, a marker for differentiated oligodendrocytes. Representative images in the core of the injected area of three animals per condition, are shown. DAPI-stained nuclei are indicated in blue. Scale bars are 50 μm.
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interferes with the polarization to classically and alternatively-activated macrophages, but that aFn-mediated inhibition of OPC maturation via secreted factors by microglia and macrophages is rescued upon co-treatment with IL-4 (chapter 4 and 5). Hence, these results open new avenues for the application of IL-4, as a strategy to overcome aFn-mediated inhibition of OPC differentiation by inducing a remyelination-supporting microglia/macrophage even when aFn is still present (Fig. 1). In addition, IL-4-activated microglia and macrophages may, upon proper activation of MMP7, contribute to ECM remodelling. A more attractive approach compared to administration of IL-4, also given the relative low numbers of microglia/macrophages in chronic MS lesions to respond to IL-4, is to apply the secreted factors(s) by IL-4-treated aFn-exposed microglia and macrophages that overcome the OPC differentiation-inhibiting effect of microglia and macrophages. Proteomic analysis of the factors present in the conditioned medium, or assessing the effect of neutralization of literature-based candidates, could facilitate the identification of the effective secreted factor(s).

Finally, other ECM molecules may prevent OPC maturation and potentially affect the remyelination-supporting function of microglia and macrophages in MS lesions (Asea et al., 2002; Back et al., 2005; Milner & Campbell, 2003; Sloane et al., 2010; Lau et al., 2012; Stoffels et al., 2013; Beachley et al., 2015; chapter 4). It is therefore important to study whether aFn is the dominant factor in remyelination failure, either via OPCs or microglia and macrophages. or that when the aFn is relieved, other inhibitory factors will stand up. In fact. ECM actions are often intertwined and dependent on how the individual components are spatially arranged. Also, stiffness of this structure is of relevance and contributes to cell behavior. Thus, it is important to establish the net effect of the ECM environment in the distinct MS lesions, i.e. active and chronic MS lesions, on glia cell behavior. This will provide knowledge on how the MS ECM environment affects microglia and macrophages, and whether this
contributes to the observed intermediate microglia/macrophage phenotype in MS lesions. Insight into the dominant ECM factor(s) will offer also means to target the relevant ECM components to overcome glia dysfunction, and accordingly regulate CNS resident cells to remodel the ECM to allow for remyelination in MS lesions. Bearing the results as described in this thesis in mind, MMP7 may be a relevant target and tool to remove the provisional ECM that is required at the onset of demyelination, but should be timely removed at the onset of remyelination.

References


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