The role of differently activated macrophages in axonal damage during multiple sclerosis

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CHAPTER 9

Summary and conclusion

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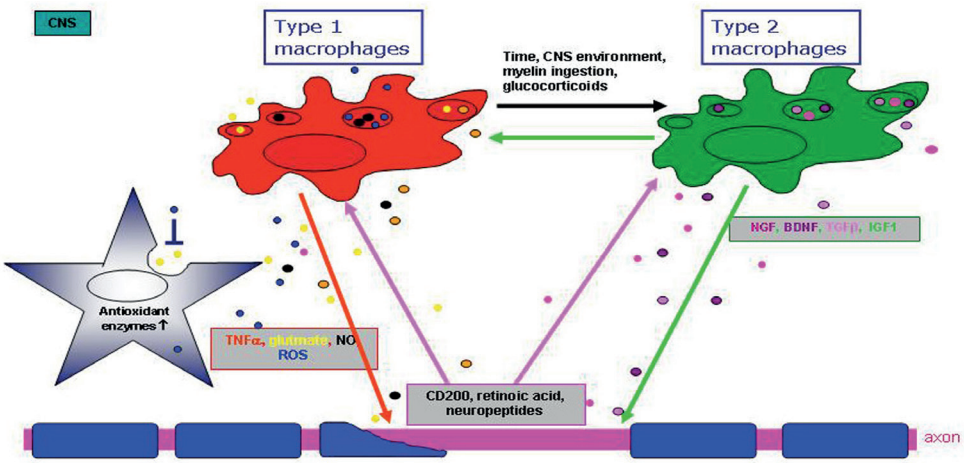
In multiple sclerosis (MS) neuronal deficits arise when damage to neurons can no longer be compensated for and/or repaired [1]. The continuous progression of axonal loss ultimately leads to irreversible clinical dysfunction, and axonal damage and loss are the main correlate of clinical disability in MS [2]. Macrophages are a major cell type infiltrating MS lesions. Macrophages may be involved in the induction of damage, due to the production of large amounts of inflammatory mediators, including cytokines and reactive oxygen species (ROS). However, macrophages have also been implicated in repair processes in MS lesions, due to the production of neurotrophic factors [3-5]. As stated in the introduction of this thesis macrophages are a heterogeneous population of cells that exist in different activational phenotypes [6-8]. The most extreme phenotypes are classically activated (CA), pro-inflammatory macrophages and alternatively activated (AA), growth promoting macrophages [6].

The studies described in this thesis aimed to elucidate the role of differently activated macrophages in axonal damage and repair. Here I will summarize the findings of my thesis, highlight their importance and give some suggestions for future research.



Proposed model for macrophages during MS. We hypothesized that in MS lesions macrophages upon entering the lesion become CA activated. This is in essence a beneficial response, because CA macrophages are efficient in the phagocytosis of cellular debris (chapter 6) and increase remyelination in an inflammatory environment 9,10. However, the CA macrophages also induce bystander damage by their production of nitric oxide (NO), ROS, glutamate and tumor necrosis factor-alpha (TNF-α) (chapter 7). These substances can all be toxic to neurons and also reduce the capacity of astrocytes to detoxicify the neuronal micro environment 11. Our hypothesis also includes that the CNS environment downregulates the CA phenotype or even switches the macrophages to an AA phenotype. The central nervous system (CNS) might modulate the macrophage phenotype by the expression of CD200, CD47, retinoic acid and neuropeptides. Finally, we also hypothesized that AA macrophages contribute to neuroprotection through the production of different species of growth factors (chapter 7)

AA macrophages have increased migratory ability and are attracted by neurons

Only few reports are available on the functions of differently activated macrophages in the context of the CNS. During spinal cord injury CA macrophages have been observed inside the lesion, while AA macrophages are present at the rim of the lesions [12]. In MS lesions similar results have been observed in that MR expression in macrophages has been observed in the perivascular space [13], while inducible nitric oxide synthase (iNOS) positive macrophages have been observed in the active rim of the MS lesion [14]. These data suggest that differently activated macrophages migrate differently in a CNS environment. Therefore, migration was studied in **chapter 3**. We found that AA macrophages were attracted in higher numbers towards neuronally conditioned medium (NCM). This was probably due to production of CXCL12 by neurons [15] and the higher expression levels of CXCR4, the receptor for CXCL12, by AA macrophages [16]. CXCL12 was found to attract AA macrophages in significantly higher numbers compared to CA macrophages. The AA macrophages were more motile and adhered less to the extracellular matrix (ECM) compared to CA macrophages. Due to the increased motility, lower adhesion and attraction towards NCM, AA macrophages might migrate better in the CNS parenchyma towards neurons and locally release growth factors stimulating repair and growth. In **chapter 7** we describe our finding that AA macrophages indeed express brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and platelet derived growth factor (PDGF) at a higher level compared to CA macrophages, indicating that a contribution of AA macrophages to axonal repair and growth is possible.

We found that the motility of CA macrophages was lower than AA macrophages, while adhesion to ECM was higher than in AA macrophages. The lower motility and higher adhesion of CA macrophages might limit migration and therefore bystander damage induced by CA macrophages by secretion of NO and pro-inflammatory cytokines. Astrocyte conditioned medium was found to significantly attract CA macrophages. Due to the production of pro-inflammatory cytokines and ROS, CA macrophages decrease glutamate uptake by astrocytes [11;17], contributing to excitotoxicity in neurons observed in MS. In MS lesions astrocytes are hypertrophic, activated and they secrete cytokines [18]. The presence of CA macrophages could further enhance the activation of astrocytes by the release of pro-inflammatory cytokines such as TNF-α, inhibiting axonal regeneration. Finally, the cytoskeletal organization also differed between AA and CA macrophages. Migration, motility and adhesion are all influenced by cytoskeletal organization [19;20].

CA macrophages phagocytose neurons and neuronal debris

Next, we determined phagocytic capacity of CA and AA macrophages. The most prominent feature of macrophages in an MS lesion is phagocytosis. We focused on the phagocytosis of neuronal debris, since neuronal debris has been found to inhibit neuronal regeneration [21;22]. In **chapter 6** we observed that the phagocytosis of neuronal debris and damaged neurons is higher in CA macrophages compared to AA macrophages. CA macrophages might therefore have beneficial effects,

since removal of axonal debris generates a regenerative environment for neurons [22], possibly due to the existence of inhibitory signals in axonal debris [22]. Inefficient clearing of cellular debris could ultimately even lead to neurodegeneration [23]. Furthermore, in MS lesions, CA macrophages could also contribute to repair, since an inflammatory environment increased remyelination [9;10;24], although the phenotype of the macrophages involved has not been clearly established. However, the continuous production of neurotoxic factors by CA macrophages results in considerable bystander damage, as described in **chapter 7**. Apparently in MS lesions of patients with progressing disease the balance shifts to the damaging effects. Altogether, it would be ideal if the phagocytic capacity of CA macrophages could be promoted and the damaging effects of ROS and NO production could be inhibited.

AA macrophages are not toxic to neurons, but are neuroprotective and growth promoting

In non-CNS tissues the diverging effects of CA and AA macrophages have been well established. However, less is known about their effects in brain and spinal cord. Only one paper directly addressed the question of the effects of CA and AA macrophages on neurons [12]. In **chapter 7** we showed that direct contact between CA macrophages and neurons was toxic, while direct contact with AA macrophages did not appear to be toxic for neurons *in vitro*. CA conditioned medium was more toxic compared to AA conditioned medium. Factors produced by CA macrophages induced a reduction of axonal growth and neuronal survival, while exposure to conditioned medium from AA macrophages did not [12]. These diverging effects lead to the need for regulation of the innate immune response, as reviewed excellently by Rivest [24]. More studies have addressed the neuroprotective effects of microglia. Conditioned medium from microglia protected against toxic insults [25-27]. Microglia are suggested to have an AA phenotype [28]. Additionally, we observed that after the induction of damage in neurons, AA conditioned medium increased neuronal vitality compared to CA conditioned medium. This could be due to the production of increased levels of PDGF, BDNF and NGF by AA macrophages compared to CA macrophages that we observed in **chapter 7**. The fact that AA macrophages produce these growth factors in higher amounts compared to CA macrophages has not been described before. Since AA macrophages are attracted towards NCM, are not neurotoxic and are able to secrete nerve growth factors, they would be beneficial in MS lesions by inducing axonal repair. This is potentially very interesting in MS lesions, since neurodegeneration is an important factor in disease progression.

Myelin phagocytosis changes the phenotype of macrophages

The phenotype of macrophages is dynamic and in response to sequential stimuli macrophages are able to change their phenotype [29;30]. In MS lesion ingestion of myelin could influence the phenotype of the macrophages present. Indeed in MS lesions foamy macrophages, containing myelin debris, were found to express anti-inflammatory cytokines, without the expression of pro-inflammatory cytokines [13]. *In vitro,* macrophages that had ingested myelin had a lowered LPS response [13]. In

chapter 4 we assessed whether the effect of the ingestion of myelin on the LPS response could be due to the activation of the Liver X receptor (LXR). We found that the expression of LXR did not increase after myelin ingestion, although LXR activity was significantly enhanced. The decrease in TNF-α mRNA expression, part of the LPS response, after myelin ingestion was no longer visible after silencing of LXR. This partial restoration of the LPS response after LXR siRNA and myelin ingestion could be indicative of the fact that myelin signals via LXR and thereby down regulates the NF-κB activity, leading to a reduced TNF-α production. However, another explanation would be that LXR is constitutively active and removing this brake on NF-κB activation might increase TNF-α production. An indication that his takes place could be observed when comparing macrophages exposed to LPS without myelin. LXR siRNA treated macrophages had higher expression levels of TNF-α compared to control macrophages. Furthermore, in **chapter 5**, we observed that not every myelin preparation had a similar effect on macrophages, as the decrease in LPS response differed between myelin preparations. We found that in our hands the myelin preparation that induced the decreased LPS response and cytokine production contained LPS. The observation that myelin can easily be contaminated with LPS and that LPS free myelin did not inhibit the LPS response indicates that myelin ingestion in itself does not lead to an inhibition of the LPS response. The phenotype of foamy macrophages is not clear cut. MR expression is a very well described marker of the AA phenotype [6;8;31;32] and in MS lesions foamy macrophages were only MR positive in the perivascular space and not in the parenchyma of the brain, although many foamy macrophages could be observed there [13]. High MR expression by perivascular macrophages and low expression by foamy macrophages in MS lesions was also observed in **chapter 8**. Since macrophages are able to switch phenotype, myelin ingestion might induce an AA phenotype, but the predominantly pro-inflammatory environment of the MS lesion might revert the phenotype. Finally, the presence of T cells in the lesions might affect the activation status of macrophages.

In MS lesions most macrophages have a CA phenotype

In MS lesions, where foamy macrophages were prominently present, we observed that all macrophages predominantly expressed markers for the CA phenotype (**chapter 8**). A panel of markers was chosen, described in literature to be differently expressed by human CA and AA macrophages. In active MS lesions the markers for the CA phenotype were highly expressed om macrophages present in the lesions, while markers for the AA phenotype were expressed at a much lower level compared to CA markers. Using the markers for AA macrophages, especially perivascular macrophages were easily identified. This leads us to conclude that the predominant phenotype in MS lesions is the CA phenotype, inducing an inflammatory environment.

This persistence of the CA phenotype was also observed in spinal cord injury [12]. The authors suggested that the persistence of the CA phenotype impairs repair and regeneration probably also due to secondary neurodegeneration [12]. In MS lesions the persistence of the CA phenotype might induce the slow burning

neurodegeneration observed. Phagocytosis, next to its beneficial effects of clearing cellular debris, is associated with an increased production of oxygen radicals [33;34], indicating an CA phenotype and leading to bystander damage. It would be beneficial if macrophages could be stimulated to acquire an activational state, characterized by an increased phagocytic capacity without the production of oxygen radicals. The persistence of the CA phenotype might lead to limited remyelination and axonal regeneration, making the research into skewing of the macrophage phenotype important in order to harness the beneficial effects of macrophages while reducing the negative aspects.

An in vitro model for MS

In order to investigate the effects of CA and AA macrophages on axonal damage during MS, we set out to develop an *in vitro* model for demyelination in **chapter 2**. We decided to use the whole brain spheroid model in which all CNS cell types are present in a 3-dimensional conformation, inducing development and spatial complexity *in vitro* similar to the *in vivo* situation [35]. In these cultures multilayered myelin is formed [36]. Lysolecithin reproducibly induced demyelination in the whole brain spheroid cultures, as measured by immunohistochemistry, electron microscopy and CNPase activity assay. Partial remyelination occurred after demyelination, probably due to differentiation of NG2 positive oligodendrocyte precursor cells present in the cultures. Since partial remyelination was visible, we concluded that this model is suitable to test the effects of novel therapeutics on remyelination, e.g. magnitude and speed. Furthermore, this model is very useful to study the mechanism of remyelination and the effects of demyelination, for example on axons. Using spheroid cultures, cells not endogenous to the CNS, like macrophages, can be introduced into the spheroids with relative ease [37-39]. In order to determine the role of macrophages on axonal damage and repair seen during MS we could add macrophages. One drawback of the whole brain spheroid culture is that it would be very difficult to determine the effect that these macrophages directly have on axons. All effects seen in these cultures would be a mix of direct effects on the axons and indirect effects via other CNS cell types. Furthermore, the extent of migration into the spheroid would play a role in the effects the differently activated macrophages would have on spheroids.

FUTURE PERSPECTIVES

We observed that in MS lesions macrophages obtain a CA phenotype and express markers for the AA phenotype at a much lower level. In MS lesions the persistence of the CA phenotype could inhibit repair, since CA macrophages have been shown to be neurotoxic while AA activation abolished the neurotoxic effects and increased expression of nerve growth factors (**chapter 7**). In MS lesions it would therefore be beneficial to reduce the CA phenotype and promote the AA phenotype. The possible mechanisms to skew the macrophage phenotype in the CNS are interesting to investigate, in order to design anti-inflammatory therapies

Glucocorticoids are an accepted therapy during MS. Treatment with glucocorticoids might be a means of skewing the activational phenotype, since

exposure to glucocorticoids induce a wound healing phenotype in macrophages *in vitro* [6;32]. By skewing the macrophage phenotype towards a more alternatively activated subtype possibly acute early neuronal damage could be reduced. A glucocorticoid receptor ligand was found to attenuate experimental autoimmune neuritis, decrease the expression of inflammatory cytokines and iNOS and induce a M2 phenotype *in vitro* [40].

Glatiramer acetate, another accepted treatment for MS, has been shown to affect monocytes/macrophages. Glatiramer acetate has been found to modulate cytokine expression in macrophages in that it induces IL-10 and reduces TNF-α expression [41]. Adoptive transfer of monocytes derived from mice treated with glatiramer actetate, with increased expression of IL-10 and TGF-β and reduced TNF-α, IL-12, IL-23 expression, ameliorated EAE [42].

The fact that substances in the periphery can influence cells present in the CNS has been observed previously. In the CNS microglia have been shown to switch cytokine profile, from relatively anti-inflammatory to pro-inflammatory CA phenotype, after systemic injection of LPS after induction of Wallerian degeneration [43]. The peripheral LPS injection also led to a decrease in neurofilament staining, indicating that neurodegeneration is increased. Similar results were found with a mouse model for prion disease, more inflammation and neuronal apoptosis after systemic LPS injection [44]. Likewise, glucocorticoids and glatiramer acetate, when applied peripherally, could influence the phenotype of microglia and macrophages inside the CNS. It would therefore be interesting to determine the effect of glucocorticoid treatment on the macrophage phenotype in the CNS. Furthermore, since in MS lesions the macrophages predominantly express markers of the CA phenotype, it would be useful to study whether AA macrophages that enter a pro-inflammatory environment would be able to keep their phenotype. Could AA macrophages be able to exert positive effects in a pro-inflammatory environment?

Another factor in the CNS that might be able to influence macrophage phenotype are neurons. Neurons exert effects on neighboring glial cells. Indications have been reported that neurons do have effects on macrophages. Neurons express molecules able to inhibit macrophage activation: CD200 and CD47 [45-47]. In MS lesions the expression of CD200 and CD47 were found to be decreased [48]. In MS lesions CD200 and CD47 could be involved in the limitation of lesion expansion, since their expression was not down regulated in the normal appearing white matter. The studies investigating the effects of CD200 and CD47 show a down regulation of activation of macrophages. Does the down regulation of the activation of macrophages also lead to an AA phenotype? An indication that CD200/CD200R could be part of the AA activational phenotypes is that the expression of CD200R is reduced during classical macrophage activation [49;50]. Furthermore, expression of CD200R was found to correlate with MR expression in macrophages after exposure to synovial fluid of spondylarthritis or rheumatoid arthritis [51]. Next to the direct interaction between neurons and macrophages via receptor ligand binding, neurons can secrete factors that influence macrophages, such as retinoic acid and neuropeptides. Macrophages are known to poses receptors for retinoic acid, both retinoid X receptor (RXR) and retinoic acid receptor (RAR). Both RXR and RAR have

been shown to modulate the immune response by reduction in the pro-inflammatory gene expression and interference with NF-κB signaling [52;52-55]. Furthermore, after activation of RXR and RAR indications have been found pointing to the induction of an AA phenotype, such as reduction of adhesion, phagocytosis by RXR [56] and inhibition of TNF-α and iNOS expression in microglia after stimulation with LPS by RAR activation [57]. All these findings are consistent with an AA phenotype, indicating that retinoic acid, which neurons can secrete, is able to induce an AA phenotype in macrophages. Neuropeptides are important tools for neurons to communicate with surrounding cells. Macrophages and microglia respond to these neuropeptides. Some neuropeptides have potent anti-inflammatory effects on macrophages, such as vasoactive intestinal peptide (VIP), α-melanocyte-stimulating hormone (α-MSH) and urocortin [58-61]. Furthermore Neuropeptide Y (NPY) has been found to modulate both phagocytosis and phagocytosis-induced production of reactive oxygen species (ROS) (reviewed in [62]). Neuropeptides are also able to increase inflammation. In macrophages substance P enhances the oxidative burst [63], induces specific chemokine expression [64] and increases phagocytosis [65]. Finally, different receptors for NPY are involved in the suppression and potentiation of the oxidative burst in macrophages, indicating that immunomodulatory properties of NPY are determined by the subtypes of receptors expressed on macrophages [66]. It would therefore be interesting to study whether CA and AA macrophages express different neuropeptide receptors. Furthermore, the effect of the addition of neuropeptides on macrophage phenotype would also be interesting to study, possibly switching the phenotype.

Finally, CD40 has been receiving more attention recently. Mainly through its role in T cell priming, CD40 is a likely candidate to be involved in autoimmunity. It has been mainly studied on dendritic cells. In mice CD40 was instrumental in the induction of a CA phenotype in that CD40 stimulation increased expression of proinflammatory cytokines, iNOS and CCL2 in macrophages. Abrogation of CD40 signaling via TRAF6 induced IL-10 expression, indicating an anti-inflammatory phenotype [67]. Expression of CD40 on microglia is essential for the progression of EAE in mice, since EAE induced in mice lacking CD40 only in microglia had reduced demyelination and T-cell infiltration [68;69]. Furthermore, treatment with anti-CD40 ligand antibody during EAE reduced clinical signs due to the blocking of the interaction between CD40 on macrophages and CD40 ligand on T cells [70]. Perhaps the blocking of this interaction leads to macrophages developing a more anti-inflammatory phenotype, thereby limiting lesion development. An antagonistic antibody for CD40 had beneficial effects during marmoset EAE, although CD40 seemed to be more critical during early phases of the disease since treatment was most beneficial if started early [71]. In MS lesions CD40 was observed mainly in macrophages and microglia, comparable to our results (**chapter 8**), in close association with CD40 ligand bearing cells [70]. Our results indicate that CD40 expression is a sign of classical activation. Apparently in MS, microglial cells are triggered to be active. Monocytes from patients with MS have increased expression of CD40 [72].

CONCLUSION

Both CA and AA macrophages can have beneficial effects in an MS lesion. The CA macrophages are very efficient in clearing debris, however, they are toxic to neurons. The AA macrophages secrete higher amounts of neurotrophins and are not neurotoxic, however they are less efficient in clearing debris. It would be of great value if an activational status in macrophages could be reached with an increased phagocytic capacity, without the production of neurotoxic substances such as ROS, NO and TNF-α, and with the secretion of growth factors. Due to the highly plastic nature of macrophages, this is feasible. Future research should therefore focus on the functional activation status of macrophages stimulated with for example glucocorticoids, neuropeptides and CD40, with respect to the secretion of growth factors, phagocytosis and secretion of neurotoxic substances.

Conclusions of the studies described in this thesis	
- Spheroids are a reproducible model for demyelination *in vitro* - Remyelination occurs in spheroids	Chapter 2
- AA macrophages are attracted towards NCM - CA macrophages are attracted towards ACM and OCM - Adhesion, motility and actin cytoskeleton differ	Chapter 3
- LXR activity is able to downregulate LPS response - Myelin ingestion increases LXR activity	Chapter 4
- LPS is a common contaminant of myelin - LPS-free myelin does not decrease LPS response in macrophages	Chapter 5
- CA macrophages phagocytose more neurons and neuronal debris compared to AA macrophages - Degradation of β-tubulin an NF occurs within 24 hours in CA and AA macrophages, MAP2 is degraded within 48 hours	Chapter 6
- CA macrophages are toxic to neurons - AA macrophages are not toxic to neurons - AA macrophages produce nerve growth factors	Chapter 7
- Markers for CA macrophages are highly expressed in MS lesions - Markers for AA macrophages are expressed at a low level in MS lesions	Chapter 8

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