Diversity of microglia

*Their contribution to multiple sclerosis lesion formation*

van der Poel, M.

Publication date
2020

Document Version
Other version

License
Other

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 11

General discussion
Microglia are recognized for their role in demyelination and inflammation and appear as foamy cells in active MS lesions\(^1,2\). Therefore, they play an essential role in MS pathology; however, the questions remain how microglia do become activated, whether they can start MS lesion formation and what is their contribution to MS lesion development and expansion. Answering these questions will expand our knowledge on the role of microglia in MS pathology and will provide information on the potential use of microglia as therapeutic target to successfully treat progressive MS. The proposed view on microglia activation and their contribution to MS lesion initiation and development, according to the results presented in this thesis, is displayed in Figure 1.

11.1 Research using primary human microglia

The use of primary human microglia will provide valuable information on their role in neurological and psychiatric diseases. Here, we optimized a protocol to rapidly isolate primary microglia within a few hours from post-mortem human brain tissue (with an average post-mortem delay of 6 hours), while retaining their viability and phenotype. We assessed the impact of ante- and post-mortem variables on the yield and phenotype of isolated microglia and showed that only the pH of the cerebrospinal fluid (CSF), reflecting brain acidity, impacts on the yield of microglia. However, brain acidity, post-mortem delay, total time until isolation and age did not impact on the expression of CD45 and CD11b on primary microglia. Therefore, changes observed in the profile of isolated human microglia reflect neuropathological differences, such as higher expression of CD45 on microglia in normal-appearing white matter MS as compared to control, indicating an alerted state of MS microglia (Chapter 2). It is important to note that primary microglia lose their signature gene profile, including \(P2RY12\) and \(CX3CR1\), very rapidly during culturing conditions, indicating that downstream applications such as gene or protein profiling should be performed acutely after isolation in order to relate microglial changes to neuropathology. However, cultured primary human microglia can still be used to perform functional assays, including phagocytosis experiments (Chapters 2-3). To conclude, the profile of acutely isolated human microglia can reliably be studied in order to identify microglia specific characteristics, and their profile in relation with neuropathology, such as MS-related changes.

11.2 Microglia core signature

The microglia core signature\(^3-6\) allows to distinguish between microglia and infiltrating monocyte-derived macrophages, which constitute the environment in active lesions and might contribute to MS lesion formation\(^7,8\). In Chapter 5, we performed RNA-sequencing analysis and observed a high expression of adhesion GPCRs by microglia in grey matter (GM) and white matter (WM) tissue, among them \(ADGRG1\) (encoding GPR56), which is one of the most abundantly expressed adhesion GPCRs on GM and WM microglia. Interestingly, this gene is not expressed by monocyte-derived macrophages that repopulate the CNS after BBB impairment\(^9\). Furthermore, we confirmed expression of GPR56 on microglia at protein level, and showed nearly absence of GPR56 expression on choroid
plexus macrophages, which makes it a relevant marker to distinguish microglia from macrophages under pathological conditions whereby monocytes/macrophages can enter the CNS.

Besides identifying microglia, GPR56 is used to identify homeostatic microglia, since the expression of GPR56 is reduced under neurodegenerative conditions in animal models\textsuperscript{10,11}. Remarkably, we demonstrate that GPR56 is the only signature gene with reduced expression on microglia in NAWM (Chapter 5) and its expression is further reduced, together with P2RY12 and TMEM119, in a subset of microglia enriched in active MS lesions (Chapter 9). In line with the reduced expression of GPR56 on activated microglia, we propose a role for GPR56 in maintaining microglia homeostasis, which is in line with our previous data that demonstrates a role for GPR56 as effector molecule in preventing cytotoxic lymphocyte activation\textsuperscript{12}. Still, more research is required to examine the microglial function of GRP56 and to confirm its microglial specificity by demonstrating absence of GRP56 on monocyte-derived macrophages that infiltrate the CNS under neurodegenerative conditions in humans.

11.3 Microglia heterogeneity

GM and WM brain regions differ in their cellular composition, as GM regions mainly contain neuronal cell bodies and WM regions mainly consist of myelinated axons, suggesting that the microglial profile might differ among brain regions. Moreover, the cellular composition of the microglial environment impacts on their signature, as we noticed drastic reduction of human microglia signature genes when microglia are removed from their environment and kept for 4 days in culture (Chapter 2). Indeed, in Chapter 5, we described profound changes in the transcriptional profile of human microglia between GM and WM brain regions. Pathway analysis revealed differences in immune response genes, whereby microglia in GM highly express interferon response genes and WM microglia highly express inhibitor genes associated with NF-κB response. High expression of interferon genes by control and MS GM microglia, might be related to the presence of neuronal cell bodies. Activation of the interferon pathway may play an essential role in the protection of neurons by limiting viral spread in the CNS\textsuperscript{13–15}. Our data is in line with previous studies on mouse, rat and human microglia, showing heterogeneity of microglia across GM and WM brain regions\textsuperscript{16–19}. Furthermore, distinct expression of immune response genes by microglia across GM and WM brain regions may have implications for their response to different immune stimuli in the CNS. Similar, we found a higher expression of CD45 protein on WM microglia as compared to GM microglia, suggesting diversity in immune activation between control WM and GM microglia (Chapter 2). Interestingly, the region-specific profile of microglia in both control and MS points to their potential distinct role in inflammation and might contribute to differences in MS lesion formation across GM and WM regions. Although we have shown that the profile of human microglia is environment-dependent, the functional properties of microglial subpopulations across brain regions and their implications in neurodegenerative diseases remains to be investigated.

Besides regional diversity, subpopulations of microglia may also appear within a brain region, for instance in response to CNS pathology, thereby reflecting diverse activation states of
microglia. In Chapter 6 we identified heterogeneous populations of microglial nodules within a MS brain region of NAWM tissue, determined by immunohistochemical staining for phagocytic and lysosomal proteins. Expression of these markers, including MSR1 and CD11c, but also the lysosomal stress enzyme CHIT1, were expressed by several nodules, indicative for demyelination in some nodules which may contribute to MS lesion formation. Similar to microglia heterogeneity in NAWM MS tissue, we identified microglia subpopulations in active MS lesions, determined by single cell mass cytometry (Chapter 9). This study showed that subsets of microglia emerge in active MS lesions, which differ in their homeostatic profile, based on P2RY12, TMEM119 and GPR56 expression, indicating that microglial subsets differ in their activation state in response to MS pathology. Summarizing, microglia heterogeneity was defined among brain regions, but also within brain regions, as subsets with diverse activation profiles appear in NAWM tissue and active MS lesions. However, the question remains which triggers are responsible for the emergence of diverse microglial subsets among and within brain regions.

11.4 Changed myelin composition in MS

Most studies described in this thesis were performed on normal-appearing MS tissue to define early MS-pathological changes. Normal-appearing tissue is histopathological characterized as a region with intact myelin, detected by immunohistochemical staining for myelin protein PLP, and expression of some HLA-DR+ ramified cells. Interestingly, we identified: 1) early signs of MS pathology related to increased expression of lipid metabolism and lysosomal genes by microglia in NAWM MS tissue (Chapter 5); 2) increased expression of scavenger receptor and lipid metabolism genes around mixed MS lesions (Chapter 8); 3) plasma and B cells together with genes for immunoglobulins appear in MS NAWM tissue that contains microglial nodules (Chapter 6); and 4) IgG-immune complexes bound to myelin that was isolated from NAWM MS tissue (Chapter 7). One of the genes that is highly upregulated in MS NAWM microglia is LPL, which facilitates myelin-derived lipid uptake by macrophages and microglia20,21. Furthermore, lipid metabolism genes CHIT1 and CHI3L1 are high expressed in NAWM tissue (Chapter 5 and 8) and several studies have highlighted these markers as important biomarkers for MS progression22–24. Together, these data provide evidence for early MS-related changes in NAWM tissue by increased uptake and processing of myelin-derived lipids, but also identified antibodies bound to myelin in NAWM MS tissue. Alterations in MS myelin might impact on lipid metabolism of microglia that take up MS myelin. In line with this, our group has previously shown that microglia take up MS myelin more efficiently than control myelin25, which might be related to the observed IgG-opsonisation of MS myelin shown in Chapter 7, since we detected IgG bound to MS myelin on the same myelin samples that were used for in vitro experiments by Hendrickx and colleagues25. Moreover, interestingly, other studies have shown alterations in myelin lipid composition that results in myelin destabilization26–28 and could even promote demyelination29. Moreover, Romanelli and colleagues provide evidence that myelin alterations were induced by antibody opsonization in cuprizone animal models30. Changes in myelin lipid composition also illustrates why NAWM is characterized as tissue with intact myelin by immunohistochemical stainings, since this method does not detect lipids but only myelin proteins, which are potentially not a-
tered yet in NAWM MS tissue. To further elucidate myelin-specific alterations in NAWM MS tissue, follow-up studies should focus on characterizing MS myelin modifications, such as myelin outfoldings (bulb-like structures of the myelin sheath)\textsuperscript{30} and g-ratio (ratio between axonal diameter and outer diameter of myelin), which serves as a measure to define myelin intactness. Both myelin outfoldings and thickness can be visualized at an ultrastructural level using high-pressure freezing techniques and subsequent electron microscopy\textsuperscript{31,32}, and could serve as a feature to visualize first signs of MS pathology. Besides myelin alterations at an ultrastructural level, lipidomic analysis of NAWM MS myelin will also provide additional information on MS myelin-lipid changes that might contribute to the start of demyelination.

In Chapter 7, we showed that MS myelin is bound by antibodies, which may target myelin lipids\textsuperscript{33} or proteins\textsuperscript{34}. However, the antigen that is targeted by these antibodies is not known yet. The cells responsible for antibody secretion are plasma and B cells, which are indeed found in NAWM MS tissue, in regions containing nodules (Chapter 6). The question still remains why plasma and B cells are present in NAWM tissue in MS and what triggers them to secrete, potentially, MS myelin-specific antibodies. Furthermore, it would be very interesting to study the effect of IgG-opsonisation on MS myelin, which, together with complement, might promote or even start demyelination\textsuperscript{35–37}. Moreover, investigating the process of myelin-lipid degradation by microglia after uptake of IgG-opsonized MS myelin in comparison to control myelin is of high interest. To conclude, MS myelin lipid alterations are observed already in NAWM tissue and may precede demyelination. When MS myelin is taken up by microglia its altered composition may increase lipid metabolism and might hamper myelin degradation, which could lead to insufficient clearance of MS myelin. Prolonged uptake of MS myelin may eventually lead to lysosomal accumulation and development of foamy microglia.

11.5 Breaking microglial tolerance in MS

For a long time, there is an ongoing debate on how microglia become activated and start to secrete pro-inflammatory cytokines in MS. In normal-appearing MS tissue, the homeostatic profile of microglia is mainly conserved (Chapter 5) and we and others showed that primary human microglia are tolerogenic to classic immune stimuli, such as the TLR4-ligand LPS\textsuperscript{38,39}. Another TLR-ligand, Poly I:C, that mimics a viral stimulus, also does not activate primary human microglia (Chapter 7). Therefore, human microglia might need an additional stimulus to break their tolerance and become immune activated. Previous studies have shown that co-stimulation with TLR-ligands and antibody immune complexes can break tolerance for immune stimuli in other myeloid cells, by promoting expression of pro-inflammatory mediators\textsuperscript{40–42}. This mechanism of tolerance breakdown depends on cross-talk between Fcγ-receptors (FcγRs) and TLR-receptors and might also be an important mechanism implicated in tolerance breakdown of primary human microglia. Indeed, in Chapter 7 we showed that microglial tolerance was broken by a combination of two stimuli, namely TLR-ligands LPS or Poly I:C combined with IgG-immune complexes, which have been identified to bind MS myelin. After tolerance breakdown, microglia secrete pro-inflammatory cytokines and chemokines such as TNF, IL-1β and IL-8. Interestingly, the most pronounced effect was observed by stimulation with IgG-immune complexes and the viral mimicking stimulus Poly I:C. Since many studies suggest
that viruses are implicated in MS pathology, either as a primary cause or as a trigger of relapses during the relapsing-remitting phase\textsuperscript{43,44}, they are an interesting topic to be studied in more detail, since neurotrophic viruses might activate microglia but might also activate T cells present in the CNS and could trigger MS lesion formation.

FcγRI and FcγRIIa are the two main responsible IgG receptors for microglial tolerance breakdown. However, whether TLR ligands and IgG-immune complexes are both present in MS and could break microglial tolerance to start MS lesion formation needs to be further elucidated. For this reason, we focussed on microglial nodules, which emerge in NAWM tissue (Chapter 6) and might be the start of an MS lesion\textsuperscript{45–47}. In line with this hypothesis, we found more active lesions and a higher number of reactive sites in MS brain donors with nodules as compared to donors without nodules. Since reactive sites contain accumulating HLA-DR\textsuperscript{+} microglia and are thought to be the first stage of MS lesion formation, a positive correlation between the number of reactive sites and the presence of nodules suggests that nodules are implicated in MS lesion initiation and microglia immune activation may start here. Interestingly, the majority of microglial nodules is phagocytic and lysosomal active, defined by expression of lesion-enriched proteins (Chapter 7) MSR1, CD11c and CHIT1, showing early signs of demyelination and lysosomal stress. Furthermore, some nodules were found in close contact to proliferating T cells, plasma and B cells, and genes for antibody production were found in nodule-containing MS tissue. These antibodies may form immune complexes on myelin and together with TLR-ligands or cytokines secreted by proliferating T cells, they could break microglial tolerance, corroborating the idea that MS lesion formation may start here. Importantly, only several MS nodules contained phagocytic active microglia and even a lower fraction is in close proximity to lymphocytes, indicating that not all nodules may start the formation of a demyelinated MS lesion. However, more in-depth analysis is required, for instance by RNA-sequencing and multiplex imaging CyTOF, to elucidate if microglial nodules that are in close contact to parenchymal lymphocytes are immune activated and contribute to MS lesion formation.

11.6 Microglial contribution to MS lesion initiation

Microglia are implicated in MS pathology by contributing to demyelination. The role of microglia in MS lesion formation has been widely studied in mice, in the experimental autoimmune encephalomyelitis (EAE) model and in models for demyelination based on cuprizone or viral infection\textsuperscript{2,48,49}. In EAE lesions, the majority of myeloid cells consist of infiltrating monocytes/macrophages, showing a key role for circulating myeloid cells, next to microglia, in EAE progression\textsuperscript{2,50,51}. In addition, lesion-associated microglia/macrophages in animal models highly express pro-inflammatory mediators, such as chemokines, radical oxygens and pro-inflammatory cytokines\textsuperscript{2,52}. An important drawback of these animal models is the lack of mimicking all aspects of MS pathology, including the heterogeneous aspect of MS regarding pathology and clinical course, and the high amount of CD4\textsuperscript{+} T cells present in EAE lesions, whereas in MS lesions the majority of T cells are CD8\textsuperscript{+} \textsuperscript{53,54}. For this reason, post-mortem brain tissue of MS donors provides us the unique opportunity to study the microglial profile in context of progressive MS pathology.
When microglia become activated, they can secrete pro-inflammatory mediators that may contribute to or even start demyelination by promoting oligodendrocyte death and myelin damage. In NAWM MS tissue, we found early signs of demyelination events identified by upregulation of lipid metabolism and lysosomal genes by homeostatic microglia, implicating that myelin degradation by microglia might precede microglial activation (Chapter 5). The question still remains if microglia start demyelination or if alterations in MS myelin occur before microglia start to demyelinate, since myelin lipid alterations and antibody-opsonization were already assessed in NAWM MS tissue (Chapter 7).

11.7 Microglial contribution to MS lesion expansion

Microglia become foamy-like cells after prolonged uptake of myelin debris and they make up the majority of HLA-DR+ cells in active MS lesions. It has been suggested that cholesterol efflux deficiency is one of the mechanisms that might lead to impaired myelin clearance and lysosomal dysfunction, and finally leads to accumulation of myelin in microglia. Interestingly, in Chapter 9 we show that these myelin-containing microglia in active MS lesions are noninflammatory, as they do not produce pro-inflammatory cytokines such as IL-1β and TNF. Their homeostatic profile is reduced, defined by decreased expression of P2RY12, TMEM119 and GPR56, and increased expression of so called disease-associated proteins, including CLEC7a, SPPI and AXL. Furthermore, several microglia highly express TNF in NAWM tissue, but these cells were absent in active lesions. TNF plays a role in immune modulation, they might have a neuroprotective function, and expression is reduced after lipid uptake, indicating that TNF expression by microglia in NAWM MS tissue might promote neuroprotection which might be impaired in active lesions after prolonged myelin uptake. Furthermore, expression of phagocytic receptors is increased in active MS lesions, such as scavenger and Fcy-receptors, implicated in uptake of myelin (Chapter 9). Similar, genes highly expressed in and around mixed lesions, containing many foamy, lipid-laden microglia/macrophages, are implicated in myelin uptake/degradation and suppression of inflammation, including scavenger receptors and lipid metabolism genes CHIT1 and GPNMB (Chapter 8). Prolonged uptake of myelin by microglia or macrophages promotes an anti-inflammatory phenotype, defined by increased expression of CCL18 and IL-10, and reduced TNF and iNOS expression in vitro. Moreover, uptake of myelin debris is crucial to promote axonal repair and eventually promotes remyelination. To conclude, myelin uptake by microglia in active lesions of progressive MS cases alters their homeostatic profile and suppresses inflammation, which might be an attempt to promote remyelination.

11.8 Infiltrating macrophages are hardly present in active MS lesions

GPR56 is highly expressed by microglia and is nearly absent on macrophages, therefore GPR56 can be used to distinguish microglia from infiltrating macrophages under neuropathological conditions. For this reason, we used GPR56 in our mass cytometry study to the profile of microglia isolated from NAWM and active lesions from progressive MS brain donors (Chapter 9). Interestingly, expression
of the macrophage markers CLEC12A, CD206 and CCR2\(^{9,18}\), were not significantly increased in active MS lesions, indicating that hardly any monocyte-derived macrophages are present in active lesions in progressive MS, probably due to an intact BBB. Contrary, other studies have shown that, next to TMEM119\(^+\)HLA-DR\(^+\) cells, TMEM119\(^-\)HLA-DR\(^+\) cells appear in active MS lesions in RRMS\(^7,8\), indicating that monocyte-derived macrophages invade MS lesions. However, importantly, the use of brain tissue derived from relapsing-remitting MS donors might explain the detection of infiltrated macrophages in these studies, highlighting that an impaired BBB in RRMS can facilitate entry of macrophages into the CNS. Therefore, microglia, and not infiltrating macrophages, are key players in de- and re-myelination events in active lesions of progressive, advanced MS.

11.9 New therapeutic approaches to treat MS

In this thesis, we have shown that microglia are not a uniform population and they can be both detrimental as protective, depending on environmental factors. In addition, probably not all microglia that encounter MS-specific triggers, such as myelin-bound antibodies, become activated. Therefore, instead of targeting all microglia, focusing on a specific microglial subset, which is pro-inflammatory, may have more promising therapeutic efficacy. Targeting microglial subsets, such as nodules, which show early signs of demyelination and activation, could be an interesting approach. Ample evidence shows that the phagocytic capacity of microglia is important to clear myelin debris, which facilitates axonal repair and remyelination\(^{58,67,68}\). Therefore, therapeutic strategies should focus on promoting efficient clearance of myelin, stimulate pro-regenerative capacities of microglia and, in addition, block full activation of microglia. The transcription factor IRF5 is an interesting candidate to mediate microglial activation; this transcription factor regulates amplification of pro-inflammatory factors in human monocytes, macrophages and dendritic cells\(^{69}\). Interestingly, IRF5 is also a risk gene for MS\(^70\) and is high expressed by microglia, but not by other CNS cells\(^7\). Inhibiting IRAK4, a kinase that blocks IRF5 translocation to the nucleus thereby blocking pro-inflammatory cytokine production\(^{71,72}\), might be an interesting target for pharmacological intervention. However, the question still remains if blocking microglial immune activation \textit{in vivo} does not interfere with the production of trophic factors and microglial phagocytic capacity, whereby it may inhibit debris clearance and remyelination.

Besides targeting microglia for the treatment of MS, further elucidating the cause of MS myelin alterations will provide information on its potential contribution to demyelination. Since antibodies can target MS myelin and might lead to myelin alterations, blocking antibody secretion by plasma and B cells could be one promising therapeutic approach to block, partly, demyelination. Taken together, preventing myelin alterations and instability could serve as a promising therapeutic approach to prevent further demyelination. Furthermore, targeting microglia for therapeutic strategies should focus on both their pro-inflammatory, phagocytic and regenerative capacities to stop demyelination and promote regeneration, thereby preventing formation of new MS lesions.
11.10 Concluding remarks

MS is one of the most common neurological diseases amongst young adults and affects 108 out of 100,000 people in Europe\textsuperscript{73}. Despite the many available therapeutic drugs, therapies to treat this complex disease have not been successful yet. Microglia are recognised as key players in MS pathology and multiple therapeutic drugs to treat MS have shown to indirectly also target inflammatory or remyelination properties of microglia\textsuperscript{67,74,75}. However, these disease-modifying therapies show limited success in patients with progressive MS. Keeping this in mind, it is important to distinguish between the role of microglia in RRMS and progressive advanced MS, since the inflammatory environment for microglia in RRMS may be different than in progressive MS, caused by absence of infiltrating immune cells form the circulation into the CNS in progressive MS\textsuperscript{76–78}. Indeed, we hardly encountered monocyte-derived macrophages in active lesions of progressive MS brains and together with the absence of peripheral lymphocytes\textsuperscript{54}, this corroborates the idea that monocytes, macrophages and T cells infiltrating from the circulation into the CNS play a less prominent role in progressive advanced MS as compared to RRMS. For this reason, microglia play a key role in de- and re-myelination in progressive MS and are interesting candidates in the search for novel therapies to treat progressive MS.

Several questions still remain to be answered to completely understand the role of microglia in MS lesion formation. First, it is still unclear what exactly triggers demyelination, but both inflammation and alterations of MS myelin might contribute to or even initiate demyelination. Second, the antigen(s) that might be present on MS myelin and evoke(s) an immune response still needs to be identified. Third, aside from functional studies that can demonstrate how microglial activation can be regulated, the potential interaction between microglia and tissue-resident lymphocytes in progressive MS that might result in microglia activation, or contrary might activate lymphocytes, needs to be further elucidated. The use of post-mortem human brain tissue will provide essential information on new approaches to target microglia and successfully treat progressive MS.
Figure 1 | Proposed view on microglial contribution to MS lesion initiation and development. A) Microglia in normal-appearing MS tissue show early changes related to MS pathology, such as increased expression of lipid metabolism genes LPL and CHI3L1 (encoding GPR56), their homeostatic profile is partly conserved (Chapter 5). B) Antibodies bound to myelin are found in normal-appearing MS tissue (Chapter 7), together with plasma cells, B cells and proliferating T cells (PCNA) that appear in nodule-containing tissue (Chapter 6). To break microglial tolerance for microbial stimuli, we identified that primary human microglia need two stimuli: IgG-immune complexes (present on MS myelin) and TLR-ligand Poly I:C (mimics a viral response), which results in production of pro-inflammatory cytokines and chemokines (TNF, IL-8, IL-1β) (Chapter 7). These two stimuli might be present in normal-appearing MS tissue near nodules and could activate microglia within nodules that can lead to MS lesion formation. Microglia within nodules are phagocytic and lysosomal active (MSR1, CD11c, CHIT1), indicative for demyelination. Furthermore, nodules contain many proliferating microglia (PCNA). C) Foamy microglia in active and mixed MS lesions show high expression of phagocytic receptors (FcγRI, FcγRII, CD11c, MSR1) and lipid metabolism gene (CHIT1) but are non-inflammatory and their homeostatic profile is partly conserved (P2RY12, TMEM119, ADGRG1/GRP56; displayed between brackets). Notably, infiltrating macrophages were hardly present in active lesions of progressive MS cases (Chapter 9).
References


Probert, L. TNF and its receptors in the CNS: the essential, the desirable and the deleterious effects. *Neuroscience* 302, 2–22 (2015).


