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DECIPHERING AND FINE-TUNING MYELOID CELLS IN CNS DEMYELINATING CONDITIONS

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Cover illustration: The illustration, created using Microsoft Bing Image Creator, portrays a woodpecker and a tree ring in an artistic style reminiscent of traditional Chinese ink painting. Woodpeckers assist in eliminating pests from trees, contributing to their overall health. However, excessive pecking can also harm the tree. This interplay between woodpeckers and trees mirrors the role of microglia in maintaining myelin health.

Deciphering and fine-tuning myeloid cells in CNS demyelinating conditions

Thesis for Doctoral Degree (Ph.D.)

By

Keying Zhu

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To my family and friends

“O ever youthful, O ever weeping.”

—Jack Kerouac (*The Dharma Bums)*

POPULAR SCIENCE SUMMARY OF THE THESIS

In our brain, there are different types of nerve cells, including neurons and various types of glial cells. In the past, glial cells were considered to be the ‘glue’ of the brain, providing connection, support, and protection for neurons, hence the name. However, new research has shown that their roles extend beyond this, playing a crucial part in the development of many brain diseases. Neurons consist of a large, star-like cell body and a long, trunk-like extension called an axon. Axons are wrapped in a protective layer called the myelin sheath, formed by oligodendrocytes. The myelin sheath protects neurons and supports their normal physiological activities, such as signal transmission, thus maintaining our daily physiological functions, including walking, talking, and various movements.

Microglia are another type of glial cell in the brain. Their primary function is to clear daily waste or metabolic substances in the brain through phagocytosis while also secreting different cytokines and growth factors to regulate and support other nerve cells, including maintaining the integrity of the myelin tissue. The abnormal accumulation of metabolic substances in the brain can lead to various neurological diseases, such as Alzheimer's and Parkinson's. Therefore, the normal function of microglia is crucial for protecting brain health. However, many neurological diseases involve inflammation in the brain, and under inflammatory conditions, microglia not only engulf harmful substances but also attack healthy myelin tissue, causing damage and loss of the myelin sheath. This process is a major pathological feature of several neurological diseases, with multiple sclerosis being the most common example. The loss of myelin can lead to a range of clinical symptoms, including muscle weakness, movement disorders, fatigue, spasms, sensory abnormalities, urinary incontinence, and impaired vision.

In my doctoral research thesis, I found that a signaling molecule called TGF-β is essential for maintaining the normal function of microglia and preserving brain health. If microglia cannot detect this signal, they become abnormally activated and attack the myelin sheath, resulting in a series of behavioral abnormalities and disease symptoms. Experiments with mice showed that female and older mice are more sensitive to the lack of TGF-β signaling, resulting in more severe disease symptoms.

Furthermore, other factors, such as brain inflammation, can directly cause myelin loss or indirectly attack myelin by activating microglia. Damaged myelin no longer protects neurons or maintains their normal function, and the accumulation of myelin debris around exposed axons hinders new myelin regeneration. This demonstrates that microglia can have both protective and harmful effects in our brain. Researchers are therefore studying new therapeutic approaches to regulate microglia, reducing their harmful effects in disease states while maintaining their beneficial physiological functions. In my doctoral thesis, I also explored and proposed some novel treatment strategies. In one of my studies, I used a mouse model of brain inflammation and found that a compound extracted from a plant called the ‘happy tree’ could inhibit the activation of microglia in an inflamed brain environment, thereby reducing their damage to normal myelin tissue. In another study, I used a mouse model with extensive myelin damage and discovered that capsaicin, the main component of chili peppers, could promote microglial clearance of myelin debris, thus creating a better environment for the growth of new myelin.

In conclusion, microglia are essential for maintaining the health of the myelin sheath. My doctoral research revealed the specific molecular mechanisms on which microglia rely to maintain myelin integrity and provided potential therapeutic strategies for modulating microglial function.

科普简要

在我们的大脑中有不同的神经细胞类型，包括称为神经元的神经细胞和不同类型的神经胶质细胞。在过去神经胶质细胞被认为是脑内的胶水，起到链接、支持和保护神经元的作用，故而得名。但是新的研究已经证明他们的作用不仅于此，并且在许多脑内疾病的发病过程中起到很关键的作用。神经元包含一个大的类似五角星形状的胞体和一个长的树干样形状的胞体延长部分，称为轴突。轴突被由少突胶质细胞形成的称为髓鞘的保护层包裹。髓鞘的包裹可以保护神经元，支持神经元的正常生理活动如神经信号的传导等，从而维持我们日常生活中的各项生理功能，包括行走、说话以及各类运动等等。

小胶质细胞是大脑中的另一类神经胶质细胞，它的主要生理功能是通过吞噬作用清除大脑中日常产生的代谢物质，同时也会分泌不同的细胞因子和营养因子对其它神经细胞起到调节和支持作用，包括对髓鞘组织完整性的维护。代谢物质和其它有害物质在脑内的异常累积会导致不同的神经系统疾病，比如阿兹海默症和帕金森症等等。因此，小胶质细胞功能的正常与否对于保护大脑的健康至关重要。然而，在许多神经系统疾病中都存在脑内炎症，而在炎症环境下，小胶质细胞不仅吞噬有害的代谢产物，也会攻击正常的髓鞘组织，导致髓鞘组织的破坏和脱落。这一过程正是一些神经系统疾病的主要病理过程，其中以多发性硬化这一疾病最为常见。髓鞘脱落会导致一系列的临床症状，包括肌肉无力、运动障碍、疲劳、抽搐、感觉异常、小便失禁、视力受损等。

在我的博士研究论文中，我发现一个叫做 TGF-β 的信号对于维持小胶质细胞的正常功能和维护大脑健康非常重要。如果小胶质细胞无法检测到该信号，它们会被异常地激活并且攻击髓鞘，从而产生一系列的行为异常和疾病症状。通过小鼠实验研究发现，雌性小鼠和大龄小鼠对于 TGF-β 信号缺失更为敏感，所产生的疾病症状也更为严重。

此外，脑内炎症等其他因素也可直接引起髓鞘损失或通过激活小胶质细胞间接攻击髓鞘。受损的髓鞘不再具有保护神经元、维持神经元正常功能的作用，并且损伤后脱落的髓鞘碎片会聚集在裸露的轴突周围，阻碍新的髓鞘再生。由此可见，小胶质细胞对于在我们大脑中既可以起到保护性作用，也可以产生有害的后果。

因此，科研人员正在研究新的用于调节小胶质细胞的治疗手段，以减少它们在疾病状态下的危害性，并同时保持甚至促进它们有益的生理功能。在我的博士论文中，我也探讨并提出了一些新的治疗策略。在我的一项研究中，我使用小鼠脑内炎症的模型，发现一类提取自喜树的化合物可以抑制脑内炎症状态下小胶质细胞的激活，从而减少它们对正常髓鞘组织的伤害。而在另一项研究中，我使用了一个产生大量髓鞘损伤的小鼠模型，发现辣椒的主要成分辣椒素能够促进小胶质细胞对髓鞘碎片的清除，从而为新的髓鞘生成创造了更好的再生环境。

综上所述，小胶质细胞对髓鞘的健康十分重要。我的博士论文研究揭示了小胶质细胞维持髓鞘完整性所依赖的具体分子机制，通过调节小胶质细胞功能提供为脑内脱髓鞘相关疾病提供了潜在的治疗策略。

ABSTRACT

Demyelination in the central nervous system (CNS) is a characteristic of various neurological disorders, such as multiple sclerosis (MS), neuromyelitis optica (NMO), subacute combined degeneration (SCD), tabes dorsalis (syphilitic myelopathy), and more. Although the causes vary, CNS demyelination is often associated with a significant buildup of inflammatory activated myeloid cells, mainly consisting of CNS resident microglia and infiltrating monocyte-derived macrophages. On one hand, these myeloid cells can contribute to inflammation in the CNS and damage myelin, but on the other hand, they play a role in clearing myelin debris and releasing substances that facilitate myelin regeneration, a process known as remyelination. Therefore, it is crucial to determine the signals that control their specific functions and develop methods for regulating their activity.

In this thesis, we investigated the role of TGF-β signaling in regulating myeloid cell function. By using cell-specific targeting mouse tools, we discovered that when the CNS lacks microglia in a specific experimental setting, where peripheral monocytes can enter the CNS and repopulate the microglia pool by transforming into microglia-like macrophages, deleting the TGF-β receptor (TGFBR2) on monocytes prevents their entry into the CNS. Furthermore, when monocyte-derived macrophages are engrafted in the CNS, the depletion of TGFBR2 causes their abnormal activation and failure to adopt a microglia-like signature, leading to spontaneous demyelination in the spinal cord and a progressive, fatal motor disease. The loss of TGF- β signaling in microglia or monocyte-derived microglia-like cells preferentially targets myelin in the dorsal column of the spinal cord, and a subpopulation of microglia closely associated with myelin loss is identified in the dorsal column. We further characterized that this microglial TGF-β signaling loss-induced disease is more severe in female and older mice and uncovered potential molecular mechanisms underlying these gender and age differences in response to the loss of TGF-β signaling.

In addition to deciphering the mechanisms governing myeloid function, we also conducted translational studies aiming to provide therapeutic insights for demyelinating diseases. By using a drug screening tool and performing *in vitro* validation, as well as experiments in mouse models with CNS inflammation and ensuing demyelination, we confirmed the regulatory effect of topotecan, a topoisomerase 1 inhibitor, on myeloid cells, leading to improved disease outcome. We further developed a DNA nanostructure-based drug delivery system to encapsule topotecan and achieve specific targeting of TOP1 in myeloid cells, demonstrating that myeloid cell-specific inhibition of TOP1 could alleviate neuroinflammation. In the final study, within a non-inflammation-driven demyelinating context, we studied the role of TRPV1 activation in remyelination and revealed that the TRPV1 activator capsaicin could enhance microglial clearance of myelin debris following demyelination and promote remyelination.

My thesis work thus provides mechanistic understanding of how myeloid cells regulate myelin health and CNS homeostasis, while also providing regulatory strategies for fine-tuning these cells.

LIST OF SCIENTIFIC PAPERS

(#equal contribution)

i. Lund H, Pieber M#, Parsa R#, Grommisch D, Ewing E, Kular L, Han J, **Zhu K**, Nijssen J, Hedlund E, Needhamsen M, Ruhrmann S, Guerreiro-Cacais A.O., Berglund R, Forteza M.J., Ketelhuth D.F.J., Butovsky O, Jagodic M, Zhang X-M#, Harris R.A.# Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling. *Nature Immunology* (2018) 19, 1-7.

ii. **Zhu K**, Min J-H, Joshua V, Yun L, Pieber M, Suerth V, Sarlus H, Harris R.A.#, Lund H#. Impaired microglial TGF-β signaling induces severe demyelinating disease with regional-vulnerability and age/gender differences. Manuscript.

iii. **Zhu K**, Wang Y#, Sarlus H#, Geng K#, Nutma E, Sun J, Kung S-Y, Bay C, Han J, Min J-H, Benito-Cuesta I, Lund H, Amor S, Wang J, Zhang X-M, Kutter C, Guerreiro-Cacais A.O., Högberg B, Harris R.A. Myeloid cell-specific topoisomerase 1 inhibition using DNA origami mitigates neuroinflammation. *EMBO Reports* (2022) 23: e54499.

iv. Sun J#, **Zhu K**#, Wang Y#, Wang D, Zhang M, Sarlus H, Benito-Cuesta I, Zhao X, Zou Z, Zhong Q, Feng Y, Wang Y, Harris R.A., J Wang. Activation of TRPV1 receptor facilitates myelin repair following demyelination via the regulation of microglial function. *Acta Pharmacologica Sinica* (2022) 0: 1–14.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

i. **Zhu K**, Pieber M, Han J, Blomgren K, Zhang X-M, Harris R.A.#, Lund H#. Absence of microglia or presence of peripherally-derived macrophages does not affect tau pathology in young or old hTau mice. *Glia* (2020) 68 (7): 1466-1478.

ii. Carlström K.E., **Zhu K**, Ewing E, Krabbendam I.E., Harris R.A., Falcão A.M., Jagodic M, Castelo-Branco G, Piehl F. Gsta4 controls apoptosis of differentiating adult oligodendrocytes during homeostasis and remyelination via the mitochondria-associated Fas-Casp8-Bid-axis. *Nature Communications* (2020) 11 (1), 4071.

iii. Han J, **Zhu K**, Zhou K, Hakim R, Sankavaram S.R., Blomgren K, Lund H, Zhang X-M, Harris R.A. Sex-Specific Effects of Microglia-Like Cell Engraftment during Experimental Autoimmune Encephalomyelitis. *International Journal of Molecular Sciences* (2020) 21 (18): 6824.

iv. Rangasami V.K., Samanta S, Parihar V.S., Asawa K, **Zhu K**, Varghese O.P., Teramura Y, Nilsson B, Hilborn J, Harris R.A., Oommen O.P. Harnessing hyaluronic acid-based nanoparticles for combination therapy: A novel approach for suppressing systemic inflammation and to promote antitumor macrophage polarization. *Carbohydrate Polymers* (2021) 254, 117281.

v. Ineichen B.V., **Zhu K**, Carlström K.E. Axonal mitochondria adjust in size depending on g-ratio of surrounding myelin during homeostasis and advanced remyelination. *Journal of Neuroscience Research* (2021) 99 (3): 793-805.

vi. Zhou K, Han J, Lund H, Boggavarapu N.R., Lauschke V.M., Goto S, Cheng H, Wang Y, Tachi A, Xie C, **Zhu K**, Sun Y, Osman A.M., Liang D, Han W, Gemzell-Danielsson K, Betsholtz C, Zhang X-M, Zhu C, Enge M, Joseph B, Harris R.A., Blomgren K. An overlooked subset of Cx3cr1wt/wt microglia in the Cx3cr1CreER-Eyfp/wt mouse has a repopulation advantage over Cx3cr1CreER-Eyfp/wt microglia following microglial depletion. *Journal of Neuroinflammation* (2022) 19 (1): 1-18.

vii. **Zhu K**, Liu J, Min J-H, Zhang J, Rorbach J, Guo Y, Benito-Cuesta I, Lewandowski S, Guerreiro-Cacais A.O., Fan R, Sarlus H, Harris RA. Microglial corepressor complex in the regulation of neuroinflammation and remyelination. In preparation.

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AGM	Aorta-gonad-mesonephros
ALS	Amyotrophic lateral sclerosis
BAM	Border-associated macrophage
BMP	Bone morphogenetic protein
CAP	Capsaicin
CMP	Common myeloid progenitor
CNS	Central nervous system
CPT	Camptothecin
CPZ	Cuprizone
CSF1	Colony stimulating factor 1
DAM	Disease-associated macrophage
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EMP	Erythro-myeloid progenitors
GMP	Granulocyte-monocyte progenitor
HSC	Hematopoietic stem cell
Iba1	Ionized calcium binding adaptor molecule 1
LAP	Latency-associated (pro)peptide
LPC	Lysolecithin/Lysophosphatidylcholine
LPS	Lipopolysaccharide
LTBP	Latent TGFβ-binding protein
MDP	Monocyte-dendritic cell progenitor
MHC-II	Major histocompatibility complex class II
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
OL	Oligodendrocyte

OPC	Oligodendrocyte progenitor cell
RNA	Ribonucleic acid
SHH	Sonic hedgehog
SMURF	SMAD ubiquitination regulatory factor
TGF-β	Transforming growth factor Beta
TOP1	Topoisomerase 1
TPT	Topotecan
TRPV1	Transient receptor potential cation channel subfamily V member 1
MS	Multiple sclerosis

1 INTRODUCTION

1.1 Dynamic myeloid trio: monocytes, macrophages, and microglia (3Ms)

The functions of the innate immune system are predominantly executed by so-called *myeloid* cells of the myeloid lineage, which comprise macrophages, monocytes, dendritic cells, granulocytes, and microglia. This thesis mainly focuses on microglia, macrophages and monocytes (denoted as the 3Ms). The 3Ms play pivotal roles in neuroinflammation, which refers to inflammatory responses within the central nervous system (CNS) and is a common feature of varied disease states including CNS degeneration. Inflammation is necessary for tissue recovery but can also lead to disease worsening if not regulated properly. Persistent neuroinflammation can subsequently exacerbate CNS disease progress. During CNS neuroinflammatory conditions such as multiple sclerosis (MS) and its rodent model experimental autoimmune encephalomyelitis (EAE), different subtypes of myeloid cells infiltrate the CNS, thereby disturbing homeostasis[1]. Resident microglia and infiltrating monocytes/macrophages become activated and rapidly expand, releasing harmful cytokines and chemokines, further exacerbating the vicious loop between myeloid cells and infiltrating lymphocytes. The 3M are closely associated with CNS pathologies and share similar molecular markers as well as functions, and when needed, monocyte/macrophages can also rapidly and dynamically invade the privileged CNS territory of microglia when homeostasis in the CNS is disrupted. The 3Ms represent very important therapeutic targets for the treatment of MS, and many other disorders with neuroinflammatory features, e.g. amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and post-operative cognitive dysfunction (POCD)[2–5]. It is therefore important to review their biology and function from an updated perspective with newly developed, advanced technologies.

Monocytes

Primitive hematopoiesis occurs during early embryonic development and erythro-myeloid progenitors (EMPs) that emerge in the yolk sac at E7.5 - E8.5 start to colonize different organs including the liver and the CNS during E9.5-E10.5[6,7]. Hematopoietic stem cells (HSCs) mainly derived from the aorta-gonad-mesonephros (AGM) region seed the fetal liver during E9.5-E10.5, where they start to expand, mature and differentiate into lymphoid, erythroid, and myeloid cells, including fetal liver monocytes. The fetal liver is therefore the major hematopoietic organ governing the immune system during mid-to-late embryonic development. Fetal liver monocytes begin to populate the circulation after the seeding of HSCs and start to colonize the developing organs from around E13.5, giving rise to other tissue-resident macrophages, such as alveolar macrophages in the lung and Langerhans cells in the epidermis. Interestingly, the enablement of fetal liver monocytes seeding to organs coincides with the formation of the blood-brain barrier[8], preventing the CNS microglial pool, established and derived entirely from the yolk sac progenitors, from being further populated by additional waves of precursor cells. During adulthood, HSCs residing in the bone marrow (BM) differentiate into monocytes following a proposed hierarchical pattern: HSCs give rise to common myeloid progenitors (CMP), which further transform into granulocyte-monocyte progenitors (GMP); GMPs give rise to granulocytes and monocyte-dendritic cell progenitors (MDP), which further differentiate into common monocyte progenitors (cMoP) and common dendritic progenitors (cDP), committing to monocyte and dendritic cell lineages, respectively[9,10]. However, utilizing modern fate mapping tools and single-cell

RNA-sequencing, this hierarchical classification is being challenged, with recent studies suggesting that GMPs and MDPs are peer progenies of CMPs and that MDPs do not give rise to cMoP but instead directly produce monocytes that are transcriptomically distinct from GMP-derived monocytes[11,12] (Figure 1).

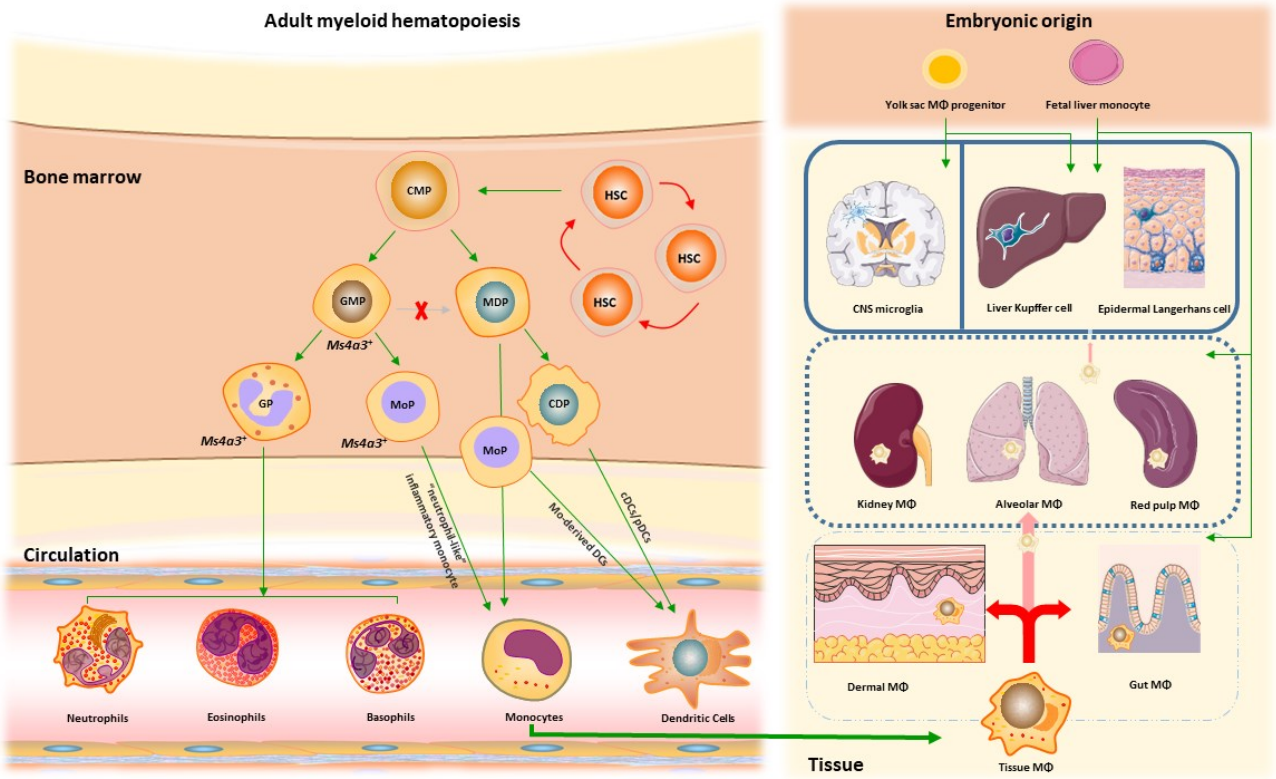

Figure 1 Revised adult myeloid hematopoiesis and its contribution to tissue resident macrophages. The figure was modified by using motifs from Servier Medical Art with a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

The classification of different subtypes of monocytes differs between humans and mice. In humans, monocytes are generally classified into 3 groups: 1) $CD14^{+}CD16^{-}$ classical human monocytes, constituting 80-90% of the monocyte pool; the remaining 10-20% are made up of 2) $CD14^{+}CD16^{+}$ intermediate monocytes, and 3) $CD14^{low}CD16^{+}$ non-classical monocytes[13–15]. In mice, there are two main populations characterized by different surface marker expressions: inflammatory monocytes ($Ly6C^{hi}CCR2^{hi}CX3CR1^{int}CD62L^{+}$) and patrolling monocytes ($Ly6C^{low}CCR2^{low}CX3CR1^{hi}CD62L^{-}$)[16]. The $Ly6C^{low}$ patrolling monocytes in mice are the counterparts of human $CD14^{low}CD16^{+}$ non-classical monocytes, and they contribute to the clearance of debris in the circulation and the resolution of inflammation[17–20]. Newly differentiated human classical monocytes are first retained in the BM for a postmitotic maturation phase of around 1.6 days and leave the BM for the circulation for around 1 day. Circulating classical monocytes can also transform into intermediate monocytes and nonclassical monocytes, with longer circulating lifespans of around 4 and 7 days, respectively[13]. The $Ly6C^{hi}$ monocytes in mice and their human CD14+ counterparts rapidly migrate to sites of injury or infection in a CCL2/CCR2-driven manner, releasing inflammatory cytokines that initiate local inflammation, and transform into inflammatory macrophages in the affected tissue[21–23]. Intermediate monocytes are involved in chronic inflammation, antigen presentation, and T cell activation/stimulation, and produce high levels of proinflammatory cytokines, whereas non-classical monocytes exhibit a patrolling

behavior (crawling on the endothelium) and are implicated in wound healing and tissue repair, bearing less potential to differentiate into tissue-resident macrophages[24,25].

With the properties of chemotaxis and cytokine production, monocytes actively mediate the initiation and pathogenesis of MS and are key contributors to CNS damage. In MS patients there is increased IL-6 and IL-12 production by proinflammatory monocytes in the blood compared to in healthy people, with elevated expression of the co-stimulatory molecule CD86, and there is a positive correlation between disease duration/severity and CD80 expression in monocytes in MS patients[26]. Within active MS lesions, $CD16^+$ monocyte infiltration and accumulation can also be detected in the perivascular area, and in an *in vitro* transendothelial migration (Transwell) experimental system, these $CD16^+$ cells facilitate the trafficking of $CD4^+$ lymphocytes. This suggests that $CD16^+$ monocytes migrate to CNS inflammatory sites and may disintegrate the blood-brain barrier, further facilitating the infiltration of peripheral immune cells into the CNS [27].

Studies using the EAE animal model of MS revealed more evidence highlighting the importance of monocytes in neurological pathogenesis. During the preclinical phase before the onset of EAE, there is a significant expansion of the $Ly6C^{hi}$ monocyte population in the circulation, and the enrichment of these cells in the blood is also positively associated with an earlier disease onset and an increased EAE severity [28]. In a study using a parabiosis chimeric system (a GFP^+ donor mouse with a GFP^- recipient mouse) and myeloablation in the GFP^- mouse by irradiation, the circulating myeloid cells in the recipient mouse were replaced by GFP^+ counterparts from the donor, whereas the CNS-resident microglia remained intact [29]. Using this system, a strong correlation was evident between the number of infiltrating GFP^+ monocytes in spinal cords and EAE paralytic symptoms as assessed by clinical scores. Importantly, inhibition of CCR2-dependent recruitment of monocytes into the CNS blocks EAE disease progression, indicating a pivotal pathogenic role of infiltrating monocytes during EAE.

GM-CSF is a crucial pathogenic cytokine largely produced by autoreactive Th17 cells during EAE that can be sensed by myeloid cells that initiate tissue inflammation[30]. During EAE, GM-CSF signaling drives $Ly6C^{hi}$ monocytes into the circulation from the bone marrow, predisposing them to develop the disease, whereas in GM-CSF-deficient mice there is a dramatic decrease in circulating $Ly6C^{hi}$ monocytes and EAE induction[28]. By employing conditional depletion of the GM-CSF receptor (*Csf2rb*) in different subpopulations of the myeloid lineage, it was discerned that $CCR2^+$ monocytes are the responsive cells mediating the pathogenic effect of GM-CSF in EAE, whereas the lack of GM-CSF signaling in neutrophils, dendritic cells, and microglia gives no beneficial effect on the disease course of EAE[31]. Deletion of *Csf2rb* on monocytes not only abrogates monocyte transmigration into the CNS and disease development, but also ameliorates established clinical EAE during the chronic phase.

Microglia and non-parenchymal CNS macrophages: origin, turnover, and functionality

Myeloid cells in the CNS represent very heterogeneous subpopulations with diverse origins, replenishment patterns, and functional properties. Microglia have for decades been a main focus of research, but emerging studies have also highlighted the importance of other CNS non-parenchymal macrophages, notably meningeal and choroid plexus macrophages, and also those that reside in the perivascular spaces (Figure 2). Microglia were firstly identified by Pío del Río-Hortega in 1919. The origin of microglia was controversial, but using the fate mapping tools developed during the last

decades, such as *Runx1*^{CreER}*R26*^{YFP} mice, it is now agreed that adult microglia are solely derived from yolk sac progenitors (EMPs) during primitive hematopoiesis during E7.5 - E8.5[7,32,33]. These yolk sac progenitors start to colonize different organs including the CNS from E9.5 onwards. Early immature microglia in the CNS continue to develop into mature microglia (E10.5) and expand with the requirement for IRF8, RUNX1, and a critical transcription factor PU.1, which is indispensable for all macrophage development[34,35]. Stimulants that sustain microglial survival and proliferation during early development include CSF1, IL-34, and TGFβ[36–39]. CSF1 and IL-34 secreted by neurons and glial cells bind to CSF1R on microglia, supporting their homing into the CNS and sustaining the development and maintenance of microglia[37,40,41]. Of note, c-Myb, the transcription factor crucial for the maturation of HSCs (definitive hematopoiesis) and the differentiation of fetal monocytes, is not required for the primitive hematopoietic wave, which also includes the early expansion and development of microglia [42]. In addition, the blood-brain barrier starts to form at E13.5 and becomes readily functional at E15.5, a time window when fetal-liver monocytes just start to seed different developing organs[33,43]. Taken together, this indicates that microglia originate exclusively from yolk sac-derived primitive myeloid progenitors without supply from the peripheral hematopoietic system, at least under physiological conditions.

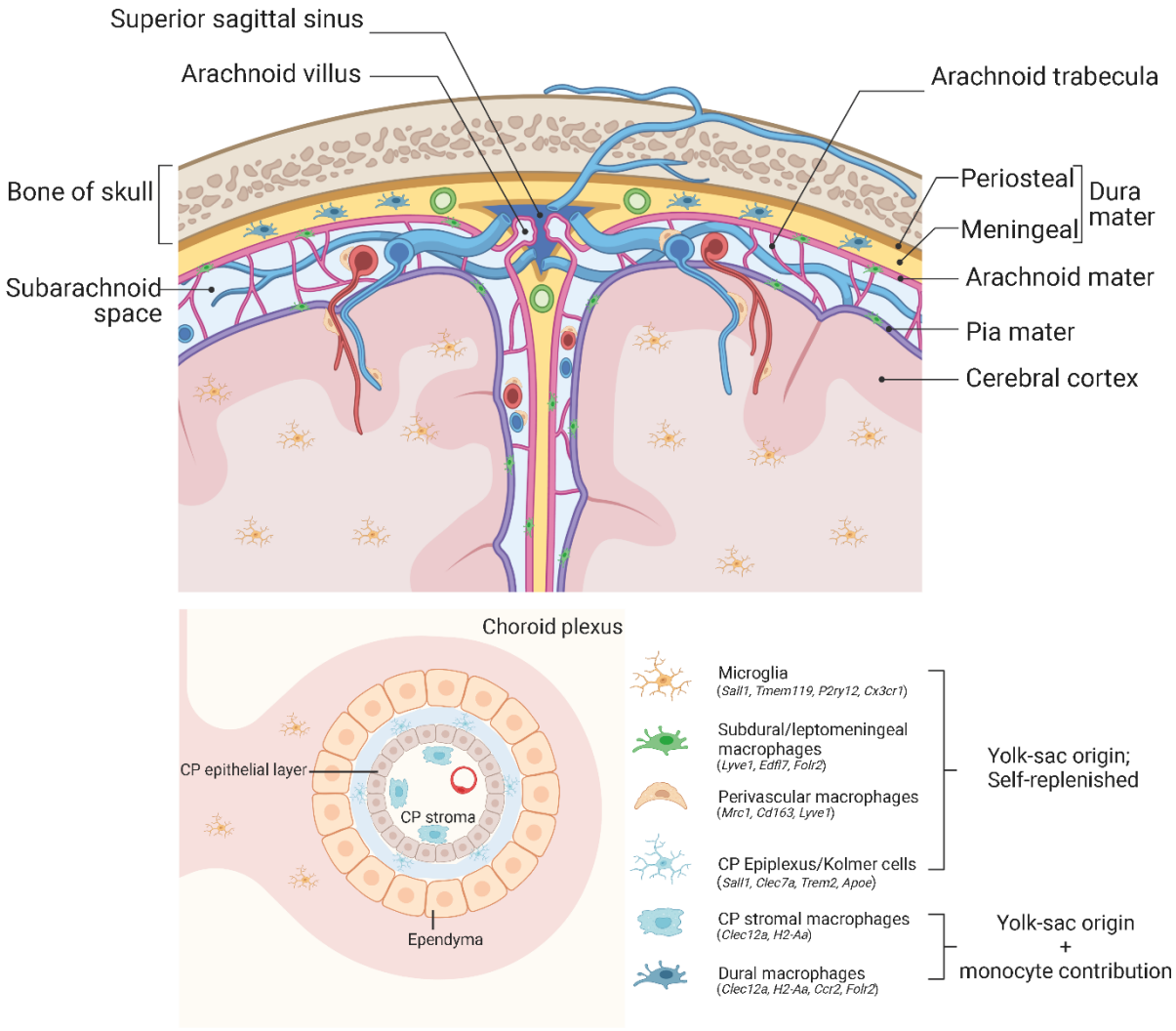

Figure 2 An overview of microglia and border associated macrophages (BAMs). The figure was modified using BioRender with references from: Mildenberger *et al.* 2022 *Current Opinion in Immunology*; *Van Hove et al.* 2019 *Nature Neuroscience*; Kierdorf *et al.* 2019 *Nature Reviews Neuroscience*; Goldmann et al. 2016 *Nature Immunology*

Unlike parenchymal microglia, the origin and development of other non-parenchymal macrophages in the CNS, also known as *border-associated macrophages* (BAMs), are more elusive yet are gaining mounting attention. It was once believed that BAMs, including perivascular and subdural macrophages, as well as macrophages in the choroid plexus, are derived from and maintained by peripheral monocytes/macrophages[44,45]. However, using a parabiosis system and fate-mapping technologies, Goldmann *et al.* demonstrated that perivascular, subdural meningeal, and choroid plexus macrophages, like microglia, are all of prenatal origin, deriving from embryonic progenitors, mostly from yolk sac macrophages, and that their development relies on similar transcription factors as microglia (as described above) [46]. Nonetheless, choroid plexus macrophages and dural macrophages have dual origins, with a partial supplement from bone marrow-derived monocyte/macrophages together with those from embryonic precursors[47,48], and they thus have a faster turnover, with up to 50% of cells being replaced gradually over 50 weeks[48]. Whether the embryonic precursors of these CNS macrophages are yolk sac primitive macrophages, or fetal HSC/monocytes or a combination of both, remains to be further elucidated, but with the advent of fast-developing fate mapping tools a definite answer should soon be forthcoming. Indeed, a recent study using single-cell RNA sequencing (scRNA-seq) profiling murine embryonic (E16.5) BAMs and microglia reveals that $CD206^+$ BAMs are also derived from early EMPs and are segregated from $CD206^-$ microglia during early development[49]. Another recent study identified three tissue-resident macrophage substructures that are conserved across tissues: self-renewing TLF^+ macrophages ($TIMD4^+/LYVE1^+/FOLR2^+$) with minimal monocyte replenishment, $CCR2^+$ ($TIMD4^-LYVE1^-FOLR2^-$) macrophages almost entirely contributed by monocytes, and MHC-II^{hi} macrophages ($TIMD4^-LYVE1^-FOLR2^-CCR2^-$) with modest but not continual replacement by monocytes[50]. In this study, using fate mapping tools to label the E8.5 yolk sac progenitor cells with TdTomato, the authors revealed that the majority of the TLF^+ and $CCR2^-$ BAMs at E19.5 are $TdTomato^+$, suggesting a yolk sac origin of BAMs. Recent studies have also unraveled some core genes that are enriched in BAMs compared to microglia under homeostatic conditions, including *Ms4a7, Cd163, Mrc1 (endocing* CD206*), Ms4a6c, Lyve1, Clec12a,* and *Clec4a1*[48,49,51,52]. Whether these genes could be used as specific markers distinguishing BAMs from microglia, and whether these gene signatures will remain unaltered under pathological changes needs to be further assessed.

Microglial turnover is slow and the microglial pool is self-replenished with minimal contribution from circulating monocytes/macrophages[7,11,32,53,54]. By detecting BrdU incorporation in $Iba1^+$ cells in the adult mouse brain, it was revealed that on average 0.69 % ($BrdU^+Iba1^+$ cells) of the total microglial cells proliferate, whereas the corresponding rate in human microglia is 2%, based on $Iba1^+Ki67^+$ double staining[54]. The turnover of the whole microglial population in rodents is on average 21 months, but the renewal rate is also region-specific, with the olfactory bulb renewing the fastest every 8 months, and the estimated turnover time of microglia in the hippocampus and cortex is 15 months and 41 months, respectively[54,55]. Another study using multiphoton microscopy to monitor microglia in living mice revealed that neocortical microglia are long-lived with a median lifetime of 15 months but under neuropathological conditions they can accelerate their proliferation by 3 fold[56]. Based on ^{14}C measurement, human microglia are determined to be 4.2 years old on average, with more than 96% of microglia being renewed throughout the lifetime, with an annual renewal rate of 28%[57]. Importantly, the turnover of microglia is not sustained by Nestin-expressing stem cells; instead, it is elegantly maintained by coupled proliferation and apoptosis[54]. However, one study claimed that following depletion the repopulation of microglia occurs from Nestin-expressing progenitor cells[58].

Nevertheless, a further study confirmed that all repopulated microglia are derived from the proliferation of the few surviving cells, and although newly forming microglia transiently express Nestin, no repopulated microglia are from Nestin-positive non-microglial cells[59]. Many microglia-specific genes have been identified through the analysis of single-cell profiling, including *Sall1, P2ry12, Tmem119, Hexb, Slc2a5, Adgrg1, F11r, Adamts1, Siglech, Sparc, Serpine 2, and Olfml3*[39,60–62]. Although microglia lose the signature of specific genes such as *P2ry12* and *Sall1* under inflammatory or pathological conditions, *Sparc*, *Olfml3,* and *Hexb* are less affected, and thus may be valuable markers for general labeling of microglia[60,63].

Under physiological conditions microglia perform important functions in the CNS, including clearance of cellular debris, release of neurotrophic factors, synaptic pruning and immune surveillance[64]. However, once the CNS homeostasis is disrupted, microglia also respond very quickly, and microglial activation has long been regarded as a hallmark of neuroinflammation. Compared to homeostatic microglia, which typically exhibit a ramified cell morphology, activated microglia display distinct morphological changes, as evidenced by an enlarged cell soma, shorter but thickened processes, and a reduced coverage area[65–67]. Activated microglia produce a set of inflammatory cytokines, among which IL-1β, IL-6, and TNF-α are thought to have critical detrimental roles in neuronal damage and neurodegenerative processes[68]. Microglial activation characterizes a wide range of neuroinflammatory conditions, including MS, ALS, and AD, and is also linked with the occurrence of postoperative cognitive dysfunction[69–72]. Through the application of ^{11}C-PK11195 (PK11195) PET imaging, we can characterize cortical microglial activation in MS patients, and it has been determined that microglial activity in the cortex of living MS patients corresponds with the progression of disability[73]. Genome-wide association studies (GWAS) have shed more light on the critical role of microglia during MS. A recent human GWAS with 47, 429 MS patients and 68, 374 control cases reported microglia and peripheral immune cells to be enriched for MS susceptibility genes, and those MS risk variants are especially enriched in microglia, but not in astrocytes or neurons, suggesting a potential but overlooked role of microglia in MS susceptibility[74]. The conclusion is that microglial alterations may play more active roles in MS development, instead of them passively interacting with adaptive immune cells.

Compared to microglia, BAMs exhibit a different molecular characterization with significantly increased expression of CD206 and MHCII, and they are proposed to be important antigen-presenting cells[51,75], possessing pivotal roles in the recruitment of peripheral immune cells and the regulation of blood-brain barrier permeability during pathological conditions[76,77]. A recent study demonstrated that LYVE1^{+} macrophages located in perivascular and leptomeningeal areas regulate arterial movement and drive cerebrospinal fluid dynamics; this homeostatic function declines with aging, which could be restored by administration of M-CSF[78]. BAMs are also significant actors in the restimulation of T cells[79,80]. These non-parenchymal CNS macrophages lying at the interface may therefore be backstage manipulators contributing to the development of neuroinflammation. It has been proposed that during inflammatory conditions, the pool of self-maintained tissue-resident macrophages decreases with a coincident influx of peripheral inflammatory monocytes[81,82]. Consistent with this notion, BAMs were also found to follow this pattern, as the pool of BAMs decreased during EAE concomitant with an increased pool of monocyte-derived cells; during the peak of EAE disease, the heterogeneity of BAMs was disrupted and almost all the cells co-expressed CD38 and MHCII[51]. In a recent study using a

Trypanosoma brucei-induced CNS infection and neuroinflammation model, BAMs prevented parasite invasion into the CNS, driving a pro-inflammatory response that subsequently recruited more peripheral immune cells to limit parasite infection, revealing a neuroprotective role of BAMs at the brain border against pathogen infection[47]. While microglia reverted to a transcriptomic profile resembling the pre-infection steady state, BAMs in the choroid plexus maintained a long-term transcriptomic alteration.

1.2 Microglial heterogeneity in the single-cell transcriptomic era

The high plasticity of microglia indicates that these cells are highly dynamic and can be reshaped towards distinct functional states, conferring neuroprotection or indulging neuroinflammation, critically depending on the microenvironment, disease context, and the stimuli or signals received. With the emergence of single-cell RNA-seq and mass cytometry technologies, the previously oversimplified M1/M2 classification of microglia has been gradually revised. Consequently, heterogeneous subclusters of microglia have now been identified.

Among these, '*Disease-Associated Microglia* (DAM)' are the most compelling subtype that arises indiscriminately in response to a wide range of neurodegenerative pathologies, irrespective of disease etiology [69,83–85]. Although the terminology of DAM was first proposed by Keren-Shaul *et al.* in 2017, additional studies have identified a similar subcluster of microglia with comparable molecular characterization, including the subcluster termed the microglial neurodegenerative phenotype (MGnD)[69,83,86]. Of all the DAM/MGnD gene signatures, TREM2-APOE signaling is the most determinant regulator for induction[83]. The induction pattern of DAM is a two-step model: 1) microglia downregulate homeostatic genes (*Cx3cr1, P2ry12, Tmem119, Hexb,* and *Cst3*) presumably by sensing neurodegeneration-associated molecular patterns (NAMP) through a set of receptors (TREM2, purinergic receptors, phosphatidylserine-sensing receptor tyrosine kinases, and others), and strongly upregulate genes such as *Tyrobp, Apoe, B2m and Trem2*, transiting to stage 1 DAM; 2) stage 1 DAM can further transit to stage 2 DAM in a TREM2 signaling dependent pattern and upregulate genes such as *Lpl, Cst7, Axl, Itgax, Spp1, Cd9, Ccl6 and Csf1*[85,87].

It is currently believed that DAM exhibit an intrinsic neuroprotective mechanism developing in response to neurodegenerative changes, with increased capacity to take up and remove damaged neurons, extracellular protein accumulation, and degraded myelin debris. The efficient removal of amyloid protein, as well as tau aggregates, is critical for the improvement of AD-like disease [88,89], and accumulation or inefficient clearance of myelin debris also hinders oligodendrocyte differentiation and remyelination following demyelination in the context of MS[90–92]. At present, DAMs are proposed to have a protective role in restricting the progression of neurodegeneration. An earlier study using electron microscopy also provided ultrastructural features of a microglial phenotype that very likely resembles DAMs, which were termed '*dark microglia*'. Although transcriptomic analysis was not performed on this ultrastructurally distinct microglial phenotype, upregulated expression of *Trem2* and downregulated *P2ry12* expression were demonstrated. These cells only appear during neuropathological conditions such as AD pathology (in APP-PS1 mice), aging, and chronic stress, being absent during homeostasis. They exhibit several signs of oxidative stress and appear as 'dark' due to their mitochondria displaying a condensed, electron-dense cytoplasm and nucleoplasm, being accompanied by a pronounced remodeling of their nuclear chromatin[93].

The functions of DAM need to be better characterized as a lot of evidence from microglial depletion studies has revealed a better disease outcome in the absence of microglia[94]. Additionally, the functional distinction (if there is one) between stage 1 DAM and stage 2 DAM should also be elaborated. Most importantly, it would be of high translational value to address if boosting DAM activity would be a potential therapeutic approach for neurodegenerative disorders, and what kind of potential therapies would mobilize DAM irrespective of disease etiology. Interestingly, in the EAE model of MS, which is endowed with exponentially higher inflammatory cytokines in the CNS compared to in the AD condition, although the gene signature of microglia resembles DAM to a certain extent, EAE microglia are homogeneously skewed toward an inflammatory phenotype with significant upregulation of MHCII and Sca-1[51]. This homogeneous activation of microglia evident in EAE is also different from neurodegenerative conditions in that only a small population of microglia (DAM) are responsive, and it could be due to a more complex cytokine network with great amounts of inflammatory mediators and disturbance from peripheral immune cells[95].

Besides studies of microglia, the heterogeneity of BAMs is also recently reported. It exists not only across different regional borders, but also BAMs in the same compartment, such as in the choroid plexus, have subpopulations separated by differential MHCII expression, and those with high MHCII expression also upregulate *Ccr2* expression[48,96]. This indicates a monocytic origin of these MHCII$^+$ BAMs, which is in agreement with the previous finding that choroid plexus macrophages are partially replenished by monocytes[46].

Appreciation of the less noted microglial spatial heterogeneity is also steadily increasing, and is more evident in the developing brain[97–99]. A subpopulation of microglia termed *Proliferative region-Associated Microglia* (PAM) has been identified, preferably in the developing white matter and corpus callosum, with the specific function of engulfing newly formed oligodendrocytes. They share a transcriptional signature with DAMs, although they appear to be independent of the TREM2-APOE axis[100]. Similar to PAM, a specialized subset defined as *Axon Tract-associated Microglia* (ATM) was also identified in the corpus callosum during a restricted postnatal period, and both of these oligodendrocyte-related microglial subsets are enriched in a set of specific genes including *Spp1, Gpnmb*, *Lgals3*, and *Igf1* [98]. This indicates that a certain subpopulation of microglia is involved in the regulation of myelination. It would be very interesting to find a way to track the lifespan of these oligodendrocyte/myelin-associated microglia, and to characterize whether they are maintained during adulthood, and if so, to determine if they have any role in remyelination following MS attack. Moreover, the two ligands for CSF1R, CSF1 and IL-34, which are critical for microglial maintenance and proliferation, are differentially expressed in and necessary for distinct brain regions[101]. Specifically, CSF-1 is predominantly expressed in the brain white matter, with minimal expression of IL-34, whereas in the gray matter, IL-34 expression is more prevalent. This discrepancy is associated with varying degrees of microglial depletion, as demonstrated by the use of function-blocking antibodies against CSF1 and IL-34. Anti-CSF1 antibodies solely depleted microglia in the white matter fimbria, whereas anti-IL-34 antibodies only targeted microglia in the gray matter.

In addition, postmortem human microglial analysis also revealed regional differences in human brains [102]. For instance, microglial activation markers (e.g. CD68, CD86, CD45, and HLA-DR) are more highly expressed in the subventricular zone (SVZ) and thalamus compared to in other regions, and

mannose receptor CD206, the previously classic M2-macrophage/microglia polarization marker, is less expressed in the temporal and frontal lobes. A recent study also described a distinct subset of choroid plexus macrophages that reside at the apical side of the choroid plexus epithelium, adopting a parenchymal microglial signature and sharing the same ontogeny and self-renewing pattern [48]. These cells were denoted as (Kolmer's) epiplexus cells and exhibit a surprising resemblance to the transcriptional profile of DAMs, with enrichment of gene sets involved in phagocytosis and lipid metabolism (e.g. the APOE-TREM2 axis). While DAMs are absent under homeostatic conditions, these choroid plexus microglia with a DAM signature are present in normal conditions without being exposed to damaged neurons, myelin debris and protein aggregates. Why and how they are triggered as well as the function of these cells should be further investigated.

Adding to the heterogeneity of microglia, studies have also revealed differences in microglia between the spinal cord and brain, and one significant phenomenon is their differential dependence on CSF1 signaling. Spinal cord microglia rely more on CSF1-dependent cell proliferation, and in *Csf1*-deficient mice the microglial density decreases dramatically by 86.4% in the spinal cord dorsal column, whereas the cerebral cortex is much less affected [103,104]. It is also reported that expression of the ligands of CSF1R, IL-34 and CSF1, are more enriched in the brain compared to the spinal cord [37,40]. In regard to this, our research group also noticed a region-specific vulnerability when CNS microglia are replaced with *Tgfbr2*-deficient peripheral derived microglia-like cells, with significantly demyelinating pathological features primarily being recorded in the white tract of the dorsal column in the spinal cord, and the mice developing fatal symptoms at around 40 days[61]. The less studied spinal cord microglia may therefore be endowed with unexpected biological features compared to brain microglia, and the potential heterogeneity in microglial subsets and their functions within the spinal cord may provide a rationale for spinal cord-specific microglial-induced pathology. The transcriptomic information of brain microglia and all known brain macrophages are almost well deciphered, and progress in understanding spinal cord microglia would add more valuable knowledge to the field.

1.3 3Ms in CNS diseases: identify the scapegoat and the saboteur?

Since resident microglia play a key role in regulating CNS inflammation, they have been regarded as being detrimental in the development of neuroinflammatory pathologies. Loss-of-function studies using CSF1R inhibitors (PLX3397, PLX5622) to deplete microglia have revealed a beneficial outcome in both the EAE model (mitigated symptomatic manifestations and reduced immune activation/demyelination) and in the 5XFAD mouse model of AD (reduced amyloid plaque and improved cognitive function) [105–107]. However, as peripheral macrophages are also phenotypically similar to resident microglia, many depletion studies rarely discriminate between the two populations. Inhibition of CSF1R also largely affects the differentiation, proliferation and survival of mononuclear phagocytes. This may also impact on the flood of monocyte-derived inflammatory macrophages into the CNS, a process highly associated with disease severity, at least in the development of EAE neuroinflammation. In a mouse surgical trauma model, depleting microglia using PLX5622 reduced hippocampal inflammation and protected mice from post-operative cognitive dysfunction, but it also abrogated the recruitment of $CCR2^{+}$ monocytes to the hippocampus[70]. Microglial depletion studies using pharmacological inhibition of CSF1R may not therefore truly only target microglia, but also affect the function of peripheral myeloid populations.

In contrast, depleting microglia also impairs the clearance of pathological TDP-43 proteins and axonal regeneration in an ALS model, and in an MS-like model it leads to inefficient clearance of myelin debris, which dampens the process of remyelination [90,108]. It had also already been proven that blocking the trafficking of peripheral monocytes/macrophages into the CNS is efficient to quench neuroinflammation [29,109–111]. An important study took advantage of serial block-face scanning electron microscopy and revealed distinct roles of monocyte-derived macrophages and microglia during EAE onset. Monocyte-derived macrophages initiatively invade the nodes of Ranvier, disrupt the axoglial units and initiate demyelination, having upregulated inflammatory and phagocytic gene profiles, whereas microglia appear to phagocytose myelin debris and demonstrate a globally suppressed cellular metabolism during disease onset [112]. Recent studies also reveal that microglia do not play a major role in antigen presentation and reactivation of encephalitogenic T cells in EAE; instead, conventional dendritic cells and monocyte-derived cells are responsible for these functions[60,113]. These observations suggest the 'saboteur' role of infiltrating monocytes/macrophages during MS, and that blocking the infiltration of pathogenic monocytes without affecting microglial function may be a therapeutic strategy.

However, the role of infiltrating monocytes may also be context dependent. Surprisingly, one study reported that in a SOD1G93A transgenic mouse model of ALS, infiltration of Ly6C^{low} non-classical monocytes into the CNS correlates with improved motor neuron survival, and that the Fc-receptor activation-dependent increased invasion of these monocytes into the CNS played a protective role delaying early disease onset [114]. Concomitantly, increased recruitment of monocyte-derived macrophages in a mouse model of tauopathy after the blockade of the PD-1/PD-L1 axis also reduced brain inflammation and improved cognitive impairment in an AD model [115]. These findings prompt us to further contemplate the role of peripheral monocytes/macrophages in CNS neuroinflammation. If modulated properly, peripheral monocytes and macrophages can thus also exert beneficial roles, improving neuroinflammation, irrespective of their peripheral origin[116,117]. Indeed, adoptive transfer of cytokine reprogrammed monocyte-derived macrophages and monocyte-derived microglia-like cells was reported to have a neuroprotective role and could efficiently combat CNS inflammation and related pathology [116,118]. With the successful induction of human monocyte-derived microglia-like cells [119] in addition to the application of microglial depletion therapy, which is currently being clinically tested[120], we are optimistic to foresee a potential therapeutic strategy using adoptive transfer of pre-modulated monocyte-derived microglia-like cells into the CNS to mitigate neuroinflammation.

A study from our research group determined that using a *Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ transgenic mouse model that replaces around 40% of resident microglia with monocyte-derived microglia-like cells, although those microglia-like cells mimic a microglia gene signature and engraft in the CNS, they still retain distinct functions such as enhanced phagocytosis[121]. Further follow-up studies are therefore warranted to assess the function of these microglia-like cells with peripheral origins in CNS disease models, before the launch of adoptive transfer therapy with microglia-like cells for MS and ALS patients.

1.4 Oligodendrogenesis: multiple origins

Oligodendrocyte precursor cells (OPCs) arising from several ventricular germinal zones of the neural tube during development give rise to oligodendrocytes (OLs), the myelin-forming cells of the CNS.

While oligodendrocytes were identified by Pío del Río Hortega in the 1920s together with microglia, it was not until the 1980s that OPCs were discovered[122]. Generally, three waves of OPC genesis contribute to the OPC/OL pool of an adult CNS[123].

In the developing spinal cord the first wave arises from the motor neuron progenitor (pMN) domain near the floor plate of the ventral neural tube at around E12.5 in mice and gestational week 6.5 (E45) in humans, under the regulation of sonic Hedgehog (SHH) signaling from the notochord that induces *Nkx6*-dependent *Olig2* and *Olig1* transcription[124,125]. Presumably, the dividing radial glial cells in the ventricular zones (VZ) give rise to OPCs. The pMN domain also generates motor neurons before OPC production. Ventrally-derived OPCs proliferate and spread quickly in the spinal cord and occupy both the white and gray matter evenly by around E15 in mice. The second wave stems from the dorsal neural tube at around E15.5 in mice, independent of SHH-signaling, and relies on transcriptional regulation by *Dbx1* and *Ascl1*[126–128]. Unlike the ventrally-derived OPCs, dorsally-derived OPCs primarily reside in the dorsal and dorsolateral white matter, making up around 20% of all OPCs in the spinal cord[129]. The third wave occurs around birth, comprising resident OPC expansion and contribution from the central canal subependyma[130]. OPCs start to differentiate to OLs shortly before birth (E18.5 in mice), and the generation of OLs accelerates during the postnatal 2-4 weeks in mice, after which the rate decreases[131](Figure 3).

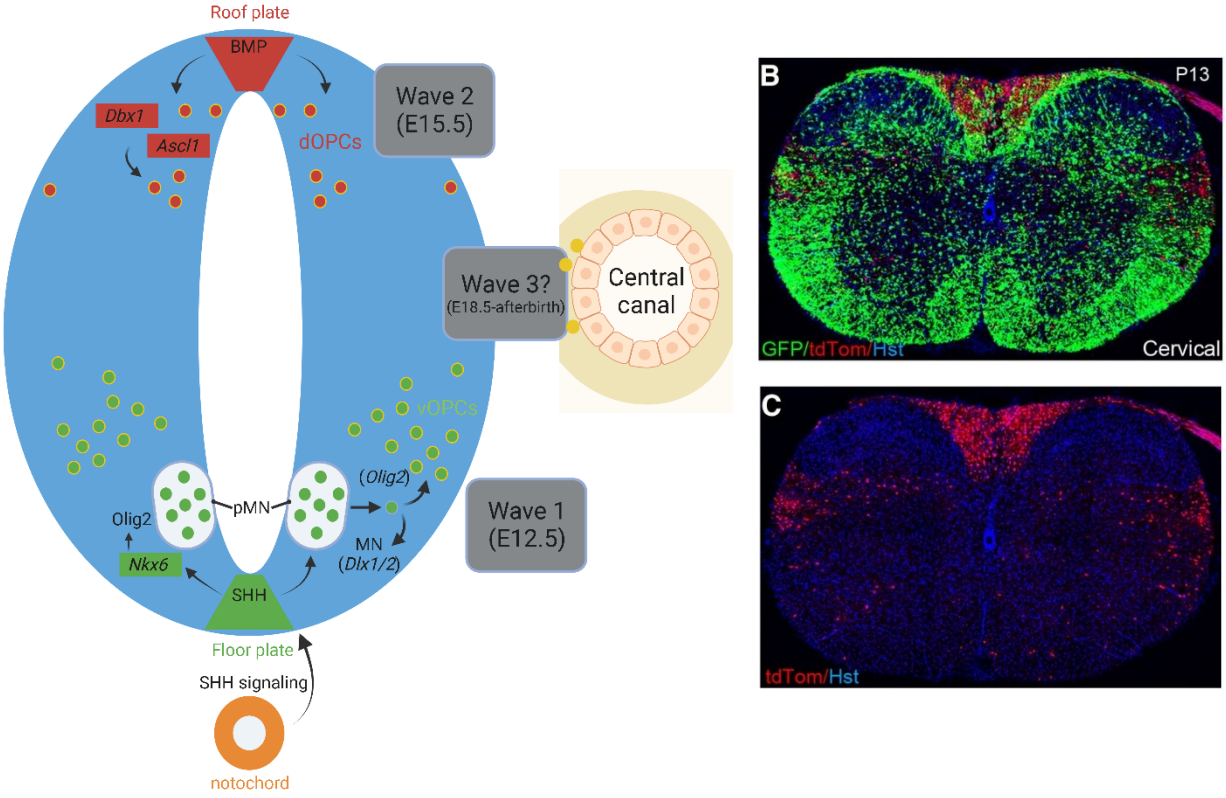

Figure 3 OPC genesis in the spinal cord. Figure generated with BioRender. Panel B and C are modified from Tripathi *et al.* 2011 *The Journal of Neuroscience* (DOI: https://doi.org/10.1523/JNEUROSCI.6474-10.2011). ventrally derived OPCs are in green and are widely distributed; dorsally derived *Msx3*-expressing OPCs are in red and found in the dorsal and dorsolateral funiculi. Copyright permission for reprinting was granted.

Similar to the developing spinal cord, OPC generation in the telencephalon (embryonic forebrain) also comprises three waves. The first wave of brain OPCs appears in the medial ganglionic eminence (MGE) progenitor domain residing at the ventral VZ at around E12.5 in mice[132,133]. The first OPC wave is driven by ventrally-derived SHH-signaling and is transcriptionally controlled by *Nkx2.1*, and the OPCs start to migrate dorsally and laterally to occupy the entire forebrain. The second OPC wave arises from the lateral ganglionic eminence (LGE) under the transcriptional control of *Gsh2* (aka *Gsx2*) and invades the developing cerebral cortex in a lateral-to-medial direction at around E16[133,134]. A third wave of OPC production occurs during birth starting from the cortical/dorsal VZ under the transcriptional regulation of *Emx1* and populates the corpus callosum and cortex[133]. Intriguingly, the MGE-derived OPCs are gradually eliminated after birth, meaning that the adult mouse cortex is mainly composed of OPCs derived from the third wave (cortical/dorsal VC) and around 20% from the second wave (LGE-derived)[129]. In adult CNS, the OPCs are derived from neural stem cells originated from the SVZ of the brain and spinal cord and migrate to other CNS areas[135,136].

OPCs of ventral origins are primarily specified by SHH signaling, but what signals specify the OPCs of dorsal origins are not well characterized; dorsally-derived BMP proteins may be involved and they also inhibit the genesis of ventral OPCs[130,137]. The OPCs/OLs that originate from different sources and are distributed throughout the CNS in varying patterns may possess distinct molecular properties and respond differently to their local environments. This can result in functional differences and variations in their capabilities. The functional heterogeneity of OPCs/OLs is determined not only by their intrinsic properties but also by extrinsic factors such as their local environment and interactions with other cells. Despite current technological advancements, linking the functional heterogeneity of OPCs/OLs with their unique developmental origins remains to be established. Additionally, understanding how local microenvironment shapes the functionality of OPCs/OLs and how other cells regulate different populations of OPCs/OLs remains to be characterized. However, transcriptomic and electrophysiological analyses have shown that OPCs or oligodendrocytes from various waves are quite similar[129,138]. Yet, it remains to be further elucidated whether they would respond differently under pathological conditions[139]. In fact, studies have shown that OPCs originating from the dorsal region demonstrate higher efficiency in recruitment and differentiation following demyelinating damage, and they surpass OPCs from the ventral region in terms of remyelination capabilities[140,141].

1.5 Myelination and its maintenance

OPCs differentiate into mature oligodendrocytes and myelin-forming cells under both intrinsic and extrinsic controls[142] (Figure 4). OPCs are enriched in transcription factors such as Olig2 and Sox10. Of note, the basic helix-loop-helix (bHLH) Olig2 is induced by the SHH signaling and is required for pMN to switch from generating motor neurons to OPCs upon Olig2 dephosphorylation[143]. OPCs express PDGF receptors, of which PDGFRa is highly involved in OPC survival and proliferation upon binding to PDGF-AA secreted from neighboring neurons and astrocytes[144]. Differentiation of OPCs to OLs requires chromatin remodeling, involving deacetylation by histone deacetylases (HDCAs) to suppress genes (e.g. Tcf4 and Id2/4) that limit OPC differentiation and ATP-dependent remodeling by SWI/SNF enzymes, such as Smarca4/Brg1, to increase the accessibility of pro-differentiating genes[145–147]. Myelin regulatory factor (Myrf) is the master regulator for OPC differentiation and is directly induced by

Sox10[148]. Conversely, Wnt signaling, bone morphogenetic protein (BMP) signaling and Notch signaling pathways are known inhibitory cues for oligodendrocyte differentiation[149–152] .

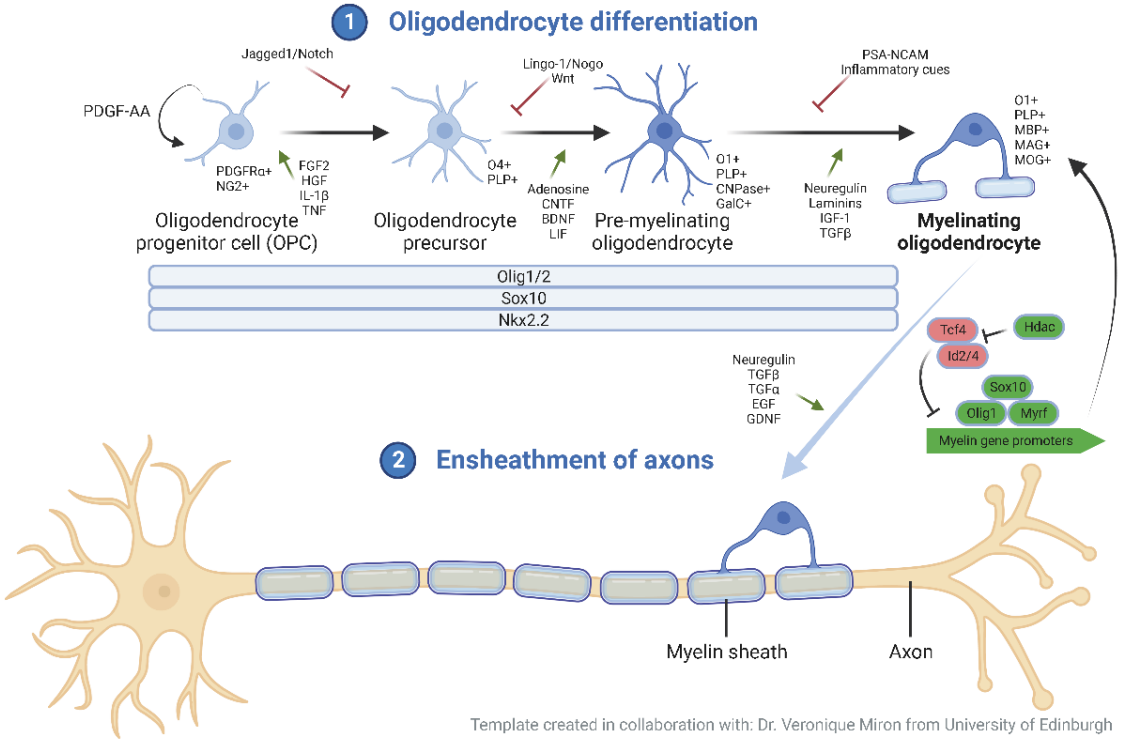

Figure 4 Regulation of oligodendrocyte differentiation. The figure was adapted from a template designed by Dr. Veronique Miron, utilizing BioRender and incorporating information sourced from the references: Emery 2010 *Science*; Kuhn et al. 2019 *Cells*; Ye et al. 2009 *Nature Neuroscience*; Zuchero *et al.* 2013 *Current Opinion in Neurobiology*; Franklin and Simons 2022 *Immunity*.

Myelination and the maintenance of myelin are complex processes requiring orchestrated cell-cell interactions involving axons, myelin-forming cells and support from microglia and astrocytes[153,154]. Firstly, the property of neurons/axons *per se* is a determinant of myelination[155,156]. Adenosine produced in axons upon action potential firing activates the adenosine receptors expressed on OPCs and induces their differentiation[157]. The polysialylated neural cell adhesion molecule (PSA-NCAM) expressed on the surface of axons is a negative regulator of myelination as it may prevent oligodendrocytes from attaching to the axons[158,159]. The axon-derived neuregulin-1 (NRG1) acting on the epidermal growth factor receptors (EGFR) such as ErbB2/3 expressed on myelin-forming cells represents one of the most well-studied and key signals of myelination and the regulation of myelin thickness, although their explicit function in Schwann cells and oligodendrocytes may be different[160–164]. Secondly, the fine-tuned microglia-astrocyte-oligodendrocyte crosstalk is fundamental for oligodendrocyte differentiation and myelin maintenance[165,166]. TGFβ1 released predominantly by microglia in the CNS represents one of the most important cytokines regulating postnatal myelin growth and integrity[102,167], and a recent study firmly shows that microglia maintain myelin health in adult mice and humans, and that disruption of TGFβR1 signaling in oligodendrocytes affects myelin integrity[168]. Cytokines released by microglia and astrocytes during pathological states, such as IL-1β, TNF, and IFN-γ, activate and recruit OPCs and facilitate their proliferation[169,170], but may also lead to

the loss of mature oligodendrocytes[171–175]. A trivial change in any of the cells involved may therefore cause a butterfly effect and disrupt myelin integrity.

1.6 CNS demyelinating pathology

CNS demyelination refers to the damage, disruption, and loss of myelin sheath and is characteristic of various disorders, with multiple sclerosis (MS) and neuromyelitis opitca (NMO) being the most common clinical indications. In MS, demyelination occurs in various patterns, from acute focal plaques of demyelination to diffuse, global demyelination accompanied by extensive CNS atrophy[176,177]. Focal demyelinating lesions could be classified into (i) active lesions, (ii) chronic active lesions, (iii) chronic inactive lesions, and (iv) shadow plaques in which partial remyelination occurs (Figure 5).

Active lesions are characteristic of a dense amount of infiltrated activated macrophages/microglia with ingested myelin components[178]. Macrophage/microglia activation is more prominent at the edge of the active demyelinating lesions, indicative of potential lesion expansion, but is also evident in the peri-lesion areas as well as normal-appearing white matter of MS patients, as compared to healthy controls. Additionally, it has been indicated that microglia create nodules before the appearance of demyelinating lesions, even in the absence of leukocyte infiltration[179,180]. This implies that microglial nodules could potentially represent the initial sign of MS lesion development. Chronic active lesions are more frequently evident in progressive MS. They are presented as activated foamy macrophage/microglia surrounding the demyelinated plaques that form a rim around the region, whereas in the center of the demyelinated area very few macrophages/microglia are detected[181]. The foamy cells in the rim highly express markers that may be involved in myelin phagocytosis, such as MSR1 and GPNMB, and these genes are already upregulated in macrophages/microglia outside the rim or active lesions, suggesting a role in lesion expansion[181]. Chronic active lesions are therefore also

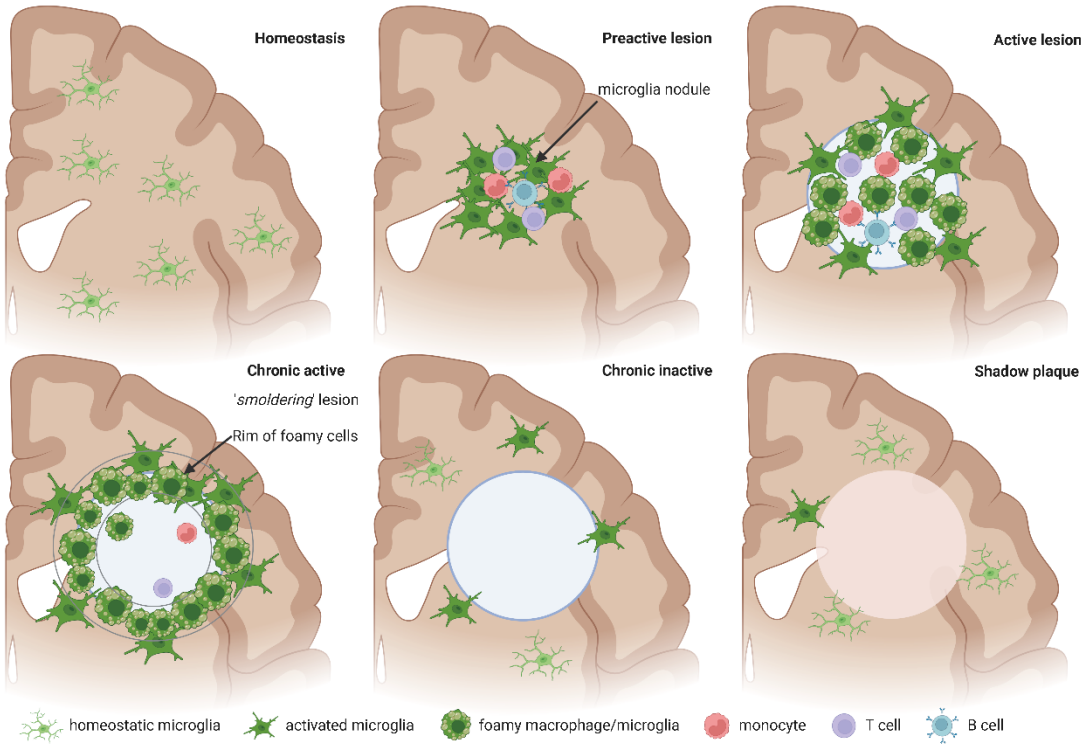

Figure 5 MS lesions. Figure was generated with BioRender.

called '*smoldering*' lesions. The most abundant lesions (around 50% of all lesions) in MS brains are the (chronic) inactive lesions, which are demyelinated plagues with few cells in the center and the formation of a dense fibrillary glial scar. In the inactive lesions, inflammation and the macrophage/microglia rim are almost absent, whereas axonal damage presented as axonal swelling is evident[178]. Shadow plaques, compared to other lesions, refer to demyelinated regions with partial remyelination and are demarcated from the surrounding normal-appearing tissues. Axons within the shadow plaques reacquire a thin myelin sheath. Shadow plaques are the desired pathological conversion goal of MS treatment, especially for progressive MS. However, only around 20% of the demyelinated plaques become remyelinated, and therefore more therapeutic interventions are urgently needed[182]. With increased duration of the disease, acute focal lesions gradually decrease with concomitant diffuse changes in the normal-appearing white matter and grey matter. This pattern is more pronounced in progressive MS patients, and diffuse inflammation, widespread macrophage/microglia activation, global axonal injury and tissue atrophy are noticeable[183].

In addition to MS, NMO is another CNS demyelinating disease with pathological sites being more evident in the optic nerves and spinal cord/medulla oblongata. The main pathological feature of some NMO cases (around 75%) is the presence of aquaporin-4 (AQP4) autoantibodies, and the binding of these autoantibodies to AQP4 triggers inflammatory responses that damage the myelin sheath[184]. Interestingly, besides grey matter pathology, posterior and lateral columns of the spinal cord seem to bear more neuropathology do than the anterior column[185–187].

1.7 Experimental CNS demyelinating models

Animal models that mimic the immunological, pathological, and clinical traits of CNS demyelinating diseases, such as MS, can serve as effective means to comprehend demyelinating processes and to evaluate novel therapeutic interventions. Nevertheless, no model can completely recapitulate the comprehensive intricacy of the actual disease in human patients and transitioning from animal-based research to human-based clinical trials is challenging. It is hence crucial to carefully select the appropriate animal model and interpret the results with discretion. Nonetheless, it is worth noting that animal models may still capture some of the pathological characteristics of MS and different models could represent different patient subgroups.

Among various rodent demyelinating models, experimental autoimmune encephalomyelitis (EAE) is the most widely used one[188]. A generic way to induce EAE is to immunize the animals with myelin antigens or peptides emulsified in Freund's adjuvant, most commonly with myelin oligodendrocyte glycoprotein (MOG) peptide. The animals are usually challenged with pertussis toxin. Pertussis toxin recruits myeloid cells to the draining lymph nodes with increased IL-1β production, and thus helps prime T cells[189]. It also increases the blood-brain barrier permeability and facilitates the migration of encephalitogenic lymphocytes into the CNS[190]. The EAE animal model capitalizes on the immunological characteristics of MS, which is characterized by autoreactive T cells infiltrating the CNS following activation by antigen-presenting cells. Typically, C57BL/6 mice are utilized to induce EAE, and the disease progression is generally segmented into three phases: induction ((Day)D0-D8), acute (D10-D18), and chronic phases (after 20 days)[191]. During the induction phase myelin antigens are presented by antigen-presenting cells to T cells located in peripheral lymphoid organs. As a result, there is a proliferation of activated Th1/Th17 cells and inflammatory monocytes, which accumulate in the

spleen and blood. During the acute phase of EAE, effector Th1/Th17 cells and monocytes infiltrate the CNS and secrete proinflammatory cytokines that cause damage to the myelin, resulting in the manifestation of clinical symptoms. However, by approximately D18-D20 the inflammatory response begins to decrease, which is then followed by a spontaneous repair phase aimed at restoring damaged myelin and axons. While the C57BL/6 mouse strain is commonly used for EAE induction, it does not develop the chronic relapsing-remitting disease pattern apparent in MS. In contrast, SJL/J mice are highly susceptible to EAE induction and develop a relapsing-remitting disease pattern that shifts to a chronic progressive disease course that more closely resembles the human form of MS. The chronic progressive disease course observed in SJL/J mice provides a longer window for evaluating potential therapeutic interventions and better recapitulates the pathology of MS, making them a more suitable model for studying this disease[192].

Toxin-induced models are also widely used to study the demyelinating process and to assess remyelination, with the lysolecithin/lysophosphotidylcholine (LPC)-induced focal demyelinating lesion and cuprizone (CPZ)-induced diffuse, non-focal demyelinating pathologies being commonly used. LPC is a detergent-like molecule that disrupts the lipid layers of myelin and can be quickly cleared following injection into the CNS[193]. The LPC model is initiated through stereotactic injection of LPC into white matter tracts such as the corpus callosum and spinal cord column. This leads to focal demyelination, which becomes evident 2-4 days post-injection and is accompanied by a significant accumulation of microglia and recruitment of OPCs. Following this (10-14 days post-LPC injection), activated microglia transition to a pro-reparative phenotype and OPCs commence differentiation, ultimately resulting in near-complete remyelination by 21 days post-injection[194]. This model induces a focal pattern of demyelination, similar to the pattern of demyelination in acute relapsing-remitting MS. Unlike LPC that targets the lipid layers, CPZ is a copper chelating agent that interferes with copper homeostasis in OLs, leading to the induction of oxidative stress and endoplasmic reticulum (ER) stress, which ultimately results in the death of OLs[195]. The CPZ model is generally induced by feeding mice with chow containing 0.2-0.3% CPZ for 4-6 weeks, followed by withdrawal of CPZ that allows for remyelination. Gliosis is also evident after 3-4 weeks of CPZ feeding. CPZ-induced demyelination yields a more diffuse pattern of demyelination, similar to the pattern of demyelination observed in progressive MS and may be useful for studying the effects of various treatments for global myelin loss.

1.8 Myelin debris clearance by microglia/macrophages

The myelin membrane is lipid-rich, consisting of approximately 70-80% lipids and 20-30% proteins. The composition of myelin lipids consists of approximately 40% cholesterol, 40% phospholipids, and 20% glycolipids. This differs from the typical composition of other biological membranes which contain approximately 25% cholesterol, 65% phospholipid, and 10% glycolipid[196]. The most abundant myelin protein is proteolipid protein (PLP), followed by myelin basic protein (MBP), 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) and MOG[197]. During demyelinating conditions, accumulation of degraded myelin debris impairs remyelination, and efficient clearance of myelin debris by microglia/macrophages represents a necessary step for sufficient remyelination[90].

Two major processes are involved in the removal of myelin debris by microglia/macrophages, which are (i) the ingestion of the debris and (ii) the intracellular processing and breakdown of myelin components.

Lipid components and the phosphatidylserine (PS) 'eat-me' signal expressed on the surface of myelin debris can be recognized by various receptors expressed on microglia/macrophages, including the scavenger receptors AI/II (SRAI/II) and MARCO, the complement receptor 3 (CR3), the fatty acid transporter CD36, the triggering receptor expressed on myeloid cells 2 (TREM2), and the tyrosine kinase receptors MERTK and AXL[198–200]. These phagocytic receptors may be compensatory to each other functionally, meaning that other receptors may adaptively upregulate and initiate phagocytosis when one receptor is dysfunctional, although the underlying cross-receptor communication is not fully understood. Phagocytes might prioritize certain receptors depending on the microenvironmental cues, and phagocytosis through different receptors may also lead to distinct downstream cellular responses and inflammatory states[198,201,202]. For example, complement-mediated phagocytosis usually activates the proinflammatory response in macrophages via NF-kB signaling, leading to elevated expression of TNF and IL-6.[203,204]; conversely, TREM2 and MERTK, which have high affinity to phosphatidylserine[205–207], facilitate a non-inflammatory phagocytosis process[199,208,209].

Ingested myelin debris is generally processed via the endosomal-lysosome pathway to break down the myelin components, and defective microglial lysosomal function induces intracellular myelin accumulation[210–212]. As cholesterol is an essential lipid of myelin and the cholesterol level in OLs is rate-limiting for synthesizing new myelin membrane following demyelination[213], effective handling of lipids by microglia/phagocytes and successful transfer of cholesterol back to OLs is critical for remyelination. In the homeostatic CNS, cholesterol synthesis mainly occurs in the OLs and astrocytes; however, during acute demyelination and early remyelination, although microglia/macrophages ingest myelin debris and accumulate intracellular cholesterol, they still increase the *de novo* cholesterol synthesis pathway within an inflammatory milieu in order to facilitate remyelination[214]. This is due to a refined intracellular mechanism to increase the production of desmosterol, an intermediate product of cholesterol synthesis, which activates the liver X receptor (LXR) signaling that upregulates genes involved in cholesterol efflux (*Abca1*, *Abcg1* and *Apoe*).

Dysfunctional lysosomal function and handling of cholesterol by microglia after ingesting myelin debris is linked to poor remyelination, which is a prevalent occurrence in aged microglia[91,211]. Cholesterol-rich myelin debris overwhelms the efflux capacity of aged microglia, and excessive free cholesterol forms cholesterol crystals that rupture the lysosomal membrane and induce inflammasome activation that dampens remyelination[91]. As mentioned above, TREM2 is involved in myelin debris clearance and contributes to remyelination[215]. In an LPC-induced demyelinating model, TREM2 facilitates the esterification of cholesterol to form lipid droplet in microglia in response to excessive cholesterol exposure, thereby reducing cholesterol accumulation in ER and alleviating ER stress. This TREM2-driven cholesterol esterification facilitates remyelination, suggesting that it may be an adaptive response following acute demyelination[216]. Paradoxically, another study using the CPZ-induced demyelinating model reported that microglial TREM2 decreases the formation of lipid-droplets and facilitates cholesterol transport[217]. Although both studies indicate a beneficial role of TREM2 in the clearance of myelin and the process of remyelination, there is some inconsistency in the underlying mechanism described. This discrepancy could be due to the use of different models of demyelination. In LPC-induced demyelination, microglia that are recruited to the lesions are suddenly exposed to a high volume of myelin debris, which exceeds their ability to remove it. To protect these cells from ER stress caused by excessive free cholesterol, transient esterification may be provoked to

reduce the overall cholesterol levels within the cells. In contrast, CPZ-induced demyelination is more chronic and diffuse, which places less of a burden on microglia to process and remove cholesterol. Therefore, esterification may not be necessary. It is possible that TREM2 has multiple roles in intracellular cholesterol processing, and further research is needed to fully understand its functions.

1.9 TGF-β signaling in microglia

One of the most important signaling pathways regulating microglial development, maturation, and homeostasis is the transforming growth factor-β (TGF-β) signaling pathway (Figure 6). The three TGFβ isoforms (TGFβ1, 2, and 3) present in mammals are functionally similar although not identical[218]; TGFβ2 and TGFβ3 are indispensable for embryonic development, whereas TGFβ1, although dispensable for normal embryonic development, playes a central role in regulating postnatal homeostasis, as evidenced by postnatal death of mice lacking TGFβ1 due to multi-organ autoimmune attacks[219–221]. TGFβ proteins are homodimeric and the two monomers are bonded via disulfide bridges to form a prodomain and a growth factor domain. Activation of TGF-β signaling in microglia is a stepwise process requiring the orchestration of extracellular elements with spatial precision, and is usually achieved through autocrine or paracrine effects[222,223]. TGFβ cytokines are released in inactive forms encased and sequestered by the latency-associated propeptides (LAPs) and form a latent complex together with the latent TGFβ-binding proteins (LTBPs). The LTBPs of the latent complex is captured by the extracellular matrix (ECM) and tethered to the cell surface by the 'anchoring' protein LRRC33 and integrins (αVβ6/8), and are eventually liberated to bioactive forms by molecules such as

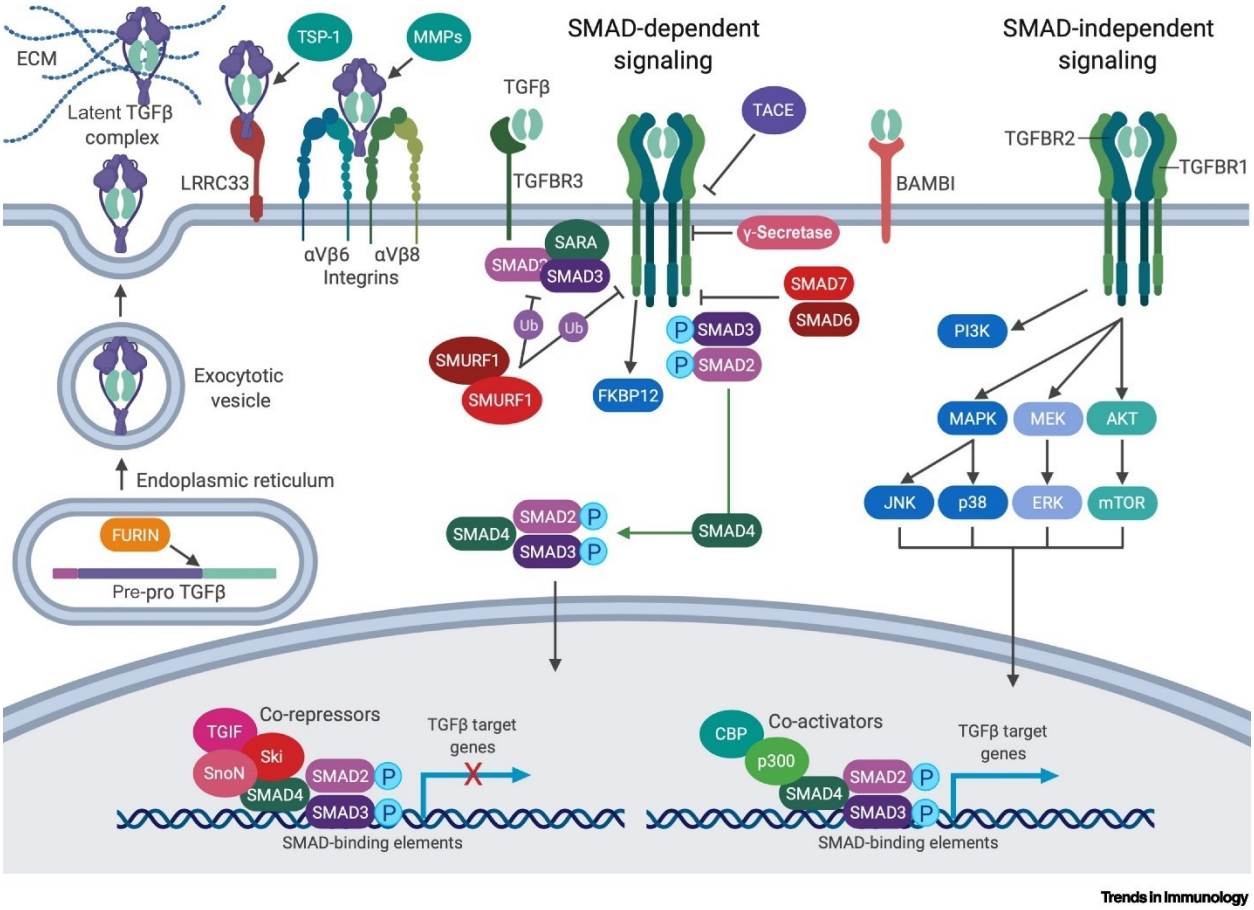

Figure 6 TGF-β signaling in microglia. Figure was modified from Spittau et al. 2020 Immunity. Copyright permission for reprinting was granted by the licensed content publisher Elsevier.

thrombospondin-1 (TSP-1), integrins, matrix metalloproteinases (MMPs) or mechanical stretching of LAPs with the exertion of force[224–230].

Upon ligand binding, the serine/threonine kinases TGFβ receptor type II (TGFBR2) phosphorylates TGFBR1 and forms a ligand-receptor complex encompassing the TGFβ dimer, two TGFBR2s, and two TGFBR1s. Activated TGFBR1 is internalized via coated vesicles to early endosomes for signaling[231,232]. This induces the phosphorylation of receptor-regulated SMADs (R-SMADs) SMAD2 and SMAD3, which further augment their affinity for the co-mediator SMAD4 to form a SMAD complex. This translocates to the nucleus and regulates TGFβ target gene expression, such as genes involved in the regulation of the cytoskeleton, cell adhesion, MAPK pathway, and WNT signaling pathway[233,234]. While TGF-β 1-3 signal through SMAD2/3, other ligands of the TGF-β superfamily, notably the bone morphogenetic proteins (BMPs), mainly signal through SMAD1/5/8[235].

The regulation of TGF-β signaling is intricate and there are several downstream inhibitory molecules that regulate TGF-β/SMAD signaling. The inhibitory SMADs (I-SMAD), such as SMAD6 and SMAD7, compete for R-Smads or SMAD4 interaction by targeting the receptors for E3 ubiquitin ligase-mediated ubiquitination and degradation with the help of SMAD ubiquitination regulatory factors (SMURFs), leading to negative regulation of TGF-β signaling[236–238]. This receptor-SMAD7-SMURF complex is internalized via caveolin-positive vesicles for degradation by proteases[232,239]. Interestingly, IFN-ɣ-induced STAT1 signaling and TNF-induced NF-κB signaling could also activate SMAD7 expression, and thereby inhibit TGF-β signaling[240,241].

In addition to the SMAD-dependent signaling, TGF-β also activates other signaling cascades independent of SMAD activities, namely SMAD-independent signaling. This involves downstream TGF-β-activated kinase 1 (TAK1)-mediated JNK and p38 MAPK signaling, and Ras-mediated signaling activation[242]. Apart from TGFβ1/2/3, there are several other ligands in the TGFβ ligand family that could be divided into two subfamilies based on their sequence similarity and downstream SMAD signaling specificity[233]. Subfamily 1 has high affinity for Type II receptors and uses downstream R-SMAD2/3 for signaling and includes TGFβ, Activin, Inhibitin, and Nodal subfamilies. Subfamily 2 includes mainly members of the bone morphogenetic proteins (BMPs); BMPs have high affinity for Type I receptor and low affinity for Type II receptors and uses R-SMAD1/5/8 for signaling.

In the CNS, TGF-β and its receptors are expressed by different cells, but with much higher abundance in microglia (according to brainrnaseq.org[243,244]). During embryonic development, TGF-β is critical for adopting microglial entity but is dispensable for BAMs[49,245]. Unlike other tissue-resident macrophages (Langerhans cells and alveolar macrophages) that rely on TGF-β signaling for development[246,247], disruption of microglial TGF-β signaling during development may not affect microglial survival, but reduces the induction of microglial specific genes and leads to microglial immaturity[39,62]. During development, mice that lack LRRC33 (the 'anchoring' protein necessary for latent TGF-β binding and activation, mainly expressed by microglia and myeloid cells), TGFBR2, or αVβ8, essential for the extracellular release of bioactive TGF-β, develop neurodevelopmental disorders with progressive paralytic symptoms and eventually die, with pathologies such as myelin loss and axon damage in the brainstem and spinal cords[225,248].

While it is well-appreciated that proper TGF-β signaling is essential for the homeostatic function of microglia, several questions remain unanswered. Arguably, many cell types in the CNS produce TGF-β; the extent to which microglial rely on their own production of TGF-β (autocrine effects) remains unclear, as well as whether TGF-β produced by other cells, such as astrocytes and neurons, can compensate for a lack of microglial TGF-β production. Furthermore, considering the previously mentioned regional preferences for CSF1 and IL-34, it is uncertain whether there is also spatial heterogeneity in the demand for TGF-β signaling within the CNS. Additionally, the interplay between different factors, such as aging and gender, and TGF-β signaling in microglia is not well understood. Further studies are thus needed to better understand the intricacies of TGF-β signaling and its impact on microglial function in the CNS.

1.10 Topoisomerase and inflammation

Topoisomerases are enzymes that play an essential role in DNA replication and transcription. They act by altering the topological structure of DNA by introducing strand breaks, disentangling the torsion ahead of the DNA/RNA polymerases, and re-ligating the strands. There are two main classes of topoisomerases: type I topoisomerase (TOP1) and type II topoisomerase (TOP2). TOP1 induces a transient single-strand break in DNA, while TOP2 breaks both strands of DNA.

Since James C. Wang's discovery of topoisomerases in the 1970s[249], much research has centered around their connection to tumors. As a result, inhibiting topoisomerases has become a strategy for treating various types of cancer. Campthothecin, a TOP1 inhibitor, together with its FDA-approved water-soluble analogs such as Topotecan and Irinotecan, show proven anticancer properties due to replication fork arrest[250,251].

Recent research has suggested that using TOP1 inhibitors at lower dosages could be a promising approach for treating inflammatory diseases[252,253]. A landmark study by Marazzi's group reveals that under infection-induced inflammatory conditions, TOP1 inhibition specifically affects the transcription of pathogen-associated molecular pattern (PAMP)-inducible inflammatory genes by suppressing RNA polymerase II activities, but does not affect the expression of housekeeping genes or cause cellular damage[252]. These PAMP-inducible genes are more dependent on chromatin remodeling by the switch-sucrose non-fermentable (SWI/SNF) nucleosome remodeling complex, and TOP1 preferably facilitates the expression of genes that require TOP1 for efficient nucleosome disassembly at the promoter regions for transcription[254–256]. Using a chem-ChIP that reveals the genomic localization of compounds, the authors show that at basal levels TOP1 inhibitors are enriched at both promoters and gene coding sequences, but during an acute inflammatory condition TOP1 inhibitors peak at the promoters of inducible genes but not into the gene bodies, suggesting that TOP1 inhibition blocks RNAPII and TOP1 from entering the productive transcriptional steps[252]. In addition, TOP1 also shows specificity in facilitating the expression of long genes (>100 kb) by resolving the topological constraints occurred on long templates[257,258]. Therefore, although TOP1 plays a ubiquitous role in gene transcription in all cells, the sensitivity of genes to TOP1 inhibition can vary depending on their individual threshold demand for TOP1 activity and the cellular states under stimulation. As a result, some genes may exhibit greater sensitivity to TOP1 inhibition than others.

TOP1 inhibition significantly suppresses inflammatory cytokine production and protects mice from lethal sepsis and SARS-CoV-2-induced severe respiratory inflammation[252,253]. Mounting evidence, including our own recent work, suggests that inhibition of topoisomerase activity in proinflammatory macrophages and microglia not only decreases the inflammatory cytokine production, but also induces the release of the anti-inflammatory cytokine IL-10, conferring beneficial and protective effects in animal models of Parkinson's disease (PD), AD, and MS[259–263]. These dual effects of TOP1 inhibition therefore represent a potential therapeutic strategy for inflammatory diseases involving dysregulated myeloid cells. In addition to the regulation of innate immune cells, topoisomerases are also indispensable for the development of mature T cells and B cells, and lack of topoisomerases may lead to immunodeficiency[264]. Although this may hold promise for the treatment of autoimmune disorders, a precise targeting approach must be devised to achieve the desired benefits while preserving normal immune function.

1.11 Regulation of microglial function through TRPV1

The transient receptor potential cation channel subfamily V member 1 (TRPV1), also known as vanilloid receptor 1 or capsaicin receptor, was first identified by David Julius and colleagues in the 1990s[265,266]. This led to the breakthrough in understanding how sensory neurons detect environmental stimuli and provides a viable therapeutic target for pain management. The TRPV1 channel can be activated by high temperature (above 43℃/109°F), low pH, capsaicin (the main compound in chili peppers), and endogenous cannabinoids such as anandamide. Upon activation, TRPV1 permits the non-selective passage of different cations, including calcium, sodium and potassium, and triggers intracellular signaling cascades. While most studies have focused on the role of TRPV1 in neurons, mounting evidence also highlights a regulatory role of TRPV1 in glial cells and immune cells. TRPV1 was predominantly detected in afferent neurons within the central nervous system with high abundance, and in certain brain regions such as the caudal hypothalamus with low-level expression[267]. However, additional research has confirmed its presence in microglia and astrocytes, which becomes more pronounced during neuroinflammatory conditions [268–272].

Activation of TRPV1 in microglia leads to divergent cellular responses depending on the cytokine milieu in which the cells are activated, and both beneficial and detrimental functions have been reported in neurodegenerative and neuroinflammatory conditions[273–276]. From a systemic point of view, it is in general believed that TRPV1 plays a pro-inflammatory role as activation of TRPV1 on afferent neurons triggers neurogenic inflammation via the release of inflammatory neuropeptides such as substance P and calcitonin gene-related peptide (CGRP), which subsequently also affects the activation of microglia[277,278]. Activation of TPPV1 in a microglial cell line by using capsaicin also induces the release of the proinflammatory mediators IL-1β, TNF, and HMGB1, but this effect is much milder than following LPS-induced proinflammatory activation[279].

In the context of EAE, TRPV1 expression is highly induced in Iba1$^+$ macrophages/microglia in the spinal cord during the peak EAE phase, and TRPV1 deficiency inhibits microglial NLRP3 inflammasome activation and alleviates disease progression[269]. However, another study reports that TRPV1 suppresses NLRP3 activation by regulating microglial autophagy in the context of cerebral ischemia-reperfusion injury[280]. In addition, capsaicin-pretreated microglia *in vitro* produce less TNF and IL-6 following LPS stimulation[281]. Despite the contradictory findings of TRPV1 activation on microglial

inflammatory responses, it is conclusive that TPRV1-activated microglia have enhanced migratory properties, possibly through MAPK activation[270,282].

Microglial metabolic reprogramming underpins the functional outcome of the cells and TRPV1 has been reported to exert key regulatory roles in it. Amyloid plaque deposition is a hallmark of AD and microglia exposed to Aβ plaques develop downregulated metabolic processes, including both oxidative phosphorylation and aerobic glycolysis via the AKT-mTOR-HIF-1α pathway. Activating TRPV1 using capsaicin rescues the metabolic defects and promotes the phagocytosis and clearance of the Aβ plaques[283]. A similar effect is also reported in the context of PD, in which the activation of TRPV1 using capsaicin boosts the defective microglial metabolic function due to the exposure to α-synuclein preformed fibrils, contributing to an enhanced phagocytic function[284]. Microglia-specific deletion of TRPV1 leads to accelerated amyloid pathology and memory impairment in the APP/PS1 mouse model of AD and worsened PD-like pathology and behavioral defects in PD mouse models, whereas administration of capsaicin improves disease outcome and preserves neuronal loss[283–286]. Capsaicin-treated microglia also undergo morphological changes and display hypertrophied morphology with enlarged cell bodies and shorter, thicker branches, which is indicative of their activation, and may imply enhanced phagocytic function[268].

To summarize, the activation of TRPV1 on microglia affects their function in various ways, such as cytokine release, metabolic reprogramming, migration and phagocytosis, resulting in different outcomes during neurological conditions (mainly studied in AD and PD). However, the impact of TRPV1 activation on microglial function during development and other neurodegenerative conditions (such as CNS demyelination and ALS) is not well understood, and the underlying mechanisms require further investigation. Nonetheless, as a TRPV1 agonist, capsaicin is a safe component of chili pepper that can be easily consumed through diet. Exploring the role of capsaicin/TRPV1 in regulating microglia and treating neurological diseases would therefore be significant in providing practical guidance for diet and disease management of patients.

1.12 Fine-tuning of macrophages using self-assembly DNA origami

Self-assembly DNA origami is a concept proposed by Paul Rothemund in 2006[287]. It creates nanostructures through the folding of a long single-stranded DNA (ssDNA) scaffold into a desired shape using precomputed short 'staple' strands to hold the scaffold ssDNA in shape (Figure 7). This process is denoted as '*DNA origami*' because the resulting ssDNA structures can resemble origami paper figures. Once the sequence of the ssDNA scaffold and different short staple DNA strands is designed, they interact and hybridize following the Watson-Crick base pairing rules under annealing conditions to form the designated DNA origami structure. Compared to other biologically active molecules, DNA is a stable and biocompatible material, and therefore programmable and addressable DNA origami represents an attractive tool for creating nanoscale structures for use in biological applications. Self-assembly DNA origami holds promise in many potential applications, including the development of nanoscale vectors for drug delivery, imaging and sensing, as well as for the study of ligand-receptor interactions[288–292].

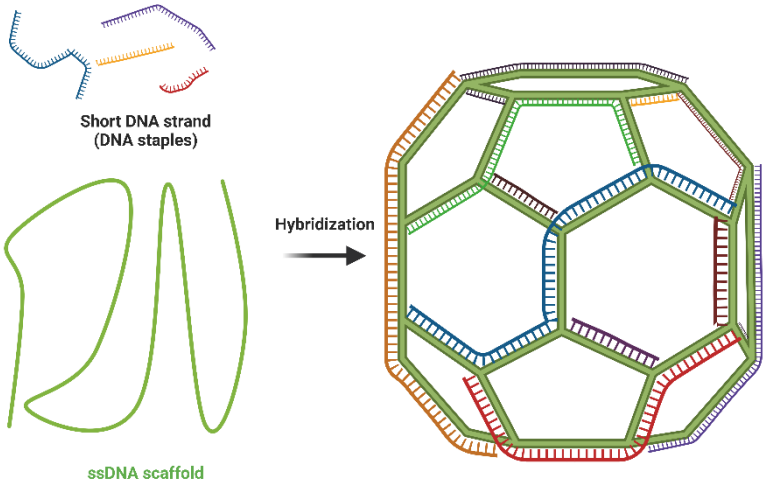

Figure 7 The assembly of DNA origami involves utilizing a long single-stranded DNA molecule combined with various short staple DNA strands.

Although it is a relatively new research field, several studies have utilized DNA origami to investigate the macrophage function. Macrophages phagocytose pathogens and cell debris using different phagocytic receptors. Upon ligand binding, transmembrane receptors form clusters to increase local concentration of signaling molecules, which leads to signaling amplification and more efficient transduction, as well as reorganization of actin structures[293]. Fcγ receptors (FcγRs) expressed on macrophages are important receptors that form clusters and mediate the phagocytosis of immunoglobulin G (IgG)-opsonized targets[294], but it is unclear what threshold of IgG opsonization is required for macrophages to initiate phagocytosis and how this regulates their phagocytic activity. A recent study reported replacement of the ligand-binding domain of the FcγR of macrophages with a synthetic chimeric antigen receptor (CAR) using SNAP-tag technology[295]. This CAR covalently binds to a ssDNA acting as a DNA receptor for a base-paired ligand DNA strand conjugated on a DNA origami pegboard. The DNA pegboard they used has 72 positions for ligand placement, with a spacing of 3.5 nm and 7 nm apart in the y and x dimensions that allows the manipulation of ligand density and spatial arrangement. The DNA ligand-CAR binding mimics the IgG-FcγR interaction and triggers downstream

SYK signaling for phagocytosis. The authors coated silica beads with the DNA pegboards featuring different ligand densities and distribution patterns and treated macrophages with the beads. Interestingly, it was demonstrated that macrophages can sense the density and spacing of the ligands and respond differently to initiate phagocytosis: dense placement of 8 ligands potently induces the clustering of the FcγRs and enhances the phagocytosis of silica beads by macrophages, and an additional increase in ligand number does not further increase phagocytosis. In addition, increasing the ligand spacing from 7 nm to 35 nm significantly abrogates the phagocytic function of macrophages. This fine-tuned system using DNA origami facilitates the understanding of how macrophages optimize their phagocytic activity.

Several studies have also examined the effect of different DNA origami structures on macrophages. Treating unstimulated macrophages with tetrahedral DNA nanostructures (TDNs) composed of 4 ssDNAs induced mild inflammatory responses. However, following LPS stimulation, TDNs also prevented the release of proinflammatory cytokines from macrophages, although the underlying mechanism remains unresolved[296,297]. Another study using flat triangle and rod-shaped DNA origami structures *in vivo* shows that DNA origami structures are preferentially internalized by $CD11b^+$ myeloid cells and elicit a modest pro-inflammatory response that diminishes quickly over time and does not induce immunogenicity. This highlights the potential of DNA origami as a promising vehicle for drug delivery[298]. In this study it also reveals that structural properties of DNA origami structures may induce shape-dependent immune responses, and therefore further studies may be warranted to better understand how and why cells may sense the spatial arrangement of the DNA nanostructure. Indeed, another study using DNA origami with different shapes and DNA density but identical molecular weights and structural flexibility reports that macrophages are more prone to phagocytose DNA origami with high DNA density, indicating that a more compacted DNA origami structure with less of a structural hollow may have better potential to be used for drug delivery to macrophages[299]. In addition, different spatial and geometric constructs may have different abilities to enter cells and induce distinct immunogenic patterns. A very recent study using gold nanoparticles reported that nanoparticles with left-handed chirality are more easily taken up by myeloid cells compared to their right-handed counterparts, with higher affinity to receptors such as CD97 and EMR1 (F4/80) [300]. These particles enter myeloid cells via endocytosis, and the left-handed enantiomers escape the lysosome more efficiently compared to the right-handed ones. It is intriguing to characterize and understand why 'nano-architecture' made of the same material but with different geometric structures leads to diverse immune responses. The utilization of DNA origami as a programmable instrument could enhance the comprehension of how macrophages detect and react to molecules exhibiting diverse spatial arrangements. This also has significant implications for disorders characterized by unresolved extracellular protein accumulation and deposition, such as AD.

DNA origami has also the advantage that it can be loaded with compounds and functional siRNAs due to complementary base-paring[301,302]. In a study the biodistribution of triangle-shaped, square-shaped, and tube-shaped DNA origami structures was compared and it was determined that triangle DNA origami has the optimal accumulation at breast tumor regions when administered *in vivo*[303]. The triangle DNA origami was then used to load the anticancer drug doxorubicin (DOX), with the base pairs in the double helices of the DNA origami serving as docking sites for drug intercalation. This DOX/DNA origami complex is transferred to lysosomes via endocytosis and exhibits controlled DOX release with

enhanced permeability and retention effects, conferring an enhanced anti-tumor effect compared to DOX when applied *in vivo,* without inducing systemic toxicity. Besides, several studies show that siRNA-loaded DNA origami structures could reprogram tumor-associated macrophages to induce the release of TNF and potentiate anti-tumor properties[304,305].

Nevertheless, it is still challenging to apply DNA origami *in vivo* to achieve the optimized effect due to compromised integrity and stability when encountering biofluids containing endonucleases such as DNases. Coating DNA origami with protective layers such as copolymers appears to increase their stability but may also induce undesired immunogenic effects[306,307]. It would therefore be of interest to develop novel methods to protect DNA origami integrity. In addition, modifying DNA origami with molecules that permits more specific targeting of certain cell types (such as myeloid cells) could further strengthen the application of DNA origami *in vivo*.

2 METHODS

Methods that are more specific for this thesis work are described below. Detailed descriptions of commonly used methods are provided in the *Materials & Methods* sections of the respective papers.

2.1 Transgenic mouse tools

We have used several transgenic mouse lines to achieve cell-specific gene targeting based on Cre-Lox recombination. The Cre-Lox recombination system was developed by crossing two types of genetically engineered mice: one with a specific gene of interest flanked by two LoxP sites (floxed), and another one expressing the Cre recombinase enzyme under the control of a cell-specific promoter. When the floxed mouse is crossed with the Cre-expressing mouse, the Cre enzyme recognizes and cuts the DNA sequence between the two LoxP sites, resulting in the excision of the gene of interest. In addition to this, tamoxifen-inducible CreER-expressing mice were also used to cross with the floxed mice[308]. In this system, the Cre is fused with a mutated ligand-binding domain of estrogen receptor (ER) that has no affinity for endogenous estrogen but shows strong binding to tamoxifen. The fused CreER protein is associated with HSP90 and retained in the cytoplasm. Upon tamoxifen administration, CreER dissociates with HSP90 and translocates from the cytoplasm to the nucleus to exert function. Several key Cre(ER)-Lox transgenic mouse tools were used in **Paper I** and **Paper II**: 1) *Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ mice (DTA mice): the *Rosa26* (*R26*) locus on chromosome 6 is ubiquitously and stably expressed across different cells in mice, and is used as a site for inserting foreign DNA without disrupting the function of other genes. In the *R26*$^{DTA/+}$ mouse genome, a STOP cassette flanked by two loxP sites is inserted downstream of the *R26* promoter, followed by the gene encoding diphtheria toxin A (DTA). When crossed with *Cx3cr1*$^{CreER/+}$ mice, tamoxifen administration leads to the translocation of Cre to the nuclei of Cx3cr1-expressing cells (mostly microglia and to a lesser extent, macrophages) and excises the floxed sites, driving the expression of DTA that causes cell death by inhibiting protein synthesis. These mice were therefore used to deplete microglia (>95% microglia were depleted 7 days post tamoxifen treatment, followed by significant repopulation by residual microglia and monocyte-derived macrophages after 28 days)[121]. 2) *LysM*$^{Cre/+}$*Tgfbr2*$^{fl/fl}$*R26*$^{YFP/+}$ mice: similar to the *R26*$^{DTA/+}$ mice, a yellow fluorescent protein (YFP) sequence is inserted in *R26*$^{YFP/+}$ mice instead of a DTA sequence. In addition, the *Tgfbr2* sequence (exon 2) is flanked with two loxP sites. When crossed with *LysM*$^{Cre/+}$ mice, the *LysM*-expressing myeloid cells become labeled with YFP and deficient of *Tgfbr2*. 3) *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$ mice: these mice were used to specifically deplete *Tgfbr2* in microglia. 4) *Cx3cr1*$^{CreER/+}$*R26*$^{DTR/+}$ (DTR mice): unlike the DTA mice, a sequence encoding the diphtheria toxin receptor (DTR) instead of DTA is inserted[309]. Tamoxifen-induced Cre-recombination leads to the expression of DTR in *Cx3cr1*-expressing cells, and subsequent diphtheria toxin administration results in around 80% cell death.

2.2 Mouse chimeras

The term 'chimera' is derived from Greek mythology and refers to a creature made up of parts from different animals. In **Paper I** we generated chimeric mice to distinguish donor derived monocyte-derived macrophages from host microglia. Chimeras were established by irradiation of host mice followed by reconstitution of bone marrow cells from donor mice. After being reconstituted, mice were utilized for experiments after a period of 6-8 weeks. In the *LysM*$^{Cre/+}$*Tgfbr2*$^{fl/fl}$*R26*$^{YFP/+}$ →

Cx3cr1$^{CreER/+}$*R26*$^{DTA/+}$ chimeric mice, LysM^{+} peripheral monocyte-derived cells became labeled with YFP and were deficient in *Tgfbr2*. Subsequent tamoxifen treatment depleted microglia in host mice and led to the recruitment of peripheral monocyte-derived cells[121]. YFP expression was used as a proxy to track *Tgfbr2* targeting. This chimera was thus used to study the function of TGF-β signaling in mediating the stepwise transmigration of monocytes through bone marrow→blood→brain. In the *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$→*Cx3cr1*$^{CreER/+}$*R26*$^{DTR/+}$ chimeric mice, the host *Cx3cr1*$^{CreER/+}$*R26*$^{DTR/+}$ mice were first administered with tamoxifen before irradiation and chimerism, rendering DTR expression in host microglia. Subsequent DT administration depleted host microglia, leading to the repopulation of the empty microglial niche by residual host microglia and macrophages from donor *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$ mice. Three weeks after DT administration, a second administration of tamoxifen in the mice led to the depletion of *Tgfbr2* in engrafted donor-derived macrophages. This chimera was used to study the function of TGF-β signaling in steadily engrafted macrophages in the CNS.

2.3 Experimental models

In **Paper III** we employed the EAE and LPS-challenge models, and in Paper **IV** we employed the CPZ-induced demyelinating model. For the induction of EAE: a previous protocol was followed to express recombinant mouse myelin oligodendrocyte glycoprotein (MOG), consisting of amino acids 1-125 from the N terminus, in *Escherichia coli* and purification to homogeneity using chelate chromatography[310]. To obtain a soluble preparation, purified MOG was dissolved in 6M urea and then dialyzed against PBS. Mice were subcutaneously immunized with recombinant mouse MOG in CFA, which involved emulsifying the recombinant protein with CFA using POWER-Kits™ (BTB Emulsions, Malmö, Sweden) following a standardized protocol[311]. In order to aid the induction of EAE, pertussis toxin was administered via intraperitoneal injection before immunization with rMOG1-125 and again 48 hours later. Mice gradually develop clinical signs around 10 days post-immunization and were assessed using the following score system: 0: no clinical score, 0.5: reduced tail tension, 1: drooping tail (no tonus), 1.5: drooping tail with clumsy gait, 2: hindlimb paraparesis, 2.5: one hindlimb dragging with paraparesis in the other hindlimb, 3: hindlimb paralysis, 3.5: hindlimb paralysis with forelimb paraparesis, 4: tetraplegia or moribund, and 5: death. Dead mice or mice reaching the ethical endpoint were scored 5 on the day of death or sacrifice and were assigned a score of 4 throughout the following experimental days. For LPS-induced neuroinflammation, intraperitoneal (5 mg/kg) or intracisternal (0.1 mg/kg) LPS was administered. For CPZ-induced demyelination, mice were fed with chow containing 0.2-0.3% CPZ powder for five weeks, followed by one-week feeding of normal food.

2.4 Behavioral tests

In **Papers I** & **II** we employed the 4-paw hanging wire test to assess the grip strength and muscle weakness. The mice were positioned on the lid of a wire cage and the lid was inverted over a cage containing soft bedding. The duration of time it took for each mouse to release its grip from the lid was noted. A cut-off time of 180 seconds was established, as normal mice could all remain on the lid for this duration. If a mouse did not remain on the lid for the full 180 seconds during the first trial, two additional trials were conducted, and the longest time taken was recorded. In **Paper III**, in order to assess cognitive function following LPS challenge, we utilized a modified version of the method described in previous studies[312] to evaluate novel object-evoked curiosity. Briefly, 24 hours after LPS challenge mice were placed in an observation cage containing a novel object (a 5cm diameter fluffy

teddy bear toy) positioned in the center of the cage. The mice's behavior was recorded for a duration of 5 minutes, and a trained observer who was blinded to the experimental conditions reviewed the video footage to determine the cumulative number of times the mouse interacted with the toy (such as touching, sniffing, and trailing). In **Paper IV** we performed a rotarod test and beam walking test to evaluate the motor coordinative function of mice. For the rotarod test, mice were subjected to an accelerating rotarod (Columbus Instruments), with a walking duration of 300 seconds, increasing from 4 to 40 rpm. The latency period was automatically noted from the beginning of the trial until the mouse fell off. The latency on the rotarod was measured three times, with a 30-minute break between each trial, and the average of the three trials was calculated. For the beam walking test we set up an apparatus consisting of a narrow wooden beam and a safety box with soft padding in a dark environment. Prior to the actual test, mice were trained to walk along the 1-meter-long wooden beam towards the protective enclosure (safety box). The duration taken to cross the beam was measured over two repetitions and an average was taken for analysis. A cut-off value of 20 seconds was introduced.

2.5 Intracisternal injection

Mice were subjected to isoflurane anesthesia, and their heads were positioned to bend forward, thus exposing the gap between the occiput and the atlas vertebra. A dental 27G needle (Terumo, DN-2721), featuring a bent tip of approximately 3.5 mm at an angle of roughly 40°, was inserted into the cisterna magna using a polyethylene tube connected to a Hamilton syringe. A maximum volume of 10µL of solution was then slowly administered over a period of around 10 seconds.

2.6 NanoString nCounter Glial Profiling

In **Paper II** we employed the NanoString nCounter Glial Profiling panel to evaluate the mRNA expression of genes involved in glial function, which is a fluorescent reporter probe-based gene expression profiling technology that allows for the direct quantification of gene expression levels without introducing amplification bias during PCR amplification. After RNA extraction of the spinal cords (n=3-5 biological samples per group), the RNA samples were hybridized to the nCounter Mouse Glial Profiling Panel CodeSet (XT-CSO-M-GLIAL-12, NanoString), which included 757 probes for genes of interest, 13 probes for internal reference genes, and 10 additional customized probes (*Smad4, Smad7, Nrros, Tgfbr2, Tgfbr3, Smurf1, Tgfb2, Cxcr4, Adgre4*, and *Clec4b1*). The probes were single target-specific, color-coded probes with approximately 100 bases in length, and no reverse transcription or amplification process was necessary. After hybridization, an automated fluidic handing nCounter Prep Station was used to purify the target-probe mix and eliminate excessive probes. The target-paired probes were immobilized in a sample cartridge, and the fluorescent reporter probes were analyzed using the nCounter Digital Analyzer. The mRNA reads were normalized with the 13 internal reference genes included in the CodeSet, and data normalization and further advanced analyses were performed using the NanoString nSolver Analysis Software according to the nSolver 4.0 analysis Software User Manual.

2.7 *Connectivity Map*-based drug screening and repurposing

Connectivity Map favors the repurposing of drugs to induce or suppress a biological/cellular state by high-throughput screening of their potential transcriptomic perturbation on a panel of human cell lines. In **Paper III** we utilized a publicly available GEO dataset, GSE76737, which includes the gene expression profile of human microglia using a human gene ST 2.0 Microarray Chip (Affymetrix). The microglia were stimulated to polarize into either pro-inflammatory (M1) or immunoregulatory (M2a/M2c/Mtgfb) subtypes using different stimuli[199]. The differential gene expression patterns from M0→Mtgfb/M2a/M2c/ and M1→Mtgfb/M2a/M2c were analyzed using the GEO2R tool, and the top 1,000 differential genes (DEGs) were categorized as either being up-regulated or down-regulated DEGs. These genes were then converted to Affymetrix HG-U133A chip identifiers and analyzed using *Connectivity Map* (*CMAP*; O2 version: broadinstitute.org/cmap). The compounds were ranked using an algorithm based on their correlation to either induce or suppress the pattern, and a *CMAP* score was assigned to each compound, with '+1' indicating the strongest positive correlation and '−1' indicating the strongest negative correlation for each pattern.

2.8 Adult primary microglia culture

Primary adult microglia were isolated from 3-month-old C57BL/6NTac mice brains through physical and enzymatic dissociation, followed by myelin removal using isotonic Percoll. After centrifugation and resuspension, the mixed glial cells were cultured and expanded in DMEM/F12 complete medium containing 10% FBS, 20ng/ml recombinant mouse M-CSF, 2mM l-glutamine, 100U/ml penicillin, and 100µg/ml streptomycin. When the cells became confluent (around 14 days), they were harvested and subjected to magnetic bead separation using anti-CD11b MicroBeads to isolate microglia.

2.9 Ethical considerations

In this thesis work, experimental animals were used with the approval of the local ethics committee. The experiments followed the guidelines set by the Swedish National Board of Laboratory Animals and the European Community Council Directive, adhering to the 3R principle (reduce, replace, and refine) for animal research. I was fully aware of the discomfort experienced by the experimental animals and treated them with respect and compassion. Measures were taken to alleviate their suffering. For instance, during the EAE experiment, mice were observed daily and given freshly prepared, easily accessible wet food along with a 'crawling frame' placed over soft padding in their cage, which assisted their movement when paralysis began. Additionally, they received care and cleaning for wounds caused by MOG immunization. In addition, their body weight was monitored every day. If a mouse reached the humane endpoint, such as losing more than 25% of its body weight or displaying severe neurological symptoms, I euthanized the mouse to prevent further suffering. The veterinarian at our animal facility maintained close communication with us to address any emergent situations involving sick mice, and I and my colleagues took quick actions to help relieve the suffering of mice.

3 RESULTS AND DISCUSSION

3.1 Paper I: Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling

In steady-state CNS, microglia remain self-replenished without monocyte engraftment. During neuroinflammatory conditions such as in the context of EAE, proinflammatory monocytes infiltrate the CNS during disease progression but do not remain in the CNS once CNS inflammation is resolved[29]. However, under certain experimental conditions (such as tissue irradiation and genetic depletion of microglia), peripheral circulating monocytes can engraft the empty CNS microglial niche and transform into monocyte-derived microglia-like cells that can reside in the CNS long-term[121,313–315]. As described before, TGF-β is a key cytokine determining microglial specificity during development and maintains microglial homeostasis. **We therefore hypothesized that TGF-β signaling is also required to drive peripherally derived monocytes to adopt a microglial signature and functionally colonize the CNS microglial niche**.

We irradiated *Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ mice (DTA mice) and reconstituted the mice with *LysM*$^{Cre/+}$*Tgfbr2*$^{fl/fl}$*R26*$^{YFP/+}$ or *LysM*$^{Cre/+}$*R26*$^{YFP/+}$ bone marrow cells. We denoted these mice as *LysM*$^{Cre/+}$*Tgfbr2*$^{fl/fl}$*R26*$^{YFP/+}$ → DTA chimeric mice. Upon tamoxifen treatment, diphtheria toxin (DT) was produced in *Cx3cr1*-expressing microglia, leading to microglial depletion and subsequent repopulation of both residual microglia and monocyte-derived macrophages/microglia-like cells[121]. We observed that, 5 weeks after microglial depletion in these chimeric mice, *LysM*$^{Cre/+}$*R26*$^{YFP/+}$ monocytes repopulated the CNS, whereas *Tgfrb2* deficient monocytes failed to engraft the CNS microglial niche. This indicates that TGF-β signaling is required for monocytes to enter the CNS and colonize the microglial niche.

We next sought to understand whether disrupting TGF-β signaling in engrafted macrophages after their entry into the CNS parenchyma would affect their identity. The fractalkine receptor CX3CR1 is lowly expressed in monocytes, but during monocyte→macrophage differentiation, cells upregulate CX3CR1 expression. We took advantage of this and produced *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$→ DTA chimeric mice in which tamoxifen treatment simultaneously depleted microglia and deleted *Tgfrb2* in the engrafted *Cx3cr1*-expressing donor macrophages post-infiltration. We observed that these chimeric mice developed motor defects and behavioral abnormalities after around 12 days post-tamoxifen treatment, starting with symptoms presented as tail and hindlimb weakness, progressing to signs such as incontinence, severe paralysis, and side-resting positions. Flow cytometric analyses revealed macrophage activation in the *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$→ DTA CNS as evidenced by high expression of MHC-II. Histological analyses revealed progressive accumulation of demyelinating lesions with macrophages ingesting myelin in the spinal cord. This pathology was predominantly located in the dorsal column white matter, with some also present in the lateral columns at disease end-stage, whereas the ventral column white matter was untouched. The demyelinating pathology was also accompanied by a gradual loss of the vesicular acetylcholine transporter (VAChT), a neurotransmitter transporter, in motor neurons in the cervical and lumbar enlargements. We also used the *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$→*Cx3cr1*$^{CreER/+}$*R26*$^{DTR/+}$ (DTR) chimeric mice and further confirmed that disrupting TGF-β signaling in steadily colonized CNS macrophages also induced a similar demyelinating disease,

but the disease progression was slower. Transcriptomic analyses revealed downregulation of genes related to microglial identity (*Tmem119*, *Siglech*, *Cx3cr1*, and *Olfml3*) in *Tgfrb2*$^{-/-}$ engrafted CNS macrophages as compared to wildtype engrafted macrophages, and upregulation of genes involved in antigen presentation, inflammation, and phagocytosis.

These results (Figure 8) thus confirm that the ability of monocytes to functionally integrate into the microenvironment of CNS relies heavily on their response to TGF-β, highlighting a critical function of TGF-β signaling in preventing macrophage/microglia-mediated CNS pathology.

An interesting observation that warrants further investigation is that the demyelinating lesions were primarily concentrated in the dorsal column of the spinal cord and were consistently degenerated during the end stage of the disease, while the ventral columns remained unaffected, suggesting that there may be region-specific vulnerability to the loss of TGF-β signaling.

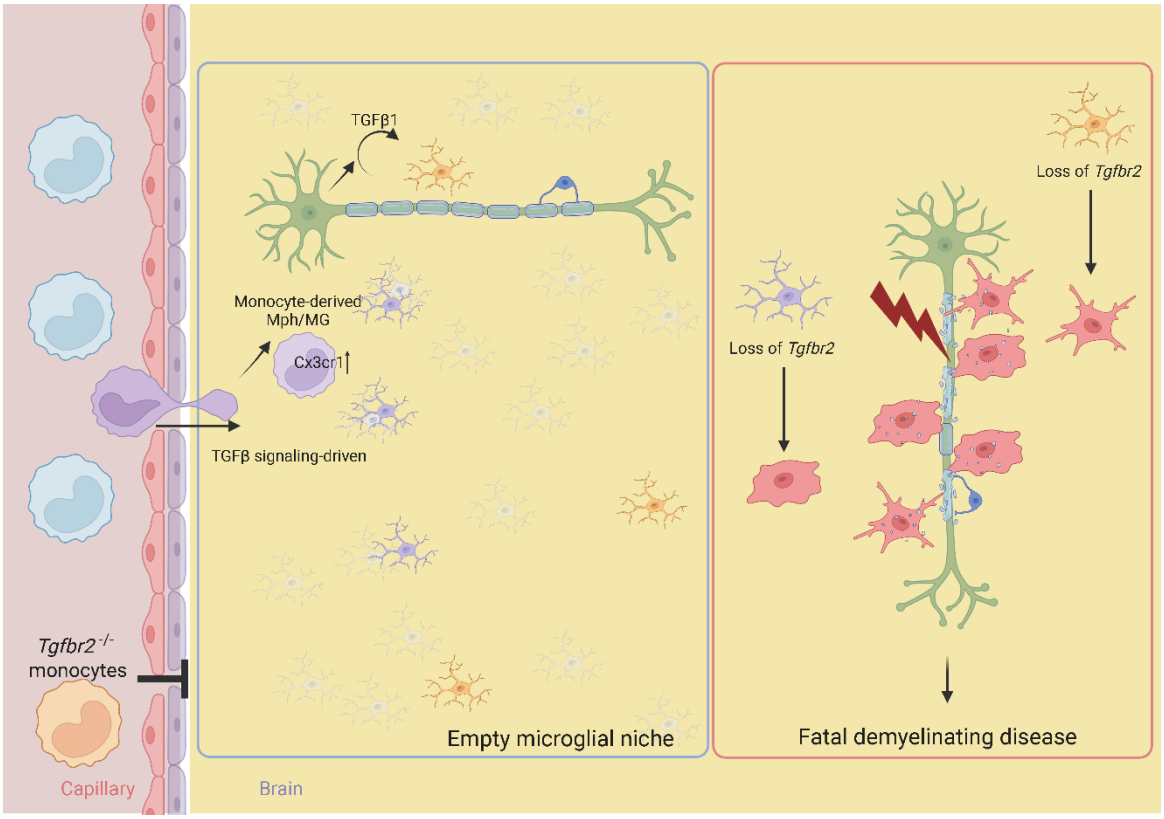

Figure 8 Graphic summary of Paper I.

3.2 Paper II: Impaired microglial TGF-β signaling induces severe demyelinating disease with regional-vulnerability and age/gender differences

The findings in **Paper I** left us the open question as to why the dorsal column in the spinal cord was more susceptible to the loss of TGF-β signaling, whereas the ventral column was barely affected. Differences in CNS regional molecular cues are known to regulate microglia in different ways. For example, microglial maintenance in the spinal cord and cerebellum is more dependent on CSF1/CSF1R signaling as compared to microglia in the forebrain[103,104], and CSF1 is more required for white matter microglia, whereas IL-34 is more indispensable for microglia in gray matter[101]. **We thus hypothesized that the microenvironment in the subregions of the spinal cord may shape microglial sensitivity to TGF-β signaling**.

To address these questions we performed detailed histological, immunophenotypic, and spatiotemporal transcriptomic profiling in the spinal cords of the *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$ mice and the *Cx3cr1*$^{+/+}$*Tgfbr2*$^{fl/fl}$ wildtype (WT) mice. We confirmed demyelinating pathologies in the dorsal column (DC) spinal cord that progressed chronologically, and no demyelination in the ventral column (VC) spinal cord. This was accompanied by a higher degree of microglial activation in the DC. NanoString Glial Profiling revealed dynamic changes in molecular pathways in the spinal cord of *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$ mice as compared to WT mice, indicating global glial activation and neurotransmission inhibition. Interestingly, genes that were key signature molecules of a previously identified 'oligodendrocyte-eating' microglia subtype (proliferative region-associated microglia, PAM) that only exist in the postnatal CNS, including *Gpnmb*, *Lgals3*, *Clec7a*, *Itgax*, *Igf1*, *Spp1* and *Fabp5*, were upregulated as the demyelinating pathology started[100]. Bulk RNA-seq analyses of microglia sorted from DC, VC and spinal cord gray matter (GM) revealed that spinal cord microglia from *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$ mice underwent transcriptomic reprogramming, similar to what we also found in **Paper I**, with induced genes involved in inflammatory and chemokine activity, antigen presentation, phagocytosis, matrix metalloproteinases, as well as cholesterol metabolism, and suppressed expression of microglia-specific genes. Cross-regional analyses revealed that DC KO microglia show elevated proinflammatory signature, as evidenced by increased *Il1b* and *Tnf* expression, compared to KO microglia from VC and GM. We further employed single nucleus RNA-sequencing (snRNA-seq) and identified a subcluster of *Gpnmb*$^{+}$*Lgals3*$^{+}$*Mmp12*$^{+}$*Mgll*$^{+}$ microglia that was only induced following microglial *Tgfbr2* deletion, and we thus denoted these microglia as TGF-β signaling-associated microglia (TbAM). Spatial mapping at the protein level revealed that these Gpnmb^{+} Galectin-3^{+} (encoded by *Lgals3*) TbAMs were predominantly located in the DC but rarely evident in the VC. Of note, the foamy cells in the rim of 'smoldering' plaques in MS brains highly express GPNMB, and it is also readily upregulated in macrophages/microglia outside the rim of active lesions[181], suggesting an active role of GPNMB in lesion expansion. These findings reveal that following *Tgfbr2* deletion, DC microglia become more inflammatory, and a subtype of microglia (TbAMs) is involved in the DC pathology.

Serendipitously, we observed that motor defects and behavioral abnormalities following microglial *Tgfbr2* deletion were more pronounced in female mice and older mice, whereas young males were less affected. This indicates age and gender differences in the development of the demyelinating disease induced by microglial loss of *Tgfbr2*. For the gender difference, we identified that in the male spinal cord, the expression of *Tmsb4x* was significantly elevated compared to that in females. *Tmsb4x* encodes thymosin β4, a biologically active peptide that has been proven to be effective in promoting

tissue regeneration and oligodendrocyte differentiation[316–319]. The concentration of thymosin β4 in the cerebrospinal fluid of patients with MS is notably reduced when compared to individuals with non-MS neurological disorders[320]. However, *Tmsb4x* is an X chromosome gene, and it therefore needs to be interpreted with caution as to whether its differential expression between males and females has any biological relevance to demyelination or remyelination. For the age difference, we found that the PAM signature genes were much more induced in older mice in comparison to young mice. To better decipher why older mice develop worsened disease following microglial deletion of *Tgfbr2*, we analyzed the spinal cord of steady-state mice of varying ages. With aging, the expression of TGFβ1 and TGFBR2 at the protein level increased significantly, but the downstream pSMAD2 signaling remained unchanged, indicating that more upstream TGFβ1/TGFBR2 binding was required in the spinal cord of older mice to induce sufficient SMAD signaling transduction and targeted gene expression. Spinal cord cross-regional analyses of genes involved in the TGF-β signaling pathway also revealed age-dependent increase in *Tgfb1* and *Tgfbr2*, and in the DC of older mice the expression of *Tgfb1, Tgfbr2, Tgfbr3* and *Smad4* was significantly higher than that in the VC and GM; this was also accompanied by an increased expression of genes acting as downstream inhibitory regulators of TGF-β signaling (*Smad7*, *Smurf1*, and *Smurf2*), implying a less efficient signaling transduction of TGF-β signaling in aged DC. Immunostaining also revealed a higher abundance of TGFβ1 in the steady-state DC compared to the VC.

Taken together, these data highlight that under steady state, the DC enriches TGFβ1 to maintain homeostasis with aging. Microglia in the DC, immersed in a TGFβ1-enriched microenvironment, are therefore more sensitive to the unavailability of TGF-β signaling following deletion of *Tgfbr2*. This could partially explain why microglia in the DC become more inflammatory activated and induce demyelination in the DC, but not their counterparts in the VC. Consistent with our findings of an increased expression of TGFβ1-TGFBR2 in the spinal cord with aging, another study using SMAD binding element–responsive luciferase reporter mice also reported increased bioluminescence of TGF-β signaling in the brain of old mice[321,322]. However, we did not see an increase in the phosphorylation of SMAD2 in the spinal cord with aging. There are several possible explanations for this. Firstly, the mice used in our study were only 11 months old, which is younger than the 18-month-old mice in the study mentioned earlier. Secondly, age-related changes in SMAD signaling in the brain and spinal cord may not occur at the same rate, implying tissue specificity. Lastly, the downstream effect of TGFβ1 in the spinal cord with aging may be driven by SMAD-independent signaling.

Although not investigated in the current study (Figure 9), I believe that the heterogeneity of oligodendrocytes in the spinal cord may also endow the DC oligodendrocytes with functional distinction relative to VC oligodendrocytes, rendering them more susceptible to the disruption of microglial TGF-β signaling. This heterogeneity may be due to different OPC generation during development, as dorsal oligodendrocytes and a portion of oligodendrocytes in the lateral columns are derived from a different wave of OPCs that is spatiotemporally different from the ventral oligodendrocytes (introduced in *Section 1.4*). Interestingly, studies have shown that following LPC-induced focal demyelination, dorsally-derived OPCs are the main source for remyelination, but their capacity to differentiate into mature oligodendrocytes declines with age and they are more susceptible to age-associated impairments[140,141]. One observation that remains to be further explored is the source of the enriched TGFβ1 in the DC with aging, and whether microglia rely on an autocrine

supply of TGFβ1, or TGFβ1 derived from other cells such as astrocytes. Reciprocally, the production of TGFβ1 by microglia may also contribute to the maintenance of myelin integrity and it is thus worth investigating whether microglia-specific depletion of TGFβ1 in adult mice also affects myelin health[168].

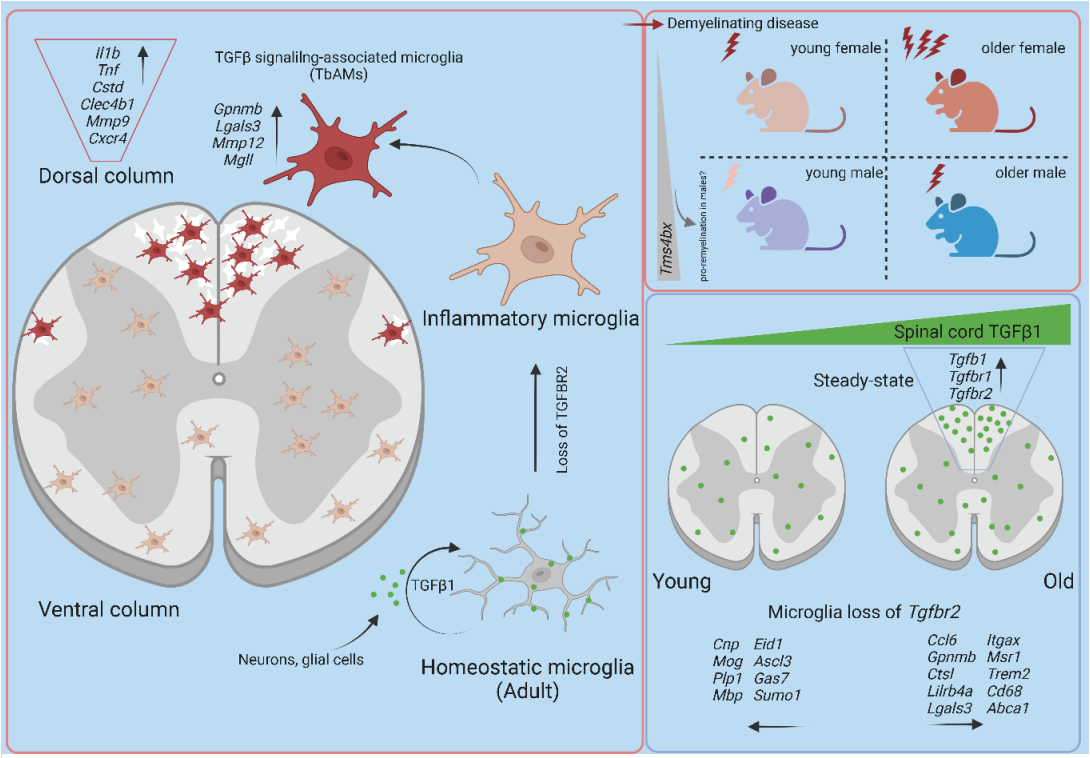

Figure 9 Graphic summary of Paper II.

3.3 Paper III: Myeloid cell-specific topoisomerase 1 inhibition using DNA origami mitigates neuroinflammation

In **Papers I** & **II** we have demonstrated that dysregulated myeloid cells could lead to myelin loss, which highlights their involvement in demyelinating diseases. Indeed, signature studies have indicated the importance of targeting myeloid cells for the treatment of MS, a devastating CNS demyelinating disease with neuroinflammatory manifestations involving different immune cells. While therapeutics are available to target T cells and B cells in relapsing-remitting MS, medications specifically developed for modulating myeloid cells are scarce. This represents an unmet medical need, especially for the untreatable progressive MS in which it is believed that the immune cells and glial cells within the CNS play a major role[323–326]. In **Paper III**, **we therefore aimed to *i)* identify clinical-applicable drugs that regulate myeloid cell function, and *ii)* develop novel therapeutic options to specifically target myeloid cells**.

To address the first aim, we analyzed the differential gene expression pattern of human microglia that were stimulated to induce different activation states and utilized the *Connectivity-map* database (method introduced in *Section 2.7*) to identify drugs that may induce the immunoregulatory microglial phenotype. This revealed that the TOP1 inhibitor camptothecin (CPT) was a potential candidate that could modulate microglial function. **We therefore hypothesized that TOP1 inhibition may regulate microglial function and represent a strategy to reduce neuroinflammation by modulating myeloid cell function**.

To test this hypothesis, we first confirmed an induction of TOP1 in inflammatory activated microglia *in vitro* and in the CNS of several neuroinflammatory conditions, including EAE spinal cord, LPS-challenged brain homogenates and microglia, and active lesions of human MS brain sections. Inhibition of TOP1 in cultured primary microglia using a non-cytotoxic concentration of CPT not only significantly reduced the production of proinflammatory mediators following LPS/IFNɣ stimulation, including nitric oxide, TNF and IL-6, but also increased the production of the anti-inflammatory cytokine IL-10. We further used the FDA-approved TOP1 inhibitor topotecan (TPT) and tested its potential to treat neuroinflammatory conditions *in vivo*. In the LPS-challenged mouse brain microglia were highly activated, whereas intracisternal administration of TPT inhibited microglial activation and resulted in an overall inhibition of cytokine production in the hippocampus and hypothalamus. This also led to improved sickness behavior of LPS-challenged mice when treated with TPT. Transcriptomic analyses of microglia sorted from the brains of control, LPS-challenged, and LPS-challenged + TPT treated mice suggested that TPT administration inhibited major inflammatory pathways (such as *TNF signaling pathway* and *NF-κB signaling pathway*) and induced genes enriched in oxidative phosphorylation and mitochondrial respiratory chain, indicating a favorable effect on mitochondrial metabolic reprogramming[327,328]. To better translate our findings for the treatment of MS, we employed the EAE model of MS, and treated the EAE mice with TPT at different disease phases, determining that prophylactic treatment and early onset treatment of TPT mitigated disease progression. However, when treating mice at EAE disease peak, a phase when the inflammation was about to be resolved and tissue regeneration started, TPT treatment did not have any beneficial effect, suggesting the timing of TPT treatment in MS-like disease is critical.

To address the second aim, we developed a DNA origami-based nanostructure that through surface modification with β-glucan could specifically target myeloid cells. We denoted this myeloid cell-targeted nanostructure *MyloGami*. TPT was incorporated into MyloGami via intercalation into the base pairs in the double helices of the original DNA origami (denoted as TopoGami). As introduced in *Section 1.12*, one hurdle for *in vivo* application of DNA origami is that they are easily degraded by nucleases. Here we showed that β-glucan coating of the DNA origami structure 'killed two birds with one stone': it not only increased the specificity to myeloid cells and facilitated their ingestion by macrophage/microglia, but also acted as an insulating layer protecting the DNA nanostructure from degradation. We further confirmed that TopoGami inhibited microglial inflammatory responses and conferred beneficial effects in the EAE model.

Our findings imply that TOP1 inhibition is a potential therapeutic strategy for neuroinflammatory conditions, and that the FDA-approved TOP1 inhibitor topotecan could be repurposed and translated for treating neuroinflammation (Figure 10). In addition, the myeloid cell-specific MyloGami we designed as a drug delivery vehicle may also be used for the treatment of other diseases with dysfunctional myeloid cells. Follow-up studies loading siRNAs into MyloGami will explore more possibilities to modulate myeloid cell function, and it would be of interest to also evaluate the effect of TopoGami in the microglia-driven disease model reported in **Paper II**.

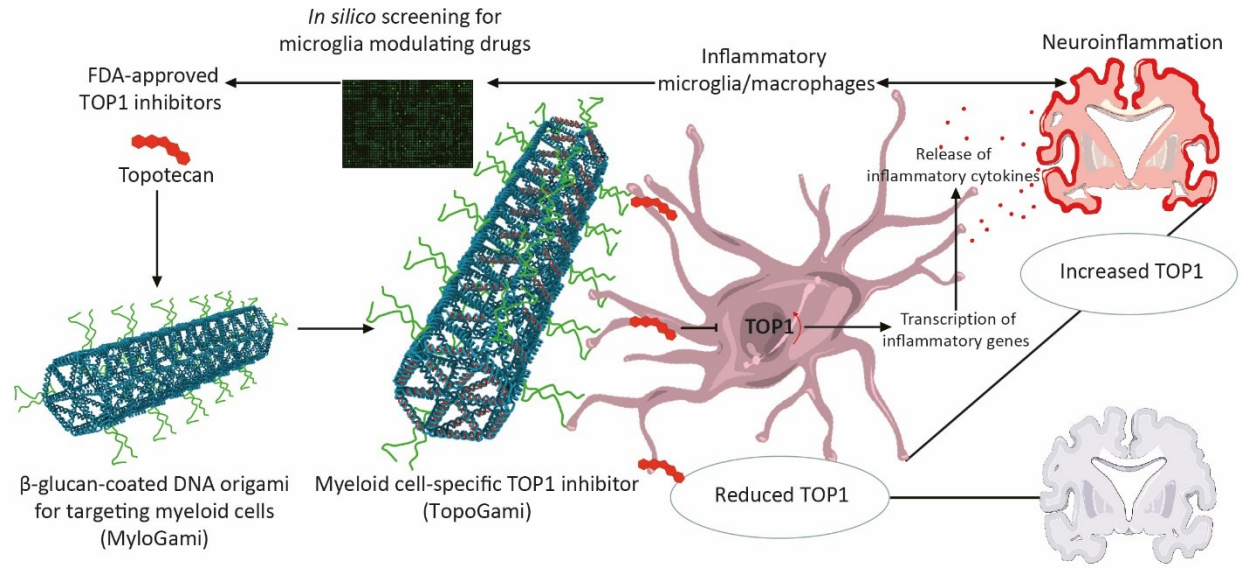

Figure 10 Graphic summary of Paper III.

3.4 Paper IV: Activation of TRPV1 receptor facilitates myelin repair following demyelination via the regulation of microglial function

In **Paper III** we presented a therapeutic approach for treating the neuroinflammatory aspect of MS. Our attention then shifted towards demyelination as the other crucial pathological feature of MS in **Paper IV**. Herein our goal was to elucidate the molecular mechanism responsible for demyelination and propose a therapeutic target for promoting remyelination. We built upon the advantageous impact of TRPV1 activation on microglia (*Section 1.11*) observed in the context of AD and PD, which includes enhancing phagocytosis of protein aggregates and metabolic reprogramming of microglia. Based on this, **we hypothesized that TRPV1 activation might also aid in the clearance of myelin debris resulting from demyelination and support the process of remyelination**.

Using the CPZ-induced mouse demyelination model we discovered that the expression of TRPV1 in the corpus callosum, the brain region most susceptible to demyelination caused by CPZ, was markedly elevated at the protein level after five weeks of treatment with CPZ. This time frame corresponds to a period of substantial demyelination and gliosis. We then first performed *loss-of-function* experiments and compared the demyelinating pathology and motor coordinative function between the *Trpv1*$^{-/-}$ (KO) and wildtype (WT) mice in response to CPZ treatment. This revealed that loss of TRPV1 aggravated demyelination and worsened the behavioral performance as evaluated by rotarod test and beam walking test. TRPV1 *gain-of-function* by administering its ligand capsaicin (CAP) to CPZ-treated WT mice demonstrated improved behavioral performance, enhanced expression of myelin genes as well as myelin basic protein, and increased the number of myelinated axons. However, this effect was absent in KO mice treated with CAP. We further confirmed that TRPV1 was expressed in microglia in the corpus callosum of CPZ-treated mice and active lesions of MS brains, suggesting that microglial TRPV1 may be involved in demyelinating processes.

To further investigate the role of TRPV1 in modulating microglial function, we first treated cultured primary microglia with CAP of varying concentrations and recorded a concentration-dependent increase in microglial migration as evidenced by scratch assay and Transwell assay. In accordance with this, we observed increased occupation by microglia in the demyelination site (corpus callosum) in CPZ-induced mice treated with CAP. These results indicated that activation of TRPV1 by CAP promotes microglial migration and recruitment to the demyelinated area. This was accompanied by a decreased accumulation of myelin debris as evidenced by staining for degraded myelin basic protein and lipid deposits following CAP treatment in CPZ-induced WT mice. In contrast to WT mice, KO mice displayed marked myelin debris accumulation in the corpus callosum after CPZ treatment. We further performed *in vitro* validation, revealing that when stimulated with CAP, primary microglia increased their uptake of dextran (~5 nm) and zymosan (~1-2 µm) bioparticles, while knocking down TRPV1 in microglia hindered their phagocytic function, suggesting TRPV1 regulates microglial phagocytic function. Mechanistically, we found that the fatty acid transporter CD36 that facilitates myelin debris clearance[201], was induced in microglia following TPRV1 activation by CAP. *Cd36* expression was also upregulated in the corpus callosum of CPZ-treated mice, and CAP treatment further enhanced *Cd36* expression. CD36-mediated fatty acid metabolism is highly linked with the Krebs cycle and regulates glucose metabolism[329]. Following CAP treatment, we observed a significant downregulation of the expression of *Hif1a*, which serves as the master regulator of glycolysis, as well as *Hk2*, which plays a rate-limiting role in glycolysis, and *Nos2*, which favors glycolysis over oxidative phosphorylation, in

inflammatory microglia. These findings suggest that the metabolic programming of microglia is altered by CAP treatment, leading to the inhibition of glycolysis (Figure 11).

This current study has a significant drawback in that mice with global TRPV1 knockout were used instead of those with microglia-specific TRPV1 knockout. As a result, the potential contribution of altered neuronal activity in regulating myelination cannot be ruled out. Additionally, when CAP is administered systemically, it may affect many cells other than microglia. A more precise administration of CAP, such as using MyloGami as developed in **Paper III**, to specifically target microglia, may thus provide stronger evidence for the direct effect of microglial TRPV1 function on remyelination.

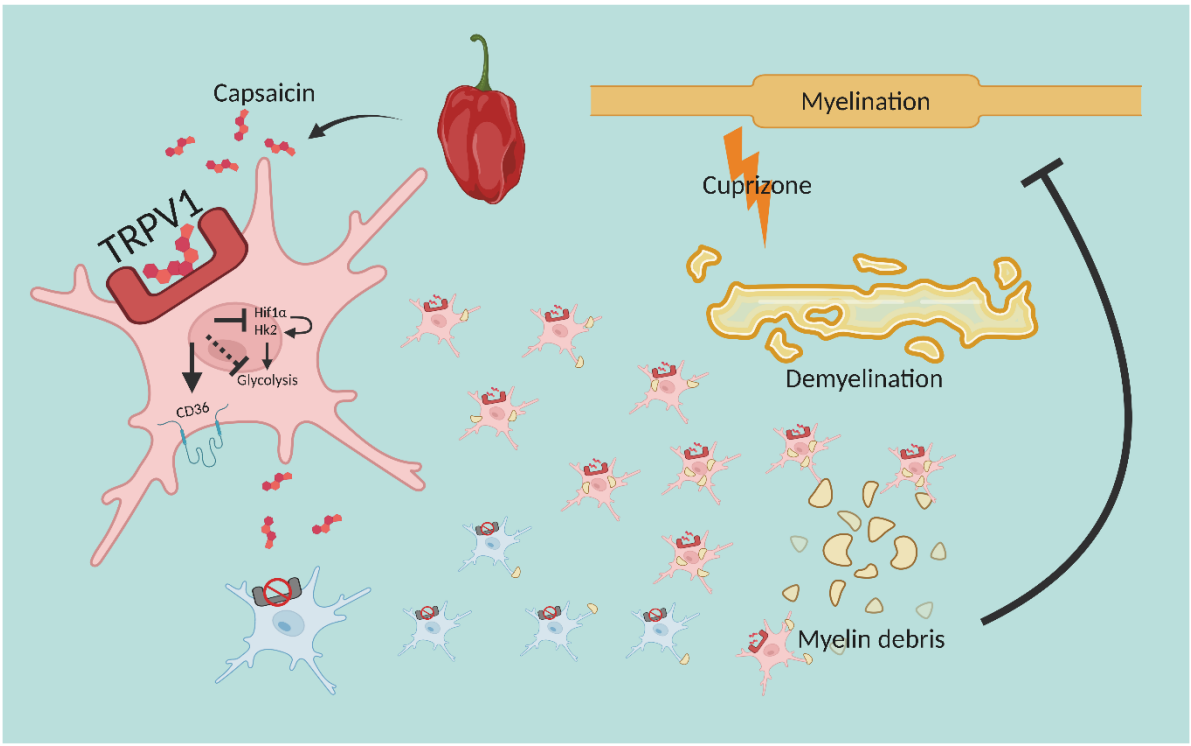

Figure 11 Graphic summary of Paper IV.

4 FUTURE PERSPECTIVES

There have been significant advancements in research regarding myeloid cell function in demyelination, leading to the development of new therapies for the treatment of MS. In light of these developments, I have identified several relevant areas that warrant further investigation, based on both the current literature and my thesis work.

In-depth understanding of region-specific and age-dependent requirement for TGF-β signaling

During the course of neurodevelopment TGF-β family (BMP) signaling regulates the formation of the neural tube and the specific patterning of dorsal-ventral axis, and is necessary for the patterning and oligodendrogenesis of the dorsal spinal cord[330–332]. We are only beginning to reveal that this developmental dorsal-ventral patterning regulated by TGF-β signaling may imprint region-specific sensitivity to the maintenance by TGF-β signaling in adulthood. We know that TGFBR1 is expressed by oligodendrocytes and that TGFβ1-TGFBR1 signaling is involved in regulating the health and integrity of myelin[168]. It is unknown why the dorsal column spinal cord increases TGFβ1 expression during aging, as well as the cellular source of increased TGFβ1 production in the dorsal column. Further research involving the depletion of TGFβ1 production in other cell types, such as microglia or astrocytes, might result in similar myelin pathology within the dorsal column. Such investigations would help determine whether the myelin disruption is caused by the aberrant activation of *Tgfrb2*-deleted microglia or by the inhibition of the TGFβ1 feed-forward loop leading to insufficient microenvironmental TGFβ1 production for myelin/oligodendrocyte maintenance, or both.

The demyelinated areas resulting from microglial *Tgfbr2* deletion, as described in Papers I & II, align well with the distribution of oligodendrocytes derived from the dorsal wave[123,129]. This suggests that oligodendrocytes of dorsal origin may be more dependent on proper intercellular communication involving TGFβ, which warrants further exploration.

Human diseases such as copper deficiency myelopathy and subacute combined degeneration (typically caused by a deficiency in Vitamin B12 or the use of nitrous oxide, also known as 'laughing gas') primarily exhibit degenerative pathologies in the dorsal column of the spinal cord[333–337]. Interestingly, histological staining of spinal cord samples from patients with subacute combined degeneration has revealed significant microglial activation (labeled with CD68) in the demyelinated dorsal area, displaying a foamy morphology[338,339]. Investigating whether TGFβ signaling in the spinal cord is disrupted in these diseases could provide valuable insights.

In accordance with our mouse study, a human study demonstrated that patients with biallelic *loss-of-function* mutations in TGFB1 develop global brain atrophy and posterior leukoencephalopathy, suggesting a protective role for TGF-β signaling in human neurological diseases[340]. However, this study did not include spinal cord imaging or histological examinations. Future research that discloses these findings or examines the spatial distribution of TGFβ1 in human spinal cords may help enhance our understanding of the region-specific response to TGF-β signaling in the spinal cord and its relevance to human health.

Refined targeting of microglial subtypes using DNA origami

The MyloGami we have developed serves as a valuable tool for increasing specificity when targeting myeloid cells. However, it lacks the ability to differentiate microglia from other myeloid cells and is unable to target specific pathological microglial subtypes as identified by various single-cell analyses. As a result, there is a need for further development of DNA origami surface modifications to improve specificity. Additionally, while blood-brain barrier permeability is elevated during active MS, the blood-brain barrier is less permeable in progressive MS and other neurological disorders. This makes it challenging for DNA origami to enter the CNS. Therefore, another essential technical enhancement involves modifying DNA origami to facilitate its entry into the CNS. We have identified Gpnmb and Lgals3 as potential targets that mediate the involvement of TbAMs in the dorsal column demyelinating pathology. Conducting loss-of-function studies with siRNAs targeting *Gpnmb* and *Lgals3*, loaded into MyloGami to treat microglial Tgfbr2-depleted mice, may further substantiate their pathological role in the demyelinating process.

Epidemiological relevance of dietary chili pepper consumption with the incidence of MS

It has been observed that individuals residing in countries with lower latitudes, such as India and Mexico, demonstrate a reduced likelihood of developing MS [341,342]. Intriguingly, these countries also exhibit some of the highest rates of chili pepper consumption[343]. Although certain environmental factors, such as lack of sun exposure and exposure to organic solvents, have been identified as risk factors for MS[344,345], the relationship between dietary habits or a preference for spicy foods and the incidence of MS remains underexplored. Capsaicin, the main component in chili peppers, is known to have a multifaceted impact on the regulation of immune cell function[346]. Given this knowledge and our findings, it would be worthwhile to conduct an epidemiological study that investigates the potential association between the consumption of chili peppers and the prevalence of MS. Such research could offer valuable insights into the role of dietary habits in the development of MS.

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