From DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

REPOPULATION OF A MICROGLIA-DEPLETED CENTRAL NERVOUS SYSTEM: MOLECULAR CHARACTERIZATION DURING HOMEOSTASIS AND DISEASE

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Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-087-2
Cover illustration: A microglia-depleted brain. Courtesy of Kai Zhou.

Repopulation of a microglia-depleted central nervous system: Molecular characterization during homeostasis and disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

CMM Lecture Hall, L8:00 024, Karolinska University Hospital, Solna, Sweden
Friday, March 12th, 2021 at 09:00

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To my parents

POPULAR SCIENCE SUMMARY OF THE THESIS

The central nervous system (CNS) is the part of the nervous system including the brain and spinal cord. Microglia are local immune cells in the CNS. They are essential for normal CNS functions by responding to foreign agents and dangers. However, uncontrolled chronic activation of these immune cells can trigger CNS disorders including multiple sclerosis (MS). The cause of MS remains largely unknown, but it is commonly acknowledged that different immune cells play a role in the development of MS. In MS, the over-active immune cells (including microglia) do not recognize normal CNS proteins as being self-proteins and attack them. Are there potential novel therapeutic strategies by precisely targeting or replacing these over-active immune cells in our CNS?

In **Study 1** we developed a mouse model to remove microglia completely from the CNS for around one week. Subsequently, new microglia appeared and gradually filled in the empty CNS. At around one month, the mouse CNS was equipped again with the new healthy microglia. This mouse model reveals a potential means of replacing dysfunctional microglia with functional microglia in the human CNS. We found that the newly replaced microglia were derived from two different sources, proliferating CNS microglia and monocyte-derived microglia.

In **Study 2** we tested if the newly replaced microglia could influence MS progression by using the same mouse model. We firstly depleted microglia, then waited for one month until new microglia had populated the CNS. We then induced a MS-like disease model in these mice. To our surprise, we found that the newly replaced microglia made MS mice more sick. Interestingly, this only happened in female mice. This study thus demonstrates sex-dependent effects of microglia on MS development.

In **Study 3** we discovered a novel but minor subset of microglia in our microglia depletion mouse model. During the period of microglial replacement following depletion, the new subset of microglia proliferated rapidly and accounted for a significant proportion of the total microglial pool. As this subset has previously been overlooked, our results raise a cautionary note regarding the use of these mice in studies of microglial depletion and replacement.

In **Study 4** we tested if some other types of immune cells in the blood and different organs would be affected when we deplete CNS microglia using currently available methods (either gene editing or drugs). This is a contentious and less studied topic, since it is generally believed that microglia depletion methods are specifically targeted for microglia. However, our results clearly showed that other types of immune cells, including blood and splenic monocytes, were also influenced. These findings remind researchers to be more careful when considering microglial depletion and replacement as a potential therapy for CNS diseases.

ABSTRACT

Microglia are predominant tissue resident macrophages within the central nervous system (CNS), and contribute to both CNS development and homeostasis. During disease conditions microglia undergo transcriptional re-programming and their dysfunction is implicated in a multitude of disorders, such as multiple sclerosis (MS). How microglia could be therapeutically targeted is a current research focus. Recent experimental microglial depletion methods using conditional genetic targeting and pharmacological therapies have broadened our perspective of these multi-tasking microglia. Newly repopulated microglia following experimental microglial ablation hold great promise for reducing neuroinflammation and treating a variety of neurological disorders.

In **Study 1** our results indicated that microglia could be ablated (approximately 95%) by systemic use of tamoxifen in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice. Microglial repopulation ensued through both the proliferation of surviving microglia in the CNS, and from the infiltration of Ly6C^{hi} monocytes. Under this condition infiltrating monocytes could be shaped into microglia-like cells by the CNS microenvironment. Furthermore, isolated newly repopulated resident microglia and infiltrating microglia-like cells following experimental depletion exhibited differential functionality *in vitro*, such as phagocytic capacity and cytokine production.

In **Study 2** we used the microglial depletion and repopulation model mentioned above and demonstrated that the presence of infiltrating microglia-like cells following ablation could exacerbate experimental autoimmune encephalomyelitis (EAE) symptoms in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ female mice. This was not evident in male mice, indicating a potential sex effect. Under this condition there was a higher expression of major histocompatibility complex class II and a greater secretion of proinflammatory cytokines during the acute period in the female mice.

In Study 3 we discovered a novel subpopulation of microglia that escape the genetic modification of *Cx3cr1* in *Cx3cr1*$^{\text{CreER-EYFP/+}}$*Rosa26*$^{DTA/+}$ mice. Following microglial depletion using tamoxifen, newly repopulated *Cx3cr1*^{high}*EYFP*$^{-}$ microglia had an advantage over *Cx3cr1*$^{\text{CreER-EYFP/+}}$ and *Cx3cr1*^{low}*EYFP*$^{+}$ microglia. We also found that microglial repopulation was tightly regulated by the CX3CL1-CX3CR1 signaling. The numbers of repopulated CNS-resident microglia were significantly decreased, while the numbers of infiltrating microglia-like cells were increased during repopulation in mice devoid of *Cx3cr1*.

In Study 4 we demonstrated that experimentally removing microglia using both *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice and PLX3397 treatment had crucial effects on circulating monocytes and splenic macrophages, a finding that had previously received little attention. We therefore proposed that clinical translation of preclinical studies using microglial depletion should take peripheral effects into consideration.

LIST OF SCIENTIFIC PAPERS

I. **Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells**

Harald Lund, Melanie Pieber, Roham Parsa, **Jinming Han**, David Grommisch, Ewoud Ewing, Lara Kular, Maria Needhamsen, Alexander Espinosa, Emma Nilsson, Anna K. Överby, Oleg Butovsky, Maja Jagodic, Xing-Mei Zhang, Robert A. Harris.

Nature Communications. 2018 Nov 19;9(1):4845

II. **Sex-specific effects of microglia-like cell engraftment during experimental autoimmune encephalomyelitis**

Jinming Han, Keying Zhu, Kai Zhou, Ramil Hakim, Sreenivasa Raghavan Sankavaram, Klas Blomgren, Harald Lund, Xing-Mei Zhang, Robert A. Harris.

International Journal of Molecular Sciences. 2020 Sep 17;21(18):6824.

III. **Microglial niche repopulation competition following genetic depletion is regulated by CX3CL1-CX3CR1 signaling**

Kai Zhou, **Jinming Han**, Harald Lund, Nageswara Rao Boggavarapu, Volker Lauschke, Shinobu Goto, Ahmed M Osman, Yuyu Wang, Asuka Tachi, Cuicui Xie, Ying Sun, Dong Liang, Wei Han, Keying Zhu, Kristina Gemzell-Danielsson, Christer Betsholtz, Xing-Mei Zhang, Changlian Zhu, Bertrand Joseph, Robert A. Harris, Klas Blomgren.

Manuscript.

IV. **Underestimated peripheral effects following pharmacological and conditional genetic microglial depletion**

Jinming Han, Yueshan, Fan, Kai Zhou, Keying Zhu, Klas Blomgren, Harald Lund, Xing-Mei Zhang, Robert A. Harris.

International Journal of Molecular Sciences. 2020 Nov 15;21(22):8603.

LIST OF SCIENTIFIC PAPERS NOT IN THE THESIS

I. **An updated assessment of microglia depletion: current concepts and future directions**

Jinming Han, Robert A. Harris, Xing-Mei Zhang.

Molecular Brain. 2017 10:25.

II. **Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling**

Harald Lund, Melanie Pieber, Roham Parsa, David Grommisch, Ewoud Ewing, Lara Kular, **Jinming Han**, Keying Zhu, Jik Nijssen, Eva Hedlund, Maria Needhamsen, Sabrina Ruhrmann, André Ortlieb Guerreiro-Cacais, Rasmus Berglund, Maria J. Forteza, Daniel F. J. Ketelhuth, Oleg Butovsky, Maja Jagodic, Xing-Mei Zhang, Robert A. Harris.

Nature Immunology. 2018 May;19(5):1-7.

III. **Enforced microglial depletion and repopulation as a promising strategy for the treatment of neurological disorders**

Jinming Han, Keying Zhu, Xing-Mei Zhang, Robert A. Harris.

Glia. 2019 Feb;67(2):217-231.

IV. **Absence of microglia or presence of peripherally-derived macrophages does not affect tau pathology in young or old hTau mice**

Keying Zhu, Melanie Pieber, **Jinming Han**, Klas Blomgren, Xing-Mei Zhang, Robert A. Harris, Harald Lund.

Glia. 2020 Jul;68(7):1466-1478.

V. **Microglial replacement therapy: A potential therapeutic strategy for incurable *CSF1R*-related leukoencephalopathy**

Jinming Han, Heela Sarlus, Zbigniew K. Wszolek, Virginija Danylaité Karrenbauer, Robert A. Harris

Acta Neuropathologica Communications. 2020 Dec 7;8(1):217.

VI. **Irreversibly re-programmed homeostatic microglia contribute to functional recovery in spinal cord injury**

Ramil Hakim, Vasilios Zachariadis, Sreenivasa Raghavan Sankavaram, **Jinming Han**, Robert A. Harris, Martin Enge, Lou Brundin, Mikael Svensson.

Submitted.

VII. **Uncovering sex differences of rodent microglia**

Jinming Han, Yueshan Fan, Kai Zhou, Klas Blomgren, Robert A. Harris.

Submitted.

VIII. **Optimisation of the synthesis and cell labelling conditions for [^{89}Zr]Zr-oxine and [^{89}Zr]Zr-DFO-NCS: a direct *in vitro* comparison in cell types with distinct therapeutic applications**

Ida Friberger, Emma Jussing, **Jinming Han**, Jeroen Goos, Jonathan Siikanen, Helen Kaipe, Mélanie Lambert, Robert. A. Harris, Erik Samén, Mattias Carlsten, Staffan Holmin, Thuy A. Tran

Submitted.

IX. **Altered numbers and volumes of secondary lysosomes in circulating monocytes in patients with *CSF1R*-related leukoencephalopathy**

Goda-Camile Mickeviciute, Lidija Smertinaite, **Jinming Han**, Maria Ntzouni, Sergei Masich, Stephan Werner, Peter Guttmann, Petter Ranefall, Anders Sandell, Robert A. Harris, Simin Mohseni, Virginija Danylaité Karrenbauer

Manuscript.

X. **Inhibiting colony stimulating factor 1 receptor (CSF1R) as a potential therapeutic strategy for neurodegenerative diseases: Opportunities and challenges**

Jinming Han, Virginija Danylaité Karrenbauer, Robert A. Harris

Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

CNS	Central nervous system
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple Sclerosis
MRI	Magnetic resonance imaging
EAE	Experimental Autoimmune Encephalomyelitis
MHC	Major histocompatibility complex
CSF-1	Macrophage colony stimulating factor-1
CFA	Complete Freund's Adjuvant
TGF-β	Transforming growth factor-beta
BDNF	Brain-derived neurotrophic factor
CX3CL1	CX3C chemokine ligand 1
CX3CR1	CX3C chemokine receptor 1
TREM2	Triggering receptor expressed on myeloid cells 2
YFP	Yellow fluorescent protein
GFP	Green fluorescent protein
TNF-α	Tumor necrosis factor-alpha
ERK	Extracellular signal-regulated kinase
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol 3-kinase
AKT	Protein kinase B

1 INTRODUCTION

1.1 MICROGLIA

Since the scientific characterization of microglia using silver carbonate staining by Pio del Rio-Hortega a century ago [1, 2], the heterogeneity and complex function of this glial cell population within the central nervous system (CNS) has been exponentially investigated using advanced technologies [3-7]. Microglia, highly specialized tissue macrophages in the CNS, have a unique origin and distinguishing features [8]. The timing of microglial ontogeny and the unique local environment in the CNS place them in a special niche when compared with hematopoietic stem cell-derived tissue macrophages [9, 10]. Specifically, microglia develop from primitive yolk sac progenitors during the embryonic period and colonize the CNS before formation of the blood brain barrier [11]. The unique yolk sac origin of microglia is further supported by requirement of transcription factor Pu.1 and independence from the transcription factor Myb [8, 12].

Under physiological conditions microglial numbers are properly maintained by the balance of local proliferation and apoptosis without contribution from peripheral immune cells in both adult mice and human brain [13]. The proliferative capacities of microglia vary widely across different brain regions [14], and recent progress has provided novel insights that some microglia live for 20 years in our body [13, 15]. Without doubt, recent research advances have changed our historic view of these cells, with *in vivo* imaging of microglia demonstrating that microglia are extraordinarily dynamic rather than remaining stationary, constantly surveying the microenvironment [16], shaping CNS development and interacting with their neighboring elements such as neurons [17], astrocytes [18, 19], oligodendrocytes [20] and neural stem cells [21] (Figure 1). Isolated microglia lose their specific signatures after several hours *in vitro*, while transplanted microglia into the mice brain may re-acquire their cellular identify due to local environmental cues [9]. Isolated microglia in culture conditions may thus not reflect the complex phenotypes of microglia *in vivo*.

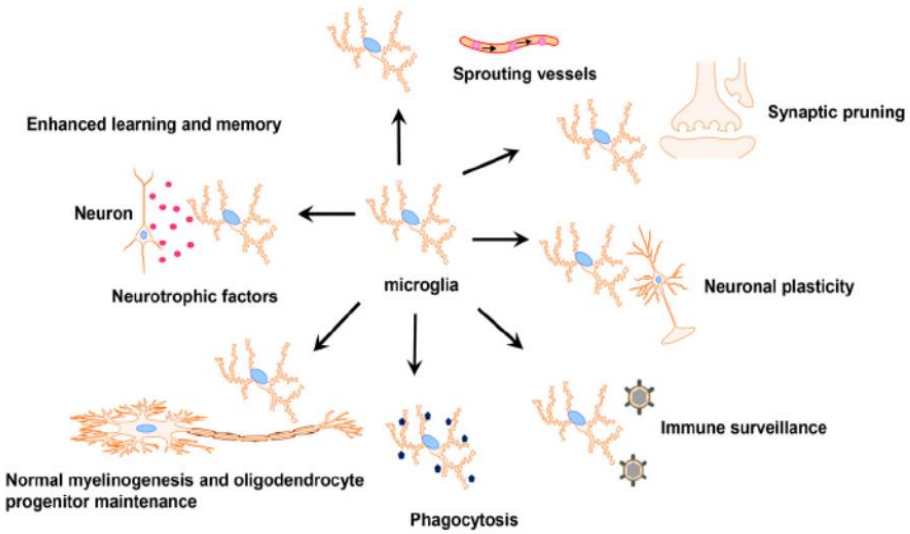

Figure 1: *The multi-tasking microglia in the CNS. Reprinted with permission from Han et al, Glia. 2019 Feb;67(2):217-231.*

Comprehensive single-cell transcriptional analysis across species has revealed that microglia have conserved morphology and transcriptional signatures in most species, while human microglia exhibit substantial heterogeneity [22]. Microglia can eliminate presynaptic and postsynaptic structures, phagocytosis debris, dead cells and neurons, produce various soluble factors [23] and migrate toward areas of injury [24]. Microglia can contribute to the forgetting of remote memories in the adult hippocampus through complement-dependent synaptic elimination, as evidenced using microglia-depleted CD11b-DTR mice as well as PLX3397-treated mice, which showed higher freezing levels than the control group after training sessions (contextual fear conditioning) [25]. Microglia can function as antigen presenting cells of bacterial or self-antigens (e.g. myelin) following upregulation of major histocompatibility complex (MHC) class II [26, 27] due to activation, the levels of which are low or absent during homeostasis [28]. Microglia do more than contribute to development and respond to injury, and emerging investigations suggest that microglia can also perform a variety of tasks in our brain such as regulating satiety [29].

In order to exercise their multiple critical functions, a number of receptors such as toll-like receptors and scavenger receptors are expressed on microglia [30]. The CX3C chemokine ligand 1 (CX3CL1)-CX3CR1 signaling and CD200-CD200R axis have been considered as the main forms of bidirectional microglia-neuron communications [31]. The signals derived from neuronal-derived factors are crucial for microglial maintenance. It has recently been convincingly demonstrated that both mice and human microglial processes can communicate with specialized nano-architectural somatic microglia-neuron junctions under non-inflammatory conditions, and that these are triggered by changes of neuronal activities and neuronal mitochondrial ATP production [17]. Murine microglia during waking conditions are less alert to the environment when compared to microglia in anesthetized conditions, since microglial process surveillance is tightly controlled by neuronal activity through noradrenergic signalling [32-34]. Growing evidence provides us with a broader and better understanding of how microglia take rapid actions under physiological conditions. It is important to note that most of our updated knowledge regarding microglia arise from rodent studies, and that some differences between rodent and human microglia may exist.

1.1.1 Microglia in disease

Microglia are considered as the first line defense against any injury of the CNS. In response to different brain insults microglia undergo morphological transformation with or without proliferation [35], alter gene expression and surface markers [36], present antigens and release cytokines, chemokines, cell adhesion molecules, reactive oxygen and nitrogen species. Upon stimulation microglia switch their metabolic features from oxidative phosphorylation to glycolysis in order to rapidly provide energy by generating ATP and to meet demands for cellular proliferation [37, 38]. Anti-inflammatory microglia/macrophages decrease their glucose consumption and utilize more oxidative metabolism for the function of tissue repair

[38]. Modulating microglial immune metabolism could thus serve as a potential means for reprogramming cellular activity in disease [39].

The activation of innate immune responses is now considered to be of major pathophysiological significance during neurological diseases [40] such as viral encephalitis [41], Huntington's disease [42], hereditary diffuse leukoencephalopathy with axonal spheroids [43, 44] and neuromyelitis optica spectrum disorder [45]. A broad repertoire of receptors is expressed on microglia [30], enabling them to sense extracellular signals via the pattern recognition receptors, which facilitates their recruitment into injury sites. Conditional depletion of P2Y12R on microglia leads to increased neuronal excitability and innate fear responses in mice [46]. Dynamic microglial responses are apparent during disease, rather than simple phenotypical changes, with multiple clusters of cells existing depending on brain regions and disease status [36]. Microglial homeostasis gene signatures such as *tmem119* and *p2ry12* can be decreased during inflammation. Furthermore, several genes (e.g. *apoe* and *trem2*) identified by genome-wide studies are associated with increased risk for neurological diseases and are highly expressed on disease-associated microglia [47]. Our colleagues have demonstrated that microglia undergo rapid and transient transcriptomic changes following a single dose of radiation when measured by single-cell RNA sequencing, suggesting a narrow time window for targeting microglia post-irradiation [48].

Accumulating evidence thus supports that microglia are central players and promising targets for treating CNS disorders [49, 50]. During cerebral hypoperfusion reactive microglia are redistributed in the striatum, adhering and phagocytosing the myelin components, then worsen white matter injury partly caused by upregulated complement C3, while either inhibiting C3aR or depleting microglia prevents the impairment [51]. It is important to note that microglia are highly adapted to brain-specific tissue microenvironments [52, 53] and are functionally modified through epigenetic modifications [54], the microbiome [55] and physical exercise [56]. In an animal model of sepsis, proinflammatory microglial markers in the hippocampus were significantly upregulated 24 hours after inducing sepsis, while the upregulation of both proinflammatory and anti-inflammatory markers co-existed for a long time period (30 days after inducing sepsis) [57]. The historically used classification of M1 (proinflammatory) and M2 (anti-inflammatory: M2a, M2b, M2c or M2d) polarized states *in vitro* [58] is now considered less valid [59], as the activation states vary between individual cells. Targeting molecular signaling pathways and microenvironmental factors controlling microglial expression, rather than their activation states, may thus be a more reasonable therapeutic approach.

1.1.2 The CX3CL1-CX3CR1 signaling pathway

CX3CL1 is a chemokine predominately synthesized by neurons in the CNS [60]. Emerging evidence suggests that there are two different forms of CX3CL1, including a membrane-bound form and a soluble cleaved form, that have distinct activities [61]. CX3CR1 is the fractalkine receptor for CX3CL1 which is mainly expressed on microglia in the CNS. Activation of CX3CR1 leads to cellular signal transduction including kinase (ERK)/mitogen-activated

protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways [60]. The CX3CL1-CX3CR1 signaling pathway is now considered to be a critical regulator of cross-communication between neurons and microglia. For example, the CX3CL1-CX3CR1 axis plays a vital role in cell-to-cell virus transmission, as evidenced by CX3CR1 antagonists inhibiting virus transmission from microglia to neighboring cells in a co-cultured system [62]. *Cx3cr1*, a highly expressed gene in microglia, plays a profound role in microglial functions and is widely used as a driver in gene reporter constructs, such as in *Cx3cr1*^{Cre} mice or *Cx3cr1*$^{CreER\text{-}EYFP/+}$ mice [52]. It has been demonstrated that microglial *Cx3cr1* deficiency in young mice results in a microglial transcriptome similar to that of an aged microglial phenotype, suggesting that *Cx3cr1*-deficient microglia accelerated the aging process [63].

1.1.3 The TGFβ signaling pathway

Transforming growth factor-beta (TGF-β) is a well-documented factor for microglial identity [64] and both TGF-β1 and TGF-β2 are highly expressed on microglia [9]. The expression of TGF-β in CNS tissues is significantly reduced during the peak of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), while TGF-β receptors remained unaffected [65]. Additional administration of TGF-β cytokine can reduce EAE disease severity [65]. We have previously indicated that long-term absence of *Tgfbr2* in CX3CR1^{+} monocyte-derived macrophages causes a fatal paralysis disorder evidenced by proinflammatory myelin-laden giant macrophages throughout the mouse spinal cord [66], and this is further supported using distinct models [67, 68]. These results suggest that TGF-β signaling may serve as a potential therapeutic target for demyelinating diseases.

1.1.4 The TREM2 signaling pathway

TREM2, a key receptor selectively expressed on myeloid cells, is now viewed as a profound regulator that switches the homeostatic microglial state into a disease-associated phenotype. Genetic variants in TREM2 may confer a risk for Alzheimer's disease in early life through subcortical alterations [69]. Higher cerebrospinal fluid levels of soluble TREM2 at baseline, serving as a valuable biomarker, is associated with reduced hippocampal volume and disease progression in patients with Alzheimer's disease [70]. The presence of human microglial TREM2 mutations causes obvious metabolic deficits by decreasing the oxygen consumption rate and reducing glycolytic capacity, suggesting that functional loss of TREM2 in microglia results in failure to perform the metabolic switch and to allow a rapid supply of energy [37].

The modulation of microglial TREM2 function within a suitable time window may benefit patients who are suffering from neurodegenerative diseases. Both *aope* and *trem2* have been viewed as major genetic risk factors for Alzheimer's disease. TREM2 can recognize a variety of ligands [71], and the recently identified TREM2-APOE pathway can function as a major mediator of suppressing microglial normal functions during neurodegenerative diseases [72]. TREM2 and DNAX-activating protein of 12 kDa (DAP12), mainly expressed on microglia, are causative genes for Nasu-Hakola disease that is characterized by early neurodegeneration in the white matter and bone lesions. The TREM2/DAP12 complexes can be formed,

consequently phosphorylating ITAM, recruiting Syk kinase, activating related downstream molecules and then regulating microglial activity [71].

1.1.5 Uncovering microglial sexual dimorphism: More still to be explored

It is well documented that there are multi-faceted sex differences of the brain and its behavior in both mice and humans [73]. Importantly, sex differences of microglia and their potential implications in disease have only recently become understood [74-76]. The impact of sex differences on microglial functions and properties are mainly through sex-specific hormonal signals, while microglia in turn participate in brain sexual differentiation [77].

It is well documented that neurodevelopmental diseases demonstrate a male bias in incidence [78]. Male microglia may be more immature in several brain regions since their development is later than in females [79, 80]. However, it is important to note that there are no consistent data showing sex-dependent effects in neonatal and adult animals in brain-based disease conditions. Previous studies reported that early sex differences of microglial responses following ischemic stroke were noted in neonatal mice, evidenced by increasing microglial immunoreactivity, more pro-inflammatory markers and infiltrating myeloid cells in male mice after inducing ischemic stroke [81]. Minocycline can protect against male neonatal ischemia by modulating microglial phenotypes, but this is not the case in female mice [82]. Our colleagues have demonstrated that neonatal microglia-depleted male *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice exhibited significantly larger infarct volumes and a higher rate of apoptotic neurons following hypoxic ischemia than in *Cx3cr1*$^{CreER/+}$ female mice [83].

Microglial sexual dimorphism following immune responses is likely to be time-dependent. These sex differences can also be influenced by estrogens, the levels of which in the circulation may vary with age and reproductive states [73]. Sex has been viewed as a prominent contributor of microglial interactions, with amyloid plaques in the EFAD mice model of Alzheimer's disease (a model that carries human APOE alleles) in female mice showing lower microglial plaque coverage and TREM2 levels than in the males [84]. Using inducible *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice to deplete adult microglia we have determined that the infiltration of microglia-like cells following ablation exerts sex-specific effects on EAE in adult mice, with female adult mice experiencing a more serious disease course than do males [85]. It has been reported that *Escherichia coli* infection in the periphery significantly decreased brain-derived neurotrophic factor (BDNF) expression in the male rat cerebellum, but not in the females [86]. Early-life infection can increase IL-6 expression in the female cerebellum, but not evidence in males [86]. The BDNF-TekB-dependent pathway potentially influenced by estrogen may account for these microglial functional sex differences, to some extent, as supported by distinct correlations between BDNF mRNA levels and depressive-like behaviors between males and females being evident in a chronic mild stress model [87]. Although there are no overall differences in microglial numbers in the rat periaqueductal gray matter between males and females, more amoeboid microglia are located at this region in females compared to males, and lower proinflammatory cytokines transcription was noted following systemic

lipopolysaccharides (LPS) challenge [88]. Furthermore, the microbiome can also exert significant effects on microglial properties in a sex-dependent manner [55, 89].

1.1.6 Future directions and challenges in microglial research

The development of new genetic animal models such as Tmem119-EGFP and Tmem119-CreERT2 mice that could target the resident microglia with a high specificity would improve our understanding of microglial biology [90]. In addition, specific microglia diagnostic methods, such as measuring soluble TREM2 in the cerebrospinal fluid, or neuroimaging of microglial signaling pathways, might facilitate the early detection of individuals at a high risk [35]. Furthermore, future microglial research needs to take potential differences between males and females into consideration.

Microglia are not easily accessed in patients. Several radioligands for positron emission tomography (PET) have been developed in order to noninvasively monitor neuroinflammation and microglial activity in patients. Specifically, the [^{11}C] PK 11195 PET tracer selectively binds to the mitochondrial translocator protein (TSPO). It is widely considered as a biomarker for neuroinflammation, interacting with reactive oxygen species and the NLRP3 inflammasome, and being upregulated expression during glial activation [91]. A degree of caution is needed when interpreting inconsistent results because TSPO as a microglial marker has additional expression on other glial cells (e.g. astrocytes) and fails to distinguish between these during complex activated glial states in distinct diseases. Recently, the novel PET tracer [^{11}C] SMW139 was developed as a radioligand for P2X7R (a member of purinergic type 2 receptor family) and as an *in vivo* marker of neuroinflammation [92]. [^{11}C] SMW139 has a high affinity to P2X7R expressed on pro-inflammatory microglia [92]. However, our preliminary results indicated that the radioligand for P2X7R may also not be specific for measuring microglial activation since P2X7 binding can be still widely noted following microglial depletion in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice (Figure 2). Such approaches for measuring microglia are thus currently limited due to low cell type specificity. Potential differences in P2X7R expression between mice and humans should thus not be ignored [93].

Since small molecules for P2Y12 do not cross the blood–brain barrier, there are no available PET radioligands for this target as yet. Recently, [^{11}C] CPPC, a novel high-affinity PET radiotracer for macrophage colony stimulating factor-1 (CSF-1) receptor has been investigated in both animal and human disease conditions [94]. The signal intensity of brain uptake of [^{11}C] CPPC was positively associated with EAE disease severity and regional distribution of demyelination, which can be significantly reduced in microglia-depleted mice [94].

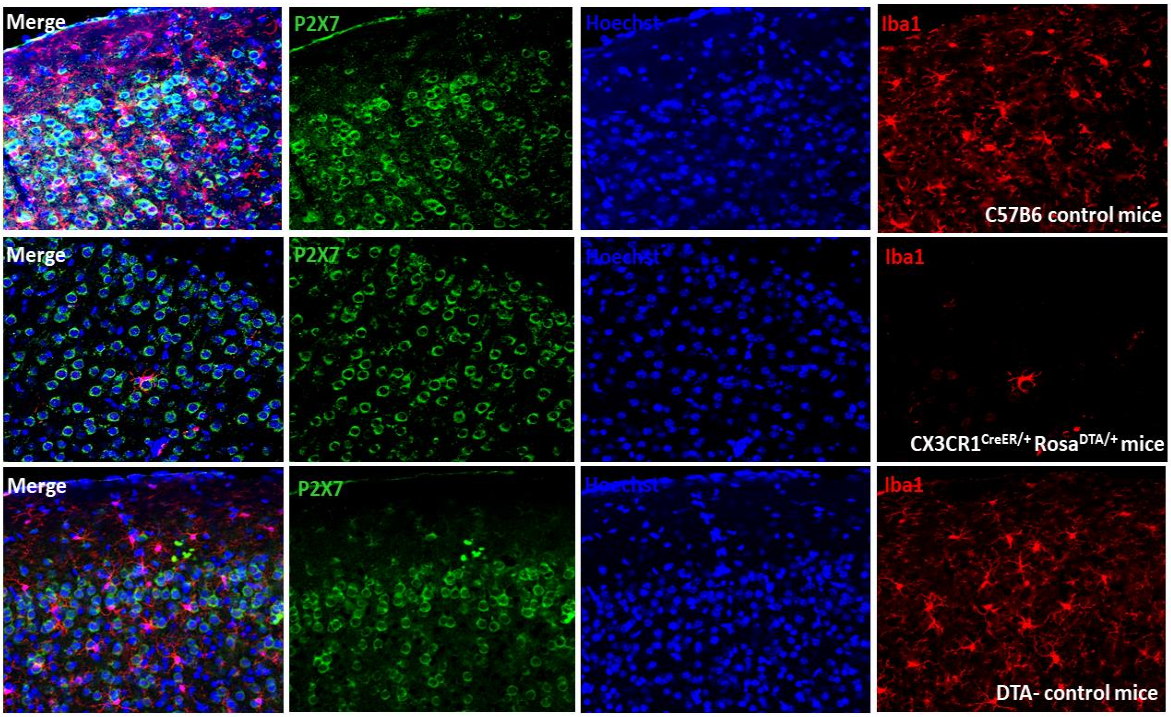

Figure 2: *Immunofluorescent staining of P2X7 (green) and Iba1 (red) in the brains of C57BL/6NTac wild type mice (without tamoxifen), Cx3cr1$^{CreER/+}$Rosa26$^{DTA/+}$ mice and Cx3cr1$^{CreER/+}$ mice with three consecutive subcutaneous tamoxifen injections.*

1.2 MONOCYTES

Both technological and ethical issues limit researchers to conduct research of fresh human microglia. Circulating monocytes are much easier to sample than human microglia obtained after autopsy [95]. Monocytes are critical mediators of the innate immunity [96]. Unlike embryonic yolk sac derived-microglia in the CNS [11], monocytes are of peripheral origin and account for approximately 10% of circulating human leukocytes [96]. Under physiological conditions peripheral immune cells are restricted from entering the CNS by multiple barriers such as the blood-brain barrier. However, monocytes can be recruited into the CNS parenchyma when the blood-brain barrier is compromised during disease conditions, thereafter developing into either macrophages or dendtiric cells with specialized functions [97]. Monocytes can be moblizated rapidly in order to perform inflammatory effector and regulation functions at inflamed sites [96]. Furthermore, peripheral monocytes may modulate other myeloid cell activities in the CNS [98]. In mice, infiltrating monocytes/macrophages can be reprogramed in the brain following ischemic stroke, upregualting efferocytosis-related genes, phagocytizing dead neurons and thus contributing to inflammation resolution [99].

Circulating human monocytes are a heterogeneous cell type that can be subdivided into three different subpopulations based on the expression of CD14 and CD16, denoted as *classical monocytes*, *non-classical monocytes* and *intermediate monocytes*. Classical monocytes ($CD14^{++}CD16^{-}$) account for 80-90 % of human blood monocytes, highly express CCR2 (an important mediator of monocyte migration) and display inflammatory properities. Non-classical monocytes ($CD14^{+}CD16^{+}$) account for 2-10 % of human blood monocytes and highly

express CX3CR1. Intermediate monocytes ($CD14^{++}CD16^{+}$) account for the remaining component and express either CCR2 or CX3CR1. It is suggested that these three isolated monocyte subsets produce different levels of cytokines following stimulation [96]. Circulating monocytes play a crucial role in mediating systemic inflammation during diverse disease conditions [100]. For example, $PD\text{-}1^{+}$ circulating monocytes (mainly intermediate monocytes) may serve as a potential biomarker for predicting vasospasm in patients with subarachnoid hemorrhage, as evidenced by $PD\text{-}1^{+}$ circulating monocytes being increased before the presence of radiographic vasospasm and correlating with the cerebral blood flow velocities [101]. In a preclinical model of subarachnoid hemorrhage, the systemic administration of soluble PD-L1 could prevent cerebral vasospasm, mainly via inhibiting the migration of $PD\text{-}1^{+}$ circulating monocytes into the brain [101]. Individual functional properties of these different monocyte subpopulations during disease conditions remain unclear, and more efforts are thus needed. Various aspects such as age and circadian rythms should not be ingored.

Circulating monocytes are differently defined in mice based on the expression of Ly6C and chemokine receptors, denoted as $Ly6C^{hi}$ inflammatory monocytes and $Ly6C^{lo}$ monocytes [102]. A gating strategy of mice circulating monocytes is presented in Figure 3.

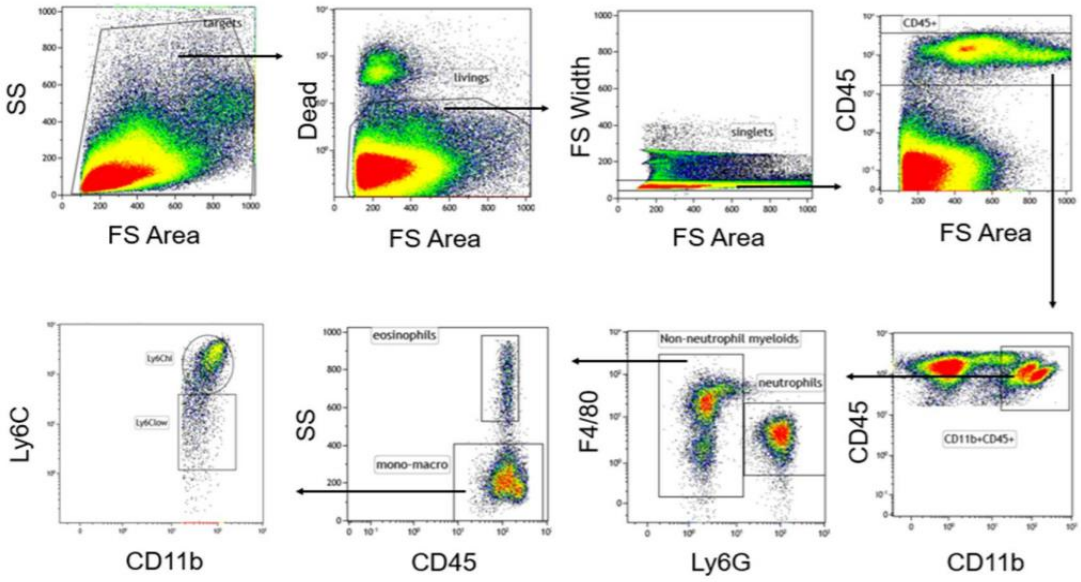

Figure 3: *Gating strategy for circulating mouse monocytes.*

1.3 EXPERIMENTAL MICROGLIAL DEPLETION

Microglial ablation through pharmacological treatments or conditional genetic depletion is now viewed as a powerful approach to induce novel microglial repopulation of the CNS niche, as we have previously reviewed [49, 103, 104]. CSF1R plays a crucial role in microglial survival and proliferation [105, 106]. This is a receptor tyrosine kinase and mediator of myeloid lineage cells and is predominately expressed on microglia in the CNS [107, 108]. Both CSF-1 and IL-34 ligands are crucial for microglial homeostasis [109]. Microglia can be experimental ablated in *csf1r*-deficient mice, while lacking the CSF-1 ligand only causes a moderate reduction of

microglial numbers [109]. Furthermore, the absence of CSF-1 in *Nes*^{Cre}*Csf1*$^{fl/fl}$ mice results in loss of cerebellar microglia, but not forebrain microglia, reducing Purkinje cells, increasing cerebellar volume and causing motor learning and sociability changes [109]. Some studies reported that activated microglia may be less dependent on the CSF1R signaling, but definitive proof is lacking as yet [110].

Pharmacological targeting of CSF1R using compounds such as PLX3397 or Pexidartinib (Plexxikon Inc.) [51], PLX5622 (Plexxikon Inc.) [111], BLZ945 (Novartis) [112] or GW2850 [113] has increased in popularity as a means to deplete microglia in research settings, such as mixed glial cultures [114] and animal models [115]. These compounds are usually integrated into rodent chow diet and do not have harmful effects on mice health and growth in adults [25]. Although diet-based PLX3397 (290 mg/kg) and PLX5622 delivery for ablating microglia is commonly used in current research, it may have limitations in depletion effectiveness for preweaning or sick mice due to reduced appetite. Intraperitoneal injection or intracerebroventricular injection of CSF1R inhibitors should thus be considered in research settings. In order to achieve a faster and higher efficiency, a higher concentration of PLX3397 (400 mg/kg) can also be used, resulting in around 90% microglial ablation in 7 days [110]. Cell death within the CNS can occur through apoptosis and phagocytosis [19], but the precise cellular mechanisms controlling experimental microglia death are not completely understood.

Microglial depletion using PLX3397 did not significantly alter adult mice behavioral performance, as measured by the elevated plus maze and open field tests [25], while ablating microglia during the neonatal period using liposomal clodronate causes long-term changes of locomotion and decreased anxiety and despair behaviors [116]. Although most previous studies reported no obvious side-effects after microglial depletion in adults [117], it is important to note that microglia also provide trophic or metabolic support in the CNS [23] and some potential effects following microglial depletion should not be ignored in research settings. For example, the electrical threshold of spreading depolarization elicitation can be significantly changed after microglia depletion using PLX5622 treatment [118].

Microglia-mediated injury is considered as a leading driver of neurodegeneration [110]. In diverse disease conditions microglial depletion has a broad range of beneficial results, mainly by reducing neuroinflammation [49]. However, the use of current experimental microglial depletion methods has yielded mixed results since immunological responses of microglia to CNS injury are complex in both space and time aspects. Removing microglia using PLX5622 decreased mRNA expression of Nox2 in the nucleus accumbens and prevented the anxiety behavior caused by nicotine withdrawal [119]. It has been reported that treatment of PLX3397 increased the rate of myelination and decreased the rate of nerve fiber destruction and the extent of the gaps formed between layers of myelin sheaths in a cuprizone-induced demyelination mouse model [115]. The rapid administration of CSF1R inhibitor PLX5622 after cell isolation *in vitro* effectively depletes microglia but required a relatively long incubation time since potential CSF-1-resistant microglia may exist in the culture from the very beginning [114]. The advent of more specific microglial markers and novel tools could provide

more clarity. Specifically targeting disease-associated microglia in neurological diseases within a suitable time window may be a potential immunotherapy for diverse neurological diseases [47].

1.4 MICROGLIAL REPOPULATION HOLDS PROMISE FOR DISEASE THERAPY

Microglia can rapidly repopulate following their depletion owing to their self-renewal ability, occurring only 1 day after withdrawal of CSF1R inhibitors [120]. Newly repopulated microglia can either stem from infiltrating microglia-like cells [52, 97] or solely from CNS-resident microglia [121, 122], depending on different models and the efficiency of microglial depletion.

Newly engrafted microglia-like cells following selective microglia depletion exhibit different gene expression and functions when compared with CNS-repopulated resident microglia [52]. This point was further supported by later studies, demonstrating that two different sources of myeloid cells including peripheral macrophages contribute to the compensation of microglial numbers independently of irradiation [97]. Microglia and microglia-like cells may thus contend for the colonization of the empty niche in the CNS, and microglia-like cells can outcompete CNS resident-microglia if resident microglia lack the capacity for CX3CL1-CX3CR1 signaling (our unpublished observations).

From the perspective of cell therapy, bone marrow-derived cells have different functions, such as clearance of amyloid beta [123]. A combined administration of intravenous and intramuscular bone marrow mononuclear cells exhibited beneficial effects in the $SOD1^{G93A}$ amyotrophic lateral sclerosis mouse model by delaying disease onset and decreasing spinal cord microgliosis [124]. These pre-clinical results remind us to take different cell sources and routes of administration into consideration when designing potential microglial replacement therapy, especially in settings of neurodegenerative diseases such as incurable *CSF1R*-related leukoencephalopathy (Figure 4).

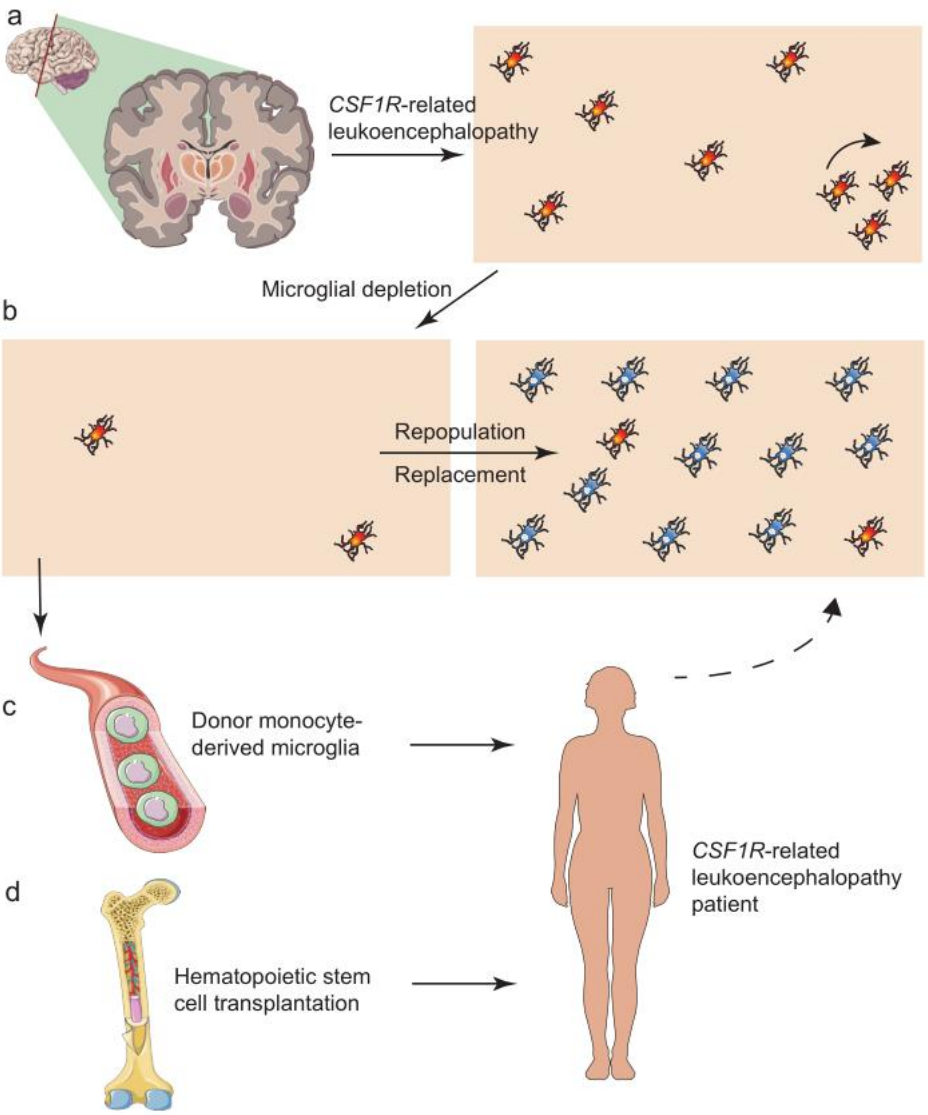

Figure 4: *Potential microglial replacement therapy for CSF1R-related leukoencephalopathy. Reprinted with the permission from Han et al, Acta Neuropathologica Communications. 2020 Dec 7;8(1):217.*

In experimental spinal cord injury condition, we have found that homeostatic spinal cord microglia undergo permanent transcriptional re-programming and transform into the disease-associated microglia, as assessed by single cell RNA-sequencing (our unpublished observations). One recent study reported that a combination of gelatin hydrogel transplantation and PLX3397 treatment could better improve disease recovery in a spinal cord injury mice model than does single PLX3397 treatment, mainly through replacing $CD68^{+}$ reactive microglia and the presence of newly repopulated microglia in the spinal cord [120].

1.5 MULTIPLE SCLEROSIS

MS is a multifocal demyelinating disease of the CNS mainly caused by environmental cues which act on genetically susceptible young individuals, causing irreversible cumulative neurological disability [125]. Diverse immune cells, neuronal and axonal loss, all likely contribute to distinct pathogenic processes in MS [126, 127]. Apart from marked demyelinating

lesions in the CNS, normal-appearing white matter is also implicated in disease pathogenesis and can be affected by the hypothalamus-pituitary-adrenal axis activity [128]. Remarkable progress has been made in disease-modifying therapies of MS during recent years. The history of disease-modifying therapies for MS started from injectable interferon beta-1a to oral immunomodulating therapies and new monoclonal antibodies targeting B cells [129]. The widely used immunotherapies for MS have been proven effective to decrease the annual relapse rate and to reduce new lesions. Early treatment for MS appears to decrease the total relapse over a long time period, especially during the first year after treatment. Most randomized controlled trials exclude MS patients aged more than 55 years old, and it remains challenging for managing these ageing individuals with MS due to unique age and MS-related comorbidities in clinical practice [130]. Currently, an expanding array of treatments for relapsing-remitting MS are available, and therapeutic options for adult progressive MS are also emerging [131]. However, a recent Phase IIb clinical trial demonstrated that potential neuroprotective drugs including Amiloride, Fluoxetine and Riluzole do not reach any primary or secondary outcomes over 96 weeks in British MS patients [132].

Among commonly used disease-modifying therapies for MS, Fingolimod has been reported to exert the strongest impact on peripheral immune cell frequencies using a highly standardized approach [133]. Cladribine has a long-term effect on peripheral immunity [129]. Monitoring circulating peripheral immune cells and opportunistic infections can be helpful for managing MS in clinical practice [134]. The right choice of disease-modifying therapies for MS depends on balancing expected efficacy, safety and tolerability [129].

The novel identification of serum/plasma neurofilament as predictors of clinical progression are broadening our perspective to predict and monitor disability in MS [135]. The baseline degree of plasma neurofilament is positively associated with the clinical Expanded Disability Status Scale score and clinical cognitive performance, and importantly, their level can be dramatically reduced by Alemtuzumab treatment [136]. Application of 7 Tesla MRI further expands our understanding of cerebral leptomeningeal enhancement and gray matter involvement in MS patients [137]. Microglial activation is a major driver of cortical demyelination. MS patients with high inflammatory profiles of cortical lesions measured by PET and 7T MRI having a worse clinical outcome than those with low cortical inflammation patients [138]. Although T cells and B cells have been intensively investigated in MS, less is known about the roles of other immune cells such as myeloid cells. Circulating monocyte numbers during the early MS period could serve as a potential candidate biomarker for predicting subsequent disease severity [139].

1.6 MOG-EAE

Despite many limitations to represent the pathogenesis of MS, EAE is still a commonly used animal model. EAE can be induced by passive transference of auto-reactive $CD4^+$ T cells or through active immunization with a myelin antigen emulsified in Complete Freund's Adjuvant (CFA). Myelin oligodendrocyte glycoprotein (MOG) is a minor protein that is expressed on the outermost lamella of the myelin sheath [140], and MOG_{33-55} serves as the most popular

antigen emulsified in CFA for inducing EAE in C57BL/6 background mice [141], especially as many research used gene specific knockout mice have been generated on this background [142]. Additional injections of pertussis toxin are also required to inhibit peripheral T cell anergy induction and to increase the permeability of the blood-brain barrier [143]. The first signs of MOG-EAE after immunization are weight loss and obvious clinical symptoms such as tail weakness and paralysis of hind limbs can be observed around 10 days after immunization [144].

In clinical practice, antibodies against MOG will directly target oligodendrocytes and the myelin sheath, which is associated with a wide variety of clinical presentations including optic neuritis, transverse myelitis and encephalitis [145]. Serum MOG-lgG1 antibodies are recommended to be preferentially detected using live cell-based assays, and high titers or persistent seropositivity of MOG-lgG antibodies may predict a relapsing disease course. It is firmly established that MOG-antibody associated disease, with less female predominance, is quite different from MS regarding immunopathological targets, clinical features and magnetic resonance imaging (MRI) findings [146]. Furthermore, a poor response to some MS drugs has been noted in some cases, while most patients with MOG-antibody-associated disease respond well to corticosteroids and intravenous immunoglobulins, often with full recovery [147]. Importantly, abundant phagocytic $CD68^+$ infiltrating macrophages (negative for Tmem119) and $CD8^+$ T cells in the gadolinium-enhancing brain lesion had been noted in one patient with histopathologically confirmed MOG-antibody-associated disease [148].

Some argue that MOG-antibody-associated disease is quite different from MS [149-151]. The complex pathogenesis of MS replies on a combination of genetic diversity, epigenetic modifications, environmental factors and a variety of immune cell types [152], while its animal models fail to represent the entire spectrum of disease characteristics. Furthermore, immunological processes of MS progression in humans are distinct from the animal model, sometimes causing disappointment when translating preclinical findings into the clinic. For example, non-selective blocking tumor necrosis factor-α (TNF-α) showed beneficial outcomes in EAE, which was detrimental for patients with MS [153]. It reminds us to choose suitable experimental animal models in order to test specific hypotheses in MS and to optimize the communication between basic and clinical research.

1.7 MICROGLIA AS A THERAPEUTIC TARGET FOR MS AND ITS ANIMAL MODEL

Inflammation contributes to the demyelination and tissue injury in both acute and progressive stages of MS [154]. Pathological studies suggested that microglial activation is most pronounced in active MS lesions, while microglial numbers are decreased in inactive MS lesions [154]. A diversity of microglial activation states as measured by single-cell RNA sequencing include previously characterized disease-associated microglia [155], and aging-associated microglia are noted in an acute local demyelination mice model, without any overlap of clear-cut M1 and M2 gene profiles [156]. Microglia are also enriched for a variety of MS susceptibility genes, which was convincingly indicated by a recent genomic map [157].

Dimethyl fumarate, the first line oral clinical treatment for MS in the clinic, may exert its neuroprotective activity by modulating microglial phenotypes and functions [158].

Several studies have demonstrated that removing microglia has beneficial effects on EAE and in the cuprizone demyelination model [112, 159, 160]. Another study inferred an opposite conclusion, that ablating microglia by administration of tamoxifen in *Cx3cr1*^{CreER}Rosa^{tdTom}Rosa26^{iDTR} mice causes higher accumulation of infiltrating macrophages in spinal cord lesions [156]. The application of microglia-specific therapeutic strategies bears great promise for CNS regeneration, particularly in progressive MS [161]. Microglia serve as a double-edged sword in MS pathology, either by exacerbating demyelination or by clearing myelin debris [162].

2 MATERIALS AND METHODS

Animal models

Cx3cr1^{CreER} and *Rosa26*^{DTA} mice were purchased from the Jackson Laboratory, and bred in the KI animal house facility in order to obtain *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ and *Cx3cr1*$^{CreER/+}$ mice (Figure 5), which were used in **studies 1, 2 and 4**.

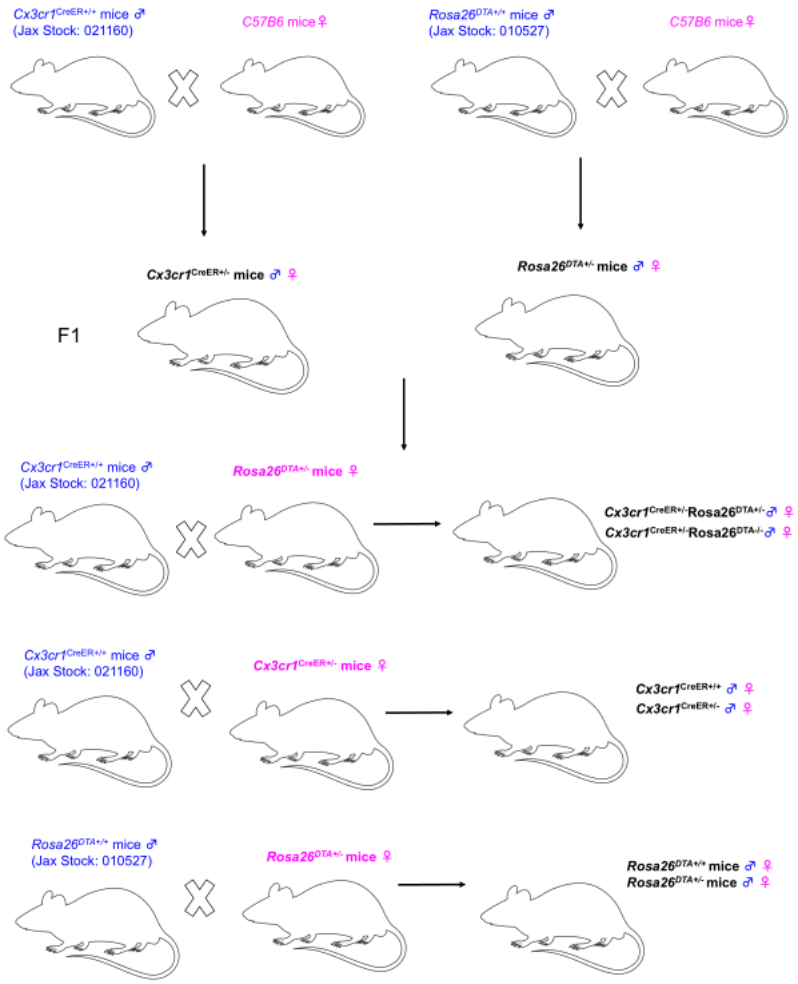

Figure 5*: An overview of breeding strategies used in studies 1, 2 and 4.*

The second generation *Cx3cr1*$^{CreER/+}$*R26*, *Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$, *Cx3cr1*$^{CreER/CreER}$*R26* and *Cx3cr1*$^{CreER/CreER}$*R26*$^{DTA/+}$ mice (Figure 6) were used **in study 3**.

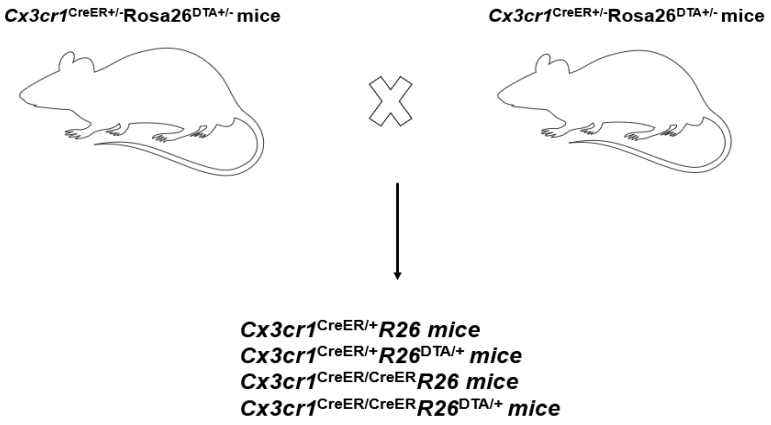

Figure 6*: An overview of breeding strategies used in study 3.*

Cx3cr1$^{GFP/GFP}$ mice breeding pairs were purchased from the Jackson Laboratory and *C57BL/6NTac* mice were received from Taconic.

Generation of bone marrow chimeras

Bone marrow chimeric mice were generated by irradiating mice with 9.5 Gray with head protection and then reconstituting on the same day with 2-5 x 10^6 bone marrow cells via tail vein injection (**used in studies 1 and 3**). The mice were used in experiments at 6-8 weeks post reconstitution.

Tamoxifen treatment

Tamoxifen was suspended in corn oil at 75°C for at least 60 min. Ault mice were administered 5mg (200µl) tamoxifen subcutaneously on three consecutive days, and then kept for varied durations to allow different degrees of microglial repopulation [52, 66] in **studies 1, 2 and 4**. **In study 3**, 125 mg/kg or 62.5 mg/kg tamoxifen was injected i.p for three (postnatal days 18, 19 and 20) or ten consecutive days (postnatal days 18–27).

PLX3397 treatment

PLX3397 (Pexidartinib, HY-16749, MedChemExpress) formulated into the standard diet (75 mg/kg and 290 mg/kg) was administered for different durations. Control mice were fed with a normal diet (**used in study 4**).

3 AIMS

Study 1: To develop an effective microglial depletion animal model followed by long-term microglial repopulation with a combination of resident microglia and engrafted microglia-like cells

Study 2: To investigate the effects of engrafted microglia-like cells after microglial depletion on autoimmune neuroinflammation

Study 3: To study the CX3CL1-CX3CR1 signaling pathway regulating microglial repopulation after conditional genetic microglial depletion

Study 4: To explore potential peripheral effects following both conditional genetic and pharmacological microglial depletion

4 RESULTS AND DISCUSSION

The main purpose of this PhD project was to assess the role of microglia in homeostasis and disease. This was accomplished by developing a novel conditional genetic model (*Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice) for depleting microglia with a higher efficiency, and by using the CSF1R inhibitor PLX3397. When the microglial niche was made available following experimental depletion, we explored if peripheral engrafted microglia-like cells could also contribute to the microglial pool during homeostasis. We then studied if replacing microglia during disease conditions could serve as a potential therapeutic strategy for MOG-EAE, and its sex difference, signaling pathways as well as potential adverse effects. The four manuscripts included in the thesis represent the combined efforts to test the hypothesis that modulation of microglial functional is both a critical underlying factor in pathogenesis, and also represents a viable therapeutic modality.

4.1 STUDY 1

Background: Microglia are the main resident immune cells in the CNS and are derived from the yolk sac during early development. Microglia can maintain their numbers during homeostasis by local self-renewal, without contribution from the periphery. The microglial niche theory has been proposed recently, suggesting that each microglia occupy its own physical space and that these niches can become available during experimental microglial depletion [163].

Hypothesis: Competitive microglial repopulation starts once the microglial niche is made available.

Methods: In this study *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$mice were systemically administered tamoxifen to deplete microglia due to intracellular DTA expression upon Cre recombination in CX3CR1-expressing cells. Microglial depletion and repopulation were mainly measured using flow cytometry and immunohistochemistry.

Results: We observed that approximately 95% of CD11b^{+}CD45^{+}Ly6C$^{–}$Ly6G$^{–}$ microglia could be depleted in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice 7 days after tamoxifen administration by flow cytometry. The findings were further supported by microglia-specific P2ry12 and CX3CR1-YFP immunostaining. Twenty-eight days later new microglia had partly repopulated the CNS of *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice, with two distinct cell subsets within the CX3CR1^{+} gate differentially expressing F4/80. Most resident microglia in *Cx3cr1*^{CreER} control mice did not express a high level of F4/80. F4/80^{hi} peripherally-derived macrophages were P2ry12$^{–}$. We then demonstrated that the repopulated microglia had dual origins, including yolk-sac derived microglia which proliferated locally and infiltrating Ly6C^{hi} monocytes which transformed into microglia-like cells within the CNS microenvironment. Embryonic yolk-sac derived microglia and microglia-like cells exhibited distinct functions *in vitro*.

Reflections: This study gave me a great opportunity to get involved in the hot research field of microglial depletion and to learn flow cytometric analyses. I also learned the breeding strategy of *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice which were used in subsequent projects.

In paper I we developed a novel conditional genetic method to deplete microglia long-lastingly and to make the microglial niche available. We concluded that following this experimental microglial depletion method peripheral monocytes could access and then adapt to the CNS microenvironment, becoming monocyte-derived microglia-like cells. Full transformation to *bona fide* microglia was excluded through transcriptional and epigenetic analyses, and these cells remained functionally different to embryonic yolk-sac derived microglia.

The unique identity of microglia is critically dependent on both ontogeny and the CNS niche-specific microenvironment [10]. Previous studies have provided evidence that microglial numbers can be maintained through their proliferation within the CNS, with no input from the periphery during homeostasis [13]. Importantly, the novel microglial niche theory suggests that each microglial cell occupies its physical space within the CNS [163, 164]. Temporarily available niches of microglia are evident in some conditions, which is tightly regulated [165]. During experimental microglial depletion as a result of tamoxifen treatment in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice, peripherally-derived monocytes could acquire microglia-like gene expression profiles and adopt microglia-like DNA methylation signatures, so that these microglia-like cells exhibited a partially different gene signature compared to resident microglia. In support of our findings, others have reported that peripherally-derived microglia-like cells can engraft the brain independently of irradiation in *Cx3cr1*$^{CreER/+}$*Csf1r*$^{Flox/Flox}$ mice [97].

Available surface markers assessed by flow cytometry for differentiating resident microglia and microglia-like cells lack the required level of specificity. For example, the expression of CD45 can be altered during inflammation [166]. F4/80 staining is also not a commonly accepted marker to discern microglia from peripheral colonizers of the CNS, either. To address these concerns, in paper I we performed a kinetic assessment of microglial depletion, demonstrating that Ly6C^{hi} circulating monocytes have already entered the CNS two days after the start of tamoxifen treatment, before the establishment of the microglia-like cells. Furthermore, adoptive transfer of Ly6C^{hi} monocytes into the microglia-depleted mice could cause specific reconstitution of the F4/80^{hi} microglia-like cell pool. Collectively, peripherally-derived cells could significantly refill the microglial niche following microglial depletion with a high efficiency in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice.

In addition, microglia were isolated from the CNS tissues into a single-cell suspension using both mechanical and enzymatic dissociations in order to facilitate culture or analyses. However, these methods may alter microglial activation status and *ex vivo* microglia may lose their key signature genes and coordinated cell-to-cell communication. We also discerned that yolk-sac derived resident microglia and infiltrating microglia-like cells exhibited different functions, such as phagocytic capacity and cytokine production *in vitro*. One previous study has demonstrated that transplantation of bone marrow-derived microglia-like cells led to an

increased phagocytic uptake of amyloid-β in an Alzheimer's mouse model, subsequently reducing amyloid-β burden and improving cognitive function [123]. However, the complicated roles of repopulating engrafted microglia-like cells in other disease conditions are not completely understood.

4.2 STUDY 2

Background: MS is a chronic demyelinating disease, predominantly in women. A wave of new research has proven that microglia exhibit sex-dependent features and play a vital role in the pathophysiology of MS. Microglia can be efficiently removed following conditional genetic depletion, with repopulating cells deriving from both CNS local microglia and the engraftment of microglia-like cells.

Hypothesis: Microglial replacement followed by experimental depletion represents a potential means of resolving neuroinflammation in MS.

Methods: Microglia were depleted by injection of tamoxifen to *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice, and retained for one month until new microglia had populated in the CNS. The MOG-EAE model was then induced in these microglia-repopulated mice. Myeloid cell populations were primarily assessed using flow cytometry.

Results: We demonstrated that the engraftment of microglia-like cells following conditional genetic ablation exacerbated EAE in female mice, but not in male mice. Female *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice had a higher chronic disease severity than male mice, while no sex-dependent EAE disease severity was noted in *Cx3cr1*$^{CreER/+}$ control mice. Our results also indicated that increased MHCII expression and cytokine production contributed to the sex-dependent severity in female mice with the engraftment of microglia-like cells.

Reflections: This study gave me a chance to plan and conduct animal studies independently. I learned how to induce the MOG-EAE model and how to monitor the clinical symptoms. I became proficient at flow cytometrical analysis, including both extracellular and intracellular stainings. I also realized that gender should be considered as a biological variable in both research and clinical settings.

In paper II, we again used the *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mouse model developed from paper I and further demonstrated that engrafting repopulating microglia-like cells reduced their ability to limit neuroinflammation when compared to self-renewing *bona fide* microglia. The peripherally-derived microglia-like cells thus perpetuate EAE disease and inhibit disease recovery. Furthermore, these effects were sex-dependent, with female mice experiencing worse EAE clinical symptoms, which is an interesting finding considering the female preponderance of MS in humans.

We are beginning to appreciate that the role of microglia is sexually dimorphic in the condition of pain, as evidenced by microglia only being involved in mediating pain hypersensitivity in males but not in females [167, 168]. It has now been gradually established that the numbers

and phenotypes of microglia in the CNS generally differ between females and males [74, 75]. Specifically, obvious sex differences in microglial numbers have been reported in several brain regions including the cortex, amygdala, hippocampus and preoptic area [75]. Furthermore, sex differences of microglia have also been noted at both RNA and protein levels [75]. From a functional viewpoint, male and female microglia may have different functions, since female rat neonatal microglia had both less basal and stimulated microglial migration than did male microglia [169]. Microglia may also exhibit sex differences in phagocytic capacity during development [170].

In clinical settings, sex differences of microglia may also exist and contribute to disease pathogenesis. For example, *CSF1R*-related leukoencephalopathy, caused by the *CSF1R* gene mutations, is now recognized as a primary CNS microgliopathy with dysfunctional microglia playing a critical role [171-173]. *CSF1R*-related leukoencephalopathy in women develop clinical signs significantly earlier than do men [174].

The sex differences of EAE severity were not attributed to different mouse strains used in the study. We observed that both male and female *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$mice and C57BL/6 mice developed similar EAE courses in conditions without the involvement of microglial depletion and repopulation. We additionally demonstrated that the sex-dependent EAE severity of mice with infiltrating microglia-like cells may be partially due to increased MHCII expression and cytokine secretion in the female CNS. However, these findings could not completely explain the apparent differences in clinical symptoms, and further studies are needed to explore the mechanisms of microglial sex differences in neuroinflammatory conditions.

4.3 STUDY 3

Background: Microglial depletion using either pharmacological treatment or conditional genetic mouse models has broadened our knowledge. However, currently available genetic depletion approaches including *Cx3cr1*$^{CreER/+}$*Rosa26*^{DTR} mice, *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice and *Cx3cr1*$^{CreER/+}$*Csf1r*$^{Flox/Flox}$ mice do not completely deplete microglia in the CNS, with some microglia remaining unabated after depletion.

Hypothesis: CX3CR1^{-} microglia may exist in the mouse brain and this subpopulation of microglia have a competitive advantage over newly repopulated microglia following experimental depletion.

Results: We reported a unique Iba-1^{+}Tmem119^{+}EYFP^{-} microglial subset in the brain of *Cx3cr1*$^{CreER\text{-}EYFP/+}$ mice under normal conditions. Isolated CX3CR1^{+}EYFP^{-} microglia did not express *Eyfp* and *Cre* mRNA, suggesting that they may escape the Cre-mediated recombination in *Cx3cr1*$^{CreER\text{-}EYFP/+}$*Rosa*26^{DTA} mice. Our results demonstrated that this unique microglial subpopulation has a competitive advantage over newly repopulated resident microglia following microglial depletion.

Reflections: This study gave me a chance to set up a collaboration beyond our own lab. Teamwork in science is of great importance. This study also enabled me to master further experimental skills such as immunohistochemistry and microscopical analysis. I learned that we should not limit ourselves to only using surface markers for phenotyping in the field of immunology.

In paper III we described a unique Iba-1^{+}Tmem119^{+}EYFP^{-} subset with a microglial morphology in the brains of *Cx3cr1*$^{\text{CreER-EYFP/+}}$ mice under homeostatic conditions. In order to further confirm that these YFP^{-} microglia-like cells were not an anomaly of distinct mouse strains, we additionally discovered that Iba-1^{+}Tmem119^{+}GFP^{-} microglia-like cells were also evident in the brains of *Cx3cr1*$^{\text{GFP/+}}$ mice. This unique microglial subpopulation could competitively repopulate the CNS following microglial depletion, and we determined that this process was tightly regulated by the CX3CL1-CX3CR1 signaling.

CX3CR1 is commonly used to label microglia by inserting EYFP or EGFP tags. In paper III we discovered that EYFP^{-} or GFP^{-} microglia may escape or silence the genetic modification and are not be ablated following tamoxifen treatment in *Cx3cr1*$^{\text{CreER-EYFP/+}}$ mice or *Cx3cr1*$^{\text{GFP/+}}$ mice. Although we do not completely understand the biological consequences of this phenomenon, our conclusion is that researchers should be cautious in their interpretation when these transgenic mice are applied in disease conditions.

We also discerned that this unique microglial population can also repopulate the CNS, independently from self-proliferating CNS resident microglia and microglia-like cells, as a result of experimental microglial depletion in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice. This process was tightly regulated by the CX3CL1-CX3CR1 signal transduction pathways. The CX3CL1-CX3CR1 pathway is now considered as a critical regulator of cross-communication between neurons and microglia. One previous study indicated that the repopulation of microglia in the retina can be delayed in the condition of CX3CR1 deficiency, while it can be enhanced after the administration of exogenous CX3CL1 [165]. We further demonstrated that resident microglia in the brain fail to compete with the peripherally-derived microglia-like cells following depletion in *Cx3cr1*$^{-/-}$ mice, suggesting that the repopulation of CNS resident microglia rather than peripherally-derived microglia-like cell repopulation is critically dependent on the CX3CL1-CX3CR1 pathway. Our study provides potential clues as to how to modulate the process of microglial repopulation in therapeutic efforts in different disease conditions.

4.4 STUDY 4

Background: Current microglial depletion approaches are usually based on microglial markers such as CX3CR1 and CSF1R. A wave of new research has demonstrated that microglial depletion can exert neuroprotective effects in diverse disease models by reducing neuroinflammation. However, other immune cells may also express these markers and potential additional targets during microglial depletion have not been comprehensively studied.

Hypothesis: Microglial depletion using current available approaches exerts effects on peripheral immune cell populations.

Results: We demonstrated that microglia can be effectively ablated after administering tamoxifen to *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice, or the CSF1R inhibitor PLX3397 to C57BL/6 mice. Numbers of red pulp macrophages and Ly6C^{hi} monocytes in the spleen were significantly reduced during the microglial depletion period using both genetic and pharmacological depletion approaches. However, in the spleens of *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice the number of monocytes was significantly higher than at baseline level. Ly6C^{hi} monocytes in the circulation were also affected during microglial depletion using both genetic and pharmacological approaches.

Reflections: This study gave me a chance to further explore microglial depletion using two different methods, including a conditional genetic mice model and using CSF1R inhibition. I also learned that current available methods to deplete microglia may still have disadvantages and potential side-effects after depletion which need to be considered in the future studies.

In paper IV we recorded that microglial depletion using a conditional genetic mouse model and pharmacological approaches has significant effects on circulating monocytes and splenic macrophages. CX3CR1 is predominately expressed on microglia in the CNS. However, non-classical monocytes in the circulation may also express CX3CR1. We found that both classical and non-classical monocyte numbers in the circulation are reduced in *Cx3cr1*$^{CreER/+}$*Rosa26*$^{DTA/+}$ mice after tamoxifen treatment. Although tamoxifen treatment itself causes a low-level systemic inflammation with an increased level of circulating Ly6G^{+} neutrophils, non-classical Ly6C^{low} monocytes are more affected than classical Ly6C^{hi} monocytes under this condition. We also observed that the levels of splenic Ly6C^{hi} monocytes are higher during the microglial depletion period (7 days after tamoxifen treatment) than at the baseline level, while a decreased level of red pulp macrophages was noted in the spleen during this period.

Expression of CSF1R is mainly enriched on microglia in the CNS and is crucial for their survival of microglia. Use of a diet containing CSF1R inhibitors such as PLX3397 or PLX5622 can ablate most microglia in preclinical models. However, CSF1R may also be expressed on circulating monocytes and peripheral tissue macrophages. For example, a high dose of PLX3397 (400 mg/kg) can alter the blood cell phenotyping [110], with red blood cells, haemoglobin, platelets, dendritic cells and Ly6C^{-} monocytes being reduced following PLX3397 treatment [110]. Furthermore, one previous study provided evidence that the weight of the spleen was decreased after PLX3397 treatment in mice [175]. We now report that the levels of splenic red pulp macrophages are significantly decreased following PLX3397 treatment (290 mg/kg). These peripheral effects of microglial depletion could be attenuated using a lower dose of PLX3397 (75 mg/kg).

Collectively, our results suggested that CSF1R inhibitors are not CNS specific. The translation of microglia depletion into the clinic should be considered with caution, particularly in disease

conditions in which perturbations of peripheral myeloid cell populations might be functionally significant.

5 FUTURE PERSPECTIVES

We strive to apply potential novel treatment strategies from preclinical studies into clinical settings. We hope to develop more effective and safe microglia-specific therapeutics for neurological diseases.

It is still difficult to specifically target microglia, perivascular macrophages or choroid plexus macrophages due to the unavailability of unique targeting markers. Microglia may exhibit broader phenotypes in different disease conditions, depending on the microenvironment. There is mounting evidence that disease-associated microglia could be either beneficial [155] or detrimental [176] for disease. Better selective characterization of disease-associated microglia within a suitable time window using novel technologies will help us to decipher their complex functions in disease and then to develop potential microglia-targeted therapies.

Recent technical progress allows researchers to generate microglia-like cells in large numbers from either pluripotent stem cells [177] or from circulating monocytes [178]. These microglia-like cells can be engrafted into the mouse brain, then maintaining microglia-specific gene signatures [52]. Niche-specific microenvironmental cues such as IL-34, CSF1 and TGF-β in the CNS are indispensable for achieving this goal [66, 67, 179]. Transplantation of these microglia-like cells into the mouse brain could therefore be a potent therapeutic intervention.

Moving forward, studying patient-specific microglia in different pathological contexts using these approaches is urgently needed. It is also important to note that microglia-like cells are distinct from embryonic yolk-sac derived microglia in terms of immune metabolism, phagocytosis and cytokine production. Furthermore, understanding specific cellular and molecular pathways that regulate or imprint repopulating microglia-like cell identity after depleting dysregulated microglia across different brain regions is of great importance.

Apart from microglia in the CNS, conventional systemic administration of compounds such as the drugs targeting CSF1R (PLX3397 or PLX5622) can also influence circulating monocytes and other tissue resident macrophages. Interpreting results and potential clinical translation using these methods should thus be considered with caution. Nanoparticles, ranging from 10 to 1000 nm, have been increasingly used in the field of drug development and they offer the ability to deliver potential drugs or molecules to specific brain regions in order to achieve therapeutic actions. Directly delivering drugs into the CNS holds promise by avoiding potential phagocytosis and other side-effects in the periphery [180].

Since a variety of brain diseases exhibit marked sex differences, and that sex differences in microglia have been recently well established, these differences cannot be ignored in future studies. Microglial research should thus include both sexes in order to understand complex intrinsic factors including immune-related genes, epigenetic modifiers and environmental influences. Better understanding of the cellular and molecular differences in microglia between sexes is thus crucial to potential development of sex-targeted therapies for improving the quality of life for those suffering from neurological disorders.

6 ACKNOWLEDGEMENTS

At the time when I am going to finish my PhD journey at KI, there is so much to be grateful for. I want to thank my main supervisor **Xingmei** for accepting me as a PhD student at the very beginning. You taught me numerous laboratory skills such as cell culture, running FACS and inducing MOG-EAE. You not only provided me professional guidance in science, but also cared for my daily life, making my time in Sweden memorable. I could not have finished my PhD study without your help. **Bob**, I can clearly remember the scene when I sat nervously in the front of my laptop in the dormitory and had the first remote Skype meeting with you. Your constant encouragement kept me moving forward and made me better myself. You have created so many opportunities for us to collaborate with other researchers. You are an excellent leader for doctoral education. I am grateful to my co-supervisor **Harald** for bringing me into the hot research field of microglial depletion. Thanks a lot for showing me how to breed the mice and how to do the genotyping. Thanks very much for helping me to modify the gating strategy, providing insightful suggestions and revising my manuscripts.

I also want to thank all *Applied Immunology and Immunotherapy* group members: **Roham,** thanks for sharing your experiences. I still remember that you said that the Post-surgery Systemic Inflammation and Neuro-immune Interactions (POSINI) project would be more interesting than the EAE project at the beginning. It seems that you were correct. **Sohel,** it was always great when I talked to you at dissertation parties. Good luck for your clinical journey. **Melanie**, thanks for your input and helping me to prepare the single cell suspensions. Working with you made everything very efficient. **Keying**, I highly rate your contribution. You are always my third hand and working together with you in the animal house made the experiments much easier and more flexible. **Sebastian**, thanks for your input on my projects in the lab meetings. I can always remember you when I read something about ALS and perivascular space in the literature. **Heela,** thanks a lot for discussing experimental procedures and sharing your experiences about cochlea, macrophages and microglia. I am also gratitude to all previous exchanged students **Yueshan**, **Marten** and **William**.

I am gratitude to all my collaborators in microglial research during my PhD study. **Joanna**, **Jingdian** and **Sarah**, it is my great pleasure to be involved in your mitochondrial scientific journey. In both the C5024T mice project and the human Leber hereditary optic neuropathy project we have made incredible progress together with all collaborators, medical doctors, nurses, patients and healthy donors. I am grateful to **Federico** and **Sigrún** for your interest in our previous work and then extending your research into microglial depletion in conditions of bacterial meningitis. **Ida**, it is my great pleasure to work with you, providing bone marrow derived-macrophages, THP-1 cells and human $CD14^+$ monocytes for the rabbit and swine experiments. **Ramil** and **Sreeni**, I will not forget the intensive time when we worked together in the lab. The role of microglia in spinal cord injury is of importance. **Lars**, **Malin**, **Marta** and **Lena**, it was really an amazing experience to work with you on the POSINI project (both mice and human). I have learned a lot from you about the clinical studies. **Virginija**, thanks for showing me around the Neurology Clinic. It will be an important step to explore potential

microglia-specific treatments for patients with *CSF1R*-related leukoencephalopathy (CNS primary microgliopathies). **Klas** and **Kai**, thanks for the great collaborations in understanding the molecular mechanisms of microglial depletion and repopulation. Thanks to people in the 9th floor of Bioclinicum **Elena**, **Wei**, **Tachi**, **Cuicui**, **Adamantia**, **Cecilia**, **Shunichiro**, **Takeo**, **Dan** and **Sailan**, for your kind help and support during my PhD study.

Thanks, **Anna** for being my mentor. You are a knowledgeable person in the fields of MS and immunology. Thanks for sending me updated literature about myeloid cells. Thanks for inviting me to review manuscripts for *Scandinavian Journal of Immunology*. I am grateful for your kind advice and support during my PhD study.

Special thanks to the Neuro-Rheuma Badminton Team. My playing skills have improved a lot during my PhD study, of course. Thanks very much for organizing these, **Leonid**. Thanks for accompanying every Tuesday evening, **Pavel**, **Manoj**, **Henna**, **Ravi** and **Charlotte**.

Thanks, **Helena** and **Mia** for organizing the National Clinical Research School in Chronic Inflammatory Disease (NCRSCID). I have learned a lot from the courses, workshops, international conferences and all members of the NCRSCID. Establishing the network with researchers in immunological fields is a precious for my career.

I would like to thank all group members in the Neuroimmunology Unit. Special thanks to **all PIs** for creating a wonderful and unique working environment to work with. **Mohsen**, thanks for your great efforts to make the lab smoothly ordered. I can always get quick responses from you. **Gunn**, thanks for accepting my late orders and ensuring enough experimental staff for use. **Majid**, you are the best and most hard-working postdoc in our PhD office. Discussing science with you is always interesting and I enjoyed the Iranian food very much. **André**, thanks a lot for your efforts for dealing with different mouse strains in the animal house. You are the only person who can help me to resolve the FACS problems at 10:00 p.m. **Rux**, thanks for your contributions to the cell rooms. I know it is a not easy task. **Lara,** you always asked questions and proposed interesting ideas in the lab meetings, which inspired me a lot. **Eliane**, thanks for scientific discussions about EAE results and sending me many related documents. **Marie**, I admired your passion for doing research and dancing. **Karl**, thanks for organizing the journal club and sending updated literature to the group. **Rasmus**, thanks for the flexible FACS running time before and after your bookings. **Mathias**, thanks for sharing your clinical experiences with me. You will be an excellent clinician sooner or later. Thanks all previous and current group members: **Faiez**, **Nada**, **Hannes**, **Venus**, **Maria**, **Galina**, **Muhammad**, **Ewoud**, **Yanan**, **Pernilla**, **Susanne**, **Ali**, **Xia**, **Jesse**, **Sunjay, Tojo, Susanna, Benjamin, Chiara**. We are a wonderful team and I have learned a lot from all of you.

I want to express my gratitude to the staff at AKM L1 and L5 animal houses for animal breeding and daily caretaking. I appreciate all my Chinese friends here in Sweden. I cannot name all of you here due to the limited space in this thesis. If so, it would be the longest part in my thesis. I appreciate my family for their encouragement and support over the years. I value the research

funding support from Neuroförbundet. I am grateful for the KI-China Scholarship Council (CSC, 201600160072) program, supporting me to pursue the doctoral education at KI.

7 REFERENCES

1. Sierra A, Paolicelli RC, Kettenmann H: **Cien Anos de Microglia: Milestones in a Century of Microglial Research**. *Trends Neurosci* 2019, **42**(11):778-792.

2. Prinz M, Jung S, Priller J: **Microglia Biology: One Century of Evolving Concepts**. *Cell* 2019, **179**(2):292-311.

3. Drummond RA, Swamydas M, Oikonomou V, Zhai B, Dambuza IM, Schaefer BC, Bohrer AC, Mayer-Barber KD, Lira SA, Iwakura Y *et al*: **CARD9(+) microglia promote antifungal immunity via IL-1beta- and CXCL1-mediated neutrophil recruitment**. *Nat Immunol* 2019, **20**(5):559-570.

4. Mancuso R, Van Den Daele J, Fattorelli N, Wolfs L, Balusu S, Burton O, Liston A, Sierksma A, Fourne Y, Poovathingal S *et al*: **Stem-cell-derived human microglia transplanted in mouse brain to study human disease**. *Nat Neurosci* 2019, **22**(12):2111-2116.

5. Sankowski R, Bottcher C, Masuda T, Geirsdottir L, Sagar, Sindram E, Seredenina T, Muhs A, Scheiwe C, Shah MJ *et al*: **Mapping microglia states in the human brain through the integration of high-dimensional techniques**. *Nat Neurosci* 2019, **22**(12):2098-2110.

6. Ruan C, Sun L, Kroshilina A, Beckers L, De Jager P, Bradshaw EM, Hasson SA, Yang G, Elyaman W: **A novel Tmem119-tdTomato reporter mouse model for studying microglia in the central nervous system**. *Brain Behav Immun* 2020, **83**:180-191.

7. Kim JS, Kolesnikov M, Peled-Hajaj S, Scheyltjens I, Xia Y, Trzebanski S, Haimon Z, Shemer A, Lubart A, Van Hove H *et al*: **A Binary Cre Transgenic Approach Dissects Microglia and CNS Border-Associated Macrophages**. *Immunity* 2021, **54**(1):176-190 e177.

8. Eyo UB, Wu LJ: **Microglia: Lifelong patrolling immune cells of the brain**. *Prog Neurobiol* 2019, **179**:101614.

9. Brioschi S, Zhou Y, Colonna M: **Brain Parenchymal and Extraparenchymal Macrophages in Development, Homeostasis, and Disease**. *J Immunol* 2020, **204**(2):294-305.

10. Bennett FC, Bennett ML, Yaqoob F, Mulinyawe SB, Grant GA, Hayden Gephart M, Plowey ED, Barres BA: **A Combination of Ontogeny and CNS Environment Establishes Microglial Identity**. *Neuron* 2018, **98**(6):1170-1183 e1178.

11. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER *et al*: **Fate mapping analysis reveals that adult microglia derive from primitive macrophages**. *Science* 2010, **330**(6005):841-845.

12. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu B, Jacobsen SE, Pollard JW *et al*: **A lineage of myeloid cells independent of Myb and hematopoietic stem cells**. *Science* 2012, **336**(6077):86-90.

13. Askew K, Li K, Olmos-Alonso A, Garcia-Moreno F, Liang Y, Richardson P, Tipton T, Chapman MA, Riecken K, Beccari S *et al*: **Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain**. *Cell Rep* 2017, **18**(2):391-405.

14. Tay TL, Mai D, Dautzenberg J, Fernandez-Klett F, Lin G, Sagar, Datta M, Drougard A, Stempfl T, Ardura-Fabregat A *et al*: **A new fate mapping system reveals context-dependent random or clonal expansion of microglia**. *Nat Neurosci* 2017, **20**(6):793-803.

15. Reu P, Khosravi A, Bernard S, Mold JE, Salehpour M, Alkass K, Perl S, Tisdale J, Possnert G, Druid H *et al*: **The Lifespan and Turnover of Microglia in the Human Brain**. *Cell Rep* 2017, **20**(4):779-784.

16. Nimmerjahn A, Kirchhoff F, Helmchen F: **Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo**. *Science* 2005, **308**(5726):1314-1318.

17. Cserep C, Posfai B, Lenart N, Fekete R, Laszlo ZI, Lele Z, Orsolits B, Molnar G, Heindl S, Schwarcz AD *et al*: **Microglia monitor and protect neuronal function through specialized somatic purinergic junctions**. *Science* 2020, **367**(6477):528-537.

18. Yun SP, Kam TI, Panicker N, Kim S, Oh Y, Park JS, Kwon SH, Park YJ, Karuppagounder SS, Park H *et al*: **Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease**. *Nat Med* 2018, **24**(7):931-938.

19. Punal VM, Paisley CE, Brecha FS, Lee MA, Perelli RM, Wang J, O'Koren EG, Ackley CR, Saban DR, Reese BE *et al*: **Large-scale death of retinal astrocytes during normal development is non-apoptotic and implemented by microglia**. *PLoS Biol* 2019, **17**(10):e3000492.

20. Hagemeyer N, Hanft KM, Akriditou MA, Unger N, Park ES, Stanley ER, Staszewski O, Dimou L, Prinz M: **Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood**. *Acta Neuropathol* 2017, **134**(3):441-458.

21. Vay SU, Flitsch LJ, Rabenstein M, Rogall R, Blaschke S, Kleinhaus J, Reinert N, Bach A, Fink GR, Schroeter M *et al*: **The plasticity of primary microglia and their multifaceted effects on endogenous neural stem cells in vitro and in vivo**. *J Neuroinflammation* 2018, **15**(1):226.

22. Geirsdottir L, David E, Keren-Shaul H, Weiner A, Bohlen SC, Neuber J, Balic A, Giladi A, Sheban F, Dutertre CA *et al*: **Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program**. *Cell* 2019, **179**(7):1609-1622 e1616.

23. Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR, 3rd, Lafaille JJ, Hempstead BL, Littman DR, Gan WB: **Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor**. *Cell* 2013, **155**(7):1596-1609.

24. Miller EB, Zhang P, Ching K, Pugh EN, Jr., Burns ME: **In vivo imaging reveals transient microglia recruitment and functional recovery of photoreceptor signaling after injury**. *Proc Natl Acad Sci U S A* 2019, **116**(33):16603-16612.

25. Wang C, Yue H, Hu Z, Shen Y, Ma J, Li J, Wang XD, Wang L, Sun B, Shi P *et al*: **Microglia mediate forgetting via complement-dependent synaptic elimination**. *Science* 2020, **367**(6478):688-694.

26. Thorsdottir S, Henriques-Normark B, Iovino F: **The Role of Microglia in Bacterial Meningitis: Inflammatory Response, Experimental Models and New Neuroprotective Therapeutic Strategies**. *Front Microbiol* 2019, **10**:576.

27. Sosa RA, Murphey C, Ji N, Cardona AE, Forsthuber TG: **The kinetics of myelin antigen uptake by myeloid cells in the central nervous system during experimental autoimmune encephalomyelitis**. *J Immunol* 2013, **191**(12):5848-5857.

28. Wolf Y, Shemer A, Levy-Efrati L, Gross M, Kim JS, Engel A, David E, Chappell-Maor L, Grozovski J, Rotkopf R *et al*: **Microglial MHC class II is dispensable for experimental autoimmune encephalomyelitis and cuprizone-induced demyelination**. *Eur J Immunol* 2018, **48**(8):1308-1318.

29. De Luca SN, Miller AA, Sominsky L, Spencer SJ: **Microglial regulation of satiety and cognition**. *J Neuroendocrinol* 2020, **32**(3):e12838.

30. Younger D, Murugan M, Rama Rao KV, Wu LJ, Chandra N: **Microglia Receptors in Animal Models of Traumatic Brain Injury**. *Mol Neurobiol* 2019, **56**(7):5202-5228.

31. Szepesi Z, Manouchehrian O, Bachiller S, Deierborg T: **Bidirectional Microglia-Neuron Communication in Health and Disease**. *Front Cell Neurosci* 2018, **12**:323.

32. Bray N: **Awake microglia are less alert**. *Nat Rev Neurosci* 2020, **21**(1):2-3.

33. Liu YU, Ying Y, Li Y, Eyo UB, Chen T, Zheng J, Umpierre AD, Zhu J, Bosco DB, Dong H *et al*: **Neuronal network activity controls microglial process surveillance in awake mice via norepinephrine signaling**. *Nat Neurosci* 2019, **22**(11):1771-1781.

34. Stowell RD, Sipe GO, Dawes RP, Batchelor HN, Lordy KA, Whitelaw BS, Stoessel MB, Bidlack JM, Brown E, Sur M *et al*: **Noradrenergic signaling in the wakeful state inhibits microglial surveillance and synaptic plasticity in the mouse visual cortex**. *Nat Neurosci* 2019, **22**(11):1782-1792.

35. Salter MW, Stevens B: **Microglia emerge as central players in brain disease**. *Nat Med* 2017, **23**(9):1018-1027.

36. Zheng ZV, Lyu H, Lam SYE, Lam PK, Poon WS, Wong GKC: **The Dynamics of Microglial Polarization Reveal the Resident Neuroinflammatory Responses After Subarachnoid Hemorrhage**. *Transl Stroke Res* 2019.

37. Piers TM, Cosker K, Mallach A, Johnson GT, Guerreiro R, Hardy J, Pocock JM: **A locked immunometabolic switch underlies TREM2 R47H loss of function in human iPSC-derived microglia**. *FASEB J* 2020, **34**(2):2436-2450.

38. Orihuela R, McPherson CA, Harry GJ: **Microglial M1/M2 polarization and metabolic states**. *Br J Pharmacol* 2016, **173**(4):649-665.

39. Lynch MA: **Can the emerging field of immunometabolism provide insights into neuroinflammation?** *Prog Neurobiol* 2020, **184**:101719.

40. Wendeln AC, Degenhardt K, Kaurani L, Gertig M, Ulas T, Jain G, Wagner J, Hasler LM, Wild K, Skodras A *et al*: **Innate immune memory in the brain shapes neurological disease hallmarks**. *Nature* 2018, **556**(7701):332-338.

41. Chen Z, Zhong D, Li G: **The role of microglia in viral encephalitis: a review**. *J Neuroinflammation* 2019, **16**(1):76.

42. Crapser JD, Ochaba J, Soni N, Reidling JC, Thompson LM, Green KN: **Microglial depletion prevents extracellular matrix changes and striatal volume reduction in a model of Huntington's disease**. *Brain* 2020, **143**(1):266-288.

43. Konno T, Yoshida K, Mizuno T, Kawarai T, Tada M, Nozaki H, Ikeda SI, Nishizawa M, Onodera O, Wszolek ZK *et al*: **Clinical and genetic characterization of adult-onset leukoencephalopathy with axonal spheroids and pigmented glia associated with CSF1R mutation**. *Eur J Neurol* 2017, **24**(1):37-45.

44. Kortvelyessy P, Krageloh-Mann I, Mawrin C, Heinze HJ, Bittner D, Wieland I, Zenker M, Nestor P: **Hereditary diffuse leukoencephalopathy with spheroids (HDLS) with a novel CSF1R mutation and spinal cord involvement**. *J Neurol Sci* 2015, **358**(1-2):515-517.

45. Chen T, Lennon VA, Liu YU, Bosco DB, Li Y, Yi MH, Zhu J, Wei S, Wu LJ: **Astrocyte-microglia interaction drives evolving neuromyelitis optica lesion**. *J Clin Invest* 2020.

46. Peng J, Liu Y, Umpierre AD, Xie M, Tian DS, Richardson JR, Wu LJ: **Microglial P2Y12 receptor regulates ventral hippocampal CA1 neuronal excitability and innate fear in mice**. *Mol Brain* 2019, **12**(1):71.

47. Priller J, Prinz M: **Targeting microglia in brain disorders**. *Science* 2019, **365**(6448):32-33.

48. Osman AM, Sun Y, Burns TC, He L, Kee N, Oliva-Vilarnau N, Alevyzaki A, Zhou K, Louhivuori L, Uhlen P *et al*: **Radiation Triggers a Dynamic Sequence of Transient Microglial Alterations in Juvenile Brain**. *Cell Rep* 2020, **31**(9):107699.

49. Han J, Zhu K, Zhang XM, Harris RA: **Enforced microglial depletion and repopulation as a promising strategy for the treatment of neurological disorders**. *Glia* 2019, **67**(2):217-231.

50. Liu CY, Wang X, Liu C, Zhang HL: **Pharmacological Targeting of Microglial Activation: New Therapeutic Approach**. *Front Cell Neurosci* 2019, **13**:514.

51. Zhang LY, Pan J, Mamtilahun M, Zhu Y, Wang L, Venkatesh A, Shi R, Tu X, Jin K, Wang Y *et al*: **Microglia exacerbate white matter injury via complement C3/C3aR pathway after hypoperfusion**. *Theranostics* 2020, **10**(1):74-90.

52. Lund H, Pieber M, Parsa R, Han J, Grommisch D, Ewing E, Kular L, Needhamsen M, Espinosa A, Nilsson E *et al*: **Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells**. *Nat Commun* 2018, **9**(1):4845.

53. Bennett ML, Bennett FC: **The influence of environment and origin on brain resident macrophages and implications for therapy**. *Nat Neurosci* 2020, **23**(2):157-166.

54. Datta M, Staszewski O, Raschi E, Frosch M, Hagemeyer N, Tay TL, Blank T, Kreutzfeldt M, Merkler D, Ziegler-Waldkirch S *et al*: **Histone Deacetylases 1 and 2 Regulate Microglia Function during Development, Homeostasis, and Neurodegeneration in a Context-Dependent Manner**. *Immunity* 2018, **48**(3):514-529 e516.

55. Thion MS, Low D, Silvin A, Chen J, Grisel P, Schulte-Schrepping J, Blecher R, Ulas T, Squarzoni P, Hoeffel G *et al*: **Microbiome Influences Prenatal and Adult Microglia in a Sex-Specific Manner**. *Cell* 2018, **172**(3):500-516 e516.

56. Mee-Inta O, Zhao ZW, Kuo YM: **Physical Exercise Inhibits Inflammation and Microglial Activation**. *Cells* 2019, **8**(7).

57. Michels M, Abatti MR, Avila P, Vieira A, Borges H, Carvalho Junior C, Wendhausen D, Gasparotto J, Tiefensee Ribeiro C, Moreira JCF *et al*: **Characterization and modulation of microglial phenotypes in an animal model of severe sepsis**. *J Cell Mol Med* 2020, **24**(1):88-97.

58. Di Benedetto P, Ruscitti P, Vadasz Z, Toubi E, Giacomelli R: **Macrophages with regulatory functions, a possible new therapeutic perspective in autoimmune diseases**. *Autoimmun Rev* 2019, **18**(10):102369.

59. Ransohoff RM: **A polarizing question: do M1 and M2 microglia exist?** *Nat Neurosci* 2016, **19**(8):987-991.

60. Korbecki J, Siminska D, Kojder K, Grochans S, Gutowska I, Chlubek D, Baranowska-Bosiacka I: **Fractalkine/CX3CL1 in Neoplastic Processes**. *Int J Mol Sci* 2020, **21**(10).

61. Winter AN, Subbarayan MS, Grimmig B, Weesner JA, Moss L, Peters M, Weeber E, Nash K, Bickford PC: **Two forms of CX3CL1 display differential activity and rescue cognitive deficits in CX3CL1 knockout mice**. *J Neuroinflammation* 2020, **17**(1):157.

62. Lannes N, Garcia-Nicolas O, Demoulins T, Summerfield A, Filgueira L: **CX3CR1-CX3CL1-dependent cell-to-cell Japanese encephalitis virus transmission by human microglial cells**. *Sci Rep* 2019, **9**(1):4833.

63. Gyoneva S, Hosur R, Gosselin D, Zhang B, Ouyang Z, Cotleur AC, Peterson M, Allaire N, Challa R, Cullen P *et al*: **Cx3cr1-deficient microglia exhibit a premature aging transcriptome**. *Life Sci Alliance* 2019, **2**(6).

64. Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, Koeglsperger T, Dake B, Wu PM, Doykan CE *et al*: **Identification of a unique TGF-beta-dependent molecular and functional signature in microglia**. *Nat Neurosci* 2014, **17**(1):131-143.

65. Xu J, Wang Y, Jiang H, Sun M, Gao J, Xie A: **TGF-beta in Mice Ameliorates Experimental Autoimmune Encephalomyelitis in Regulating NK Cell Activity**. *Cell Transplant* 2019, **28**(9-10):1155-1160.

66. Lund H, Pieber M, Parsa R, Grommisch D, Ewing E, Kular L, Han J, Zhu K, Nijssen J, Hedlund E *et al*: **Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-beta signaling**. *Nat Immunol* 2018, **19**(5):1-7.

67. Zoller T, Schneider A, Kleimeyer C, Masuda T, Potru PS, Pfeifer D, Blank T, Prinz M, Spittau B: **Silencing of TGFbeta signalling in microglia results in impaired homeostasis**. *Nat Commun* 2018, **9**(1):4011.

68. Qin Y, Garrison BS, Ma W, Wang R, Jiang A, Li J, Mistry M, Bronson RT, Santoro D, Franco C *et al*: **A Milieu Molecule for TGF-beta Required for Microglia Function in the Nervous System**. *Cell* 2018, **174**(1):156-171 e116.

69. Lancaster TM: **Associations between rare microglia-linked Alzheimer's disease risk variants and subcortical brain volumes in young individuals**. *Alzheimers Dement (Amst)* 2019, **11**:368-373.

70. Ewers M, Franzmeier N, Suarez-Calvet M, Morenas-Rodriguez E, Caballero MAA, Kleinberger G, Piccio L, Cruchaga C, Deming Y, Dichgans M *et al*: **Increased soluble TREM2 in cerebrospinal fluid is associated with reduced cognitive and clinical decline in Alzheimer's disease**. *Sci Transl Med* 2019, **11**(507).

71. Konishi H, Kiyama H: **Microglial TREM2/DAP12 Signaling: A Double-Edged Sword in Neural Diseases**. *Front Cell Neurosci* 2018, **12**:206.

72. Krasemann S, Madore C, Cialic R, Baufeld C, Calcagno N, El Fatimy R, Beckers L, O'Loughlin E, Xu Y, Fanek Z *et al*: **The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases**. *Immunity* 2017, **47**(3):566-581 e569.

73. Sheppard PAS, Choleris E, Galea LAM: **Structural plasticity of the hippocampus in response to estrogens in female rodents**. *Mol Brain* 2019, **12**(1):22.

74. Villa A, Gelosa P, Castiglioni L, Cimino M, Rizzi N, Pepe G, Lolli F, Marcello E, Sironi L, Vegeto E *et al*: **Sex-Specific Features of Microglia from Adult Mice**. *Cell Rep* 2018, **23**(12):3501-3511.

75. Guneykaya D, Ivanov A, Hernandez DP, Haage V, Wojtas B, Meyer N, Maricos M, Jordan P, Buonfiglioli A, Gielniewski B *et al*: **Transcriptional and Translational Differences of Microglia from Male and Female Brains**. *Cell Rep* 2018, **24**(10):2773-2783 e2776.

76. Villa A, Della Torre S, Maggi A: **Sexual differentiation of microglia**. *Front Neuroendocrinol* 2019, **52**:156-164.

77. Xie D, He M, Hu X: **Microglia/macrophage diversities in central nervous system physiology and pathology**. *CNS Neurosci Ther* 2019, **25**(12):1287-1289.

78. Polyak A, Rosenfeld JA, Girirajan S: **An assessment of sex bias in neurodevelopmental disorders**. *Genome Med* 2015, **7**:94.

79. Hanamsagar R, Alter MD, Block CS, Sullivan H, Bolton JL, Bilbo SD: **Generation of a microglial developmental index in mice and in humans reveals a sex difference in maturation and immune reactivity**. *Glia* 2017, **65**(9):1504-1520.

80. Lenz KM, Nelson LH: **Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function**. *Front Immunol* 2018, **9**:698.

81. Villapol S, Faivre V, Joshi P, Moretti R, Besson VC, Charriaut-Marlangue C: **Early Sex Differences in the Immune-Inflammatory Responses to Neonatal Ischemic Stroke**. *Int J Mol Sci* 2019, **20**(15).

82. Li LZ, Huang YY, Yang ZH, Zhang SJ, Han ZP, Luo YM: **Potential microglia-based interventions for stroke**. *CNS Neurosci Ther* 2020, **26**(3):288-296.

83. Tsuji S, Di Martino E, Mukai T, Tsuji S, Murakami T, Harris RA, Blomgren K, Aden U: **Aggravated brain injury after neonatal hypoxic ischemia in microglia-depleted mice**. *J Neuroinflammation* 2020, **17**(1):111.

84. Stephen TL, Cacciottolo M, Balu D, Morgan TE, LaDu MJ, Finch CE, Pike CJ: **APOE genotype and sex affect microglial interactions with plaques in Alzheimer's disease mice**. *Acta Neuropathol Commun* 2019, **7**(1):82.

85. Han J, Zhu K, Zhou K, Hakim R, Sankavaram SR, Blomgren K, Lund H, Zhang XM, Harris RA: **Sex-Specific Effects of Microglia-Like Cell Engraftment during Experimental Autoimmune Encephalomyelitis**. *Int J Mol Sci* 2020, **21**(18).

86. Osborne BF, Turano A, Caulfield JI, Schwarz JM: **Sex- and region-specific differences in microglia phenotype and characterization of the peripheral**

immune response following early-life infection in neonatal male and female rats. *Neurosci Lett* 2019, **692**:1-9.

87. Liu LL, Li JM, Su WJ, Wang B, Jiang CL: **Sex differences in depressive-like behaviour may relate to imbalance of microglia activation in the hippocampus**. *Brain Behav Immun* 2019, **81**:188-197.

88. Doyle HH, Eidson LN, Sinkiewicz DM, Murphy AZ: **Sex Differences in Microglia Activity within the Periaqueductal Gray of the Rat: A Potential Mechanism Driving the Dimorphic Effects of Morphine**. *J Neurosci* 2017, **37**(12):3202-3214.

89. Dodiya HB, Kuntz T, Shaik SM, Baufeld C, Leibowitz J, Zhang X, Gottel N, Zhang X, Butovsky O, Gilbert JA *et al*: **Sex-specific effects of microbiome perturbations on cerebral Abeta amyloidosis and microglia phenotypes**. *J Exp Med* 2019, **216**(7):1542-1560.

90. Kaiser T, Feng G: **Tmem119-EGFP and Tmem119-CreERT2 Transgenic Mice for Labeling and Manipulating Microglia**. *eNeuro* 2019, **6**(4).

91. Betlazar C, Middleton RJ, Banati R, Liu GJ: **The Translocator Protein (TSPO) in Mitochondrial Bioenergetics and Immune Processes**. *Cells* 2020, **9**(2).

92. Hagens MHJ, Golla SSV, Janssen B, Vugts DJ, Beaino W, Windhorst AD, O'Brien-Brown J, Kassiou M, Schuit RC, Schwarte LA *et al*: **The P2X7 receptor tracer [(11)C]SMW139 as an in vivo marker of neuroinflammation in multiple sclerosis: a first-in man study**. *Eur J Nucl Med Mol Imaging* 2020, **47**(2):379-389.

93. Cao F, Hu LQ, Yao SR, Hu Y, Wang DG, Fan YG, Pan GX, Tao SS, Zhang Q, Pan HF *et al*: **P2X7 receptor: A potential therapeutic target for autoimmune diseases**. *Autoimmun Rev* 2019, **18**(8):767-777.

94. Horti AG, Naik R, Foss CA, Minn I, Misheneva V, Du Y, Wang Y, Mathews WB, Wu Y, Hall A *et al*: **PET imaging of microglia by targeting macrophage colony-stimulating factor 1 receptor (CSF1R)**. *Proc Natl Acad Sci U S A* 2019, **116**(5):1686-1691.

95. Hamatani M, Yamashita H, Ochi H, Ashida S, Hashi Y, Okada Y, Fujii C, Kawamura K, Kitazawa R, Nakagawa M *et al*: **Altered features of monocytes in adult onset leukoencephalopathy with axonal spheroids and pigmented glia: A clue to the pathomechanism of microglial dyshomeostasis**. *Neurobiol Dis* 2020, **140**:104867.

96. Ozanska A, Szymczak D, Rybka J: **Pattern of human monocyte subpopulations in health and disease**. *Scand J Immunol* 2020, **92**(1):e12883.

97. Cronk JC, Filiano AJ, Louveau A, Marin I, Marsh R, Ji E, Goldman DH, Smirnov I, Geraci N, Acton S *et al*: **Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia**. *J Exp Med* 2018, **215**(6):1627-1647.

98. Greenhalgh AD, David S, Bennett FC: **Immune cell regulation of glia during CNS injury and disease**. *Nat Rev Neurosci* 2020, **21**(3):139-152.

99. Zhang W, Zhao J, Wang R, Jiang M, Ye Q, Smith AD, Chen J, Shi Y: **Macrophages reprogram after ischemic stroke and promote efferocytosis and inflammation resolution in the mouse brain**. *CNS Neurosci Ther* 2019, **25**(12):1329-1342.

100. Wen M, Cai G, Ye J, Liu X, Ding H, Zeng H: **Single-cell transcriptomics reveals the alteration of peripheral blood mononuclear cells driven by sepsis**. *Ann Transl Med* 2020, **8**(4):125.

101. Jackson CM, Choi J, Routkevitch D, Pant A, Saleh L, Ye X, Caplan JM, Huang J, McDougall CG, Pardoll DM *et al*: **PD-1+ Monocytes Mediate Cerebral Vasospasm Following Subarachnoid Hemorrhage**. *Neurosurgery* 2020.

102. Han J, Fan Y, Zhou K, Zhu K, Blomgren K, Lund H, Zhang XM, Harris RA: **Underestimated Peripheral Effects Following Pharmacological and Conditional Genetic Microglial Depletion**. *Int J Mol Sci* 2020, **21**(22).

103. Han J, Harris RA, Zhang XM: **An updated assessment of microglia depletion: current concepts and future directions**. *Mol Brain* 2017, **10**(1):25.

104. Han J, Sarlus H, Wszolek ZK, Karrenbauer VD, Harris RA: **Microglial replacement therapy: a potential therapeutic strategy for incurable CSF1R-related leukoencephalopathy**. *Acta Neuropathol Commun* 2020, **8**(1):217.

105. Green KN, Crapser JD, Hohsfield LA: **To Kill a Microglia: A Case for CSF1R Inhibitors**. *Trends Immunol* 2020, **41**(9):771-784.

106. Du X, Xu Y, Chen S, Fang M: **Inhibited CSF1R Alleviates Ischemia Injury via Inhibition of Microglia M1 Polarization and NLRP3 Pathway**. *Neural Plast* 2020, **2020**:8825954.

107. Kerkhofs D, van Hagen BT, Milanova IV, Schell KJ, van Essen H, Wijnands E, Goossens P, Blankesteijn WM, Unger T, Prickaerts J *et al*: **Pharmacological depletion of microglia and perivascular macrophages prevents Vascular Cognitive Impairment in Ang II-induced hypertension**. *Theranostics* 2020, **10**(21):9512-9527.

108. Son Y, Jeong YJ, Shin NR, Oh SJ, Nam KR, Choi HD, Choi JY, Lee HJ: **Inhibition of Colony-Stimulating Factor 1 Receptor by PLX3397 Prevents Amyloid Beta Pathology and Rescues Dopaminergic Signaling in Aging 5xFAD Mice**. *Int J Mol Sci* 2020, **21**(15).

109. Kana V, Desland FA, Casanova-Acebes M, Ayata P, Badimon A, Nabel E, Yamamuro K, Sneeboer M, Tan IL, Flanigan ME *et al*: **CSF-1 controls cerebellar microglia and is required for motor function and social interaction**. *J Exp Med* 2019, **216**(10):2265-2281.

110. Shi Y, Manis M, Long J, Wang K, Sullivan PM, Remolina Serrano J, Hoyle R, Holtzman DM: **Microglia drive APOE-dependent neurodegeneration in a tauopathy mouse model**. *J Exp Med* 2019, **216**(11):2546-2561.

111. Ali S, Mansour AG, Huang W, Queen NJ, Mo X, Anderson JM, Hassan Ii QN, Patel RS, Wilkins RK, Caligiuri MA *et al*: **CSF1R inhibitor PLX5622 and environmental enrichment additively improve metabolic outcomes in middle-aged female mice**. *Aging (Albany NY)* 2020, **12**(3):2101-2122.

112. Beckmann N, Giorgetti E, Neuhaus A, Zurbruegg S, Accart N, Smith P, Perdoux J, Perrot L, Nash M, Desrayaud S *et al*: **Brain region-specific enhancement of remyelination and prevention of demyelination by the CSF1R kinase inhibitor BLZ945**. *Acta Neuropathol Commun* 2018, **6**(1):9.

113. Gerber YN, Saint-Martin GP, Bringuier CM, Bartolami S, Goze-Bac C, Noristani HN, Perrin FE: **CSF1R Inhibition Reduces Microglia Proliferation, Promotes**

Tissue Preservation and Improves Motor Recovery After Spinal Cord Injury. *Front Cell Neurosci* 2018, **12**:368.

114. Hupp S, Iliev AI: **CSF-1 receptor inhibition as a highly effective tool for depletion of microglia in mixed glial cultures**. *J Neurosci Methods* 2019, **332**:108537.

115. Tahmasebi F, Pasbakhsh P, Mortezaee K, Madadi S, Barati S, Kashani IR: **Effect of the CSF1R inhibitor PLX3397 on remyelination of corpus callosum in a cuprizone-induced demyelination mouse model**. *J Cell Biochem* 2019, **120**(6):10576-10586.

116. Nelson LH, Lenz KM: **Microglia depletion in early life programs persistent changes in social, mood-related, and locomotor behavior in male and female rats**. *Behav Brain Res* 2017, **316**:279-293.

117. Elmore MRP, Hohsfield LA, Kramar EA, Soreq L, Lee RJ, Pham ST, Najafi AR, Spangenberg EE, Wood MA, West BL *et al*: **Replacement of microglia in the aged brain reverses cognitive, synaptic, and neuronal deficits in mice**. *Aging Cell* 2018, **17**(6):e12832.

118. Varga DP, Menyhart A, Posfai B, Csaszar E, Lenart N, Cserep C, Orsolits B, Martinecz B, Szlepak T, Bari F *et al*: **Microglia alter the threshold of spreading depolarization and related potassium uptake in the mouse brain**. *J Cereb Blood Flow Metab* 2020:271678X19900097.

119. Adeluyi A, Guerin L, Fisher ML, Galloway A, Cole RD, Chan SSL, Wyatt MD, Davis SW, Freeman LR, Ortinski PI *et al*: **Microglia morphology and proinflammatory signaling in the nucleus accumbens during nicotine withdrawal**. *Sci Adv* 2019, **5**(10):eaax7031.

120. Ma D, Zhao Y, Huang L, Xiao Z, Chen B, Shi Y, Shen H, Dai J: **A novel hydrogel-based treatment for complete transection spinal cord injury repair is driven by microglia/macrophages repopulation**. *Biomaterials* 2020, **237**:119830.

121. Huang Y, Xu Z, Xiong S, Sun F, Qin G, Hu G, Wang J, Zhao L, Liang YX, Wu T *et al*: **Repopulated microglia are solely derived from the proliferation of residual microglia after acute depletion**. *Nat Neurosci* 2018, **21**(4):530-540.

122. Rice RA, Pham J, Lee RJ, Najafi AR, West BL, Green KN: **Microglial repopulation resolves inflammation and promotes brain recovery after injury**. *Glia* 2017, **65**(6):931-944.

123. Kawanishi S, Takata K, Itezono S, Nagayama H, Konoya S, Chisaki Y, Toda Y, Nakata S, Yano Y, Kitamura Y *et al*: **Bone-Marrow-Derived Microglia-Like Cells Ameliorate Brain Amyloid Pathology and Cognitive Impairment in a Mouse Model of Alzheimer's Disease**. *J Alzheimers Dis* 2018, **64**(2):563-585.

124. Gubert F, Bonacossa-Pereira I, Decotelli AB, Furtado M, Vasconcelos-Dos-Santos A, Mendez-Otero R, Santiago MF: **Bone-marrow mononuclear cell therapy in a mouse model of amyotrophic lateral sclerosis: Functional outcomes from different administration routes**. *Brain Res* 2019, **1712**:73-81.

125. Olsson T, Barcellos LF, Alfredsson L: **Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis**. *Nat Rev Neurol* 2017, **13**(1):25-36.

126. Zhou Y, Zhang Y, Han J, Yang M, Zhu J, Jin T: **Transitional B cells involved in autoimmunity and their impact on neuroimmunological diseases**. *J Transl Med* 2020, **18**(1):131.

127. Cheng Y, Sun L, Xie Z, Fan X, Cao Q, Han J, Zhu J, Jin T: **Diversity of immune cell types in multiple sclerosis and its animal model: Pathological and therapeutic implications**. *J Neurosci Res* 2017, **95**(10):1973-1983.

128. Melief J, Orre M, Bossers K, van Eden CG, Schuurman KG, Mason MRJ, Verhaagen J, Hamann J, Huitinga I: **Transcriptome analysis of normal-appearing white matter reveals cortisol- and disease-associated gene expression profiles in multiple sclerosis**. *Acta Neuropathol Commun* 2019, **7**(1):60.

129. Derfuss T, Mehling M, Papadopoulou A, Bar-Or A, Cohen JA, Kappos L: **Advances in oral immunomodulating therapies in relapsing multiple sclerosis**. *Lancet Neurol* 2020, **19**(4):336-347.

130. Vaughn CB, Jakimovski D, Kavak KS, Ramanathan M, Benedict RHB, Zivadinov R, Weinstock-Guttman B: **Epidemiology and treatment of multiple sclerosis in elderly populations**. *Nat Rev Neurol* 2019, **15**(6):329-342.

131. Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, de Seze J, Giovannoni G, Hartung HP, Hemmer B *et al*: **Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis**. *N Engl J Med* 2017, **376**(3):209-220.

132. Chataway J, De Angelis F, Connick P, Parker RA, Plantone D, Doshi A, John N, Stutters J, MacManus D, Prados Carrasco F *et al*: **Efficacy of three neuroprotective drugs in secondary progressive multiple sclerosis (MS-SMART): a phase 2b, multiarm, double-blind, randomised placebo-controlled trial**. *Lancet Neurol* 2020, **19**(3):214-225.

133. Cellerino M, Ivaldi F, Pardini M, Rotta G, Vila G, Backer-Koduah P, Berge T, Laroni A, Lapucci C, Novi G *et al*: **Impact of treatment on cellular immunophenotype in MS: A cross-sectional study**. *Neurol Neuroimmunol Neuroinflamm* 2020, **7**(3).

134. Schweitzer F, Laurent S, Fink GR, Barnett MH, Hartung HP, Warnke C: **Effects of disease-modifying therapy on peripheral leukocytes in patients with multiple sclerosis**. *J Neurol* 2020.

135. Lizarraga AA, Weinstock-Guttman B: **Multiple sclerosis in 2019: predicting progression**. *Lancet Neurol* 2020, **19**(1):12-14.

136. Delcoigne B, Manouchehrinia A, Barro C, Benkert P, Michalak Z, Kappos L, Leppert D, Tsai JA, Plavina T, Kieseier BC *et al*: **Blood neurofilament light levels segregate treatment effects in multiple sclerosis**. *Neurology* 2020, **94**(11):e1201-e1212.

137. Zurawski J, Tauhid S, Chu R, Khalid F, Healy BC, Weiner HL, Bakshi R: **7T MRI cerebral leptomeningeal enhancement is common in relapsing-remitting multiple sclerosis and is associated with cortical and thalamic lesions**. *Mult Scler* 2020, **26**(2):177-187.

138. Herranz E, Louapre C, Treaba CA, Govindarajan ST, Ouellette R, Mangeat G, Loggia ML, Cohen-Adad J, Klawiter EC, Sloane JA *et al*: **Profiles of cortical inflammation in multiple sclerosis by (11)C-PBR28 MR-PET and 7 Tesla imaging**. *Mult Scler* 2019:1352458519867320.

139. Akaishi T, Takahashi T, Nakashima I: **Peripheral blood monocyte count at onset may affect the prognosis in multiple sclerosis**. *J Neuroimmunol* 2018, **319**:37-40.

140. Perez CA, Agyei P, Gogia B, Harrison R, Samudralwar R: **Overlapping autoimmune syndrome: A case of concomitant anti-NMDAR encephalitis and myelin oligodendrocyte glycoprotein (MOG) antibody disease**. *J Neuroimmunol* 2019, **339**:577124.

141. Karpus WJ: **Cytokines and Chemokines in the Pathogenesis of Experimental Autoimmune Encephalomyelitis**. *J Immunol* 2020, **204**(2):316-326.

142. Glatigny S, Bettelli E: **Experimental Autoimmune Encephalomyelitis (EAE) as Animal Models of Multiple Sclerosis (MS)**. *Cold Spring Harb Perspect Med* 2018, **8**(11).

143. Sanabria-Castro A, Flores-Diaz M, Alape-Giron A: **Biological models in multiple sclerosis**. *J Neurosci Res* 2020, **98**(3):491-508.

144. Teixeira NB, Sant'Anna MB, Giardini AC, Araujo LP, Fonseca LA, Basso AS, Cury Y, Picolo G: **Crotoxin down-modulates pro-inflammatory cells and alleviates pain on the MOG35-55-induced experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis**. *Brain Behav Immun* 2020, **84**:253-268.

145. Wang L, ZhangBao J, Zhou L, Zhang Y, Li H, Li Y, Huang Y, Wang M, Lu C, Lu J *et al*: **Encephalitis is an important clinical component of myelin oligodendrocyte glycoprotein antibody associated demyelination: a single-center cohort study in Shanghai, China**. *Eur J Neurol* 2019, **26**(1):168-174.

146. Leite MI, Sato DK: **MOG-antibody-associated disease is different from MS and NMOSD and should be considered as a distinct disease entity - Yes**. *Mult Scler* 2019:1352458519868796.

147. Salama S, Khan M, Pardo S, Izbudak I, Levy M: **MOG antibody-associated encephalomyelitis/encephalitis**. *Mult Scler* 2019, **25**(11):1427-1433.

148. Kwon YN, Waters PJ, Kim M, Choi YS, Kim JW, Sung JJ, Park SH, Kim SM: **Peripherally derived macrophages as major phagocytes in MOG encephalomyelitis**. *Neurol Neuroimmunol Neuroinflamm* 2019, **6**(5).

149. Fujihara K: **MOG-antibody-associated disease is different from MS and NMOSD and should be classified as a distinct disease entity - Commentary**. *Mult Scler* 2019:1352458519895236.

150. Cobo-Calvo A, Marignier R: **MOG-antibody-associated disease is different from MS and NMO and should be considered as a distinct disease entity - No**. *Mult Scler* 2019:1352458519863135.

151. Bronge M, Ruhrmann S, Carvalho-Queiroz C, Nilsson OB, Kaiser A, Holmgren E, Macrini C, Winklmeier S, Meinl E, Brundin L *et al*: **Myelin oligodendrocyte glycoprotein revisited-sensitive detection of MOG-specific T-cells in multiple sclerosis**. *J Autoimmun* 2019, **102**:38-49.

152. Yang MG, Sun L, Han J, Zheng C, Liang H, Zhu J, Jin T: **Biological characteristics of transcription factor RelB in different immune cell types: implications for the treatment of multiple sclerosis**. *Mol Brain* 2019, **12**(1):115.

153. Gobel K, Ruck T, Meuth SG: **Cytokine signaling in multiple sclerosis: Lost in translation**. *Mult Scler* 2018, **24**(4):432-439.

154. Lassmann H: **Multiple Sclerosis Pathology**. *Cold Spring Harb Perspect Med* 2018, **8**(3).

155. Keren-Shaul H, Spinrad A, Weiner A, Matcovitch-Natan O, Dvir-Szternfeld R, Ulland TK, David E, Baruch K, Lara-Astaiso D, Toth B *et al*: **A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease**. *Cell* 2017, **169**(7):1276-1290 e1217.

156. Plemel JR, Stratton JA, Michaels NJ, Rawji KS, Zhang E, Sinha S, Baaklini CS, Dong Y, Ho M, Thorburn K *et al*: **Microglia response following acute demyelination is heterogeneous and limits infiltrating macrophage dispersion**. *Sci Adv* 2020, **6**(3):eaay6324.

157. International Multiple Sclerosis Genetics C: **Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility**. *Science* 2019, **365**(6460).

158. Pagani F, Testi C, Grimaldi A, Corsi G, Cortese B, Basilico B, Baiocco P, De Panfilis S, Ragozzino D, Di Angelantonio S: **Dimethyl Fumarate Reduces Microglia Functional Response to Tissue Damage and Favors Brain Iron Homeostasis**. *Neuroscience* 2020, **439**:241-254.

159. Nissen JC, Thompson KK, West BL, Tsirka SE: **Csf1R inhibition attenuates experimental autoimmune encephalomyelitis and promotes recovery**. *Exp Neurol* 2018, **307**:24-36.

160. Heppner FL, Greter M, Marino D, Falsig J, Raivich G, Hovelmeyer N, Waisman A, Rulicke T, Prinz M, Priller J *et al*: **Experimental autoimmune encephalomyelitis repressed by microglial paralysis**. *Nat Med* 2005, **11**(2):146-152.

161. Wilbanks B, Maher LJ, 3rd, Rodriguez M: **Glial cells as therapeutic targets in progressive multiple sclerosis**. *Expert Rev Neurother* 2019, **19**(6):481-494.

162. Luo C, Jian C, Liao Y, Huang Q, Wu Y, Liu X, Zou D, Wu Y: **The role of microglia in multiple sclerosis**. *Neuropsychiatr Dis Treat* 2017, **13**:1661-1667.

163. Guilliams M, Thierry GR, Bonnardel J, Bajenoff M: **Establishment and Maintenance of the Macrophage Niche**. *Immunity* 2020, **52**(3):434-451.

164. Guilliams M, Scott CL: **Does niche competition determine the origin of tissue-resident macrophages?** *Nat Rev Immunol* 2017, **17**(7):451-460.

165. Zhang Y, Zhao L, Wang X, Ma W, Lazere A, Qian HH, Zhang J, Abu-Asab M, Fariss RN, Roger JE *et al*: **Repopulating retinal microglia restore endogenous organization and function under CX3CL1-CX3CR1 regulation**. *Sci Adv* 2018, **4**(3):eaap8492.

166. Bennett ML, Bennett FC, Liddelow SA, Ajami B, Zamanian JL, Fernhoff NB, Mulinyawe SB, Bohlen CJ, Adil A, Tucker A *et al*: **New tools for studying microglia in the mouse and human CNS**. *Proc Natl Acad Sci U S A* 2016, **113**(12):E1738-1746.

167. Mapplebeck JC, Beggs S, Salter MW: **Molecules in pain and sex: a developing story**. *Mol Brain* 2017, **10**(1):9.

168. Sorge RE, Mapplebeck JC, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin JS, Sotocinal SG, Chen D *et al*: **Different immune cells mediate mechanical pain hypersensitivity in male and female mice**. *Nat Neurosci* 2015, **18**(8):1081-1083.

169. Yanguas-Casas N, Crespo-Castrillo A, de Ceballos ML, Chowen JA, Azcoitia I, Arevalo MA, Garcia-Segura LM: **Sex differences in the phagocytic and migratory activity of microglia and their impairment by palmitic acid**. *Glia* 2018, **66**(3):522-537.

170. Bilbo SD: **Sex differences in microglial appetites during development: Inferences and implications**. *Brain Behav Immun* 2017, **64**:9-10.

171. Konno T, Kasanuki K, Ikeuchi T, Dickson DW, Wszolek ZK: **CSF1R-related leukoencephalopathy: A major player in primary microgliopathies**. *Neurology* 2018, **91**(24):1092-1104.

172. Kempthorne L, Yoon H, Madore C, Smith S, Wszolek ZK, Rademakers R, Kim J, Butovsky O, Dickson DW: **Loss of homeostatic microglial phenotype in CSF1R-related Leukoencephalopathy**. *Acta Neuropathol Commun* 2020, **8**(1):72.

173. Chen J, Luo S, Li N, Li H, Han J, Ling L: **A Novel Missense Mutation of the CSF1R Gene Causes Incurable CSF1R-Related Leukoencephalopathy: Case Report and Review of Literature**. *Int J Gen Med* 2020, **13**:1613-1620.

174. Tian WT, Zhan FX, Liu Q, Luan XH, Zhang C, Shang L, Zhang BY, Pan SJ, Miao F, Hu J *et al*: **Clinicopathologic characterization and abnormal autophagy of CSF1R-related leukoencephalopathy**. *Transl Neurodegener* 2019, **8**:32.

175. Tanabe S, Saitoh S, Miyajima H, Itokazu T, Yamashita T: **Microglia suppress the secondary progression of autoimmune encephalomyelitis**. *Glia* 2019, **67**(9):1694-1704.

176. Rangaraju S, Dammer EB, Raza SA, Rathakrishnan P, Xiao H, Gao T, Duong DM, Pennington MW, Lah JJ, Seyfried NT *et al*: **Identification and therapeutic modulation of a pro-inflammatory subset of disease-associated-microglia in Alzheimer's disease**. *Mol Neurodegener* 2018, **13**(1):24.

177. Speicher AM, Wiendl H, Meuth SG, Pawlowski M: **Generating microglia from human pluripotent stem cells: novel in vitro models for the study of neurodegeneration**. *Mol Neurodegener* 2019, **14**(1):46.

178. Sellgren CM, Sheridan SD, Gracias J, Xuan D, Fu T, Perlis RH: **Patient-specific models of microglia-mediated engulfment of synapses and neural progenitors**. *Mol Psychiatry* 2017, **22**(2):170-177.

179. T'Jonck W, Guilliams M, Bonnardel J: **Niche signals and transcription factors involved in tissue-resident macrophage development**. *Cell Immunol* 2018, **330**:43-53.

180. Nally FK, De Santi C, McCoy CE: **Nanomodulation of Macrophages in Multiple Sclerosis**. *Cells* 2019, **8**(6).