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Elucidation of neuronal and glial cell phenotypes and functions utilizing mass spectrometry techniques

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–The inspiring mentor–

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2.2.2. **Boettcher C,** Fellermeier M, Boettcher C, Dräger B, Zenk MH.
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Proc Natl Acad Sci USA, 102:8495-500 (2005).

Abbreviations

ANXA2	Annexin A2
ApoC1	Apolipoprotein C1
ApoE	Apolipoprotein E
AXL	A member of receptor tyrosine kinase family
C	Carbon
CCL2	CC-chemokine ligand 2
CCL4	CC-chemokine ligand 4
CCL5	CC-chemokine ligand 5
CNS	Central nervous system
CST3	Cystatin 3 or Cystatin C
CTSD	The gene encoding cathepsin D
CyTOF	Cytometry by Time-of-Flight
CXCL10	C-X-C motif chemokine 10
eGFP	Enhanced green fluorescent protein
FISH	Fluorescence in situ Hybridization
GCL	Ganglion cell layer
GPNMB	Glycoprotein non-metastatic b
hCMEC/D3	Human cerebral microvessel endothelial cells/D3
hiPSCs	Human-induced pluripotent stem cells
HLA-DR	Major histocompatibility complex class II cell surface receptor
IBA1	Ionized calcium binding adaptor molecule 1
IGF1	Insulin like growth factor 1
INL	Inner nuclear layer
IPL	Inner plexiform layer
ITGAX	Integrin α-X (or CD11c)
LGALS1	Lectin or Galactose binding, soluble 1
LYZ2	Lysozyme 2
MAFB	V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
mRNA	messenger Ribonucleic acid
MS	Mass spectrometry

N	Nitrogen
O	Oxygen
ON	Optic nerve
OPL	Outer plexiform layer
PKCα	Protein kinase C alpha
RPE	Retinal pigment epithelium
SILAC	Stable isotope labelling by amino acid in cell culture
SH-SY5Y	Human neuroblastoma cell line
SPARC	Secreted protein, acidic and rich in cysteine
SPP1	Secreted Phosphoprotein 1 or osteopontin
TBI	Total body irradiation
TDP-43	Transactive response DNA binding protein 43 KDa
TMEM119	Transmembrane protein 119
TNFα	Tumor necrosis factor alpha

1. Introduction and Aims

1.1. Diversity of the non-neuronal cell compartment of the CNS

Resident macrophages in the central nervous system (CNS) are key players in CNS homeostasis and during CNS pathology. In the brain, this cellular compartment comprises parenchymal microglia and non-parenchymal macrophages that reside in the perivascular (Virchow-Robin) spaces, choroid plexus, and meningeal compartments [Prinz & Priller 2014]. Brain macrophages play critical roles in regulation of immune responses at the boundaries and in the parenchyma of both healthy and diseased brain. In addition, microglia are involved in the clearance of apoptotic neurons and the refining of synaptic connectivity, and thus play an important role in maintaining CNS integrity and function [Sierra et al., 2010; Parkhurst et al., 2013]. Other brain macrophages sense danger signals at brain interfaces such as the blood-brain barrier (BBB; perivascular macrophages) or the blood-cerebrospinal fluid barrier (choroid plexus macrophages). Brain microglia originate from yolk sac-derived precursors that enter the CNS during early embryonic development. Under homeostasis, microglia expansion depends on local self-renewal of the resident population throughout life [Ginhoux et al., 2010; Kierdorf et al., 2013; Schulz et al., 2012; Ajami et al., 2011; Ajami et al., 2007; Saederup et al., 2010; Mizutani et al., 2012]. Likewise, the other CNS macrophage populations (these are meningeal, perivascular and choroid plexus macrophages) are also derived from yolk sac precursor, which largely relied on similar transcription factors such as PU.1 [Goldmann et al., 2016]. Similarly, the retina is populated by both perivascular macrophages and parenchymal microglia. Although the contribution of microglia in retinal homeostasis remains unclear, they are a key regulator in a variety of retinal diseases [Rathnasamy et al., 2019].

During CNS diseases, hematogenous cells such as monocytes can enter the CNS, differentiate into brain parenchymal and/or non-parenchymal macrophages. On the one hand, these cells share some phenotypical characteristics with the resident macrophage population, on the other hand, their phenotypes are slightly differ from parenchymal microglia. For example, the monocyte-derived macrophages can be characterized as $CD45^{hi}CD11c^{+}F4/80^{+}MHCII^{+}$, whereas the parenchymal microglia are $CD45^{lo}CD11c^{lo}F4/80^{lo}MHCII^{-}$ [O'Koren et al., 2016]. Moreover, resident and hematogenous brain macrophages differentially contribute to local immune responses and the regulation of CNS inflammation [Hoogerbrugge et al., 1988; Priller et al., 2001; Corti et al., 2004;

Malm et al., 2005; Priller et al., 2006; Simard et al., 2006; Solomon et al., 2006; Djukic et al., 2006; Schechter et al., 2009; Derecki et al., 2012]. It has been suggested in mouse models of neurodegenerative disorders that hematogenous brain macrophages engrafted preferentially at the lesioned sites of the brain provide therapeutic advantages over resident populations [Simard et al., 2006; Corti et al., 2004; Derecki et al., 2012; Hoogerbrugge et al., 1988; Schechter et al., 2009]. Whether the monocytes can also target the damaged retina and provide such therapeutic potential remains to be investigated. Furthermore, the above-mentioned findings are obtained from mouse models of neurodegenerative diseases that were applied to total body irradiation (TBI) and bone marrow (BM) transplantation. In these mouse models, the immune compartment of the recipients is diminished and, subsequently, TBI-induced brain damage triggers the engraftment of transplanted BM-derived cells, independently of neurodegenerative conditions [Mildner et al. 2007], thus the migratory capability of hematogenous cells to the CNS remained questioned. Therefore, to precisely determine engraftment potential of peripheral cells into the CNS, an experimental protocol avoiding lethally irradiation and systemic collapse of host immunity is required.

Importantly, spatially heterogeneity and functional differences between resident brain macrophages, in particular microglia, has been displayed at mRNA level in the mouse brains [Grabert et al., 2016]. These differences may explain region-dependent vulnerability of microglia and regional differences in the involvement in neurological and psychiatric diseases. In human, overall transcriptomic signature of microglia has been provided [Gosselin et al. 2017 & Galatro et al. 2017] at the bulk system analysis. However, a comprehensive profiling of human microglia transcriptomes and phenotypes at the single-cell level has remained to be investigated. In general, the phenotypic profiling on the basis of marker protein expressions on human microglia relied on either immunohistochemistry of post-mortem brain tissue or flow cytometric analysis of acutely isolated microglia cells [Melief et al. 2016, Mizee et al. 2017, Mildner et al. 2017, Moore et al. 2015]. However, these approaches are limited to the high autofluorescent background of post-mortem tissue and the limitation of investigated markers that can be simultaneously analysed in one measurement (generally less than 20). To unravel the phenotypic and functional diversity of the macrophage/microglial compartment of the CNS and to investigate how they interact with other CNS cells, a comprehensively high-dimensional analysis at the single-cell level is required. Better understanding of the phenotypic and functional differences between CNS macrophage populations,

as well as the differences between brain regions, may have clinical implications for the treatment and diagnosis of neurological and psychiatric disorders.

1.2. Mass spectrometry (MS) techniques in neurosciences

MS techniques are widely applied for the elucidation, identification and quantification of molecules, including proteins. MS determines the mass-to-charge ratio of ionized species of molecules in simple and/or complex samples. MS can be applied to a broad spectrum of research fields and applications, including proteomics and metabolomics in neurosciences.

As previously described in **1.1.**, the CNS hosts various cell types including neurons, glial cells and infiltrating immune cells, studying dynamic changes of CNS cell phenotypes and functions requires high-dimensional single-cell analytical methods. A complementary method for cell identification and quantification is needed to better understand cellular heterogeneity of the CNS during disease pathogenesis and progression. Cytometry by Time-of-Flight (CyTOF) or mass cytometry, a cell profiling technique that combines metal isotope-labelling technology, flow cytometric analysis with time-of-flight mass spectrometry, has been introduced for the real time high-dimensional single-cell immune profiling [Bandura et al., 2009]. Using CyTOF technology, cellular targets are labelled with metal-conjugated antibodies, and detected and quantified by time-of-flight mass spectrometry (**Figure 1**).

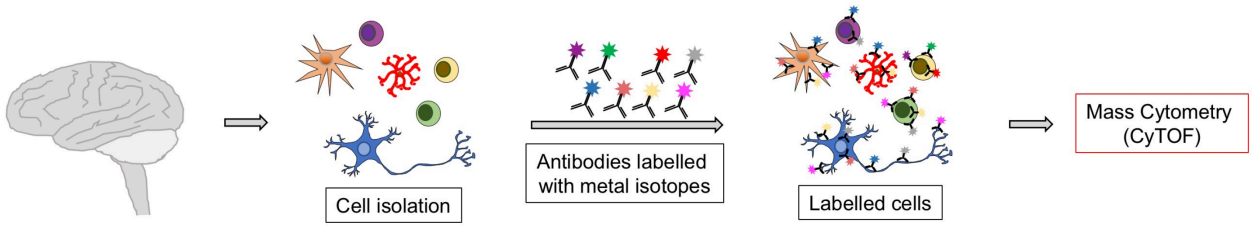

Figure 1 Mass cytometry in neurosciences. CNS cells are isolated from the brain tissue and subsequently stained with stable metal isotope-labelled antibodies. The antibody-labelled cell mixture can be then identified and quantitatively analysed by mass cytometry (CyTOF), a combination of flow cytometry and mass spectrometry techniques.

Taking advantage of the low signal overlap between metal isotopes, CyTOF allows the simultaneous cell identification and quantification on the basis of more than 45 marker targets on a single cell. This technique can also overwhelm difficulties of investigating samples with the high background signal such as the post-mortem brain samples. Data obtained from CyTOF measurement are processed and analysed in an unsupervised manner using algorithm-based data analysis.

The combination of a comprehensive array of protein markers and unsupervised data analysis provides a powerful strategy for cellular identification and quantification in a complex system of the CNS. Of note, CyTOF is however not a high-throughput-omics technique, since only up to 40-45 selected molecules can be simultaneously assessed, and CyTOF assesses cellular phenotypes and functions using an antibody-based approach, thus it is not really an un-biased method. Nonetheless, CyTOF analysis reveals the comprehensive information on phenotypes and functions at the single-cell level, which is to date the only high-dimensional array of protein markers that could greatly complement the transcriptomic signature of various cell types in the complex environment of the CNS.

Apart from the cell phenotypic heterogeneity revealed by CyTOF, the real-time cellular responses to the environment as well as the cell-cell interaction are also crucial information needed to understand the maintenance and regulation of CNS function. This dynamic interaction can be assessed using for example a mass spectrometry technique – metabolomic analysis –. Unlike classical proteomics, metabolomics reveal the alteration of abundance and/or characteristics of the downstream metabolites in a dynamic system, which result from environmental, genomic and/or proteomic factors as well as disease conditions. Although the methodology is still limited as bulk system investigations, the information obtained from metabolomic analyses during disease progression can provide a functional readout of target cells at different states, which is invaluable complementary information to phenotypic information obtained from CyTOF analysis, and thus will facilitate the investigation of cell signalling and/or biomarkers specifically involved in disease pathogenesis or disease progression and severity.

One of the methodological approaches to study metabolomics is the *stable isotope-resolved metabolomics*, a supervised investigation in which selected isotopically labelled metabolites are dynamically traced in order to unequivocally assess metabolic alterations of the selected signalling pathway [Patti et al., 2012a; Patti et al., 2012b]. To apply this analytical approach, the metabolic

or signalling pathways are pre-selected prior to labelling experiments. Subsequently, cells of interest are cultured in the medium containing the isotope-labelled precursors of the pre-selected pathway. After some hours/days *in vitro*, cells are harvested, isotope-labelled molecules yielded from the labelling experiment are then isolated and subsequently digested prior to elucidation and quantification using MS.

The most well-known stable isotope-metabolomic analysis is the stable isotope labelling by amino acid in cell culture (SILAC). In SILAC experiments, cells are cultured in the medium containing stable isotope-labelled amino acids. Commonly, amino acids lysine and arginine such as $^{2}H_4$-lysine with $^{13}C_6$-arginine or $^{15}N_2{}^{13}C_6$-lysine and $^{15}N_4{}^{13}C_6$-arginine were used for the labelling experiments. The labelled proteins are then isolated, digested and measured by MS (**Figure 2**).

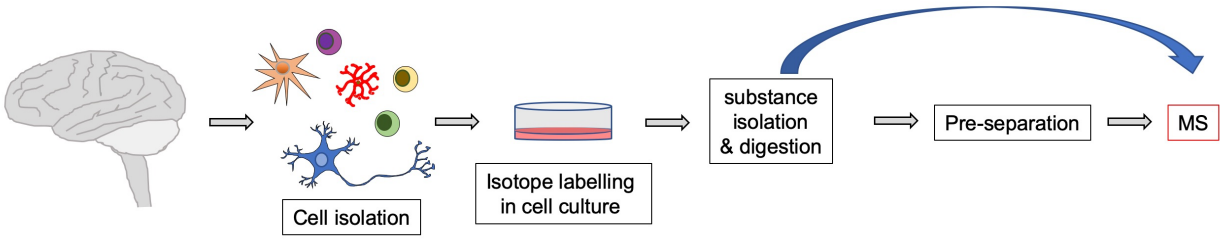

Figure 2 Schematic representation of stable isotope-resolved metabolomics. Isolated CNS cells or CNS cell lines are cultured in the presence of stable isotope-labelled amino acid or precursors of the signalling pathway of interest. The resulting isotope-labelled protein is then isolated, digested and subsequently analysed by mass spectrometry with or without a pre-separation step.

SILAC has been widely applied for the investigation of disease-associated changes in proteomic profiles during neurological and psychiatric diseases, including glioblastoma [Formolo et al., 2011; Kozuka-Hata et al., 2012; Narushima et al., 2016], ischemia [Llombart et al., 2016], Alzheimer's disease [Klegeris et al., 2008; McGeer et al., 2010; Tan et al., 2014] and Parkinson's disease [Sarraf et al., 2013], TDP-43 proteinopathy [Seyfried et al., 2010]. Various CNS cell types can be used for SILAC experiments, for example the brain endothelial cell line hCMEC/D3 [Llombart et al., 2016], neuroblastoma SH-SY5Y cell line [Klegeris et al., 2008; Gokhale et al., 2012], human induced pluripotent stem cell (hiPSC)-derived neurons [Brennand et al., 2015], primary murine microglia

[Pinho et al., 2017; Zhang et al., 2017; Zhang et al., 2016] or primary cultures of mouse cerebellar granule neurons [Thouvenot et al., 2012].

For example, Brennand *et al.* (2015) performed a discovery study on an alteration of cellular proteomics during schizophrenia using SILAC quantitative proteomic mass spectrometry analyses. In this particular study, the hiPSC neuronal progenitor cells (NPCs) were differentiated from fibroblasts, and were subsequently cultured in isotope ($^{13}C^{15}N$)-enriched arginine and lysine. Protein including isotope-labelled protein was then isolated from the cultured cells. The protein identification, quantification and analyses were performed using mass spectrometry and proteomics data analysis platform. In the comparison with the hiPSC-NPCs from healthy controls, cells from four schizophrenia patients showed abnormal protein expressions related to cytoskeletal remodelling and oxidative stress.

Similar to SILAC, other precursor molecules instead of amino acids can be applied *in vitro* and/or *in vivo*, to elucidate the metabolomic phenotypes of the target cells. In this particular case, the labelled molecules obtained from the labelling experiment are not necessary to be proteins. For example, to demonstrate evidence for endogenous morphine biosynthesis in human neuronal cells, the human neuroblastoma cells, SH-SY5Y, were cultured in the medium containing ^{18}O-labelled oxygen ($^{18}O_2$), [*ring*-$^{13}C_6$]-tyramine, (*S*)-[1-^{13}C,*N*-$^{13}CH_3$]-reticuline or [*N*-C^2H_3]-thebaine, the precursors of the morphine biosynthetic pathway [Poeaknapo et al., 2004]. After seven days *in vitro*, morphine (the final product) and other intermediates of the morphine biosynthetic pathway were isolated from the cultured SH-SY5Y cells and were further elucidated and quantified by gas chromatography coupled with ion-selective tandem MS (GC/MS/MS). The results showed specific incorporation of the ^{18}O, ^{13}C or ^{2}H isotopes into endogenous morphine and it's intermediates, thus unequivocally proved the capability of synthesizing morphine of human neuroblastoma cells [Poeaknapo et al., 2004] (**Figure 3**).

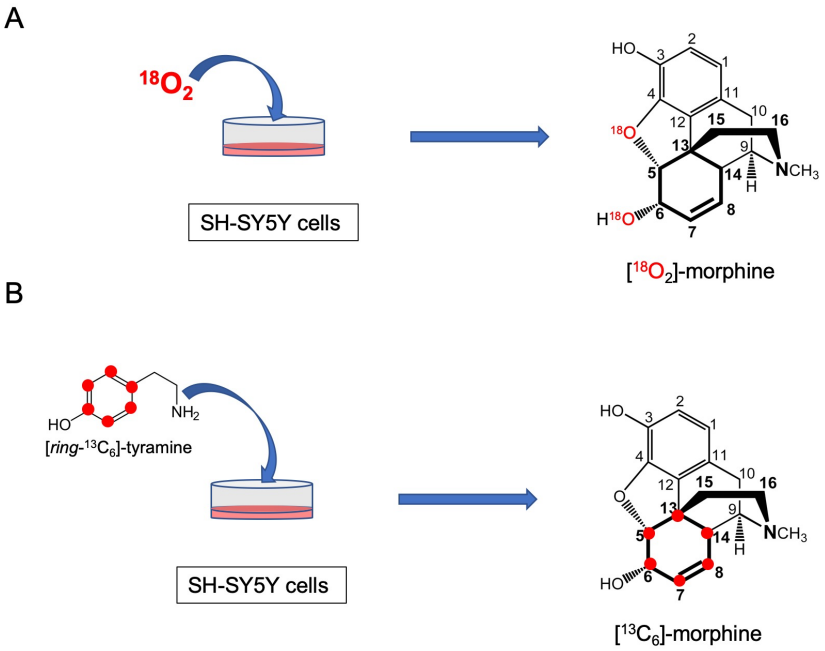

Figure 3 Schematic representation showing two examples of stable isotope-resolved metabolomic experiments from *Poeaknapo et. al. 2004*. Incubation of SH-SY5Y cells in the presence of ^{18}O-oxygen (**A**) or [*ring*-$^{13}C_6$]-tyramine (**B**) resulted in an incorporation of two atom of ^{18}O per morphine molecule (**A**) or six atom of ^{13}C at specific positions per morphine molecule (**B**).

1.3. Aims

This study attempts to reveal cellular complexity of the myeloid compartment of the CNS including the retina in mouse models, as well as in the human system. Furthermore, analytical protocols for phenotypic and functional characterization of neuronal and non-neuronal cells (in particular microglia) in the CNS during homeostasis and diseases shall be established and evaluated.

First, the study aims to investigate the migratory and engraftment capability of peripheral cells into the retina of mouse models for retinal degeneration. It should prove whether the retina processes the cellular complexity similar to that was observed in the brain. An integration of blood-derived macrophages into the network of the retinal macrophages/microglia will be determined.

Second, this study shall demonstrate an induction of the engraftment of blood-derived cells under an experimental protocol avoiding lethally irradiation and systemic collapse of host immunity, which may in the future serve as a tool to precisely evaluate the migratory capacity of the circulating cells into the CNS and/or as a tool for development of therapeutic strategy in CNS diseases.

Finally, analogue to mouse models, heterogeneous phenotypes and functions of the human CNS cells will be investigated. In this study, exploratory experiments aim to comprehensively characterise various CNS cell types and their responses during diseases will be demonstrated utilizing multi-parameter analysis including mass spectrometry techniques such as mass cytometry and stable isotope-resolved metabolomics.

2. Selected own work

Summary

Microglia and brain macrophages are increasingly evidenced as key regulators in CNS development, homeostasis and pathology. A better understanding of the cellular dynamics and mechanisms that regulate microglial homeostasis and function will provide the means to manipulate these cells for therapeutic purposes.

In the first section of the selected own works (**2.1.**), the cellular complexity of CNS myeloid compartment were unraveled using an adoptive transfer experiment with gene-modified bone marrow cells (**2.1.1.**), bone marrow transplantation in a mouse model of facial nerve axotomy (**2.1.2.**) or high-throughput techniques such as single-cell RNA sequencing (scRNA-Seq) (**2.1.3.**). These findings demonstrated that, followed CNS conditioning, bone marrow-derived cells are recruited to the CNS including the retina, preferentially to the lesioned sites of the CNS. These infiltrating bone marrow-derived macrophages stably integrated into the CNS myeloid cell compartment of the lesioned brain. Similarly, the results obtained from scRNA-Seq revealed spatial and temporal microglial heterogeneity in both mouse and human brain. In diseased brain, the composition of microglial sub-populations was altered, and their microglial signatures could be rapidly changed during neurodegeneration (such as facial nerve axotomy) and/or neuroinflammation (such as multiple sclerosis).

In the second part of the selected own works (**2.2.**), microglial heterogeneity was investigated in human post-mortem brain tissue and fresh brain biopsies (**2.2.1.**) at the single-cell protein level. Again, these results highlight the cellular complexity of the CNS myeloid compartment including the microglia subpopulations described in the first section (**2.1.**), which complemented the transcriptomic signatures revealed by scRNA-Seq. Moreover, the findings translated mouse microglial phenotypes to the human system, emphasizing the translational potential of the methodology for further investigation in clinical applications. Besides single-cell phenotypic and functional characterization by mass cytometry, functions and metabolomics of CNS cells can be assessed using mass spectrometry. For this approach, the human neuroblastoma cell line SH-SY5Y was used for the establishment of the methodology (**2.2.2.**). The study demonstrated that the SH-SY5Y cell line was capable of synthesizing targeted metabolites (in this case "morphine"). Briefly, SH-SY5Y cells were cultured in the presence of ^{13}C-, ^{2}H- or ^{18}O-labelled precursors of the morphine biosynthetic pathway. SH-SY5Y cells *de novo* incorporate the stable isotope-labelled

precursors into endogenously synthesized morphine. The finding unequivocally proved the capacity of *de novo* morphine synthesis of human neuroblastoma cells. This established methodology can be applied for future metabolomic study of CNS cells including microglial cells.

2.1. Cellular complexity of CNS myeloid compartment

2.1.1. **Boettcher C,** Ulbricht E, Helmlinger D, Mack AF, Reichenbach A, Wiedemann P, Wagner HJ, Seeliger MW, Bringmann A, Priller J.

Long-term engraftment of systemically transplanted, gene-modified bone marrow-derived cells in the adult mouse retina.

Br J Ophthalmol, 92:272-275 (2008).

http://dx.doi.org/10.1136/bjo.2007.126318

The study provided evidence for the engraftment of BMDCs in the retina, preferentially around sites of retinal damage, and hence provided proof-of-principle of an application of BMDCs as vehicles for gene delivery to the retina.

Similar to the brain, after retinal damage, BMDCs can be targeted to the retina, precisely into the inner (IPL) and outer (OPL) plexiform layers and the ganglion cell layer (GCL), as well as into the optic nerve (ON). These cells differentiated into retinal macrophages.

These observations were also true for BMDCs that were transduced with a retroviral vector to express the enhanced green fluorescent protein (eGFP) prior to transplantation. The eGFP-expressing BMDCs engrafted in the central retina (i.e. IPL, OPL and GCL), where the retinal vascular plexuses are present. Rarely, the gene-modified BMDCs were found in the inner nuclear layer (INL), but virtually none of them engrafted to the photoreceptor layer or retinal pigment epithelium (RPE). The engrafted cells were immunoreactive for the myeloid markers CD11b and Iba1, but did not express vimentin (marking astrocytes, retinal Müller glial cells and retinal horizontal cells), calbindin (marking retinal horizontal cells), PKCα (marking retinal bipolar cells) or NeuN (marking ganglion cells). Strikingly, the gene-modified BMDCs engrafted in the retina and expressed the delivered gene *eGFP* for up to 15 months after transplantation.

In mouse models of retinal degeneration (i.e. *rd1* mutant FVB/N mice and spinocerebellar ataxia type 7 mice), eGFP-expressing BMDCs were found at high frequency, predominantly at the sites of retinal degeneration both in central and peripheral retina. These cells also expressed the GSA lectin, a marker for activated microglia and macrophages.

In sum, the findings provided evidence for the cellular complexity of the retinal myeloid compartment and the capability of BMDCs to enter the retina, in particular the degenerated retina. These findings are in line with previously mentioned observations in the brain (**see 2.1.2.**).

2.1.2. **Böttcher C,** Fernández-Klett F, Gladow N, Rolfes S, Priller J.
Targeting myeloid cells to the brain using non-myeloablative conditioning.
Plos One, 8:e80260 (2013).
https://doi.org/10.1371/journal.pone.0080260

Besides microglia, brain macrophages are involved in the maintenance of brain homeostasis. In neurogenerative diseases like Alzheimer´s disease, brain macrophages are capable of phagocytosing β-amyloid, whereas the resident microglia seem to be ineffective in this function. Ontogenetically, microglia are derived from immature yolk sac macrophages that enter the brain during early embryogenesis, while brain parenchymal macrophages originate from bone marrow-derived cells (BMDCs). Since the phenotypes of resident microglia and brain parenchymal macrophages are overlapping, it is challenging to distinguish between these two cell types.

In this particular study, a mouse model of bone marrow transplantation (BMT) was used as a tool to study bone marrow-derived brain macrophages. Unlike the classical mouse model of BMT that uses total body irradiation (TBI) or chemotherapy for host conditioning prior to the transplantation of eGFP-expressing BMDCs, non-myeloablative focal head irradiation (HI) was applied in this study to target the BMDCs to sites of brain damage in mice. This mild treatment induced recruitment of BMDCs to the sites of motoneuron degeneration in the brainstem as early as 7 days after facial nerve axotomy despite much lower levels of blood chimerism (i.e. <5% of donor-derived cells in the peripheral blood of the recipients) compared with TBI (>95%). Of note, the HI regimen showed comparatively lower inflammatory responses in the CNS than TBI or chemotherapy, as revealed by lower mRNA expression of CCL2, CXCL10, TNF-α and CCL5. The findings demonstrated that peripheral BMDCs can be targeted to the CNS, in particular after neuronal damage, even at the very low chimerism in the peripheral blood. At the sites of brain damage, these cells differentiated to brain parenchymal macrophages. The established conditioning regimen is an alternative protocol for recruiting BMDCs to the CNS with minimal disturbance of the hematopoietic compartment, and thus can be used to selectively study BM-derived brain macrophages in animal models of neurodegenerative diseases.

PLOS ONE

Targeting Myeloid Cells to the Brain Using Non-Myeloablative Conditioning

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Abstract

Bone marrow-derived cells (BMDCs) are able to colonize the central nervous system (CNS) at sites of damage. This ability makes BMDCs an ideal cellular vehicle for transferring therapeutic genes/molecules to the CNS. However, conditioning is required for bone marrow-derived myeloid cells to engraft in the brain, which so far has been achieved by total body irradiation (TBI) and by chemotherapy (e.g. busulfan treatment). Unfortunately, both regimens massively disturb the host's hematopoietic compartment. Here, we established a conditioning protocol to target myeloid cells to sites of brain damage in mice using non-myeloablative focal head irradiation (HI). This treatment was associated with comparatively low inflammatory responses in the CNS despite cranial radiation doses which are identical to TBI, as revealed by gene expression analysis of cytokines/chemokines such as CCL2, CXCL10, TNF-α and CCL5. HI prior to bone marrow transplantation resulted in much lower levels of blood chimerism defined as the percentage of donor-derived cells in peripheral blood (< 5%) compared with TBI (> 95%) or busulfan treatment (>50%). Nevertheless, HI effectively recruited myeloid cells to the area of motoneuron degeneration in the brainstem within 7 days after facial nerve axotomy. In contrast, no donor-derived cells were detected in the lesioned facial nucleus of busulfan-treated animals up to 2 weeks after transplantation. Our findings suggest that myeloid cells can be targeted to sites of brain damage even in the presence of very low levels of peripheral blood chimerism. We established a novel non-myeloablative conditioning protocol with minimal disturbance of the host's hematopoietic system for targeting BMDCs specifically to areas of pathology in the brain.

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Introduction

Microglia are the key immune effector cells of the central nervous system (CNS), mediating local inflammatory and innate immune responses. This CNS cell population has recently been shown to derive from immature yolk sac macrophages that infiltrate the brain during early embryogenesis [1-3]. Thus, microglia are ontogenetically distinct from hematopoietic stem cell (HSC)-derived tissue macrophages that originate from the bone marrow continuously throughout adult life. In line with these findings, no engraftment of myeloid cells was observed in the CNS of parabiotic mice, in which blood from two different animals is chronically shunted, suggesting that circulating hematopoietic progenitors and circulating monocytes do not significantly contribute to microglia homeostasis after birth [1-5].

However, bone marrow-derived cells (BMDCs) are able to colonize the adult CNS under certain conditions. Transplantation of genetically labelled BMDCs into total body-irradiated hosts has demonstrated that circulating myeloid cells engraft in the CNS and contribute to the pool of brain macrophages, both in the absence and during overt brain pathology [6-21]. Irradiation-induced changes in the CNS, as well as the introduction of hematopoietic stem/progenitor cells into the circulation have been suggested as necessary conditions for the recruitment of myeloid cells into the brain [1,4,21].

In line with their different origin, bone marrow-derived myeloid cells and microglia appear to exert differential functions in the CNS. In a mouse model of Alzheimer's disease, myeloid cells were able to phagocytose β-amyloid, whereas resident microglia appeared to be rather ineffective in this task [13,14]. Similarly, BMDCs were found to attenuate or even arrest pathology in mouse models of neuropsychiatric disorders including Rett syndrome, amyotrophic lateral sclerosis, Krabbe's disease and Parkinson's disease

[19,20,22,23]. Hence, myeloid cells have a tremendous therapeutic potential for neurological and psychiatric diseases. However, establishing a clinical conditioning regimen remains a challenge. Although total body irradiation (TBI) is an effective conditioning protocol to target the myeloid cells to the brain, this myeloablative treatment induces massive CNS inflammation and disturbance of the host's hematopoietic system [21,24]. Recently, conditioning with the alkylating chemotherapeutic agent, busulfan, has been suggested as an alternative [24]. Indeed, myeloablation with busulfan is being used in the clinical setting [25]. However, myeloid cell engraftment at sites of CNS damage after busulfan conditioning in mice was either absent [26] or dramatically reduced compared to irradiation [24].

Here, we established a protocol for CNS conditioning using focal head irradiation (HI) that avoids myeloablation and minimally disturbs the host's hematopoietic system. Regardless of the low presence of donor-derived cells in the peripheral circulation, BMDCs rapidly and selectively engrafted at sites of neurodegeneration. This conditioning regimen may serve as an alternative protocol for targeting myeloid cells to the CNS with minimal impairment of the hematopoietic compartment.

Materials and Methods

Mice

C57BL/6 wild type mice were purchased from Charles River (Sulzbach). C57BL/6 mice expressing the enhanced green fluorescent protein (GFP) under the control of β-actin promoter (*ACTβ-EGFP*) [27] were obtained from breeding facility of Charité. All recipient mice were 7-12 weeks old at the time of bone marrow transplantation (BMT).

All animal experiments were performed in strict accordance with national and international guidelines for the care and use of laboratory animals (Tierschutzgesetz der Bundesrepublik Deutschland, European directive 2010/63/EU, as well as GV-SOLAS and FELASA guidelines and recommendations for laboratory animal welfare). The experiments were specifically approved by the committee on the ethics of animal experiments of Berlin (Landesamt für Gesundheit und Soziales, Berlin, Germany, Permit Number: G0364/10).

Conditioning

Mice were anesthetized by subcutaneous injection of a mixture of ketamine (50 mg/kg) and xylazine (7.5 mg/kg) prior to either total body irradiation (TBI) or focal head irradiation (HI) with a single dose of 11Gy. Irradiation was performed using a $Caesium^{137}$ source (Gammacell 40 Exactor, Theratronics). During HI, the body was protected from irradiation with lead bars (3 cm thick). Dosimetric studies revealed a shielding efficieny >90% (cumulative dose < 1 Gy in protected areas). Busulfan-treated animals received two intraperitoneal (i.p.) injections of 50 mg/kg busulfan (Busilvex®, Pierre Fabre Pharma) at 5 and 3 days before BMT.

Bone marrow transplantation

After conditioning with either TBI or HI, wild type mice were intravenously (i.v.) injected with 2 x 10^7 unsorted bone marrow cells from *ACTβ-EGFP* mice within 24 hr after irradiation. Animals were examined at 1, 2, 4 and 16 weeks post-transplantation.

In the case of facial nerve axotomy (FNA), conditioning with HI was performed at 2 weeks and with busulfan at 5 and 3 days prior to BMT (i.v. injection of 2 x 10^7 unsorted bone marrow cells from *ACTβ-EGFP* mice). FNA was performed 24 hr before BMT and animals received daily i.p. injections of 2 mg/kg rapamycin (Enzo Life Science) thereafter. Groups with HI or FNA alone served as controls. Animals were examined at 7 and 14 days post-transplantation.

Facial nerve axotomy

Facial nerve axotomy (FNA) was performed as described previously [6]. Briefly, mice were anesthetized by subcutaneous injection of a mixture of ketamine (50 mg/kg) and xylazine (7.5 mg/kg). The right facial nerve was transected at the stylomastoid foramen, resulting in ipsilateral whisker paresis. The left facial nerve served as control.

Immunohistochemistry

Mice were anesthetized and perfused transcardially with cold phosphate-buffered saline (PBS). Brains were dissected, post-fixed in 4% paraformaldehyde (PFA) and cryoprotected with 30% sucrose. Coronal brain sections (30 µm) were obtained on a cryostat.

Sections were blocked at room temperature for 1 hr with 20% normal goat/donkey serum (Biozol) in Tris-buffered saline (TBS) containing 0.3% Triton X-100. After three washes in TBS, sections were incubated with primary antibodies diluted 1:200 [anti-Iba-1 (Wako), anti-F4/80 (Invitrogen) or anti-GFP (Nacalai Tesque, Invitrogen)] at 4°C overnight. After washing with TBS, sections were incubated with Alexa-conjugated secondary antibodies diluted 1:250 (Alexa 488-IgG and Alexa 594-IgG, Invitrogen) at room temperature for 3 hr. All antibodies were diluted in TBS containing 1% normal goat/donkey serum and 0.3% Triton X-100. Nuclei were counterstained with 4,6-diamidino-2-phenylindole diluted 1:10,000 (DAPI, Sigma). The immunostained sections were examined using a conventional fluorescence or laser-scanning confocal microscope (Leica TCS SP5, Leica Microsystems).

Flow cytometric analysis

Spleens were excised and pushed through a 70-µm strainer, bone marrow cells were flushed from both femurs and tibias. All samples were collected in Dulbecco's PBS (Gibco) containing 2% fetal bovine serum (Biochrom) and were stored on ice during staining and analysis. Blood was collected from the inferior vena cava using a citrate-coated syringe. Red blood cells were lysed in Pharm Lyse™ buffer (BD Biosciences).

Following Fc blocking, cells were stained with anti-CD115 (AFS98), Ly6C (AL-21), CD11b (M1/70), CD4 (RM4-5), CD8 (53-6.7), Ly6G (1A8) and CD19 (6D5). All antibodies were purchased from Biolegend. Fluorescence-activated cell sorting

(FACS) was performed using a Canto II (Becton Dickinson). Forward- and side-scatter parameters were used for exclusion of doublets from analysis. Data were analyzed with the FlowJo software (TreeStar).

Quantitative real-time PCR (qPCR)

Animals were anesthetized and perfused transcardially with cold PBS at 1, 2, 4 and 16 weeks after irradiation/BMT. Brains were dissected and immediately shock-frozen in liquid nitrogen. Total RNA was isolated from the brain using RNeasy Plus Mini kit (Qiagen). RNA (approximately 2 µg) was transcribed into cDNA using Amplitaq® DNA Polymerase kit (Applied Biosystems, Roche). PCR reactions were carried out using the LightCycler FastStart DNA Master Kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. The following primer pairs were used: CCL5 (forward 5'-TGC CCA CGT CAA GGA GTA TTT-3', reverse 5'-TCT CTG GGT TGG CAC ACA CTT-3'), CXCL10 (forward 5'-TGC TGG GTC TGA GTG GGA CT-3', reverse 5'-CCC TAT GGC CCT CAT TCT CAC-3'), TNF-α (forward 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3', reverse 5'-TGG GAG TAG ACA AGG TAC AAC CC-3') and CCL2 (forward 5'-TCT GGG CCT GCT GTT CAC C-3', reverse 5'-TTG GGA TCA TCT TGC TGG TG-3'). qPCR was performed using a LightCycler 2.0 (Roche).

Statistical analysis

Results were analyzed with Prism 4.0 (GraphPad) and statistical differences were evaluated using a non-paired Student's *t* test or one-way ANOVA with Posthoc Bonferroni correction. Significance was accepted for $p<0.05$. Data are shown as means ± SEM.

Results

Focal head irradiation induces delayed BMDC engraftment in the brain

Irradiation has been described to be a necessary condition for BMDC engraftment in the brain [21]. We hypothesized that non-myeloablative conditioning using HI would induce CNS microenvironment changes that suffice to trigger the recruitment of circulating myeloid cells to the CNS in the absence of full hematopoietic reconstitution. To this end, we transplanted adult mice with unsorted GFP-expressing bone marrow cells following either HI or TBI (Figure 1A). Thereafter, we analyzed the induction of different cytokines and chemokines in the brain (Figure 1B). Irradiation-induced increases in gene expression of monocyte chemoattractant protein-1 (MCP-1 or CCL2), interferon gamma-induced protein 10 (IP-10 or CXCL10), regulated on activation, normal T cell expressed and secreted (RANTES or CCL5) and tumor necrosis factor (TNF)-α were observed in the brains of both TBI and HI mice (Figure 1B). However, the induction of CCL2 and CXCL10 mRNAs was strongly reduced in HI compared with TBI animals although the doses of radiation to the brain were identical (Figure 1B). CCL5 and TNF-α mRNAs were also reduced at 2 weeks after HI compared with TBI (Figure 1B).

As expected, chimerism (determined as the percentage of GFP^+CD45^+ cells among all $CD45^+$ cells) was significantly lower in peripheral blood of HI mice compared with TBI animals at 16 weeks post-BMT (TBI: 97 ± 0.6%, HI: 3 ± 0.3%; Figure 2A). When analyzing the brains of the chimeras, no donor-derived GFP^+ cells were detected up to 12 weeks after BMT in HI animals, whereas TBI animals showed engraftment of ramified BMDCs in all brain regions (data not shown). At 16 weeks after BMT, clusters of donor-derived GFP^+ cells appeared in the cortex of HI mice (Figures 2B,C). At this time point, olfactory bulb, cortex and cerebellum were populated by ramified GFP^+ cells in the TBI group (Figures 2B,C). Quantitative analysis revealed reduced BMDC engraftment in the brains of HI mice compared with TBI animals (Figure 2B). The numbers of ramified GFP^+ cells were 5 ± 1/ mm^2 (TBI) versus 0/ mm^2 (HI) in the olfactory bulb, 25 ± 1/ mm^2 (TBI) versus 5 ± 1/ mm^2 (HI) in the cortex, and 18 ± 3/ mm^2 (TBI) versus 0/ mm^2 (HI) in the cerebellum. These results suggest that low blood chimerism and reduced expression of chemoattractants like CCL2 in the brain result in reduced and delayed engraftment of BMDCs in the HI protocol.

Enhanced recruitment of myeloid cells to sites of brain damage

We next tested whether HI in combination with neuronal damage accelerates the recruitment of BMDCs to the brain. To this end, we performed facial nerve axotomy in chimeric mice, which results in motoneuron degeneration in the absence of blood-brain barrier disruption [6]. Given that some potential precursors of brain macrophages, such as bone marrow-derived $Ly6C^{hi}$ inflammatory monocytes [21] or $Cx3CR1^+$ progenitors [5], do not self-renew and have a short life span in the blood stream, we performed FNA one day before BMT (Figure 3A). HI was carried out 14 days prior to transplantation to match the peak of chemokine/cytokine expression after irradiation (cf. Figure 1B) with the time points of analysis at 7 and 14 days after BMT (i.e. 3 and 4 weeks after irradiation). For comparison, we also used the chemotherapeutic agent busulfan for conditioning (Figure 3A). Busulfan has recently been demonstrated to trigger the entry of BMDCs into the brain with reduced CNS inflammation [24]. Mice receiving either HI or FNA alone prior to BMT served as control groups (Figure 3A).

As expected given the myelotoxic properties of busulfan, HI treatment resulted in lower blood chimerism compared to busulfan conditioning (HI + FNA: 5 ± 0.7% versus busulfan + FNA: 50 ± 4%; Figure 3B). FNA had no impact on blood chimerism (HI + FNA: 5 ± 0.7% versus HI: 8 ± 1%; Figure 3B), and conditioning was required to establish blood chimerism (FNA: <0.5%; Figure 3B). Notably, the vast majority of GFP^+ cells in peripheral blood of HI, HI + FNA and busulfan + FNA chimeras were identified as $CD11b^+$ myeloid cells (> 90% of GFP^+ cells; Figure 3C). These included $Ly6C^{hi}$ monocytes (HI: 8 ± 1%; HI + FNA: 9 ± 1% and busulfan: 10 ± 2% of GFP^+CD11b^+ cells; Figure 3C) and $Ly6G^+$ neutrophils (HI: 60 ± 3%; HI + FNA: 62 ± 2% and busulfan: 67 ± 7.0% of GFP^+CD11b^+ cells; Figure 3C). No significant differences in the contribution

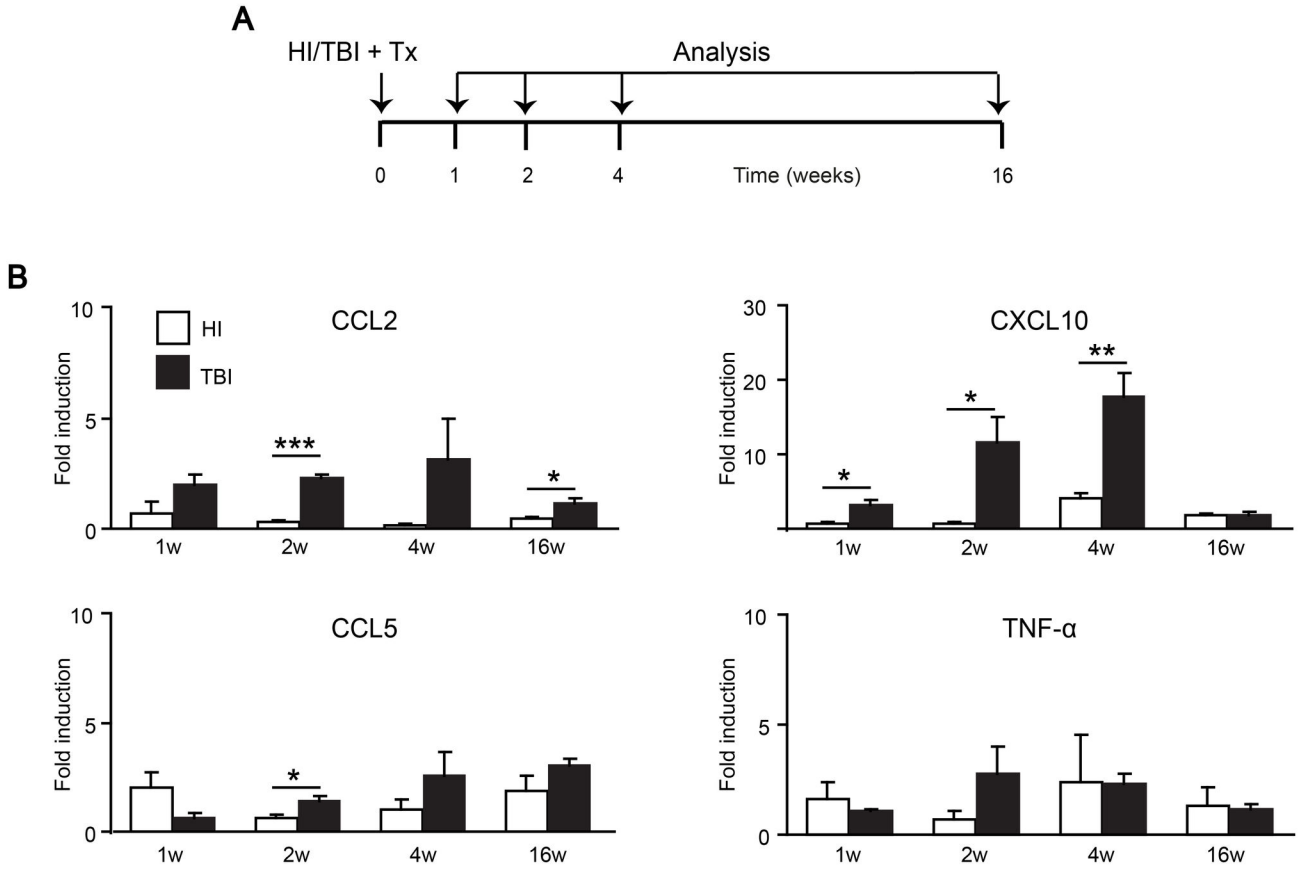

Figure 1. Gene expression profiles of cytokines and chemokines in the brain after HI and TBI. **A**) Overview of the experimental protocol. *HI*, *TBI* and *Tx* denote focal head irradiation, total body irradiation and bone marrow transplantation, respectively. **B**) Quantitative real-time PCR of CCL2, CXCL10, CCL5 and TNF-α mRNA expression in brains of HI (grey columns) and TBI (white columns) animals at 1, 2, 4 and 16 weeks after irradiation and BMT. The mRNA expression levels were normalized to GAPDH mRNA and compared to naïve mice (fold induction). Reduced cytokine/chemokine mRNA levels were observed in HI brains compared to the TBI paradigm. Data are means + SEM from 3-5 animals per group. Statistical significance is indicated by asterisks (*p<0.05; **p<0.01; ***p<0.001).

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of donor-derived myeloid cells were observed between the groups.

As early as 7 days after BMT, GFP$^+$ cells were specifically detected in the lesioned facial nucleus of HI + FNA mice (26 ± 7 cells/facial nucleus; Figures 4A,B). These cells were amoeboid and localized in the brain parenchyma. They were immunoreactive for Iba-1 and F4/80, which are markers of macrophages (Figure 4A). At 14 days after BMT, GFP$^+$ cells with a characteristic ramified morphology and expression of Iba-1 and F4/80 were detected in the lesioned facial nucleus of HI + FNA mice (40 ± 12 cells/nucleus, Figures 4A,B). The unlesioned contralateral facial nucleus was devoid of GFP$^+$ cells at all time points (Figure 4A). Notably, no donor-derived GFP$^+$ cells were observed in the lesioned facial nucleus of busulfan + FNA mice at 7 and 14 days after BMT despite high levels of blood chimerism (Figure 4B). Similarly, FNA and HI alone also failed to recruit donor-derived myeloid cells into the lesioned facial nucleus at 7 and 14 days after BMT (Figure 4B). This is in line with the delayed engraftment kinetics of myeloid cells after HI treatment (Figures 2B,C), and with published evidence documenting the necessity of conditioning for myeloid cell engraftment in the CNS [7,21].

Since myeloid cell recruitment into the CNS has been suggested to correlate with the induction of CCL2 and CXCL10 mRNAs [21], we used real-time PCR to quantify the expression levels of both chemokines in the facial nucleus of mice with FNA, HI and HI + FNA. The expression of CXCL10 and CCL2 mRNAs was potentiated in the HI + FNA group at 14 days after BMT (Figure 4C). The results suggest that HI and FNA act synergistically to induce chemokines, which may accelerate the recruitment of myeloid cells into the CNS.

Discussion

We established a novel non-myeloablative conditioning protocol for targeting BMDCs to the brain using focal head

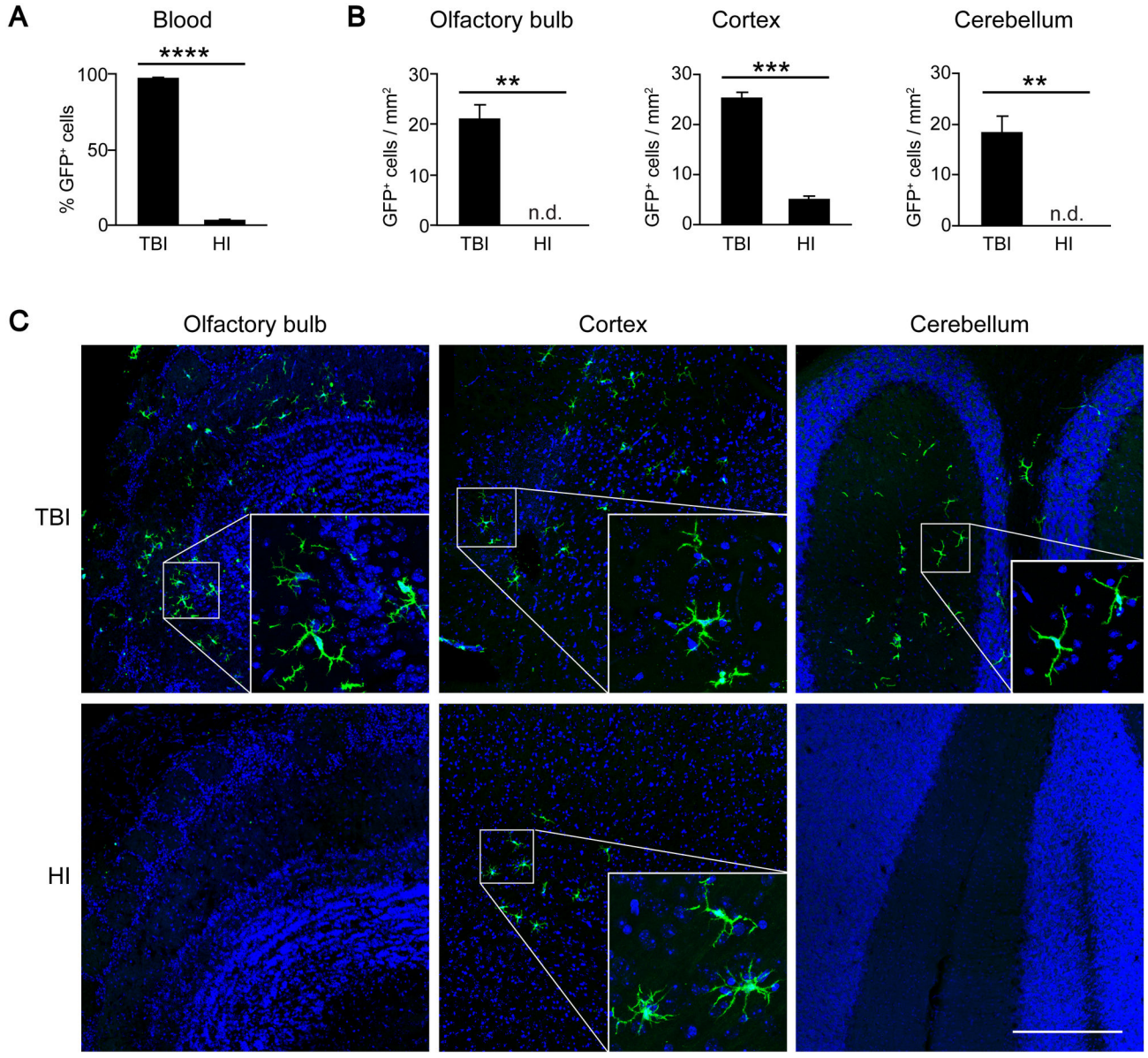

Figure 2. Delayed engraftment of BMDCs after HI. **A**) Flow cytometric analysis of GFP expression in peripheral blood leukocytes of HI and TBI chimeras. The level of chimerism was significantly lower in HI compared with TBI animals at 16 weeks after transplantation. Data are means + SEM from 3-5 animals per group. Statistical significance is indicated by the asterisk (****p<0.0001). **B**) Quantification of BMDC engraftment in the brains of HI and TBI chimeras. Data are expressed as GFP^+ cells per area in three different brain regions (olfactory bulb, cortex and cerebellum) at 16 weeks after BMT. Data are means + SEM from 3-5 animals per group. n.d. = none detected. Statistical significance is indicated by asterisks (**p<0.01; ***p<0.001). **C**) Representative laser confocal microscopic images of ramified donor-derived GFP^+ cells in the brains of HI and TBI animals at 16 weeks after BMT. Note that GFP^+ cells were restricted to the cortex in the HI group, but distributed throughout the grey and white matter in TBI animals.

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irradiation plus rapamycin. In contrast to TBI or busulfan treatment, this conditioning regimen minimally disturbs the host's hematopoietic system and enables rapid transmigration of adoptively transferred myeloid cells into the CNS.

BMDCs have emerged as promising treatment vehicles in neurological and psychiatric disorders [6,14,18,19,22,23]. However, the transmigration of BMDCs into the brain is tightly regulated. In animal models, in which peripheral blood chimerism was obtained by parabiosis, no engraftment of

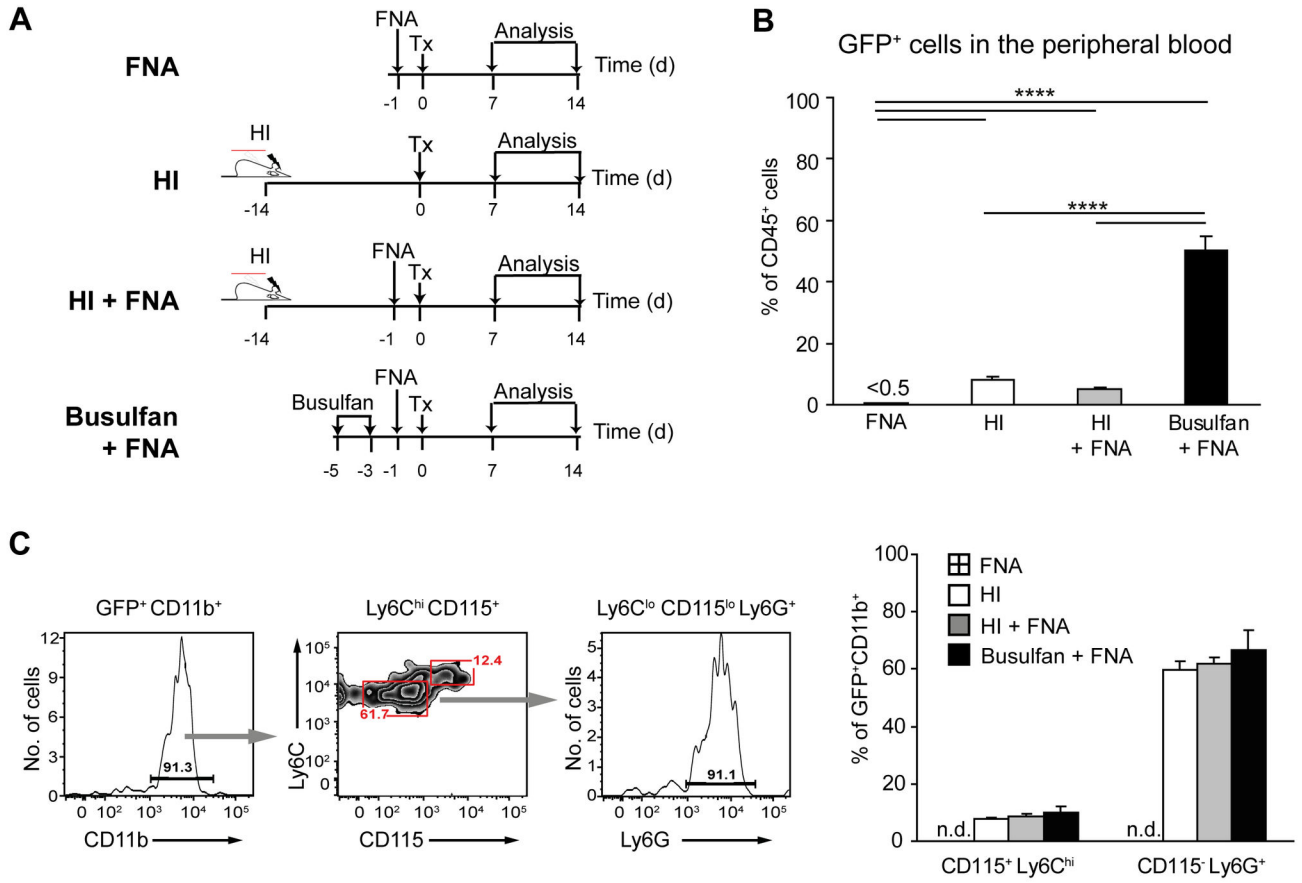

Figure 3. Blood chimerism in HI- and busulfan-conditioned mice with FNA. **A**) Overview of the experimental protocol. *HI*, *FNA* and *Tx* denote focal head irradiation, facial nerve axotomy and bone marrow transplantation, respectively. **B**) Flow cytometry of GFP expression in peripheral blood CD45$^+$ cells at 2 weeks after BMT. The level of chimerism was significantly higher in the busulfan + FNA group compared with the HI + FNA group. FNA had no impact on blood chimerism. Data are means + SEM from 3-5 animals per group. Statistical significance is indicated by asterisks (****p<0.0001). **C**) Flow cytometric characterization of GFP-expressing cells in peripheral blood at 2 weeks after BMT. The vast majority of GFP$^+$CD45$^+$ cells express CD11b (>90%). Gating of this cell population reveals predominantly CD115$^+$Ly6C^{hi}Ly6G$^-$ monocytes and CD115$^-$Ly6C$^+$Ly6G$^+$ neutrophils. Data are means + SEM from 3-5 animals per group. n.d. = none detected. No statistical differences were observed between the groups.

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BMDCs in the adult brain was observed [4]. Similary, no CNS engraftment of BMDCs was observed after irradiation with brain protection [4,21]. In contrast, TBI is a highly effective conditioning regimen for targeting BMDCs to the rodent brain [6,24,26].

In the past, TBI has played an important role in patients undergoing HSC transplantation. However, TBI may result in serious acute graft-versus-host disease (GVHD) causing transplant-related morbidity and mortality, as well as damage to non-target tissues, which may predispose to GVHD or enhance the clinical manifestations of acute GVHD [28-30]. Targeted irradiation with selective delivery of myeloablative doses to bone and marrow resulted in reduction of tissue damage and allowance for dose escalation compared with conventional TBI [28]. Thus, focal irradiation may serve as an alternative regimen for host conditioning that causes less irradiation/transplant-related morbidity and mortality. Furthermore, the availability of a computed tomography (CT) image-guided radiotherapy, such as helical tomotherapy, provides the opportunity to deliver highly conforming dose distributions to complex target shapes while simultaneously avoiding excessive doses to critical normal tissue [28].

In this study, we established a conditioning protocol in mice using targeted head irradiation that induced comparatively low inflammatory responses in the CNS despite cranial radiation doses which were identical to TBI. Moreover, HI minimally perturbed the host's hematopoietic compartment. In contrast to TBI and busulfan chemotherapy, the HI regimen achieved very low chimerism in peripheral blood. Nevertheless, HI conditioning enabled rapid and selective recruitment of myeloid cells to sites of brain damage. Note that HI alone did not trigger any CNS engraftment of BMDCs in the absence of additional

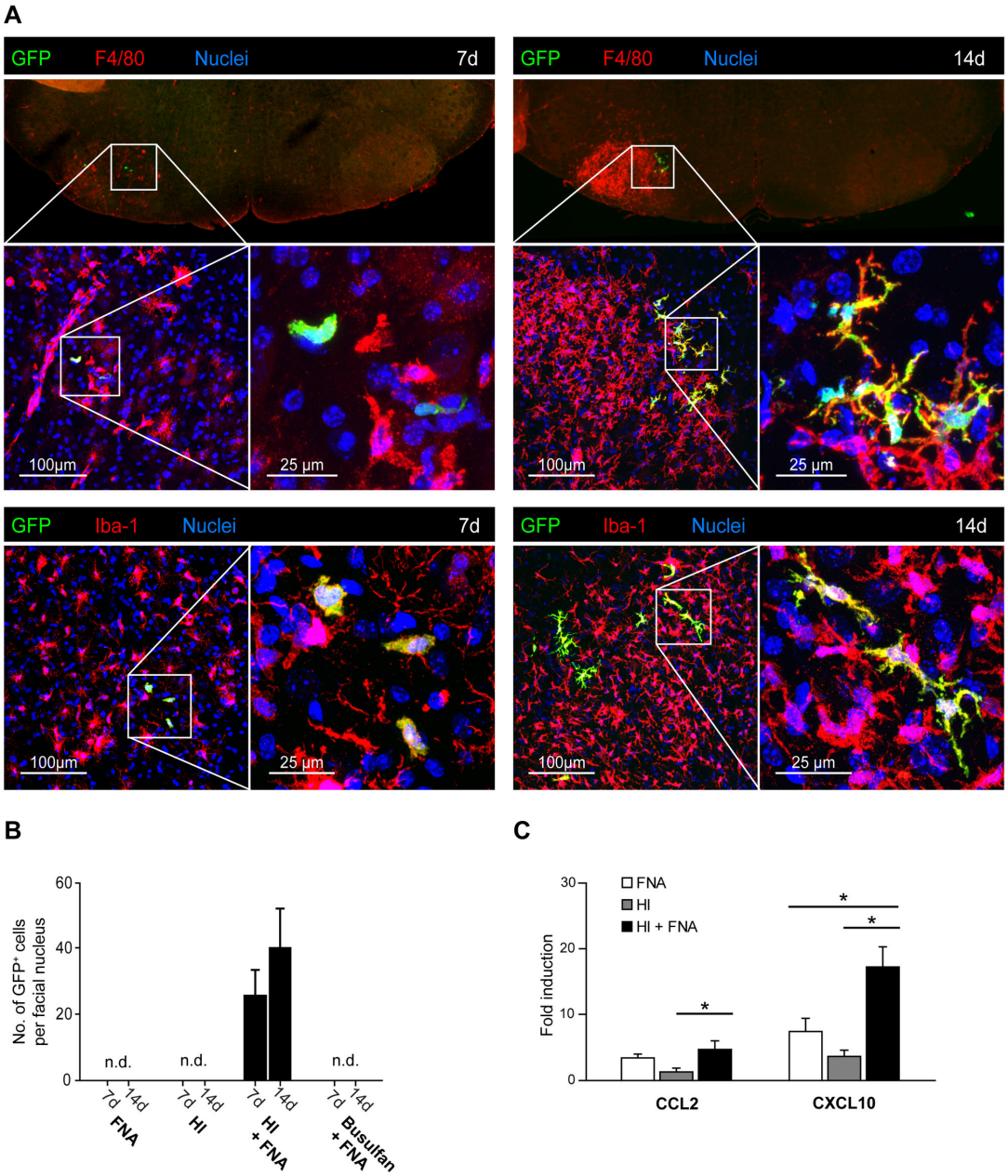

Figure 4. Selective engraftment of donor-derived myeloid cells in the lesioned facial nucleus after HI. **A**) Identification of GFP$^+$ myeloid cells in the lesioned facial nucleus of HI chimeras at 7 and 14 days after BMT. Note the increase in F4/80 immunoreactivity at day 14 compared with day 7, indicating increased inflammation. The contralateral unlesioned facial nucleus is devoid of donor-derived GFP$^+$ cells and shows minimal F4/80 immunoreactivity. Laser confocal microscopic images of areas of interest (white squares) are shown at increasing magnifications (scale bars: 100 µm – 25 µm). Seven days after BMT, amoeboid GFP$^+$F4/80$^+$ and GFP$^+$Iba-1$^+$ cells were detected in the lesioned facial nucleus. At 14 days after BMT, GFP$^+$F4/80$^+$ and GFP$^+$Iba-1$^+$ cells in the lesioned facial nucleus were highly ramified. All donor-derived GFP$^+$ cells expressed the macrophage markers, F4/80 and Iba-1. Nuclei were counterstained with DAPI. **B**) Quantification of myeloid cell engraftment in the lesioned facial nucleus at 7 and 14 days after BMT in FNA, HI, HI + FNA and busulfan + FNA animals. Note that donor-derived GFP$^+$ cells were only detected in HI + FNA mice. Data are means + SEM from 3-5 animals per group. n.d. = none detected. **C**) Quantitative real-time PCR of CXCL10 and CCL2 mRNA expression in the facial nucleus of animals with FNA (white bars), HI (grey bars) and HI + FNA (black bars) at 14 days after BMT. The mRNA expression levels were normalized to GAPDH mRNA and compared to naïve mice (fold induction). Increased chemokine mRNA levels were observed in the facial nucleus of HI + FNA mice compared to HI animals. Data are means + SEM from 3-5 animals per group. Statistical significance is indicated by asterisks (*p<0.05).

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brain damage. Engraftment occurred with a short latency after BMT (7 days). In contrast, busulfan treatment failed to target myeloid cells to the CNS within 14 days after BMT. Although myeloablative chemotherapy with busulfan is often used clinically in allogeneic HSC transplantation, the effectiveness of busulfan conditioning in triggering BMDC recruitment to the rodent brain remains controversial. Lampron et al. did not observe any donor-derived cells in the CNS after the administration of 80 mg/kg of busulfan plus 200 mg/kg cyclophosphamide before BMT [26]. Other reports showed reduced [24,31] or enhanced [32] BMDC engraftment compared with TBI using 80 mg/kg, 90 mg/kg and 125 mg/kg of busulfan, respectively. In our study, the myelosuppressive dose of 100 mg/kg busulfan failed to rapidly target BMDCs to the CNS within the first two weeks after BMT.

In order to prevent transplant rejection, we administered the immunosuppressant drug, rapamycin, in the FNA, HI, HI+FNA and busulfan+FNA groups. Thus, the differences in CNS engraftment of BMDCs between the groups cannot be attributed to rapamycin. Moreover, rapamycin has been used to stabilize the blood-brain barrier [33].

Cytokines and chemokines are among the signals which may mediate the transmigration of BMDCs into the brain. Notably, TBI and busulfan treatment were found to induce CCL2, CXCL10, CCL3, CCL5, TNF-α and IL-1 gene expression in the murine brain [21,24,32]. We observed that HI induces the same pattern of cytokines/chemokines, but to a much lower degree than TBI. CCL2 is *de novo* expressed in facial motoneurons after axotomy [34]. Interestingly, HI increases CCL2 mRNA expression in the axotomized facial nucleus, which may trigger myeloid cell engraftment.

The receptor for CCL2, CCR2, was suggested to be necessary for the egression of monocytes from the bone marrow (BM) to the spleen [35] and from the blood stream to the tissue [36], including the brain [21]. Results obtained in chimeric mice generated by TBI and transplantation of CCR2-deficient BM cells suggested that BM-derived myeloid cells in the adult brain originate from circulating $Ly6C^{hi}CCR2^{+}$ inflammatory monocytes [21]. In contrast, using parabiotic mice, Ajami et al. showed that the progenitors of BM-derived myeloid cells in the brain do not spontaneously enter the blood stream, but need to be artificially administered into the circulation. The authors proposed HSCs and CX_3CR1^{+} myelomonocytic progenitors (a mixture of non-self-renewing progenitor populations) as sources of microglia and brain macrophages, respectively [4,5]. However, these results were based on hematopoietic reconstitution of the TBI recipients by transplantation of partially purified BMDCs. Since selective transplantation of purified BMDC populations is not possible in a myeloablative setting, the precise characterization of the precursors of brain myeloid cells has posed a significant challenge. The optimized conditioning and transplantation regimen presented in this study allows for the first time to adoptively transfer selected BMDC populations into wild-type recipients and to perform short-term analysis of the transmigration of BMDCs into the CNS without hematopoietic reconstitution. Thus, the HI protocol provides a valuable tool for tracking the fate of short-lived BMDCs and for identifying the precursors of brain macrophages, a promising cell population for the treatment of neurodegenerative disorders.

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Author Contributions

Conceived and designed the experiments: CB JP. Performed the experiments: CB FFK NG SR. Analyzed the data: CB FFK NG SR JP. Wrote the manuscript: CB FFK JP.

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Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution.

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The study used multiple advanced techniques including single-cell RNA sequencing (scRNA-Seq), single-molecule FISH, immunohistochemistry and computational modelling to comprehensively characterize microglial heterogeneity both spatially and temporally. To investigate the single-cell transcriptional profiles, microglia were isolated from different regions of embryonic, juvenile and adult mouse brains. Transcriptomic landscapes were comparatively analysed using scRNA-Seq technique. The results demonstrated spatial and temporal heterogeneity of microglia across developmental stages. The transcriptomic landscape of microglia isolated from cerebellum was comparable over the course of development. In contrast, the microglial signatures elucidated in the cortex and hippocampus were different between embryonic and postnatal microglia. For example, the $CST3^{+}SPARC^{+}IBA1^{+}$ microglia subpopulation was prominent in the cortex of the postnatal brain, whereas the embryonic forebrain lacked of this population. The major microglial subset in embryonic forebrain was negative for both CST3 and SPARC expression. During demyelination and remyelination, disease-dependent microglial transcriptomic signatures were characterized by an induction of mRNA expression of *Apoe*, *Axl*, *Igf1*, *Lyz2*, *Itgax*, *Gpnmb* and *Apoc1*. Subsets that associated with demyelination ($SPP1^{+}CD74^{-}IBA1^{+}$ and $TMEM119^{-}CD74^{-}IBA1^{+}$) and remyelination ($SPP1^{-}CD74^{-}IBA1^{+}$ and $TMEM119^{-}CD74^{+}IBA1^{+}$) were identified. Analysis of human microglial signatures revealed similarities with gene expression profiles of mouse microglia in the homeostatic brain. However, different gene expression profiles between species were also identified, including the expression of *Ccl4* mRNA in human microglia but not in mouse cells. In the brains of patients with multiple sclerosis, disease-associated microglial subpopulations, which were distinct from homeostatic microglia, were identified. These particular microglial subtypes showed increased expression levels of *APOE, MAFB, CTSD, APOC1, GPNMB, ANXA2, LGALS1, CD74, SPP1* and *HLA-DR*, while microglial homeostatic markers such as TMEM119 were strongly reduced. The findings suggested heterogenous responses and/or regulation of the microglial population in the CNS, as well as immunoregulatory functions of microglia during CNS diseases.

2.2. Phenotypic and functional characterization of CNS cells utilizing mass spectrometry technique

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Human microglia regional heterogeneity and phenotypes determined by multiplexed single-cell mass cytometry.

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The cellular complexity of the CNS myeloid compartment has been extensively investigated, both in the context of cell ontogeny and phenotypic heterogeneity, as well as in response to brain pathology. However, these studies were mostly performed in the rodent models, and thus human CNS myeloid cells remained largely unexplored. Although some comparative analyses of the transcriptomic landscapes of microglia and CNS myeloid cells revealed similarities between mouse and man, species-specific patterns of gene expression, and differences in the responses of human and mouse microglia to altered CNS conditions were observed.

In this study, the comprehensive characterization of microglial phenotypes and regional heterogeneity was performed using high-dimensional single-cell cytometry by time-of-flight mass spectrometry (mass cytometry or CyTOF). Microglial characteristics were compared with other immune cells in the brain including perivascular macrophages. To do so, human microglia (huMG) were isolated from different brain regions of post-mortem brain tissues and from fresh brain biopsies, and at the same time peripheral immune cells were isolated from peripheral blood and cerebrospinal fluid (CSF). Subsequently, the isolated cells were fixed and cryopreserved according to a newly established protocol, which allowed for a long-term storage of huMG, which are otherwise strongly susceptible to cryopreservation-induced damage. To simultaneously determine multiple samples from different individuals and compartments, a cellular barcoding technology was applied for multiplexing samples prior to CyTOF analysis.

Based on the expression of 57 selected markers, human microglial core signature was identified, which were distinct from myeloid cells in the peripheral blood and CSF, as well as different from the infiltrating immune cells (**Figure 4**).

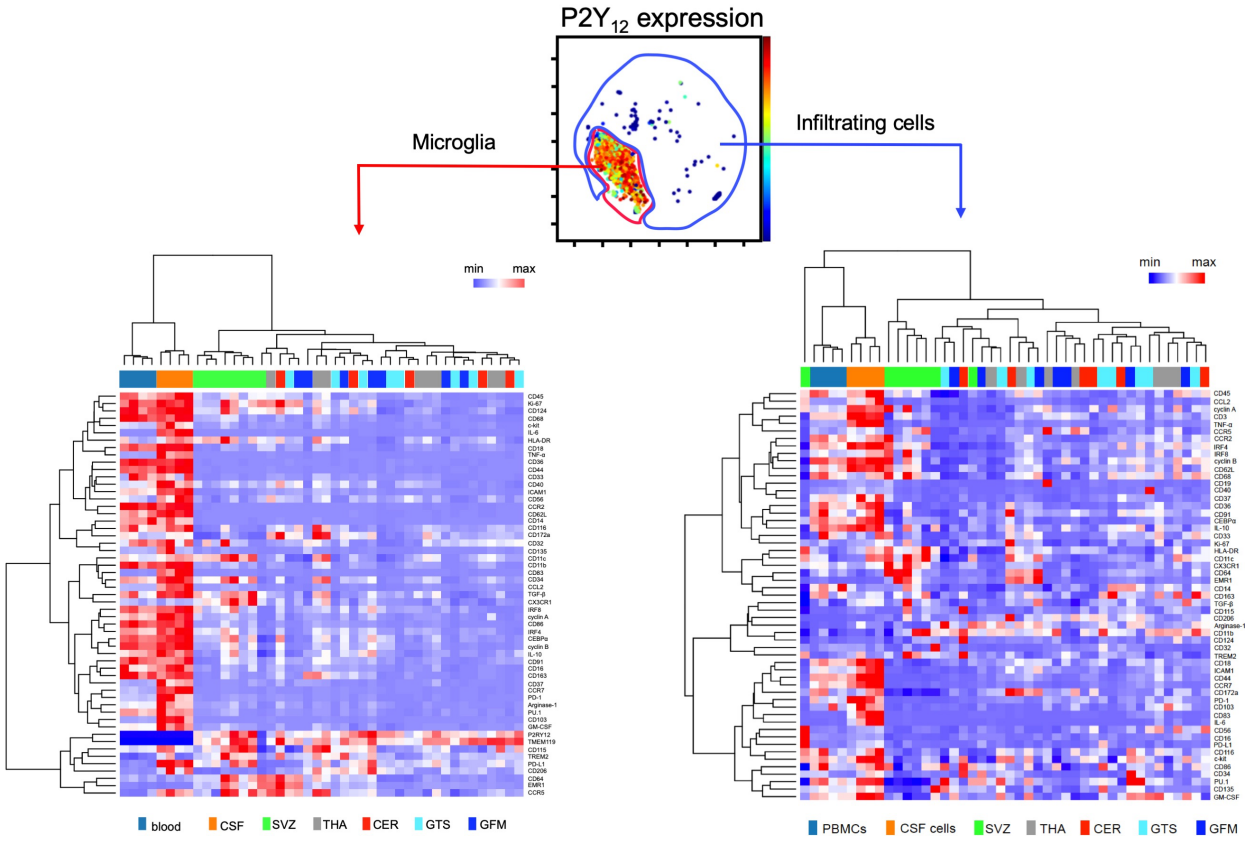

Figure 4 Representative two-dimensional t-SNE map of brain mononuclear cells (n = 36). Each dot represents one cell. The color spectrum represents expression of $P2Y_{12}$ (a microglia-specific marker; red denotes high expression, blue denotes no expression). $P2Y_{12}^{+}$ cells were gated as microglia (red gate) and $P2Y_{12}^{-}$ cells were gated as different infiltrating immune cells (blue gate). Heat maps and cluster analysis of all samples based on the mean expression of 57 markers. Similarities between PBMCs (blue), CSF cells (orange) and huMG (green), as well as the similarities between microglia from different brain regions (SVZ (bright green) = subventricular zone; THA (grey) = thalamus; CER (red) = cerebellum; temporal lobe (GTS, bright blue); frontal lobe (GFM, dark blue) samples and expression levels are indicated by dendrograms. Heat colours of expression levels have been scaled for each marker individually (to the 1st and 5th quintiles), while red denotes high and blue low expression.

Post-mortem huMG were phenotypically comparable to fresh biopsy huMG. Using algorithm-based data analysis, microglia clustered separately from perivascular brain macrophages, another cell type of CNS tissue resident macrophages, which are phenotypically very similar to microglia. Furthermore, regional heterogeneity of microglia in the human brain was identified. These findings are in line with the previous mentioned study (**2.1.3.**). Four region-dependent microglial subpopulations were identified. The microglial subpopulation that is predominantly found in the

subventricular zone (SVZ) and thalamus (THA) highly expressed CD11c, CCR5, CD45, CD64, CD68, CX3CR1, EMR1 and HLA-DR, whereas cortical microglia expressed higher levels of CD206 compared with the other subpopulations. Taken together, the identification of microglial heterogeneity in the human brain suggests region-specific functions and/or regional vulnerability to brain diseases, and thus encourages the development of the more specific therapeutic approaches targeting subpopulation of human microglia.

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How human neuroblastoma cells make morphine.

Proc Natl Acad Sci USA, 102:8495-500 (2005).

https://doi.org/10.1073/pnas.0503244102

Dynamic interactions between neurons and non-neuronal cells (in particular microglia) are necessary for CNS homeostasis and function. Microglia dynamically regulate neuronal activity and synaptic transmission by clearance of cellular and subcellular elements of damaged neurons and/or providing factors with trophic or neuroprotective properties, such as BDNF, ATP and glutamate. *Vice versa*, neuronal activity can modulate microglial behavior by secreting for example chemokines such as CCL21, CX3CL1. However, the molecular cues that regulate the dynamic interaction between both cell populations remain largely unknown, which has previously resulted from the lack of high-resolution analytical techniques to reveal dynamic biosynthesis of key regulators responsible for the neuron-glial crosstalk.

In this study, targeted metabolomic analysis was performed in human SH-SY5Y neuroblastoma cells, the cell line that was proven to be capable of synthesizing morphine [Poeaknapo et al. 2004]. The entire morphine biosynthetic pathway was investigated in SH-SY5Y cells by applying various isotope-labelled precursors of morphine including L-[1,2,3-$^{13}C_3$]- and [*ring*-2′,5′,6′-$^{2}H_3$]-dopa, [2,2-$^{2}H_2$]-dopamine, (*S*)-[1,3,4,-$^{2}H_3$]-norlaudanosoline, [7-^{2}H]-salutaridinol, [7-^{2}H,*N*-C$^{2}H_3$]-oripavine, and [6-^{2}H]-codeine (**Figure 5**).

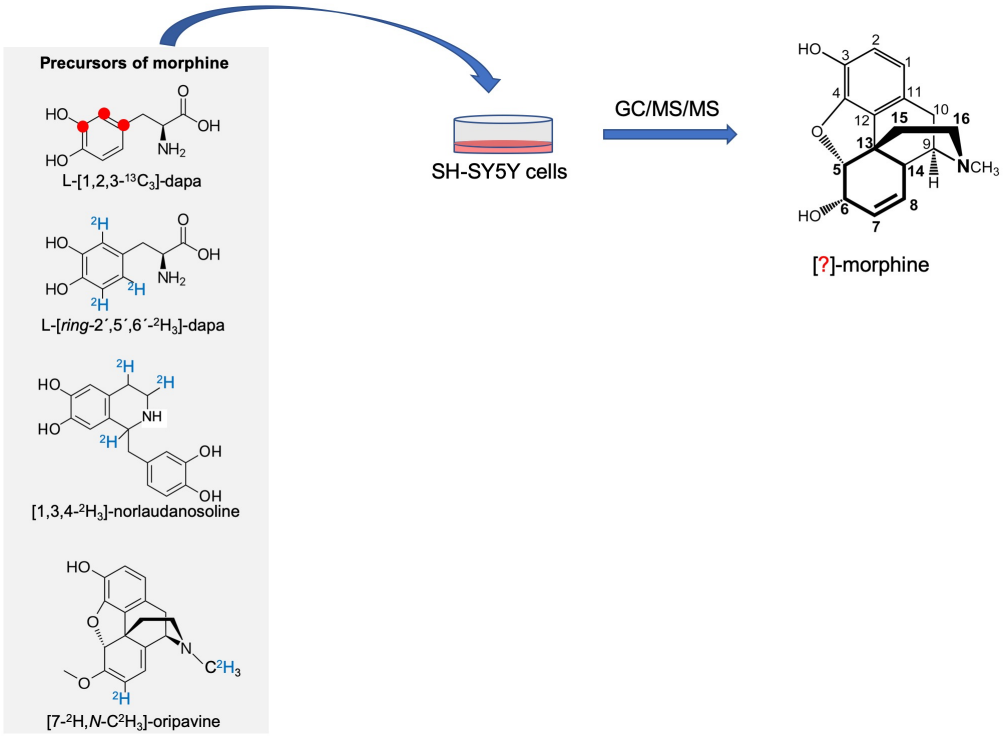

Figure 5 Schematic representation of stable isotope-resolved metabolomic experiments elucidating morphine biosynthesis in SH-SY5Y cells.

Five days after the feeding experiment, isotope-labelled morphine was isolated from SH-SY5Y cells and analysed by gas chromatography-tandem mass spectrometry (GC/MS/MS). Based on the positions where the applied isotopes incorporated into morphine, 19 chemical steps of morphine biosynthesis in human neuroblastoma cells were detected. Unlike morphine biosynthesis in the poppy plant, human morphine was synthesized via the tetraoxygenated initial isoquinoline alkaloid (*S*)-norlaudanosoline and not via trioxygenated (*S*)-norcoclaurine.

The study demonstrated the feasibility of studying targeted metabolomics in a human neuroblastoma cell line using an isotope-labelling approach, and thus serves as a platform for metabolomic analysis in primary neuronal or non-neuronal cells in the CNS at the homeostasis or during diseases.

3. Discussion

The CNS is a highly dynamic compartment with complex cellular networks. Apart from neurons, microglia and infiltrated immune cells play crucial roles in the regulation of CNS homeostasis and function. Microglia perform dynamic phenotypic and functional changes throughout the lifespan, depending upon the cue signals from other CNS cells. Better understanding characteristics of these cells and how they interact with each other may lead to more effective strategy for treatment development in neurological and psychiatric disorders.

Infiltrating immune cells integrate into the myeloid cell compartment of the retina and the brain

Adoptive transfer experiments of gene-modified bone marrow-derived cells (BMDCs) in mouse models of retinal [selected own works **2.1.1**] and brain [selected own works **2.1.2.**] degenerations performed in this study unequivocally provides evidence for capability of circulating cells from the periphery to engraft into the CNS, preferentially into the damage sites. These engrafted cells stably integrate into myeloid cell compartment of the CNS and express their transferred gene, even at more than one year after adoptive transfer. These findings suggest therapeutic potential of circulating cells like transplanted BMDCs as live Trojan horse for delivery of therapeutic gene to the damage sites of damage retina and brain. However, circulating cells have been shown to be able to trigger neuroinflammation and/or involve in resolution of neuroinflammation in CNS diseases [Schwartz & Baruch 2014; Ajami et al., 2011], and hence therapeutic effects of these engrafted bone-marrow-derived cells in CNS diseases remain to be precisely evaluated.

The other concern for clinical application of gene-modified BMDCs is the host-precondition and the route of administration, especially an application route for treatment in retinal degenerative diseases. Our study in mouse models of retinal degeneration [selected own works **2.1.1**] demonstrated that BMDCs engrafted only into particular layers of the retina, these were the inner (IPL) and outer (OPL) plexiform layers and the ganglion cell layer (GCL). None of them were detected in the photoreceptor cell layer, where the degeneration actually occurred, thus an alternative route of application remains to be investigated for treatment development. Furthermore, in mouse models, total body irradiation (with a lethal irradiation dosage) has been normally applied to precondition the host immune system prior to adoptive transfer of gene-modified donor-derived cells. It has already been demonstrated that BMDCs were not capable of entering the non-

conditioned brain, and thus required host-preconditioning [Ajami et al., 2007; Mildner et al., 2007]. This precondition leads to systemic collapse of host immunity, which avoids the immune rejection of the donor cells. However, this strategy is not a viable option for a clinical application in human patients. Herein, an alternative protocol for host-precondition that avoids a complete ablation of the host immunity was demonstrated. This non-myeloablative precondition using focal-head irradiation effectively targeted the BMDCs from the periphery to the damage brain. Although the number of engrafted cells were much lower compared to the classical precondition using lethally total body irradiation, the engraftment in this precondition model was more specific and restricted to the damage sites. Therefore, it may be used to precisely identify the cell population of BMDCs that are capable of entering the CNS. Whether this low number of infiltrating cells would effectively result in subtle therapeutic effects also remains further evaluation.

Spatial and temporal heterogeneity of microglia in homeostasis and during brain disease

Microglia are highly plastic cells. They provide multifaceted effects on neuronal as well as non-neuronal cells in homeostasis and during disease. In an *in vitro* environment, microglia can express a variety of neuroactive agents that are considered as pathological factors, including TNF-α or ligands for chemokine receptors such as CCR1, 3, 5, and 7 and CXCR1 or 3, but they can also express trophic factors like brain-derived neurotrophic factor (BDNF), the gaseous transmitter NO or neurotransmitters (ATP and glutamate) [Kettenmann et al., 2011; Biber et al., 2001; de Jong et al., 2005]. These substances rapidly modulate neuronal function and/or mediate the regulation of synapse integrity and plasticity in the healthy brain.

In the selected own work **2.1.3.**, we identified the spatial and temporal transcriptomic heterogeneity of mouse and human microglia using single-cell RNA sequencing. These findings were in line with the previous observations by Grabert et al. [2016], which were shown in the RNA-bulk system analysis that, under CNS homeostasis, various microglial subpopulations have different characteristics and differential functions in a region-specific manner. During diseases, microglia respond to the local inflammation and undergo activation, and thereby can either resolve the neuroinflammation or escalate the tissue toxicity and subsequently induce neuronal degeneration. In this study (**2.1.3.**), we showed that disease-associated microglial responses were also temporally heterogenous. Early after neurodegeneration induced by facial nerve axotomy, homeostatic microglial population rapidly changed their phenotypes to disease-specific signatures,

characterized by higher expression of *Apoe*, *Axl*, *Igf1*, *Lyz2*, *Itgax*, *Gpnmb* and *Apoc1*. At recovery phase, strongly reduced microglial activation was observed. Moreover, microglial signatures at this late disease phase was comparable to homeostatic signatures, thus confirmed high plasticity of microglial population. Similarly, we could also identify disease-specific microglial signatures in human patients with multiple sclerosis. However, in this study (**2.1.3.**), the correlation between phenotypic alteration of microglia and the number and phenotypic of infiltrating cells during disease remains undiscovered. Since circulating cells have been shown to be able to trigger neuroinflammation and/or involve in resolution of neuroinflammation in CNS diseases [Schwartz & Baruch 2014; Ajami et al., 2011], investigating the correlation between changes in microglial subpopulations and infiltrating cells may lead to better understanding of disease pathogenesis and/or regulation. Nonetheless, microglial heterogeneity at the single-cell protein level remains to be elucidated.

Elucidating CNS cell phenotype and function using mass spectrometry techniques

In the selected own work **2.2.1.**, mass cytometry (CyTOF) was used to simultaneously characterize circulating immune cells in the peripheral blood and cerebrospinal fluid, the infiltrating immune cells in the brain and the perivascular brain macrophages, in comparison to the parenchymal microglia isolated from different brain regions. In this study (**2.2.1.**), we confirmed unique microglial signature, which was distinct from peripheral and infiltrating immune cells. Moreover, similar to the selected own work **2.1.3.**, regional microglial heterogeneity could be confirmed by CyTOF analysis. However, it remains to be investigated whether these proteomic signatures of microglia can be rapidly altered during disease, as it has been observed at the transcriptomic signatures (selected own work **2.1.3.**). For the first time, CyTOF allows a comprehensive investigation of multiple cell subtypes at the single-cell protein level. Although these results were in line with and nicely complement the previously reported transcriptomic profiles in the human system, many transcriptomic signatures could not yet be proven, which largely due to the limitation of availability of CyTOF antibodies and stable metal isotopes. Adding more antibodies for the characterization of other CNS cells such as anti-NeuN antibody for neurons, anti-GFAP and anti-GLAST antibody for astroglia, or anti-CD31 (or platelet endothelial cell adhesion molecule-1, PECAM-a) antibody for endothelial cells can expand the phenotypic spectrum of detected cell types. However, finding a validated protocol for simultaneous isolation of all different cell types

from the brain tissue or the retina, and optimizing a staining protocol that is suitable for all cell types of interest are major challenges of such objectives. Open questions also include the spatial characteristics and dynamic crosstalk between each cell population in the CNS. Imaging mass cytometry (IMC) has recently become commercially available and may serve as the technique of choice for uncovering spatial phenotypic and functional heterogeneity in the CNS, since this technique allows multi-parameter profiling of a wide spectrum of cell types on tissue sections using the same principle as mass cytometry. In IMC, a total of about 37 markers can be simultaneously determined on the brain tissue section [Giesen et al., 2014].

Yet phenotypic profiles *per se* merely outline the characteristics of the cells, whereas metabolomics reflect dynamic responses of living cells, which can be rapidly altered to maintain their energy, cycling and communication with others in the complex system. Thus, metabolomics reveal gene expression products, protein outputs and metabolites/small molecules reflecting their responses to environment changes. Exquisitely sensitive methods are required to quantify rare compounds whose abundances can change rapidly in a living system. In this study (selected own work **2.2.2.**), a stable isotope-labelling approach was successfully used for metabolomic analysis of human neuronal cells. This methodology can be optimized for *in vitro* metabolomic studies of other CNS cell types, both cell lines and isolated primary cells. Although it is still limited as a bulk system analysis, this method complements the results obtained from transcriptomic analysis (such as single-cell RNA-sequencing) and phenotypic profiling, for example utilizing mass cytometry. And *vice versa* the transcriptomic analysis and/or phenotypic profiling at the single-cell level can be used to identify the targeted metabolites and their precursors a priori. Nonetheless, concerns should be taken into account in the context of differential characteristics of the targeted cells *in vitro* and *in vivo*, which could lead to differences in phenotypes and metabolomics between cells from different experimental conditions.

Perspectively, it is important to further explore this CNS myeloid cell compartment under disease conditions along with neurons and other non-neuronal cells in the CNS, *how they interact with each other*, *how they response to disease* and *how they regulate or obliterate CNS homeostasis during pathology*. In sum, comprehensive analysis using *in vivo* isotope-resolved metabolomics (**Figure 6A**) and single-cell multiplexed mass cytometry of the isolated CNS cell populations (**Figure 6B**) will provide invaluable information on dynamic interaction between multiple cell types in homeostasis and during diseases. The obtained results can be then confirmed, for example,

in *in vitro* isotope-resolved metabolomic experiment with and without co-culturing between different cell types (**Figure 6C**). Although the *in vitro* system could not completely represent the *in vivo* conditions, it provides possibility to directly study interaction (especially cell-cell signalling) between two or more CNS cell population under modified conditions. Finally, to achieve subcellular spatial information of dynamic changes detected, imaging mass cytometry (Hyperion) can be applied on the brain (or the retina) tissue to investigate up to 37 markers simultaneously.

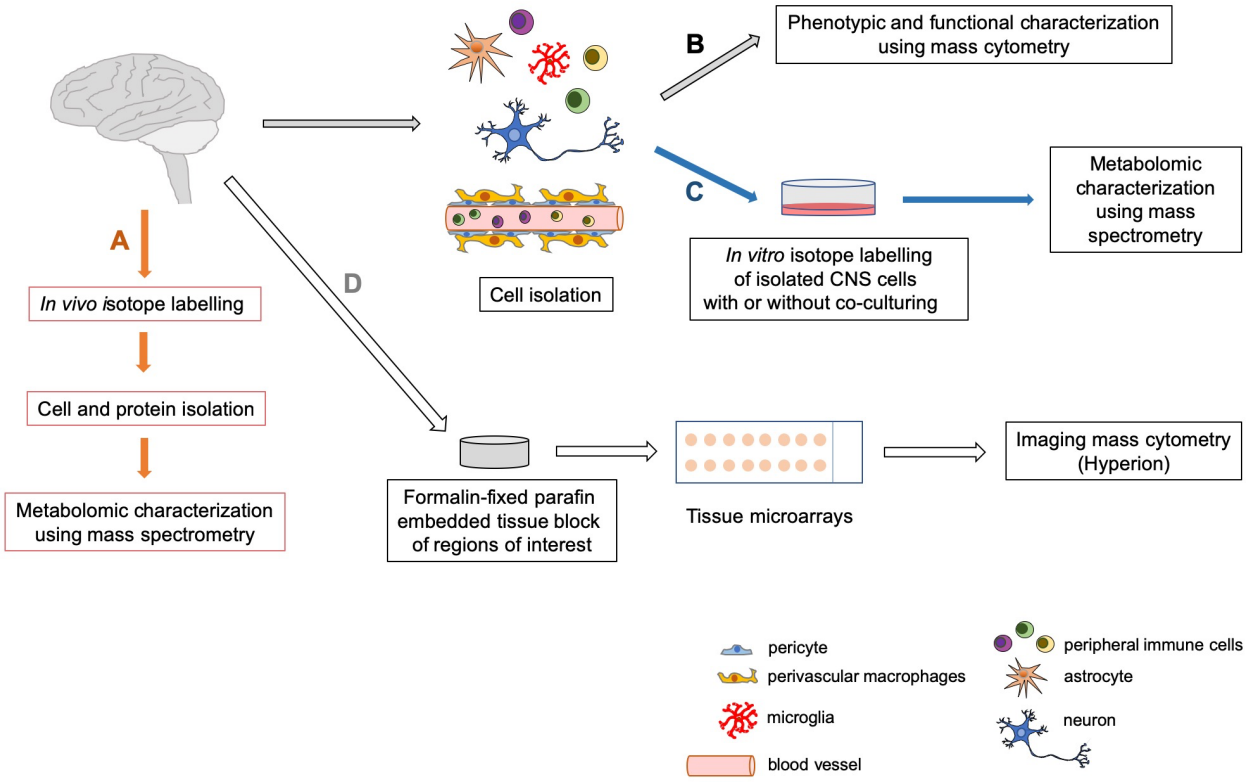

Figure 6 Suggestive combination of mass spectrometry techniques for simultaneous characterization of multiple cell types of the CNS. (**A**) Isotope labelling can be performed *in vivo* (for example in patients with brain tumour prior to tumour biopsy [Charidemou et al., 2017]). CNS cells can be then isolated and analysed for their metabolomic characteristics using mass spectrometry. (**B**) Multiple isolated CNS cell populations including perivascular macrophages, microglia, astrocytes and neurons can be directly and simultaneously characterized by mass cytometry (CyTOF). (**C**) Isolated CNS cells can be cultured *in vitro* for further metabolomic characterization using mass spectrometry. Using this experimental approach two or more cell types can be co-cultured for further metabolomic analysis. (**D**) High-dimensional immunohistochemistry (for determination of up to 37 markers on one tissue section) of formalin-fixed paraffin embedded (or frozen) brain tissue can be performed to obtain multiplexed data with subcellular spatial resolution.

4. Conclusion

In sum, this study combined multiple techniques including flow cytometry, immunohistochemistry, RT-PCR and mass spectrometry to comprehensively characterize CNS microglia/myeloid cell compartment in mouse and man. The findings confirmed the complexity of this cell population in the CNS comprising various cell types of both CNS-resident cells and infiltrating immune cells. Our results suggested their importance in the regulation of CNS homeostasis. Furthermore, we provided herein evidence for possibility to use infiltrating myeloid cells as cellular vehicle to deliver therapeutic gene/protein to the sites of brain lesion.

We demonstrated the power of two mass spectrometry techniques, *mass cytometry* and *stable isotope-resolved metabolomics* for comprehensive elucidation of CNS cell phenotypes and functions, both in *in vitro* and *in vivo* system. The combination of both mass spectrometry techniques, as well as the novel imaging mass cytometry, for phenotypic and functional elucidation will make the leap to unravel complex systems biology of the CNS cellular compartment. In addition, beyond the scope of this study, the number of available high-throughput techniques that can be applied for simultaneous characterization of various CNS cell populations (such as the cutting edge technology of single-cell metabolomics that may make the leap to unravel [Fessenden 2016]) has already increased. Again, the combination of these techniques is required to provide unprecedented insights into the complex biology of the CNS.

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
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- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

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