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Different means of glial activation in neurodegenerative diseases

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Summary

Neurodegenerative disorders, such as multiple sclerosis (MS), are described as one of the leading cause for clinical disabilities and death worldwide. Besides peripheral immune cells, activated glial cells are a relevant effector in the pathology of neurodegenerative diseases. However, the pathomechanism and etiology of neurodegenerative disorders are still far from being understood. So far, immune inhibitory therapies were shown to improve disease symptoms in the short term, but effectiveness decreases in the long run. To this end, there is a clear scientific need to further characterize neurodegenerative diseases in order to establish new therapeutic approaches. Furthermore, recent findings have demonstrated that human endogenous retroviruses (HERVs), such as HERV-W, might play a regulatory role in the onset and progression of neurodegenerative diseases. In general, HERVs are endogenous viral entities that were integrated into the genome of superior primates million years ago. Especially the envelope protein (ENV) is regularly described to induce immune activation and inflammation, particularly in MS.

This thesis therefore aims to identify new mechanisms in the complex pathology of neurodegenerative diseases, by characterizing different aspects of glial activation, primarily in reactive microglial and astroglial cells. Furthermore, a contribution to the understanding on HERV's mode of action in disease onset and progression will be made, focusing on the characterization of reactive astroglial and microglial cells, but also on myelination and neurodegeneration.

In order to improve the understanding of the contribution of glial activation to neurodegenerative diseases, different MS mouse models were analyzed immunohistochemically. Additionally, diverse primary glial cell cultures experiments were performed in order to gain knowledge on the polarization mechanisms of neuroglia in the context of neurodegeneration but also in the context of HERVs. In order to characterize the effects of HERV-W ENV in MS, a novel HERV-W ENV expressing transgenic mouse line was established and different MS mouse models were applied, in which the four major cell populations: microglia, astrocytes, oligodendrocytes and oligodendroglial precursor cells (OPCs) were analyzed.

Initially, reactive microglia in the enlarged choroid plexus of MS patients as well as of murine MS models were found indicating that activated microglial cells play important roles in the choroid plexus enlargement and blood-brain-barrier integrity. Furthermore, siponimod, an orally administered drug used to treat progressive forms of MS, was shown to modulate microglial activation to an immune modulatory phenotype. Regarding the activation of HERV expression in health and diseases, it was found, that these viral entities are buried behind a

strong epigenetic barrier that might lead to more complex activation mechanisms. Before HERV expression can be initiated, these epigenetic barriers need to be unlocked and then might lead to the onset of neurodegenerative diseases. Lastly, HERV-W could be shown to block OPC differentiation in a direct and/or indirect manner, as HERV-W induced microglial and astroglial activation *in vitro* and *in vivo*, resulting in increased demyelination and neurodegeneration.

This thesis therefore provides new insights into the complex pathomechanisms of glial activation in neurodegenerative disorders. Both, the microglial contribution to the loss of blood-brain-barrier (BBB) integrity as well as the immune modulatory effects of siponimod, display new findings that improve our understanding in the role of glial cells in neurodegenerative diseases. Furthermore, a long expected functional proof on HERV-W's mode of action in the context of MS is offered as a strong overall glial activation that is associated with the occurring demyelination and neurodegeneration could be identified. The here presented data about HERV-W's mode of action provide a proof of principle for the recently completed phase 2b clinical study (ANGEL-MS). Similar to the here presented results, the outcome of the clinical trial using a neutralizing anti-HERV-W ENV antibody (Temelimab) also suggested glial pathological reactions to be responsible for the reduced brain atrophy rates as well as preserved myelin integrities that was observed.

Zusammenfassung

Neurodegenerative Erkrankungen, wie Multiple Sklerose (MS), gelten weltweit als eine der häufigsten Ursachen für klinische Behinderungen und Todesfälle. Neben peripheren Immunzellen sind aktivierte Gliazellen ein relevanter Effektor in der Pathologie neurodegenerativer Erkrankungen. Der Pathomechanismus und die Ätiologie neurodegenerativer Erkrankungen sind jedoch noch lange nicht aufgeklärt. Bisher zeigte sich, dass immunhemmende Therapien die Krankheitssymptome kurzfristig verbessern, die Wirksamkeit allerdings langfristig abnimmt. Aus diesem Grund besteht ein klarer wissenschaftlicher Bedarf, neurodegenerative Erkrankungen weiter zu charakterisieren, um neue Therapieansätze zu etablieren. Darüber hinaus haben neuere Erkenntnisse gezeigt, dass humane endogene Retroviren (HERVs), wie HERV-W, möglicherweise eine regulatorische Rolle bei der Entstehung und dem Fortschreiten neurodegenerativer Erkrankungen spielen. Im Allgemeinen handelt es sich bei HERVs um endogene virale Entitäten, die sich vor Millionen Jahren in das Genom höherer Primaten integrierten. Vor allem das Hüllprotein (ENV) wurde regelmäßig beschrieben Immunaktivierung und Entzündungen zu induzieren, insbesondere bei MS.

Ziel dieser Arbeit ist es daher, neue Mechanismen in der komplexen Pathologie neurodegenerativer Erkrankungen zu identifizieren, indem verschiedene Aspekte der glialen Aktivierung, vor allem in reaktiven Mikroglia- und Astrogliazellen, charakterisiert werden. Darüber hinaus soll ein Beitrag zum Verständnis der Wirkungsweise von HERVs bei Krankheitsentstehung und -progression geleistet werden, wobei der Schwerpunkt auf der Charakterisierung reaktiver Astroglia- und Mikrogliazellen, aber auch auf Myelinisierung und Neurodegeneration, liegt.

Um das Verständnis des Beitrags der glialen Aktivierung zu neurodegenerativen Erkrankungen zu verbessern, wurden verschiedene MS-Mausmodelle immunhistochemisch analysiert. Darüber hinaus wurden diverse Experimente mit primären Gliazellkulturen durchgeführt, um Erkenntnisse über die Polarisationsmechanismen von Neuroglia im Kontext von Neurodegeneration, aber auch im Kontext von HERVs, zu gewinnen. Um die Auswirkungen von HERV-W ENV bei MS zu charakterisieren, wurde eine neuartige HERV-W ENV-exprimierende transgene Mauslinie etabliert und verschiedene MS-Mausmodelle angewendet, in denen die vier Hauptzellpopulationen Mikroglia, Astrozyten, Oligodendrozyten und oligodendrogliale Vorläuferzellen (OPCs) analysiert wurden.

Zunächst wurden reaktive Mikroglia im vergrößerten Plexus choroideus von MS-Patienten sowie in murinen MS-Modellen gefunden, was darauf hindeutet, dass aktivierte Mikrogliazellen eine wichtige Rolle bei der Vergrößerung des Plexus choroideus und der

Integrität der Blut-Hirn-Schranke spielen. Darüber hinaus wurde gezeigt, dass Siponimod, ein oral verabreichtes Medikament zur Behandlung progressiver Formen der MS, die Mikroglia-Aktivierung zu einem immunmodulatorischen Phänotyp moduliert. Im Hinblick auf die Aktivierung der HERV-Expression bei Gesundheit und Krankheiten wurde herausgefunden, dass diese viralen Einheiten hinter einer starken epigenetischen Barriere verborgen sind, die zu komplexeren Aktivierungsmechanismen führen könnten. Bevor die HERV-Expression eingeleitet werden kann, müssen diese epigenetischen Barrieren durchbrochen werden, was dann zum Ausbruch neurodegenerativer Erkrankungen führen kann. Schließlich konnte gezeigt werden, dass HERV-W die OPC-Differenzierung auf direkte und/oder indirekte Weise blockiert, da HERV-W *in vitro* und *in vivo* eine mikrogliale und astrogliale Aktivierung induzierte, was zu einer erhöhten Demyelinisierung und Neurodegeneration führte.

Diese Arbeit liefert daher neue Einblicke in die komplexen Pathomechanismen der glialen Aktivierung bei neurodegenerativen Erkrankungen. Sowohl der mikrogliale Beitrag zum Verlust der Blut-Hirn-Schranken (BHS)-Integrität als auch die immunmodulatorischen Wirkungen von Siponimod stellen neue Erkenntnisse dar, die unser Verständnis der Rolle von Gliazellen bei neurodegenerativen Erkrankungen verbessern. Darüber hinaus wird ein seit langem erwarteter funktioneller Beweis für die Wirkungsweise von HERV-W im Zusammenhang mit MS erbracht, da eine starke allgemeine gliale Aktivierung identifiziert werden konnte, die mit der vorkommenden Demyelinisierung und Neurodegeneration verbunden ist. Die hier präsentierten Daten zur Wirkungsweise von HERV-W liefern einen Grundsatzbeweis für die kürzlich abgeschlossene klinische Phase-2b-Studie (ANGEL-MS). Ähnlich wie die hier präsentierten Ergebnisse deutete auch das Ergebnis der klinischen Studie mit einem neutralisierenden Anti-HERV-W-ENV-Antikörper (Temelimab) darauf hin, dass gliale pathologische Reaktionen für die verringerten Hirnatrophieraten sowie die beobachtete Erhaltung der Myelinintegrität verantwortlich sein könnten.

Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ALV	avian leucosis virus
ApoE	apolipoprotein E
APP	amyloid precursor protein
ASCT1	neutral amino acid transporter A
ASCT2	neutral amino acid transporter B(0)
Aβ	β-ameloid
BBB	blood-brain-barrier
BD	bipolar disorder
C1qa	complement C1q subcomponent subunit A
C3	complement C3
CD	cluster of differentiation
ChP	choroid plexus
CIDP	chronic inflammatory demyelinating polyneuropathy
Clec7a	C-type lectin domain family 7 member A
CNS	central nervous system
CPZ	cuprizone
Cre	Cre recombinase
CSF	cerebrospinal fluid
DAM	disease associated microglia
EAE	experimental autoimmune encephalomyelitis
EBV	Eppstein-bar virus
ENV	envelope protein
ERV	endogenous retroviruses
FUS	RNA-binding protein FUS/TLS
GM	grey matter
HERV	human endogenous retrovirus
HHV	human herpesvirus
HIV	human immunodeficient virus
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
iNOS	inducible NO synthetase
ITAM	immunoreceptor tyrosine-based activation
Lcn	lipocalin
LINE	long interspersed nuclear elements
LONG	long interspersed nuclear elements
LPS	lipopolysaccharide
LTR	long terminal repeats
MBP	myelin basic protein
MCT	monocarboxylat-Transporter
MFSD	major facilitator superfamily domain-containing protein
MHC	major histocompatibility complex
MLV	murine leukemia virus
MOG	myelin oligodendrocyte glycoprotein
MRI	magnet resonance imaging
MS	multiple sclerosis
MSRV	multiple sclerosis associated retrovirus
NF-H	neurofilament heavy chain
NF-κB	nuclear factor kappa light chain enhancer of activated B-cells
NG2	nerve/glial antigen 2
NO	nitric oxide
NSC	neural stem cell
OPC	oligodendroglial precursor cell
PD	Parkinson's disease
PET-CT	positron emission tomography-computed tomography
PNS	peripheral nervous system
PP	primary progressive
PSEN	presenilin
ROS	reactive oxygen species
RR	relapse-remitting
RT	reverse transcriptase
S1PR	sphingosine-1-phosphate receptor
SAg	superantigen
SCZ	schizophrenia
SINE	short interspersed nuclear elements
SOD	superoxide dismutase 1
SP	secondary progressive
SYK	spleen associated tyrosine kinase
SYP	synaptophysin
TDP	TAR DNA binding protein
TE	transposed elements
Th1	T helper cell type 1
TLR	toll-like receptor
TNF	tumor necrosis factor
WM	white matter

Content

1. Introduction

1.1 Cellular composition of the central nervous system

The mammalian nervous system can be divided into a peripheral and a central nervous system (PNS and CNS, respectively). The PNS senses environmental and organ specific stimuli and forwards the information via afferent nerve fibers to the CNS, where the information is processed. Subsequently, efferent signals propagate back into the periphery, transmitting the processed information back to the muscles and organs. The CNS consists of the eye, spinal cord and cerebrum, which can be furthermore classified into multiple sub regions such as the cerebellum, hippocampus, corpus callosum, motor- and sensory cortex. Although in both compartments, information are forwarded by electro-chemical signaling, the cellular composition is different. While the PNS is mainly composed of neurons and Schwann cells, the CNS consists of a variety of different cells types. A fact that was first recognized in the 20th century by Golgi and Cajal, two neuroanatomists that were awarded with the Nobel Prize for Physiology or Medicine in 1906. For the first time, they divided central nervous system cells into neurons and a “variety of supporting cells”. These cells are meanwhile known as neuroglia that can be subdivided into micro- and macroglia, while the latter can be further differentiated in oligodendrocytes, NG2-glia and astrocytes.

Neurons are typically characterized by a cell body harboring the nucleus and other fundamental organelles, a connected dendritic tree and often a single axon that transmits the electrochemical signal to other neurons or muscles. The dendritic tree but also the cell body itself serves as input regions to receive information from neighboring neurons. This signal transmission between neurons is conducted by so-called synapses, membrane protuberances, which are designed to release neurotransmitters from the presynapsis that eventually bind to specific postsynaptic receptors, initiating downstream signal transmission. This downstream signaling leads at the axon hillock to action potential generation, characterized by the active depolarization of the membrane. The resulting electric signal will then be propagated along the axon and might lead to the release of neurotransmitters at the next synapse. In order to conduct complex brain functions, the propagation of action potentials needs to be quick, which can be ensured by an increased axon diameter as seen in giant squids or by a so-called saltatory signal transduction (Zalc et al., 2008). The saltatory signal transduction is warranted due to the insulation of axons with myelin sheaths produced by oligodendrocytes or Schwann cells (Cohen et al., 2020).

In the CNS, mature oligodendrocytes extend multiple processes, each of which repeatedly enwraps axonal segments leading to a dense insulation, which manly consist of lipids and

myelin proteins (Bunge, 1968). Of note, the equivalent of axon-ensheathing cells in the PNS are Schwann cells, which in contrast to oligodendrocytes, are only capable of ensheathing a single axon. Besides the isolating role of oligodendrocytes, it is meanwhile known that these cells also support the neuronal integrity as well as facilitate trophic support (Nave, 2010). In the adult CNS of vertebrates, regions can be classified as grey and white matter (GM and WM respectively). In this context, the WM mainly consist of myelinated axons and insulating oligodendrocytes, which is leading to the “white” color. In contrast, the unmyelinated cell bodies of neurons are resident in the GM.

In general, the regeneration of the CNS is very limited. Although, recent studies have shown that the number of oligodendrocytes continues to expand until adulthood, indicating that the formation of newly generated oligodendrocytes is continuously taking place (Hill et al., 2018), the regeneration of functional oligodendrocytes but also neurons in a damage scenario is inefficient (Fawcett, 2020). This leads to certain problems, since the loss of function often cannot be restored completely or only very slowly. However, when the renewal of myelination is taking place the primary source may be oligodendrocyte precursor cells (OPCs) or nerve/glial antigen (NG2)-glia. OPCs or NG2-glia are a very dynamic cell type that has the capability to self-renew but also to differentiate into other CNS cells. In contrast to oligodendrocytes, OPCs can migrate throughout the whole brain and are equally present in all brain regions. In the adult brain, OPCs only give rise to oligodendrocytes, however, during development of the brain, as well as *in vitro* it is described that NG2-glia can also give rise to astrocytes (Dimou & Gallo, 2015). Although this function will eventually get lost during oligodendrocyte differentiation, OPCs are, in general, immune competent cells as they express a variety of immune regulatory proteins including pattern recognition receptor toll-like receptor (TLRs), antigen presenting proteins such as major histocompatibility complex (MHC) class I and II and are also capable to phagocytose (Falcao et al., 2018; Kirby et al., 2019). In the event of an infection or injury, OPCs are therefore able to react by the expression of various cytokines and chemokines and thereby get recruited to the injury site, which induces the migration, proliferation and eventually differentiation into newly myelinating oligodendrocytes (Zeis et al., 2016).

Astrocytes are the second major macroglial cell type that provide structural and metabolic support to neurons. Furthermore, by contributing to the function of synapses as well as in the formation of the blood-brain-barrier (BBB; Sofroniew & Vinters, 2010), astroglia play important roles in the development of the CNS. Similar to OPCs, astroglial cells are evenly distributed in the brain and spinal cord, however, already in the late 19th century, astrocyte morphologies have been characterized into two main subtypes: protoplasmic and fibrous. These two main types keep their validity as protoplasmic astrocytes can be found in the gray matter, exhibiting

a morphology of numerous stem branches that branch out to many finely branching processes in an even globoid distribution and fibrous astrocytes can be found throughout all white matter, displaying a morphology of many long fiber-like processes (Sofroniew & Vinters, 2010). In contrast to the healthy CNS, astroglial morphology is characterized by a larger cell body and a thickening of processes in disease states often referred as astrogliosis and/or glial scar (Zhou et al., 2019). This already indicates that astroglia are immune competent cells and they express a variety of receptors involved in innate immunity, such as TLRs, nucleotide-binding oligomerization domains, double-stranded RNA-dependent protein kinase, scavenger receptors, mannose receptor etc. (Farina et al., 2007). Furthermore, astrocytes are a major source of the complement system proteins, such as of the third complement component (C3) and they stand out as a very prominent player of the complement-mediated innate immune processes (Pekna & Pekny, 2021). These pathways can get activated by damage and/or infection, leading to a change in astroglial polarization that is often referred as reactive astroglia. In the past, these activated astrocytes were characterized in anti-inflammatory/neuroprotective, A2 astrocytes and pro-inflammatory/neurotoxic A1 astrocytes. However, it is meanwhile proposed that the boundaries of this classification are fluent (Escartin et al., 2021) – probably an achievement of the advances in transcriptome analysis.

Microglial cells are the macrophages of the CNS and their main task is the constant surveying and scavenging of CNS tissue. They have a ramified morphology, a small soma with fine cellular processes in the healthy mature CNS and, similar to OPCs and astrocytes, are evenly distributed in all regions of the CNS. In contrast to neurons and macroglia, they are of mesodermal origin and originate from the yolk sac. Microglia enter the brain during prenatal development before the BBB is closed and instead of constantly being replaced by myeloid progenitor cells, they maintain their *status quo* by itself. Of note, in the mid-20th century Sir Peter Medawar showed, that the BBB limits the entrance of immune cells by their adhesion molecules, cytokines and chemokines, and their receptors (Carson et al., 2006; Owens et al., 2008), which first described the so-called “immune privilege” of the CNS. Since then, microglia are described as the primary immune cells of the CNS. A cardinal dogma that has been revised, as multiple studies were able to provide evidences that the CNS is no longer an immune privileged organ (Negi & Das, 2018), although microglia still represent the majority of immune cells within the CNS. To this end, infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity, anything that leads to the disturbance or loss of brain homeostasis evokes a rapid and profound change in the microglial cell shape, gene expression and the functional behavior, which summarily is defined as “microglial activation” (Block et al., 2007; Colton & Wilcock, 2010; Streit et al., 2005). Phenotypically, the complexity of the cellular processes is reduced, a microglial morphology that is referred to as amoeboid.

Furthermore, microglial cells can become motile and actively migrate towards a lesion or herd of infections following chemotactic gradients. In the past, this activated microglia were classified by M1 (pro-inflammatory), M2 (anti-inflammatory) and resting microglia, however also this classification is meanwhile outdated (Ransohoff, 2016). Instead, large scale transcriptome analyses are continuously uncovering of disease specific as well as physiological microglial genetic signatures (Paolicelli et al., 2022; Stratoulias et al., 2019; Wishart et al., 2023) and future publications will show whether a particular key disease signature can be established.

1.2 Neurodegenerative diseases

Neurodegenerative disease are characterized by a progressive loss of neural function most often as a result of neuronal cell death within the CNS. Since the mammalian CNS has lost most of its ability to regenerate damaged neurons and severed axons (reasons for this are reviewed by (Blackshaw, 2022)), neurological deficits in human often show only limited improvements over time, resulting in permanent clinical disabilities and progressive worsening. In general, neurodegenerative disorders are often classified by their primary pathological characteristic, leading to the scientific characterization of amyloidoses, primarily associated with prion disease and Alzheimer's disease (AD); taupathies, also characterized by AD; synucleinopathies, associated with Parkinson's disease (PD) and TAR DNA-binding protein (TDP)-43 proteinopathies, which is associated with amyotrophic lateral sclerosis (ALS; Dugger & Dickson, 2017; Mey et al., 2023). Although demyelination is described as the primary cause for neuronal death in multiple sclerosis (MS), this disease also belongs to neurodegenerative disorders (Trapp & Nave, 2008).

1.2.1 Prevalence of neurodegenerative diseases

Neurological disorders, in general, are currently seen as the leading cause of disability and the second leading cause of death worldwide. In the past 30 years, the absolute numbers of deaths and people with disabilities owing to neurological diseases have risen substantially, resulting in about 276 million people suffering from disabilities and nine million people dying (Feigin et al., 2020). Additionally, it is proposed, that the number of non-communicable neurological disorders, in particular of neurodegenerative nature, are increasing significantly (Feigin et al., 2020; Fereshtehnejad et al., 2019). In Europe, four of five neurological disorders are of neurodegenerative nature such as Alzheimer's disease (prevalence increase between 1996 and 2017: ~61%), Parkinson's disease (prevalence increase between 1996 and 2017: ~64%), motor neuron disease (prevalence increase between 1996 and 2017: ~69%) and multiple sclerosis (prevalence increase between 1996 and 2017: ~39%). In comparison, stroke, which is still responsible for more than 50% of deaths due to neurological disorders,

only shows an increase in prevalence of about 32% (Deuschl et al., 2020). Since the leading risk factor for the development of neurodegenerative disease such as AD and PD is still increasing age, it is proposed that the cause for the prevalence increase in these neurological disorders is based on the fact that the world population is currently changing and people become much older than before (Lee & Gilbert, 2016; Mayeux & Stern, 2012).

Besides aging, there are many more risk factors associated with the development of neurodegenerative diseases. Other risk factors include gender, endocrine conditions, oxidative stress, inflammation, stroke, hypertension, diabetes, smoking, head trauma, depression, infection, tumors, vitamin deficits, immune and metabolic conditions and exposure to chemicals (summarized by (Brown et al., 2005)., indicating the complexity of neurodegenerative disorders. Furthermore, most of the neurodegenerative diseases also show a genetic component in their pathology, as many diseases have a sporadic as well as familial form of the disease as well as show implications of genetic polymorphisms (Brown et al., 2005).

In conclusion, neurodegenerative diseases are a leading cause for progressing disabilities and death. Besides their effects onto the patients suffering from these diseases, it also highly affects the health system. In order to mitigate the effects of neurodegenerative diseases, it should be a duty of future science to further research the complex pathomechanisms leading to the permanent neuronal disabilities.

1.2.2 Pathologies of neurodegenerative diseases

Although neurodegenerative disorders have similar risk factors, the primary pathomechanism differs between the diseases. To this end, one of the most complex and multiclausal neurodegenerative disease is proposed to be Alzheimer’s disease (AD). AD is currently the major cause of dementia. Besides the memory loss in AD patients, common symptoms can include problems with language, disorientation, mood swings, loss of motivation, self-neglect, and behavioral issues. Yet, pathological studies have generated overwhelming evidence for the complexity and multicausality of dementia (Boyle et al., 2013). However, the strongest evidence is still pointing to β-amyloid (Aβ) accumulation and tau inclusions although the causative comes from studies of familial Alzheimer's disease cases with mutations in amyloid precursor protein (APP), presenilin (PSEN) 1, or PSEN2 (Karch & Goate, 2015). On the one hand, mutations in the APP gene affect Aβ cleavage that can lead to aggregation of the protein. On the other hand, mutations in the PSEN1 and PSEN2 genes, that account as the catalytic subunit to the γ-secretases and cleave APP, lead to less efficient processing of APP and the generation of longer and more hydrophobic Aβ peptides (Karran et al., 2011). However, Tau is also known as a prerequisite for the diagnosis of AD, although mutations in

the tau gene cause frontotemporal dementia without amyloid plaques (Small & Duff, 2008). Tau is a microtubule-associated phosphoprotein present in axons involved in promoting polymerization and stabilization of microtubules (Binder et al., 1985; Buee et al., 2000; Mandelkow & Mandelkow, 2012). In addition to phosphorylation, tau undergoes other posttranslational modifications, such as ubiquitination, nitration, glycation, and acetylation, all of which have been linked to abnormal tau that can accumulate within neurons (Alonso et al., 2008; Cook et al., 2014; Martin et al., 2011; Morishima & Ihara, 1994). Although pathological modifications of tau were thought to be downstream events of Aβ aggregation, it is also plausible that tau and Aβ act in parallel pathways, both causing AD and enhancing each other's toxic effects (Small & Duff, 2008). However, in this context it needs to be highlighted, that the so called Aβ hypothesis is highly debated (Makin, 2018; Piller, 2022). It is criticized that the whole theory is based only on findings in familial forms of AD (Makin, 2018) and some inconsistencies were found in the analysis of key data that cast doubt on the theory (Piller, 2022), leading to great criticism of this dogma.

Another leading neurodegenerative disease is Parkinson’s disease (PD). PD is a chronic neurodegenerative disorder that is characterized by the loss of neurons in certain brain regions (Dickson et al., 2009). Neurodegeneration in PD is proposed in certain subsets of neurons, primarily dopaminergic neurons in the substantia nigra (Kordower et al., 2013), but also glutaminergic neurons in the presupplementary cortex (MacDonald & Halliday, 2002) and glutaminergic neurons in the caudal intralaminar thalamus (Henderson et al., 2000). Early symptoms include tremor, rigidity, slowness of movement, and difficulty with walking (although, non-motor symptoms can emerge in later stages). The neuropathology of PD is characterized by the formation of so called Lewis bodies due to the accumulation of the presynaptic protein α-synuclein within the neuron but also within oligodendrocytes (Dugger & Dickson, 2017). Furthermore, a defined set of peptides from α-synuclein can act as possible antigenic epitopes and drive helper and cytotoxic T-cell responses as well as microglial activation (Kim et al., 2013; Sulzer et al., 2017). However, the α-synuclein theory appears to be critical, since α-synuclein levels in the brain of PD patients do not show any substantial increase compared to other synucleinopathies (McCann et al., 2016; Tong et al., 2010; Zhou et al., 2011). Furthermore, only about 40% of PD patients show immune responses against α-synuclein (Sulzer et al., 2017), further underscoring the concerns about therapy.

In contrast to PD, motor neuron diseases are characterized by the progressive loss of motor neurons in different parts of the brain and/or spinal cord (Wijesekera & Leigh, 2009). In general, they are described as a group of diseases in which amyotrophic lateral sclerosis (ALS) is the most common. Classical symptoms of ALS are muscle weakness, atrophy and muscle spasms, whereas sensory nerves are unaffected. About 5% of all ALS cases are

classified as familial forms of ALS mostly having autosomal dominant pattern (Gros-Louis et al., 2006). 20% of these show mutations in superoxide dismutase (SOD)-1 or RNA-binding protein FUS (FUS; Chio et al., 2018). Furthermore, mutations in the TDP gene were also found and linked to familial as well as sporadic forms of ALS (Kabashi et al., 2008; Sreedharan et al., 2008; Yokoseki et al., 2008). The major histopathological features of ALS are intra neuronal inclusion such as: i) Bunina bodies: small eosinophilic, hyaline intracytoplasmic inclusions that stain positive for cystatin and transferring bodies (Mizuno et al., 2006; Okamoto et al., 2008); ii) Ubiquitinated inclusions: major constituent is TDP-43 (Arai et al., 2006; Neumann et al., 2006; Tan et al., 2007) and iii) a depletion of ≥50% of spinal motor neurons in combination with microglial and astroglial activation in grey and white matter of the spinal cord (Hardiman et al., 2017).

In contrast to the previous neurodegenerative diseases, multiple sclerosis (MS) is characterized primarily by the progressive loss of myelination as a result of oligodendroglial cell death. The occurring neuronal death is seen as a result from the massive loss in oligodendroglia, although recent studies suggest that neurodegeneration can also occur directly mediated by inflammatory microglia and astrocytes (Muzio et al., 2021; Zhou et al., 2019). Demyelination and neurodegeneration arise focally in MS and are often referred as MS lesions. Because these lesions can occur everywhere in the CNS, the symptoms of MS are very diverse and therefore include the loss of sensitivity, muscle weakness, blurred vision/blindness, problems with speech and/or swallowing etc. (Lassmann et al., 2012). In principle, demyelinated areas have the potential to remyelinate due to the differentiation of resident OPCs and neural stem cells (NSCs), however, this process remains overall inefficient (Kotter et al., 2011). Furthermore, MS is characterized by different progressive forms, most commonly in which patients suffer from alternating relapses followed by phases of recovery known as relapsing-remitting MS (RRMS). Approximately 60-70% of those patients will develop a secondary progressive MS (SPMS), leading to a progressive worsening of the symptoms. About 10% of MS patients suffer from a primary progressive form of MS (PPMS), which is characterized by a progressive course of the disease already in the early stages (Dutta & Trapp, 2014; Hauser & Oksenberg, 2006; Trapp & Nave, 2008). Furthermore, MS is mostly affecting young adults in the age between 20 and 40 years and the ratio of incidence between men and women is 1:2 (Koch-Henriksen et al., 2018; Kurtzke, 2000). In contrast to AD and PD, MS is therefore not seen as an age related disease, however, similar to all neurodegenerative disorders, the etiology of MS is far from being understood. Interestingly, prevalence and migration data clearly show that environmental influences play a role (Kurtzke, 2000; Pugliatti et al., 2002; Rosati, 2001) and viral infections, such as with Epstein-Barr virus

(EBV) and human herpesvirus (HHV) 6, have also been suggested to trigger MS development (Alvarez-Lafuente et al., 2004; Wagner et al., 2004).

1.2.3 Parallels in the pathomechanism of neurodegenerative diseases

Although different neurodegenerative diseases develop in diverse brain sites and exhibit distinct primary pathologies, they are characterized by similar cellular and molecular mechanisms.

One of these mechanisms is the activation of either the innate as well as the adaptive immune system (Amor et al., 2010). The activation of the innate immune system is a crucial first line of defense, to opsonize and clear apoptotic cells. However, innate immune responses can also lead to the recruitment of the adaptive immune system. In this process, cytokines and chemokines lead to the induction of adhesion molecules expression on the BBB, supporting peripheral immune cells to enter the CNS (Amor et al., 2010). In general, this notion, which is regularly taking place in the CNS, is crucial for the elimination of infectious agents as well as for clearing debris and should be considered as beneficial (Campbell, 2004). However, in neurodegenerative diseases, it often comes to a complete breakdown of the BBB, leading to massive immune cells infiltrates and the loss of protection from the periphery (Amor et al., 2010). Furthermore, the increasing expression and secretion of cytokines leads to strong inflammation that over time turns into a chronic state and thereby losing its beneficial purpose (Block & Hong, 2005; Tansey et al., 2007). Although chronic inflammation and immune activation in neurodegenerative diseases are triggered by different primary pathologies, similar features are well described. In this context, it is shown for all neurodegenerative diseases that peripheral immune cell infiltrates can be found as a hallmark in CNS pathology (Doty et al., 2015; Rezai-Zadeh et al., 2009). However, the role of peripheral immune cell infiltration is still highly debated, since either beneficial as well as destructive functions are described in the context of neurodegenerative diseases (Yang et al., 2020).

In detail, recent advances in transcriptome analysis show three main players in relation to neurodegenerative diseases: microglia, astrocytes and lymphocytes, which influence each other within the framework of a complex interactome (Absinta et al., 2021). A central player of this interactome is C1q, a known protein complex of the complement pathway. Furthermore, a comparative bioinformatics approach, analyzing multiple myeloid transcriptomic datasets, was able to identify 192 common macrophage genes as well as 119 microglial genes to be upregulated upon all neurodegenerative diseases and animal models of neurodegeneration (Wishart et al., 2023). One of these genes is meanwhile seen as a key marker for reactive microglia in neurodegenerative diseases, named C-type lectin domain family 7 member A (Clec7a; Stratoulias et al., 2019). Of note, C-type lectin receptors belong to the class of

signaling pattern recognition receptors, which are involved in immune responses to a broad repertoire of pathogens such as bacteria, viruses, but also nematodes and fungi.

Another feature that is common to neurodegenerative diseases is oxidative stress, which has been proposed as one of the key factors inducing neurodegeneration (Niedzielska et al., 2016). In general, oxidative stress is the result of the disruption in the pro-/antioxidant homeostasis, leading to the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite anions ($ONOO^-$), superoxide (O_2^-), and the highly reactive hydroxyl radicals ($HO^\bullet$; Metodiewa & Koska, 2000; Popa-Wagner et al., 2013). Moderate concentrations of ROS play a regulatory role as mediators in signaling processes, as it is able to regulate vascular tone, sense oxygen tension, enhance the signal transduction from various membrane receptors including the antigen receptor of lymphocytes, and modulate oxidative stress responses in order to maintain redox homeostasis (Dröge, 2002). However, high oxygen consumption, relatively low antioxidant levels and low regenerative capacity result in pathophysiological levels of ROS, which can promote tissue damage by directly activating apoptosis via intrinsic mitochondrial pathways (Circu & Aw, 2010; Sinha et al., 2013).

In addition, oxidative stress is often associated with immune defense mechanisms, as the immune cells use oxidative radicals as part of their instrument to kill pathogens. Indeed, reactive oxygen species are proposed to be critically involved in the inflammatory processes of multiple sclerosis (Ohl et al., 2016), but also other neurodegenerative diseases (Teleanu et al., 2022). However, certain neurodegenerative diseases also show different associations with oxidative stress than mediated via immune defense mechanisms. In AD, β-amyloid accumulation as well as the build-up of intracellular tau neurofibrillary tangles originated from oxidative stress generation (Li et al., 2013). In this regard, three mechanisms were proposed that affect cells homeostasis, ROS generation and the up-regulation of Aβ and phosphorylated tau formation: macromolecule peroxidation, Aβ metal ion redox potential, and mitochondrial dysfunction (LoGerfo et al., 2014). Of note, a characteristic feature of the substantia nigra is that neurons accumulate neuromelanin with age (Marsden, 1983). In PD, however, these neuromelanin-containing cells are the first that get lost during progression of the disease (Barnham et al., 2004; Hirsch et al., 1988). Although the precise mechanism of neuromelanin formation is not known, yet, it was shown, that it consists primarily in products from dopamine redox chemistry (Wakamatsu et al., 2003; Zecca et al., 2003). In ALS, mutations of SOD1 are described to be the reason for many cases (Hardiman et al., 2017). Interestingly, SOD1 is a cupro-enzyme that detoxifies the ROS superoxide, when mutated, however, it can convert the protein from an anti-oxidant to a pro-oxidant that might lead to oxidative insults. Furthermore,

this evidence is supported by the observation that copper chelators inhibit the course of the disease in both cell culture and mouse models (Azzouz et al., 2000; Hottinger et al., 1997).

In conclusion, understanding the joint process of chronic inflammation, immune activation and oxidative stress will be an important step towards fully elucidating the pathologies of these life-threatening diseases.

1.3 Endogenous Retroviruses in health and disease

In 1950, Edward B Lewis and Barbara McClintock first described (retro)-transposable elements in Drosophila melanogaster and maize as DNA sequences that are capable of transposition (Lewis, 1950; Mc, 1950). At that time, no one suspected that 50 years later, with the completion of the human genome project (Lander et al., 2001), it would be found that 40% - 50% of the human genome consists of transposed elements (TEs). In general, TEs are (retro)-transposable elements that have been rendered immobile due to truncation and mutation (Faulkner & Carninci, 2009) and primarily consists of long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), and DNA transposons. A fourth group of TEs is described as Long Terminal Repeat (LTR)-retrotransposons in particular endogenous retroviruses (ERVs; Venter et al., 2001).

Endogenous retroviruses were first described in the late 1960s and early 1970s (Weiss, 2006). Within a few years, three types of ERVs were reported by independent groups: avian leucosis virus (ALV; Weiss, 1969a, 1969b), murine leukemia virus (MLV; Aaronson et al., 1971), and murine mammary tumor virus (MMTV; Bentvelzen et al., 1970). Human endogenous retroviruses (HERVs), on the other hand, were not discovered until ten years later (Martin et al., 1981). While ERVs comprise approximately 10% of the mammalian genome, the human genome consists of 5% - 8% (Lander et al., 2001; Stocking & Kozak, 2008). Furthermore, ERVs are usually classified into three classes (I, II and III) based on their similarities to the exogenous *gammaretrovirus*, *betaretrovirus* and *spumaretrovirus* (Gifford et al., 2018). In the past years, TEs including ERVs were often referred as “junk DNA”, due to the fact that these genetic elements are highly mutated and no protein expression could be detected. However, it is meanwhile known that some of these elements were domesticated and can fulfill important functions within the human body. On the other hand, activation of certain pathological retroviral elements can lead to immune activation and thereby contribute to the development of certain neurodegenerative and neuropsychiatric diseases (Küry et al., 2018).

1.3.1 Human endogenous Retroviruses

To date, over 20 HERV families have been discovered during the past two decades (Nelson et al., 2003). The tRNA binding to the viral primer binding site to initiate reverse transcription had traditionally been used for HERV classification and the current nomenclature still often relies on the tRNA type associated recognition of different HERV types (e.g., HERV-K for lysine tRNA, HERV-W for tryptophan tRNA, etc.). However, if the primer binding site sequence of the retroviral entities was not available, the names based on neighboring genes (e.g. HERV-ADP), clone number (e.g. HERV-S71), or amino acid motifs (e.g. HERV-FRD) were used (Gifford et al., 2018). The current knowledge suggests that over time multiple independent infection events have taken place, which created a distinctive genomic ERV content in different species. Additionally, genetic recombination has taken place, resulting in more than 100.000 ERV loci identified in humans and led to extensive interindividual variations (Nellaker et al., 2012; Thomas et al., 2018). Although many of these retroviral elements accumulated defects within their coding sequence, such as mutations, deletions, and termination signals, a limited number of HERVs have still the potential to produce viral proteins and even viral-like particles (Küry et al., 2018).

However, the fact that these retroviral elements have survived in our genome also suggests a certain degree of symbiosis or domestication (Küry et al., 2018). The best examples for domesticated retroviral elements are syncytin-1 and -2, as the two proteins are thought to be derived from previous human endogenous retroviruses (HERV-W and HERV-FRD, respectively). Both proteins play regulatory roles in the placentogenesis and are proposed to be involved in the fetal-maternal immune tolerance (Xiang & Liang, 2021). Many researchers have also emphasized the role of HERVs in shaping the evolution of the human genome solely through their retrotransposonal properties (Grandi & Tramontano, 2018). In this regard, the thousands of HERV sequences that are integrated in our DNA provide an abundant source of regulatory elements. It is well known that our genetic information is organized in regulatory networks, involving both *cis*- regulatory sequences and *trans*-acting genes, and that their interaction is at the base of cellular plasticity and evolution (Göke & Ng, 2016; Hua-Van et al., 2011). In fact, HERVs also participate to this complex interplay, being able to regulate the host genes' activity in several ways and at different expression levels (Grandi & Tramontano, 2018). Additionally, HERVs are also thought to shape the immunological landscape of an individual, as e.g. cells infected with a certain virus become resistant to superinfections. It is therefore proposed that ERV expression could potentially influence to inherent host defense mechanism, leading to resistance against superinfections (Villarreal, 2011). Such beneficial effects of HERVs are possible due to the fact that endogenous and exogenous entities reveal strong parallels in their protein and nucleic acid sequences (Grandi & Tramontano, 2017) and

might therefore compete with the same receptors exogenous viral proteins (Spencer et al., 2003). In contrast to HERVs' beneficial roles, numerous studies described the activation and expression of these viral entities in different neurodegenerative as well as neuropsychiatric diseases such as MS, ALS, but also AD as well as schizophrenia (SCZ) and bipolar disorder (BD; Römer, 2021).

Although small leaks at the transcriptional level can still occur (Leung & Lorincz, 2012), under physiological conditions most HERVs are silenced due to various epigenetic processes that are relevant to the control of ERV expression (Deaton & Bird, 2011; Lavie et al., 2005; Ohtani et al., 2018; Szpakowski et al., 2009). Moreover, HERVs flanking LTR sequences are described to feature strong regulatory properties by containing binding sites for the pro-inflammatory transcription factor nuclear factor kappa light chain enhancer of activated B-cells (NF-κB; Manghera & Douville, 2013; Thompson et al., 2016). Given that NF-κB regulates various aspects of innate and adaptive immunity and can be activated by numerous pro-inflammatory cytokines such as tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6 and interferon (IFN) γ, binding of NF-κB to flanking LTR sequences may readily provide a direct mode of action of how inflammation can drive HERV transcription (Liu & Wang, 2017).

While inflammation is one of the factors that can stimulate ERV expression, endogenous retroviral proteins can induce inflammatory responses in different cell types as well. One of the first studies supporting this notion showed that the HERV-W ENV protein is able to activating human monocytes in a toll-like receptor (TLR) 4 dependent manner (Rolland et al., 2006). Furthermore, dendritic cells were similarly triggered by HERV-W ENV protein, leading to an increased T helper cell type 1 (Th1) differentiation. This concept was later supported by another study that used a genetically modified HEK-Blue cell line to prove that HERV-W ENV signaling is mediated by TLR4 (Charvet et al., 2018).

Taken together, although some HERVs have been domesticated and perform beneficial functions in the human body, the ever-increasing number of pathology-associated HERVs suggests that they may also have harmful effects.

1.3.2 Pathology of the Human endogenous retrovirus type W in multiple sclerosis

One of the first associations between HERVs and neurodegenerative disorders was made by the initial discover of HERV-W viral particles in leptomeningeal cell cultures from MS patients (Perron et al., 1989). While it was initially termed multiple sclerosis associated retrovirus (MSRV), it was later identified as a human endogenous retroviruses and named HERV-W according to its tryptophan tRNA binding site (Dolei, 2018). Follow-up investigations showed that RNA- as well as protein levels of HERV-W ENV are increased in the cerebrospinal fluid (CSF) and serum of MS patients compared to healthy individuals (Garson et al., 1998; Mameli

et al., 2007; Mameli et al., 2009; Perron et al., 2012). Of note, similar correlations were observed in chronic inflammatory demyelinating polyneuropathy (CIDP), an inflammatory, demyelinating disease of the PNS (Faucard et al., 2016).

In general, HERV-W belongs to the *gammaretroviruses* and in contrast to its pathological form, syncytin-1 exerts physiological roles in placentogenesis. The pathological HERV-W, however, might therefore be either a non-ubiquitous replication-competent member, or a partly defective, non-ubiquitous copy of the HERV-W family. Either way, Charvet and colleagues have recently identified important biochemical differences between syncytin-1 and HERV-W ENV and could thereby clearly demonstrate that the HERV-W ENV protein isolated from MS lesions is not syncytin-1 (Charvet et al., 2021).

It is meanwhile proposed, that the activation and expression of otherwise silenced HERV-W and the following production of the ENV protein can trigger an immune response (Perron et al., 2001; Rolland et al., 2006). In particularly, HERV-W ENV protein can activate TLR4 and cluster of differentiation (CD) 14 on human monocytes, inducing to the production and secretion of pro-inflammatory cytokines (Rolland et al., 2006). In addition, ENV protein triggered dendritic cells to promote Th1 differentiation. Further activation of the immune system was found, when HERV-W ENV protein was used as an adjuvant in a model of experimental autoimmune encephalomyelitis (EAE), leading to autoimmune reactions against myelin producing cells. This, in turn, was rescued by the treatment of a HERV-W ENV-neutralizing therapeutic IgG4 antibody called Temelimab (Perron et al., 2013). Histopathological analysis of autopsy material from MS patients revealed that the HERV-W ENV protein is most likely expressed by myeloid cells and some astrocytes (Kremer et al., 2013; van Horssen et al., 2016). Subsequent *in vitro* studies revealed that HERV-W ENV protein has an influence onto TLR4- expressing OPCs that are present in the rim of MS lesions, leading to impaired differentiation capacity (Kremer et al., 2013). This effect was mediated by the induction of nitrosative stress, resulting in a subsequent reduction in myelin protein expression.

In conclusion, particularly for MS, there is strong evidence that HERV-W ENV plays a crucial role in the onset as well as progression of the neurodegenerative disease by blocking OPC differentiation as well as by activating peripheral immune cells.

1.3.3 Pathology of the Human endogenous retrovirus K in amyotrophic lateral sclerosis

ALS is another neurodegenerative disease that is associated with the expression of human endogenous retroviruses, in particular HERV-K. Phylogenetically, the HERV-K group belongs to the ERV2, Class II or *betaretrovirus*-like supergroup. Currently, the HERV-K clade contains ten subgroups (from HML-1 to HML-10) of which HML-2 is primarily associated with the onset

and progression of ALS. However, the nomenclature of the HERV-K family is very complex and has many different designations for the same, ALS-associated group: HLM-2, HML-2, HERV-K10, HTDV/HERV-K, HERV-K (HML-2), HERV-K, HERVK or ERVK (Garcia-Montojo et al., 2018). Apart from its role in cancer (Grabski et al., 2019), HERV-K is proposed to be associated with the onset and progression of subpopulation of patients with sporadic ALS. Initially, several groups found increased reverse transcriptase (RT) activities in the serum and CSF of human immunodeficiency viruses (HIV)-negative patients diagnosed with ALS (MacGowan et al., 2007; McCormick et al., 2008; Steele et al., 2005). The first direct proof of HERV-K's implication in ALS pathology was provided three years later as qPCR analysis of brain autopsy tissue identified an increased expression of HERV-K transcripts in cortical and spinal neurons of ALS patients compared to healthy control individuals (Douville et al., 2011). Although the evidence for such an involvement of HERV-K in ALS is increasing (Meyer et al., 2017), it was recently challenged by an independent study that did not find increased HERV-K RNA levels in cortical ALS tissue (Garson et al., 2019). Despite the contradictory statements regarding HERV-K detection, it must be highlighted that HERV-K expressing transgenic mice display progressive motor dysfunction and motor cortex volume loss (Li et al., 2015). Furthermore, and in contrast to HERV-W, two loci were identified in the 7q34 and 7q36.1 regions that can lead to the expression of HERV-K elements (Frank et al., 2005). Additional evidence comes from the fact that HIV infected patients rarely develop ALS. Although HIV infection has been associated with increasing levels of HERV-K, when these patients were treated with antiretroviral drugs early in the course of the neurological manifestations, ALS symptoms could be reversed or slowed in a subset of patients (Alfahad & Nath, 2013). Furthermore, the activation of HERV-K found in the blood of some of these patients decreased following treatment with antiretroviral drugs (Bowen et al., 2016).

1.4 Aim of this thesis

Neurodegenerative diseases are among the most debilitating life-threatening diseases worldwide that lead to permanent neurological deficits. Despite their prevalence for the human being, their pathologies are far from being understood. Multiple environmental as well as genetic factors might play a role, of which human endogenous retroviruses have been repeatedly associated with onset and progression of neurodegenerative diseases as well with immune modulating function. In order to gain knowledge in the understanding of the underlying pathomechanism, the aim of this work is the analysis and characterization of neurodegenerative associated processes particularly in the context of multiple sclerosis. Since resident immune competent glial cells are the first line of defense, this work will investigate the four major glial cell types: microglia, astroglia, oligodendroglia and OPCs. Therefore, this thesis will focus on: I.) The characterization of novel mechanisms related to microglial

activation responses in neurodegenerative diseases, and in particular multiple sclerosis, as well as how these responses can be modulated using immunomodulating therapeutics. II.) In order to gain a deeper insight into the effects of HERV-W on glial cells, this thesis investigates how this viral entity contributes to the onset and progression of neurodegenerative diseases such as multiple sclerosis. In this context, this work will address the complex (re)awakening processes necessary for the expression of endogenous retroviruses and focus on the analysis of HERV-W ENV-specific glial responses leading to reactive phenotypes, associated with demyelination and neurodegeneration.

2. Results – Publications

2.1 pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis

David Kremer, Joel Gruchot, Vivien Weyers, Lisa Oldemeier, Peter Göttle, Luke Healy, Jeong Ho Jang, Yu Kang T Xu, Christina Volsko, Ranjan Dutta, Bruce D Trapp, Hervé Perron, Hans-Peter Hartung, Patrick Küry

Abstract

Axonal degeneration is central to clinical disability and disease progression in multiple sclerosis (MS). Myeloid cells such as brain-resident microglia and blood-borne monocytes are thought to be critically involved in this degenerative process. However, the exact underlying mechanisms have still not been clarified. We have previously demonstrated that human endogenous retrovirus type W (HERV-W) negatively affects oligodendroglial precursor cell (OPC) differentiation and remyelination via its envelope protein pathogenic HERV-W (pHERV-W) ENV (formerly MS-associated retrovirus [MSRV]-ENV). In this current study, we investigated whether pHERV-W ENV also plays a role in axonal injury in MS. We found that in MS lesions, pHERV-W ENV is present in myeloid cells associated with axons. Focusing on progressive disease stages, we could then demonstrate that pHERV-W ENV induces a degenerative phenotype in microglial cells, driving them toward a close spatial association with myelinated axons. Moreover, in pHERV-W ENV-stimulated myelinated cocultures, microglia were found to structurally damage myelinated axons. Taken together, our data suggest that pHERV-W ENV-mediated microglial polarization contributes to neurodegeneration in MS. Thus, this analysis provides a neurobiological rationale for a recently completed clinical study in MS patients showing that antibody-mediated neutralization of pHERV-W ENV exerts neuroprotective effects.

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Contribution on experimental design, realization and publication

Establishment, execution and analysis of an *ex vivo* co-culture model via qPCR, enzyme-linked immunosorbent assay and immunocytochemistry. Contribution to the analysis of human immunohistochemistry. Preparation of manuscript (Material and Method) and figure design.

pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis

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Axonal degeneration is central to clinical disability and disease progression in multiple sclerosis (MS). Myeloid cells such as brain-resident microglia and blood-borne monocytes are thought to be critically involved in this degenerative process. However, the exact underlying mechanisms have still not been clarified. We have previously demonstrated that human endogenous retrovirus type W (HERV-W) negatively affects oligodendroglial precursor cell (OPC) differentiation and remyelination via its envelope protein pathogenic HERV-W (pHERV-W) ENV (formerly MS-associated retrovirus [MSRV]-ENV). In this current study, we investigated whether pHERV-W ENV also plays a role in axonal injury in MS. We found that in MS lesions, pHERV-W ENV is present in myeloid cells associated with axons. Focusing on progressive disease stages, we could then demonstrate that pHERV-W ENV induces a degenerative phenotype in microglial cells, driving them toward a close spatial association with myelinated axons. Moreover, in pHERV-W ENV-stimulated myelinated cocultures, microglia were found to structurally damage myelinated axons. Taken together, our data suggest that pHERV-W ENV-mediated microglial polarization contributes to neurodegeneration in MS. Thus, this analysis provides a neurobiological rationale for a recently completed clinical study in MS patients showing that antibody-mediated neutralization of pHERV-W ENV exerts neuroprotective effects.

multiple sclerosis | axonal degeneration | endogenous retrovirus | myeloid cells | demyelination

As early as 1868, Jean-Martin Charcot described axonal degeneration in multiple sclerosis (MS). However, this histopathological hallmark of the disease was only rediscovered in the late 20th century (1). Even though neurodegeneration is already present in relapsing-remitting (RR) MS, it predominates in later progressive MS stages, leading to severe neurological disability (1–6). Among myeloid cells, microglia that originate from the yolk sac are part of the innate immune system of the central nervous system (CNS) and survey its parenchyma responding to various pathogens (7). However, they are not the only population of myeloid cells that play a role in MS, as blood-borne monocytes invading the brain through a leaky blood–brain barrier (BBB) are present in MS lesions as well (8). In the inflamed MS brain, it is therefore challenging to delineate microglia and invading monocytes (9). In this regard, it remains to be shown whether the recently identified transmembrane protein 119 (TMEM119) might facilitate efforts in this direction (10), since there is evidence that it is exclusively expressed in only a subset of microglial cells (11). In addition, we here investigated predominantly progressive MS cases where the BBB is assumed to be mostly intact, restricting peripheral cell infiltration (12). We therefore focused on the brain-resident myeloid cell population of microglia for our functional in vitro analyses. In MS, microglia participate in both autoimmune inflammation and neurodegeneration by producing proinflammatory cytokines and molecules (4, 13–15). This crucial role is underlined by experiments which found that blocking microglial activation represses the experimental MS model experimental autoimmune encephalomyelitis (16). Microglia also seem to be linked to disease progression in MS: Positron emission tomography (PET) studies in relapsing and progressive MS using the mitochondrial translocator protein TSPO, which is up-regulated in activated microglia, demonstrated that microglial activation is a significant predictor of disability in progressive MS (15, 17). However, microglia can also contribute to neurorepair, *inter alia*, via myelin debris clearance, which is key for remyelination and neuroprotection (18). Therefore, over the years, a distinct nomenclature was established to capture the complex role of this cell population in disease: A phenotype classically categorized as "M1" produces reactive oxygen and nitrogen species, as well as

Significance

There is a broad repertoire of immunomodulatory drugs that effectively treat the inflammatory aspects of relapsing multiple sclerosis (MS). However, axonal degeneration, which occurs mainly in progressive MS, is still not understood and cannot be treated pharmaceutically. As it is the major factor contributing to clinical disability in MS, it represents an unmet clinical need. A recently completed phase IIb study has demonstrated that anti-pathogenic human endogenous retrovirus type W (pHERV-W) envelope protein (ENV) treatment results in a significant decrease of neurodegenerative brain atrophy in treated MS patients. For these results, the work presented here offers an explanation by demonstrating that, via myeloid cells, pHERV-W ENV directly harms axons.

Author contributions: D.K., J.G., H.P., and P.K. designed research; D.K., J.G., V.W., L.O., P.G., L.H., J.H.J., Y.K.T.X., and C.V. performed research; P.G., L.H., C.V., R.D., and B.D.T. contributed new reagents/analytic tools; D.K., J.G., V.W., L.H., J.H.J., Y.K.T.X., R.D., B.D.T., H.P., H.-P.H., and P.K. analyzed data; and D.K. and P.K. wrote the paper.

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proinflammatory cytokines, leading to myelin destruction and subsequent neurodegeneration. In contrast, the so-called "M2" phenotype is associated with the production of antiinflammatory molecules and the clearance of myelin debris (18, 19). However, M1 and M2 states never occur as pure phenotypes in vivo. As a result, while this nomenclature may serve as a tool to simplify data interpretation, this concept remains artificial and is increasingly viewed as insufficient in the field (20). Accordingly, we refrained from using this nomenclature in the work presented here. In this study, we set out to investigate if the envelope protein (pathogenic human endogenous retrovirus type-W [pHERV-W] ENV, formerly MS-associated retrovirus [MSRV]-ENV) of the HERV-W can drive microglia to promote axonal degeneration in MS. HERVs represent 8% of the human genome and originate from mammalian germ-line retroviral infections millions of years ago (21, 22). Usually epigenetically silenced, transactivation by exogenous viral infection such as Epstein–Barr virus or other viruses epidemiologically associated with MS may lead to their (re)expression (23–25). Accordingly, in both clinically isolated syndrome (CIS) and clinically definite (CD) MS, elevated concentrations of pHERV-W ENV protein, RNA, and/or DNA can be found in the serum, cerebrospinal fluid (CSF), and brain (26–28). In addition, pHERV-W positivity is correlated with a more rapid clinical disease progression and increased conversion rate to secondary progressive (SP) MS (29). In previous studies, we demonstrated that the pHERV-W ENV protein interferes with myelin repair by inhibiting oligodendroglial precursor cell (OPC) differentiation via the induction of nitrosative stress through activation of Toll-like receptor 4 (TLR4) (27). This process can be rescued by the humanized anti-ENV IgG_4 monoclonal antibody GNbAC1 (30). In a recently completed phase IIb clinical study, GNbAC1 was shown to exert neuroprotective effects in MS patients (Clinical Trial Assessing the HERV-W pHERV-W ENV Antagonist GNbAC1 for Efficacy in MS [CHANGE-MS]; ClinicalTrials.gov identifier NCT02782858). MRI data demonstrated that anti–pHERV-W ENV treatment results in a significant 31% reduction of cortical atrophy and a 72% reduction of thalamic atrophy. Moreover, GNbAC1 reduced the number of T1 hypointense lesions (so-called "black holes") by 63% within 1 y of treatment. Black holes are considered an MRI correlate of permanent tissue destruction in the brain.

In the study presented here, we found that pHERV-W ENV protein is present on TLR4-positive microglia in MS lesions tightly associated with myelinated axons. In primary microglia, pHERV-W ENV induces ameboid cell morphologies, increases cell proliferation, promotes the secretion of proinflammatory agents, reduces the expression of neuroprotective factors, and diminishes myelin clearance capacity. Furthermore, in ENV protein-stimulated myelinated cocultures, microglia are driven to associate themselves with axons, resulting in the leakage of intraaxonal and myelin proteins. These observations suggest that in the MS brain, pHERV-W ENV may induce myeloid cells to cause damage of myelinated axons. Our work suggests that pHERV-W ENV-mediated modulation of microglial cell polarization fuels and contributes to axonal damage and neurodegeneration in MS. Thereby, this study provides a biomedical rationale for the results of the above-mentioned CHANGE-MS study.

Results

In MS Lesions, pHERV-W ENV Is Present in Myeloid Cells and the Extracellular Space. We studied pHERV-W ENV protein localization in brain tissue sections of 5 MS patients, 2 amyotrophic lateral sclerosis (ALS) patients, and 2 healthy controls (HCs; Table 1) using immunohistological analyses. In a first step, using 3,3′-diaminobenzidine (DAB) staining, we found that pHERV-W ENV-positive cells were absent in HC brains (Fig. 1*B*), while numerous pHERV-W ENV-immunoreactive cells could be detected in chronic and acute active MS lesions (exemplarily shown for primary progressive [PP] MS case MS 36 in Fig. 1 *A*, *C*, *D*, and *E*). pHERV-W ENV-positive cells were found within the lesion parenchyma (Fig. 1 *A* and *E*), but also as part of perivascular cuffs (Fig. 1 *C* and *D*). Anti-major histocompatibility complex class II (MHCII) DAB staining of serial sections confirmed that the majority of pHERV-W ENV-positive cells featured a myeloid phenotype (Fig. 1*F*). To confirm this morphology-based hypothesis, we next performed fluorescent double immunostaining demonstrating that pHERV-W ENV was present in a subpopulation of ionized calcium binding adaptor molecule 1 (Iba1)/MHCII-positive myeloid cells (Fig. 1 *G*–*G*″ and *H*–*H*″). Moreover, abundant extracellular pHERV-W ENV, conceivably from demised immune cells (27), could be found in the lesion parenchyma (Fig. 1 *G*, right lower corner). Furthermore, we found that pHERV-W ENV-positive myeloid cells also expressed the pHERV-W ENV receptor TLR4 (Fig. 1 *I*–*I*″). In contrast, Iba1-positive myeloid cells in ALS brains, which were used as an other neurological disease (OND) control, were pHERV-W ENV-negative (Fig. 1 *J*–*K*′).

In a second step, we studied how pHERV-W ENV-positive myeloid cells associate themselves with proteolipid protein (PLP)-positive myelinated axons, focusing on the edges of chronic and acute active lesions (exemplarily shown for case MS 36 in Fig. 2), where scarce PLP reactivity allows for closer analysis of cell/cell interactions (Fig. 2 *A* and *A*′). Fluorescent double immunostaining of serial sections of the same lesion demonstrated that pHERV-W ENV-positive myeloid cells were in direct contact with PLP-positive axons (Fig. 2 *B*–*D*) and even wrapped around them as revealed by confocal microscopy (Fig. 2 *B*′, merged z-stack and *C* and *D*, single-layer images). Furthermore, pHERV-W ENV-positive cells could be found in direct vicinity to bulb-like axonal structures (Fig. 2*B*″, merged z-stack). Taken together, these data suggested that in the MS brain, pHERV-W ENV modulates myeloid cell behavior,

Table 1. Clinical features of MS and ALS patients whose brain tissue was used for immunohistochemistry

Case designation	MS subtype	Age, y	Sex	Disease duration, y	EDSS	Tissue (no. of lesions analyzed)
MS 25	SPMS	56	M	33	9.5	Chronic active (3)
MS 36	PPMS	63	F	9	7.5	Chronic active (4)
MS 141	SPMS	57	M	13	8	Chronic active (3)
MS 147	RRMS	50	F	31	1	Acute active (4)
MS 150	SPMS	51	F	23	7	Chronic active (3)
MS 100	HC	47	F	n/a	n/a	n/a
MS 61	HC	90	F	n/a	n/a	n/a
ALS 15	OND	61.6	M	3.0	n/a	n/a
ALS 16	OND	63.7	F	2.1	n/a	n/a

F, female; M, male; n/a, not applicable.

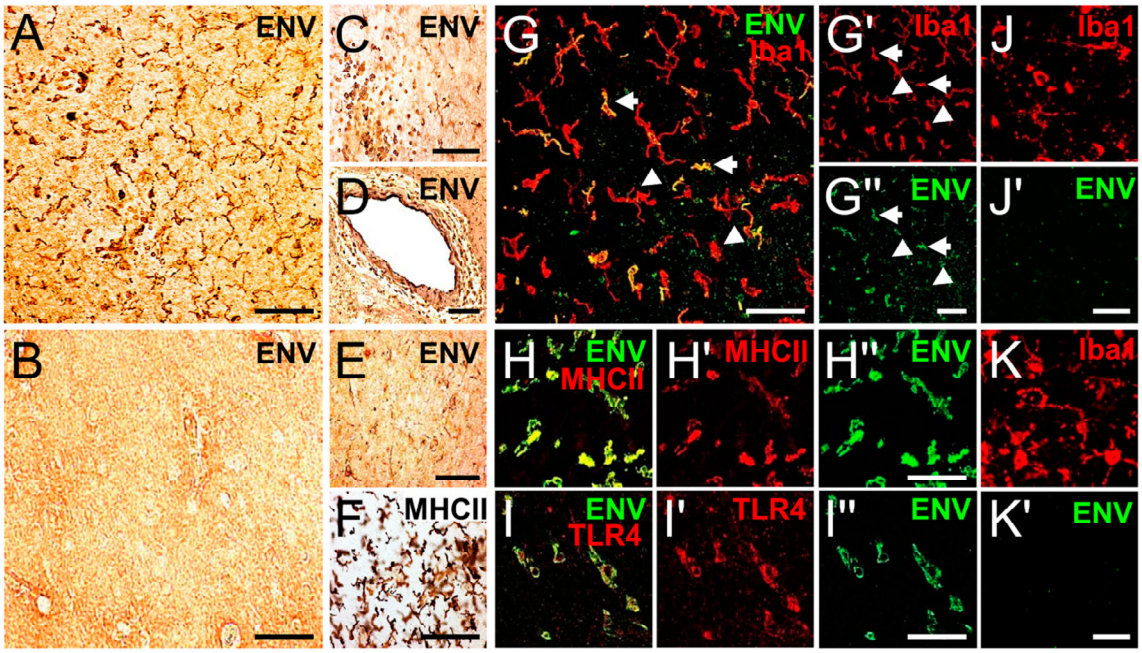

Fig. 1. pHERV-W ENV is present in myeloid cells in MS lesions. (*A* and *C–E*) Anti–pHERV-W ENV DAB staining of an MS lesion (case MS 36) showing pHERV-W ENV-positive cells within the lesion parenchyma (*A* and *E*) and as part of perivascular cuffs (*C* and *D*). (*B*) Control tissue (case MS 100) negative for pHERV-W ENV. A minority of cells feature astrocytic morphologies (*E*), but anti-MHCII DAB staining of a serial section reveals that the majority of pHERV-W ENV-positive cells feature a myeloid phenotype (*F*). (Scale bars: *D*, 80 μm; *A–C*, *E*, and *F*, 50 μm.) (*G–H''*) Double staining demonstrating that pHERV-W ENV (green) is present in a subpopulation of Iba1/MHCII-positive (each in red) myeloid cells in MS lesions (case MS 36). (*G*) Note the extracellular pHERV-W deposits in the right lower corner. Arrows in *G* indicate double-positive cells, and arrowheads point to pHERV-W ENV-negative cells. (Scale bars: *G* and *H''*, 30 μm; *G''*, 50 μm.) (*I–I''*) Double staining demonstrating the expression of pHERV-W ENV receptor TLR4 (red) in pHERV-W ENV-positive cells (green). (Scale bar: *I''*, 30 μm.) (*J–K'*) Double staining demonstrating that pHERV-W ENV (green) is absent in Iba1-positive (red) myeloid cells in ALS tissue (case ALS 15 in *K* and *K'*, case ALS 16 in *J* and *J'*). (Scale bars: *J'* and *K'*, 25 μm.)

driving the myeloid cells toward a physical interaction with axons. Such axon-wrapping myeloid phenotypes were, however, significantly less frequent in Iba1/PLP double-stained ALS tissue sections (Fig. 2 *E–G*).

pHERV-W ENV Induces a Proinflammatory Phenotype in Cultured Microglia. Against the backdrop of the detection of myeloid cell/axon interactions in chronic active lesions of progressive MS cases where the BBB is mostly intact, we used primary rat and human microglial cells for further functional analyses. Immunofluorescent staining of purified CD11b-positive rat microglia confirmed expression of the pHERV-W ENV receptor TLR4 (Fig. 3 *A* and *A'*). Based on previous studies, we stimulated cultured microglia with 1,000 ng/mL recombinant full-length pHERV-W ENV protein for various periods. Note that to confirm the observed effects were ENV- and TLR4-specific, several control experiments have already been performed in previous studies (27, 30, 31). Gene expression analysis revealed a strong induction of proinflammatory markers such as tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin (IL)-6, and IL-1β (Fig. 3 *B*, *C*, *F*, and *G*). We then corroborated these results using a TNF-α enzyme-linked immunosorbent assay (ELISA) and nitric oxide (NO) spectrometry, respectively, which confirmed increased quantities of TNF-α protein (Fig. 3*D*) and elevated NO levels (Fig. 3*E*) in pHERV-W ENV-stimulated cell culture supernatants. NO and TNF-α are well-established mediators of axonal injury and demyelination (32–35). Further gene expression analysis showed that pHERV-W ENV also reduced the gene expression of triggering receptor expressed on myeloid cells 2 (TREM2; Fig. 4*A*) and protooncogene tyrosine-protein kinase MER (MerTK; Fig. 4*B*), which are key for microglial phagocytosis (36–38). This translated to a functional deficit in myelin uptake capacity in pHERV-W ENV-stimulated microglia as evidenced by diminished in vitro phagocytosis of phRodo-decorated bovine myelin (Fig. 4 *D–F*). As outlined further above, it is known that myelin debris is a major obstacle to neurorepair both as a physicospatial impediment via the expression of axon growth inhibitory molecules and also via an inhibition of OPC differentiation (18, 37–39). In doing so, myelin debris inhibits remyelination (18) so that this microglial core ability has recently become a new target for potential therapeutic approaches (40, 41). Moreover, we observed that pHERV-W ENV stimulation led to a pronounced morphological shift to ameboid microglial phenotypes typical of proinflammatory activation already apparent after 1 and 2 d of pHERV-W ENV stimulation (Fig. 4 *G–I*). In addition, we found that pHERV-W ENV strongly induced microglial proliferation as revealed by Ki-67 expression (Fig. 4*C*).

pHERV-W ENV Treatment Decreases Microglial Expression of Neuroprotective Molecules. In addition to its properties as a proinflammatory agent, we found that stimulation of microglia with pHERV-W ENV protein led to a significant reduction of neuroprotective features as exemplified by gene expression analysis of insulin-like growth factor 1 (IGF-1; Fig. 5*A*), colony stimulating factor 1 (CSF-1; Fig. 5*C*), and fibroblast growth factor 2 (FGF-2; Fig. 5*D*). This also

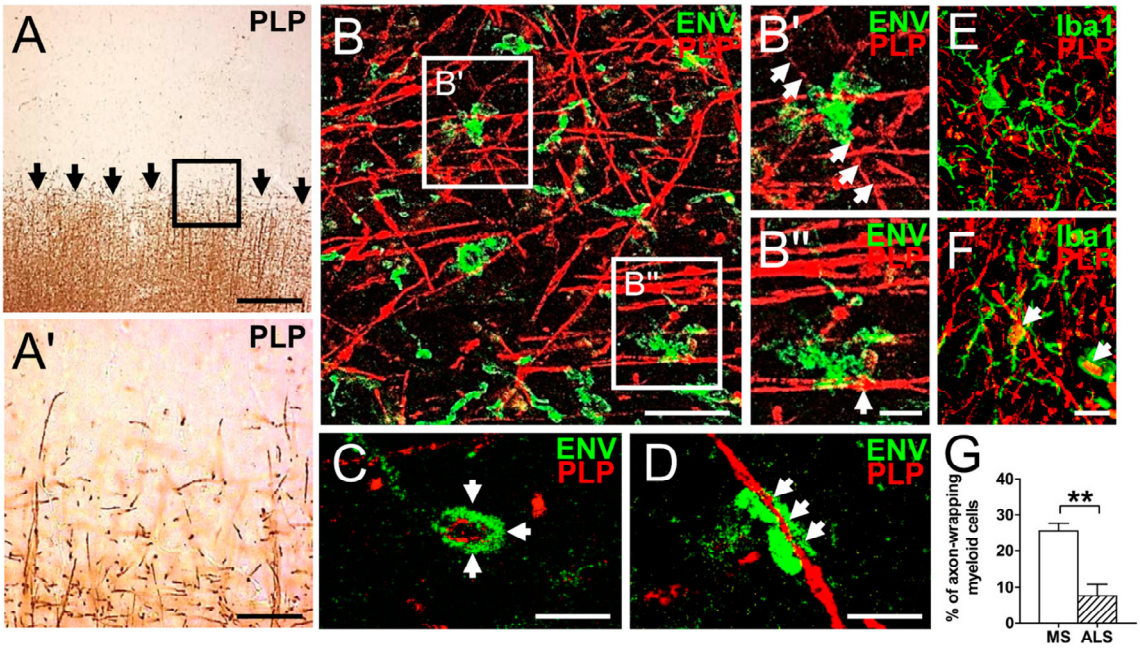

Fig. 2. pHERV-W ENV-positive myeloid cells interact with PLP-positive axons in MS lesions. (*A* and *A′*, *Inset*) Anti-PLP DAB staining of the edge of a chronic active MS lesion (case MS 36). Arrows in *A* indicate the lesion border. (Scale bars: *A*, 200 μm; *A′*, *Inset*, 100 μm.) (*B*–*B′′*) Double immunostaining (merged z-stack) of serial sections of the same lesion (PLP, red; pHERV-W ENV, green). (*B′* and *B′′*, *Insets*) pHERV-W ENV-positive myeloid cells are in direct contact with PLP-positive axons. Arrows in *B′* point to an axon completely wrapped by pHERV-W ENV-positive myeloid cells. The arrow in *B′′* points to a bulb-like axonal structure indicating damage. (Scale bars: *B*, 30 μm; *B′* and *B′′*, 10 μm.) (*C* and *D*) Single-layer confocal imaging showing that pHERV-W ENV-positive cells completely wrap around PLP-positive axons (arrows). (Scale bars: 20 μm.) Representative photographs of axon-wrapping (*F*) and nonwrapping (*E*) myeloid cells in ALS sections (case ALS 15) are revealed by double immunostaining (Iba1, green; PLP red). (Scale bar: 10 μm.) (*G*) Quantification of myeloid cells with axon-wrapping morphologies in MS and ALS tissue sections. Student's 2-tailed *t* test, unpaired: $^{**}P < 0.01$ ($n = 5$ for MS, $n = 2$ for ALS).

translated to decreased protein levels as exemplarily corroborated by IGF-1 ELISA of cell culture supernatants (Fig. 5*B*). IGF-1 has been described to protect OPCs from cell death (42) and to amplify the positive effects of FGF-2 on OPC proliferation (43). Furthermore, it has been described as a stimulator of myelin synthesis (44). CSF-1, on the other hand, decreases proinflammatory cytokine expression, protects oligodendrocytes from apoptosis (45), and exerts neuroprotective effects on neurons (46).

pHERV-W ENV Protein Induces a Proinflammatory Antiregenerative Phenotype in Human Adult Microglia. To translate our findings to the human paradigm, we investigated the key effects of pHERV-W ENV on purified human adult microglia. As revealed by phalloidin-fluorescein isothiocyanate (FITC) staining, stimulation of these cells with pHERV-W ENV resulted in morphological changes such as elongated and amœboid cell morphologies (Fig. 6 *A* and *B*). This morphological shift was accompanied by increased TNF-α and IL-6 transcript levels (Fig. 6 *C* and *D*), which translated to significantly increased respective protein levels in the culture supernatants (Fig. 6 *G* and *H*). In line with previous observations which have substantiated that iNOS/NO is not easily induced in human myeloid cells (summarized in ref. 47), we found no iNOS expression in human microglial cells following pHERV-W ENV stimulation. In contrast, microglial expression of the phagocytosis-associated genes TREM2 and MerTK was significantly decreased in pHERV-W ENV-stimulated microglia (Fig. 6 *E* and *I*). This was also the case for the neuroprotective growth factors IGF-1 and CSF-1 as demonstrated by gene expression analysis (Fig. 6 *F* and *J*). Taken together, these observations in human adult microglia confirmed the results generated in rodent cells.

pHERV-W ENV Induces a Microglia/Axon Association Resulting in Axonal Injury. Taking into account that no ideal animal model for progressive MS exists, and given the fact that transgenic expression of this particular HERV element is currently not established, we used a primary rat coculture system to study interactions between microglia and myelinated axons. To this end, primary rat microglia were added to myelinated neuron/oligodendrocyte cocultures shortly after the peak of their myelination (Fig. 7*A*). On the following day, pHERV-W ENV protein was added to the medium. As revealed by anti-Iba1 immunostaining, pHERV-W ENV stimulation of microglia-containing cocultures led to a shift of microglial phenotypes (Fig. 7 *B*–*D′′′*) similar to the morphological changes observed in rodent and human microglial monocultures (Figs. 4 and 6). Double labeling of myelin basic protein (MBP)-positive myelinated axons and Iba1-positive microglial cells demonstrated a significant increase in activated microglia physically associated with myelinated axons following pHERV-W ENV stimulation throughout the whole observation period (Fig. 7 *E*–*G′*) providing an in vitro recapitulation of the observations made in MS tissue (Fig. 2). Of note, pHERV-W ENV stimulation did not lead to a reduction of the overall percentage of either Iba1-positive microglia or MBP-positive myelinated axons in comparison to controls (Fig. 7 *E′* and *E′′*). In addition, pHERV-W ENV-stimulated microglia were found to induce neurofilament light chain (NFL), synaptophysin (SYP), and MBP leakage from axons as revealed by ELISA of supernatants from

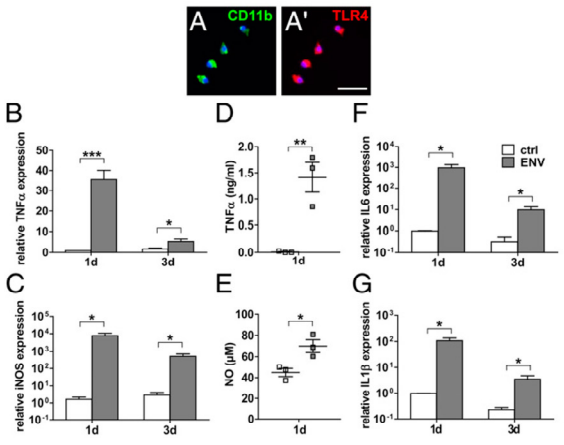

Fig. 3. pHERV-W ENV induces a proinflammatory phenotype in TLR4-positive microglial cells. (*A* and *A'*) TLR4-positive primary rat microglial cells stimulated with pHERV-W ENV protein over a total period of 3 d feature a strong induction of the proinflammatory markers TNF-α (*B*), iNOS (*C*), IL-6 (*F*), and IL-1β (*G*) compared with controls (ctrl). (Scale bar: 20 μm.) Multiple Student's 2-tailed *t* test, unpaired: $*P < 0.05$, $***P < 0{,}001$ ($n = 6$). (*D* and *E*) ELISA-based quantification of TNF-α levels and spectrometry of NO in cell culture supernatants of pHERV-W ENV-stimulated microglia confirms increased levels of these proinflammatory agents after 24 h of pHERV-W ENV stimulation. Student's 2-tailed *t* test, unpaired: $*P < 0.05$, $**P < 0.01$ ($n = 3$). Data are presented as mean ± SEM.

pHERV-W ENV-stimulated cocultures (Fig. 8 *A*, *B*, and *D*). Of note, ENV alone did not lead to such effects, pointing to the key role of microglia. This effect was likely mediated by increased TNF-α concentrations (Fig. 8C).

Discussion

Based on histopathological studies, axonal injury is today widely accepted as a core hallmark of MS (48). Mechanistically, repeated demyelination during the course of MS is thought to lead to the degeneration of axon fibers (49). Accordingly, NFL levels in the CSF of MS patients correlate directly with Expanded Disability Status Scale (EDSS) scores (50), supporting histopathological observations (3). MRI further corroborates the importance of axonal degeneration in MS as gray matter atrophy correlates with disability progression (51). Even though the exact mechanisms underlying axonal degeneration in MS are currently elusive, Wallerian degeneration initiated at lesion sites (52), dysregulation of calcium homeostasis (53), cytotoxic CD8-positive T cells (54), glutamate excitotoxicity (55), and direct NO toxicity (56) are being discussed in this context. As both histological studies and PET-computed tomography (CT) imaging point to the relevance of myeloid cells such as microglia in MS pathology, research has recently focused on their role in axonal degeneration. As a continuation of our previous studies in which we described how pHERV-W ENV inhibits oligodendroglial differentiation (27, 30), in this study, we propose a mechanism by which axonal injury is driven by pHERV-W ENV-activated microglia. While we found that pHERV-W ENV leads to the loss of both myelin and axon integrity, it remains to be demonstrated which of these structures constitutes the primary target of this pathophysiological process. Despite this aspect, we offer a biomedical rationale for the results of the clinical phase IIb CHANGE-MS study (ClinicalTrials.gov identifier NCT02782858) in which anti-pHERV-W ENV (GNbAC1, temelimab) treatment of RRMS patients resulted in significant neuroprotective effects. Among these effects, the 63% reduction in the number of "black holes," an MRI correlate of permanent tissue damage, is particularly striking as, according to PET-CT studies, these structures contain large numbers of microglia. Of note, the primary endpoint of the CHANGE-MS study was the cumulative number of gadolinium-enhancing lesions seen on brain MRI scans after 6 mo (24 wk) of temelimab treatment. This endpoint was not met, and all 48-wk neurodegenerative MRI analyses, including the above-mentioned reduction in the number of "black holes," as well as brain volume and magnetization transfer ratio measurements, were secondary/exploratory. That is why additional studies in a more appropriate population (i.e., nonactive progressive MS patients) at higher doses are currently in a planning stage.

In previous studies, pHERV-W ENV-positive myeloid cells have been reported to be present in MS lesions (27, 28). Going beyond that, we found here that pHERV-W ENV/TLR4–double-positive myeloid cells are abundantly present in MS lesions, where they are tightly associated with myelinated axons at sites of axonal damage. Whether these cells in the MS brain

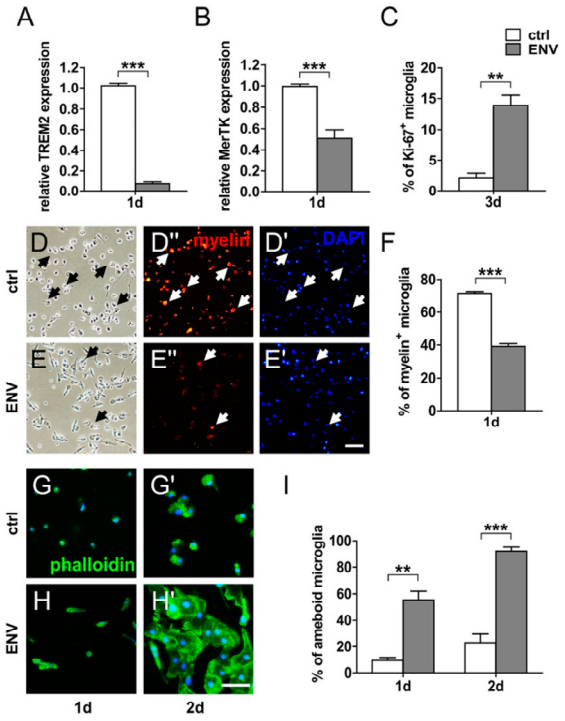

Fig. 4. pHERV-W ENV stimulation decreases the expression of phagocytosis-associated microglial genes, results in diminished myelin phagocytosis capacity, promotes cell proliferation, and induces ameboid microglial morphologies. (*A* and *B*) Gene expression analysis of microglia stimulated with pHERV-W ENV protein shows a significant decrease of the phagocytosis-associated genes TREM2 and MerTK in comparison to controls (ctrl) after 1 d of stimulation. Student's 2-tailed *t* test, unpaired: $***P < 0.001$ ($n = 6$). (*D–F*) Phagocytosis assays with rhodamine-labeled bovine myelin confirm a decreased phagocytosis capacity of microglia stimulated with pHERV-W ENV protein in comparison to ctrl. Arrows indicate rhodamine/myelin-positive cells. Student's 2-tailed *t* test, unpaired: $***P < 0.001$ ($n = 3$). (Scale bar: 40 μm.) (*G–I*) Phalloidin-FITC–based analysis demonstrating that pHERV-W ENV induces an ameboid microglial phenotype already after 1 d of stimulation. Multiple Student's 2-tailed *t* test, unpaired: $**P < 0.01$, $***P < 0.001$ ($n = 3$). (Scale bar: 30 μm.) (*C*) Ki-67–based analysis following 3 d of stimulation reveals that pHERV-W ENV increases microglial proliferation. Student's 2-tailed *t* test, unpaired: $**P < 0.01$ ($n = 3$). Data are presented as mean ± SEM.

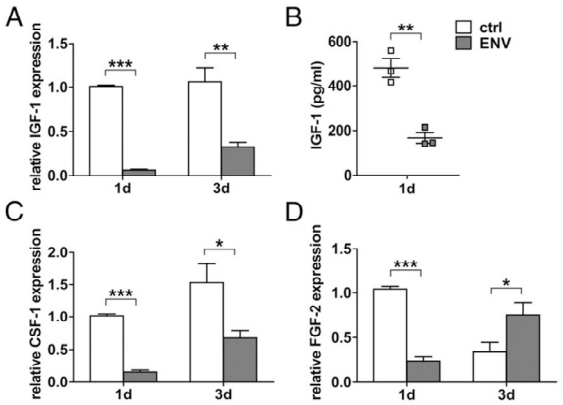

Fig. 5. pHERV-W ENV decreases microglial expression of regenerative factors. Stimulation of microglia with pHERV-W ENV protein results in a significant decrease of the transcription of the regenerative genes IGF-1 (*A*), CSF-1 (*C*), and FGF-2 (*D*) exemplarily confirmed by IGF-1 ELISA showing decreased levels of this protein in cell culture supernatants (*B*). Student's 2-tailed *t* test, unpaired: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (*A*, *C*, and *D*, $n = 6$; *B*, $n = 3$). Data are presented as mean ± SEM. ctrl, controls.

express pHERV-W ENV themselves or if they phagocytose extracellular pHERV-W ENV, possibly previously imported into the CNS by other cells, is currently unclear. Mechanistically however, this aspect is probably less important, given the fact that the pHERV-W ENV receptor TLR4 has been described as both a surface and intracellular receptor (57, 58). Of note, TLR4 is also involved in the innate immune response to respiratory syncytial virus through an interaction with the viral envelope fusion protein (59, 60). The decisive finding of this study is that pHERV-W ENV-activated microglia cause a breakdown of both axonal and myelin sheath integrity leading to leakage of intraaxonal and myelin proteins. Mechanistically, this can be explained by our in vitro experiments in both rodent and human microglia demonstrating that pHERV-W ENV induces a cellular phenotype secreting noxious molecules such as TNF-α and NO that are harmful to axons. Furthermore, we could demonstrate that pHERV-W ENV drives microglia to associate themselves with axons in myelinated neuron/oligodendrocyte cocultures in vitro. In this context, the question arises as to whether, in MS, pHERV-W ENV-positive microglia/monocytes are a primary mediator of axonal injury or if they are merely secondarily chemoattracted to axons that are already injured or (partially) demyelinated. However, even if pHERV-W ENV-positive activated cells were only to associate themselves with axons "at risk" secondarily or parallel to occurring damage, they contribute to further axonal demise as we demonstrate here. It therefore remains to be shown whether future MS model systems mimicking pHERV-W reactivation and expression will be able to shed further light on the exact underlying kinetics. Finally, it is intriguing that the CHANGE-MS trial results were obtained in an RRMS population where classically inflammation is assumed to outweigh degeneration. The results of this study therefore underline once more the relevance of neurodegeneration even in early MS. Mirroring this, beyond PPMS and SPMS brains, we also found pHERV-W ENV-positive myeloid cells adjacent to axons in an RRMS brain. Therefore, while future clinical studies will have to assess the effect of anti-ENV treatment in progressive MS, targeting ENV in relapsing subtypes seems to be a worthwhile approach as well.

Materials and Methods

Experimental Design. The primary objective of this study was to establish pHERV-W ENV as a mediator of axonal degeneration in MS. In this regard, we could confirm its presence in myeloid cells in SPMS, PPMS, and RRMS brains by immunohistochemical methods. Functional experiments demonstrating that pHERV-W ENV induces a proinflammatory and antiregenerative microglial phenotype were performed on both rodent and human primary microglia. To clarify the observations made in the MS brain, we used myelinated neuron/glia cocultures to study the interaction between microglia and axons. The number of replicates (*n*) per experiment is noted in each figure legend. The quantitative analyses were performed blinded. One of the limitations of this study consists of the fact that, unfortunately, an animal model featuring transgenic expression of human pHERV-W ENV in microglia is still unavailable. Such a model would have been ideal to study the interaction between pHERV-W ENV-positive microglia and axons in the brain, which, instead, had to be performed in neuron/glia cocultures. In addition, and as a general limitation of most studies in our field, there is no established animal model for progressive MS. A further limitation of this study lies in the fact that it is not a comprehensive retrospective analysis systematically investigating pHERV-W ENV positivity in different MS disease variants and lesion subtypes. For instance, we did not study chronic inactive lesions where the inflammatory process has virtually died out. However, neurodegeneration in these regions has previously been reported to be comparable to levels seen in non-MS control patients (61). Of note, this study was designed as a first neurobiological proof-of-principle approach to establish the basic mechanisms of pHERV-W ENV-induced axonal degeneration. Moreover, a definite distinction between genuine microglia and infiltrating monocytes based on cell surface markers is still challenging despite the recent description of TMEM119, as there is evidence that it is expressed in only a subset of microglial cells.

Immunohistochemistry of Human Tissue Sections. Tissue sections from the brains of 5 MS patients (3 SPMS, 1 PPMS, and 1 RRMS), 2 ALS patients, and 2 HCs were studied (Table 1). All brains were collected as part of the tissue procurement program approved by the Cleveland Clinic Institutional Review Board. All donors or their surrogates gave informed consent for their brains

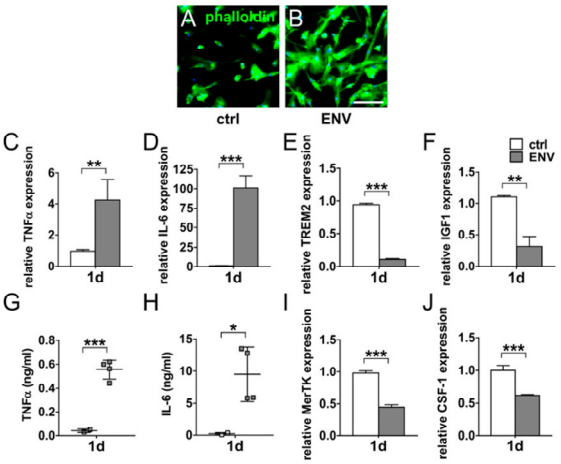

Fig. 6. pHERV-W ENV leads to a proinflammatory antiregenerative phenotype in human adult microglia. (*A* and *B*) Phalloidin-FITC–based analysis of human adult microglia stimulated with pHERV-W ENV protein demonstrating an induction of ameboid phenotypes. (Scale bar: 100 μm.) pHERV-W ENV induces the transcription of proinflammatory genes TNF-α and IL-6 (*C* and *D*), which is mirrored by increased supernatant levels of the respective proteins (*G* and *H*). Student's 2-tailed *t* test, unpaired: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ ($n = 3$ controls [ctrl], $n = 4$ ENV). (*E* and *I*) pHERV-W ENV stimulation decreases the expression of phagocytosis-associated microglial genes TREM2 and MerTK. (*F* and *J*) pHERV-W ENV also reduces the expression of the regenerative genes IGF-1 and CSF-1. Student's 2-tailed *t* test, unpaired: $**P < 0.01$, $***P < 0.001$ ($n = 3$ ctrl, $n = 4$ ENV). Data are presented as mean ± SEM.

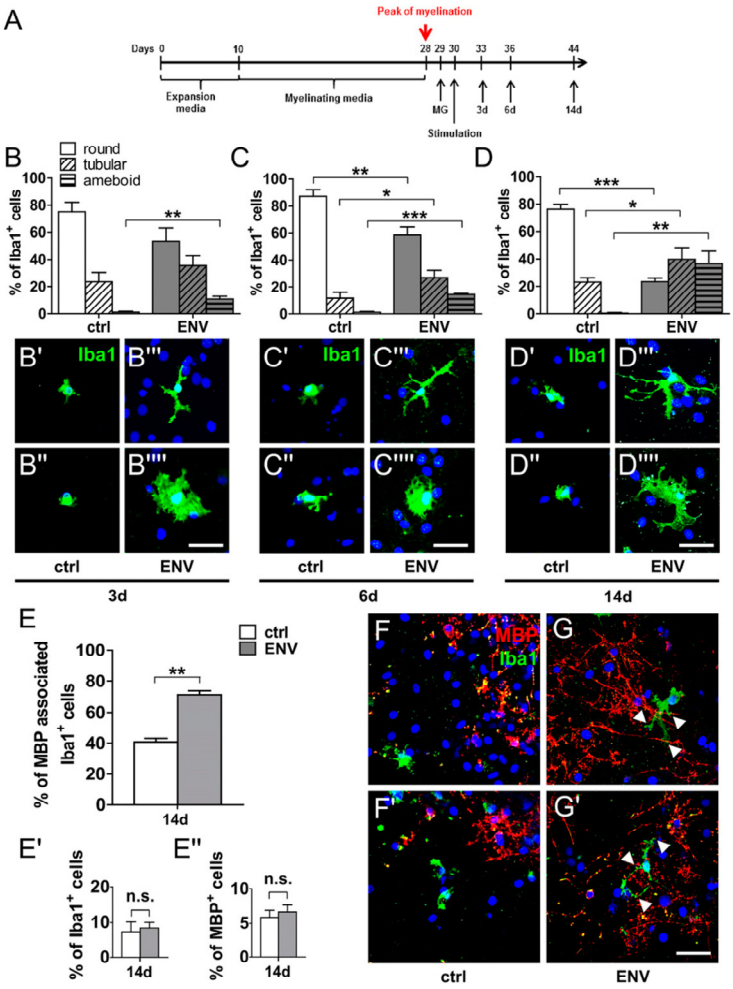

Fig. 7. pHERV-W ENV stimulation induces microglia to associate themselves with myelinated axons. (*A*) Rat primary microglia (MG) were added to neuron/oligodendrocyte cocultures shortly after the peak of myelination, and cultures were stimulated with pHERV-W ENV. (*B–D''''*) Anti-Iba1 immunostaining shows that, mirroring monoculture experiments, pHERV-W ENV leads to a persistent induction of tubular and ameboid microglial phenotypes after as early as 3 d of stimulation in comparison to controls (ctrl). Multiple Student's 2-tailed *t* test, unpaired: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ ($n = 3$). (*D–D''''*) This effect is sustained over a total period of 14 d. (Scale bars: 50 μm.) (*E–G'*) Double immunostaining of MBP-positive myelinated axons (red) and Iba1-positive microglia (green) in cocultures following 14 d of pHERV-W ENV stimulation demonstrating that pHERV-W ENV leads to a significant increase in microglia associating themselves with axonal structures (arrowheads in *G* and *G'*), mirroring the findings in MS tissue. Student's 2-tailed *t* test, unpaired: $**P < 0.01$ ($n = 3$). n.s., not significant. (Scale bar: 25 μm.) Data are presented as mean ± SEM.

to be used for research studies. All human immunohistochemistry experiments were carried out in accordance with the Cleveland Clinic Institutional regulations and guidelines.

Briefly, MS tissue was fixed in 4% paraformaldehyde (PFA), protected in 70% sucrose, placed on the stage of a sliding microtome, and frozen. Free-floating sections (16–30 μm thick) were cut without exposure to solvents or other embedding mediums. Sections were rinsed in phosphate-buffered saline (PBS) 4 times for 5 min each time, microwaved once for 5 min in 10 mM citrate buffer (pH 6.0), incubated in 3% hydrogen peroxide and 10% Triton X-100 for 30 min, and immunostained by the avidin–biotin complex procedure and with DAB (Sigma–Aldrich) as described previously (62). Sections for confocal fluorescence microscopy were pretreated as described above, incubated with 2 primary antibodies, and then incubated with secondary antibodies (Abcam) conjugated to either Alexa Fluor 594 or Alexa Fluor 488 (Thermo Fisher Scientific). The following primary antibodies were used: mouse anti-MHCII (1/250; Research Resource Identifier [RRID]:AB_2313661; Dako, Agilent Technologies), rat anti-PLP (1/250, hybridoma; a gift from W. Macklin, Department of Cell and Developmental Biology, University of Colorado School of Medicine, Aurora, CO), mouse anti–pHERV-W ENV (GN-mAB_03 [3B2H4], 1/1,000; provided by GeNeuro SA), rabbit anti-TLR4 (1/1,000; RRID:AB_300457; Abcam), and rabbit anti-Iba1 (1/500; RRID:AB_839504; WAKO Pure Chemical Corporation). Anti-ENV/MHCII double staining was carried out sequentially with intermediate blocking and washing steps. Sections were analyzed on a Leica Aristoplan laser scanning microscope (Leitz) and on a Zeiss confocal CLSM 510 microscope. Analysis was performed using ImageJ and Zen 2012 software (Zeiss), respectively. Individual confocal optical sections represented an axial resolution of 0.5 μm. The entire thickness of the section was scanned. Images consisted either of single layers or merged z-stacks combining 16–32 single layers. Fluorescence was collected individually in the green (Alexa 488) and red (Alexa 594) channels to eliminate "bleed-through" from either channel.

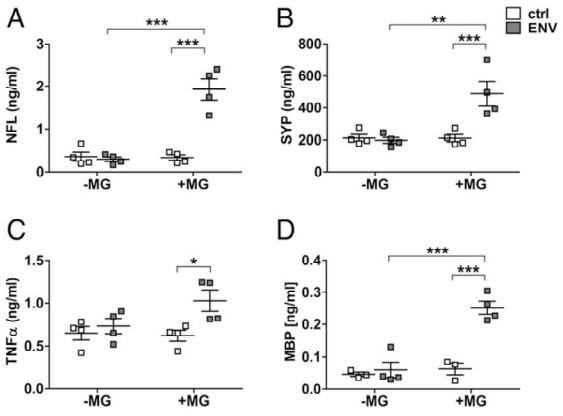

Fig. 8. pHERV-W ENV stimulation induces microglial-dependent injury of myelinated axons. pHERV-W ENV protein-stimulated microglia (MG) induce axonal NFL (*A*), SYP (*B*), and MBP (*D*) leakage as revealed by ELISA of co-culture supernatants. Note that pHERV-W ENV protein and nonstimulated MG alone were unable to exert such effects. (*C*) This was most likely mediated by increased TNF-α levels as revealed by ELISA. Two-way ANOVA, followed by Bonferroni's post hoc test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (n = 3–4). ctrl, controls.

Primary Rat Microglial Cell Culture. All animal procedures were performed in compliance with the experimental guidelines approved by the regional authorities (state agency for Nature, Environment and Consumer Protection of North Rhine Westphalia) and conform to the NIH *Guide for the Care and Use of Laboratory Animals* (63). The Institutional Review Board (IRB) of the ZETT (Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben) at the Heinrich Heine University Düsseldorf has approved all animal procedures under licences O69/11 and O82/12. Briefly, dissociated postnatal day 1 (P1) Wistar rat cortices were cultured on poly-D-lysine–coated cell culture flasks in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) substituted with 10% fetal calf serum (FCS; Lonza), 4 mM L-glutamine (Invitrogen), and 50 U/mL penicillin/streptomycin (Invitrogen) as previously described (30, 64–67). After 10 d, flasks were shaken at 180 rpm for 2 h. Microglia-containing supernatants were transferred to bacterial dishes and kept in the incubator, allowing for cell attachment to the surface. Culture flasks were again loaded with fresh DMEM and shaken for another 24 h to increase the final cell yield. Afterward, supernatants were again transferred to bacterial dishes to allow for attachment. Microglia-containing bacterial dishes from the first and second shaking steps were checked for viability via bright-field microscopy, medium was discarded, and cells were rinsed with PBS. Microglia were dislodged by accutase (Thermo Fisher Scientific), which was stopped by FCS-containing DMEM. Microglial cell suspensions were then centrifuged for 5 min at 1,500 rpm at 4 °C. Cell-free supernatants were discarded. Cell pellets were then resuspended in 80 μL of magnetic activated cell sorting (MACS) buffer containing 0.5% bovine serum albumin (BSA) in PBS, and 20 μL of CD11b/c microbeads (Miltenyi Biotec) was added for 15 min at 2–8 °C to allow for binding. Cells were then washed adding 2 mL of MACS buffer and spun down for 5 min at 1,500 rpm at 4 °C. Supernatants were again discarded, and pellets were resuspended in 500 μL of MACS buffer and subjected to MACS sorting according to the manufacturer's protocol (Miltenyi Biotec). The resulting cell suspension was again spun down for 5 min at 1,500 rpm at 4 °C, pellets were resuspended in 1 mL of DMEM, and cell viability and numbers were quantified using trypan blue staining. Average cell purities as assessed by Iba1 positivity were consistently *ca.* 98%. Microglia were seeded on cell culture dishes at different concentrations in DMEM containing 10% FCS and 2 mM L-glutamine.

Microglial Culture Experiments. pHERV-W ENV stimulation of both human and rat primary cells was carried out at a concentration of 1,000 ng/mL recombinant full-length ENV protein with respective buffer volumes as controls as previously described (27, 30). Recombinant ENV protein was produced by Protein'eXpert according to quality control specifications of GeNeuro SA. Endotoxin levels were below the detection limit (<5 endotoxin units [EU]/ml) as measured by the limulus amebocyte lysate test. Control experiments to confirm ENV- and TLR4-specific effects were previously published (27, 30, 31). NO spectrometry and TNF-α and IGF-1 ELISAs of rat microglial cell culture supernatants were performed after 24 h of pHERV-W ENV stimulation using a Nitric Oxide Assay Kit (Merck), a rat TNF alpha ELISA Kit (Abcam), and a Quantikine ELISA Kit (R&D Systems), respectively, according to the manufacturers' protocols. ELISAs of human microglial cell culture supernatants were performed after 24 h of pHERV-W ENV stimulation using a TNF-α ELISA (catalog no. 555212; BD Biosciences), an IL-6 ELISA (catalog no. 555220; BD Biosciences), and an IGF-1 ELISA (catalog no. DY291; R&D Systems) according to the manufacturers' protocols. Immunocytochemistry was performed using previously described protocols (27, 30). Briefly, microglia were fixed with 4% PFA, washed with PBS, blocked for 45 min using 2% normal goat serum (Sigma–Aldrich) and 0.5% Triton X-100 (Sigma–Aldrich) in PBS, and subjected to incubation at 4 °C overnight in 2% normal goat serum (Sigma–Aldrich) and 0.1% Triton X-100 (Sigma–Aldrich) in PBS with mouse anti-CD11b (1/500; RRID:AB_395560; BD Biosciences), mouse anti-TLR4 (1/1,000; RRID:AB_300457; Abcam), and rabbit anti-Ki67 (1/250; RRID:AB_302459; Abcam). Following PBS washes, secondary anti-mouse and anti-rabbit antibodies conjugated with Alexa Fluor 594 (1/500; Thermo Fisher Scientific) were added for 2 h at room temperature. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Roche). Cells were mounted using Citifluor (Citifluor) and analyzed with an Axio Cam HRc microscope (Zeiss). For morphology experiments, microglial F-actin was visualized with FITC-conjugated phalloidin (Sigma–Aldrich) according to the manufacturer's protocol. Myelin phagocytosis experiments were carried out using purified bovine myelin (68) and phRodo (Thermo Fisher Scientific). Briefly, 1 mg of myelin was resuspended in 1 mL of PBS at pH 8. Afterward, 10 μL of phRodo was added and the mix was incubated on a shaker for 1 h at room temperature. The phRodo/myelin was then centrifuged at 1,500 rpm for 10 min. The pellet was resuspended in 1 mL of fresh PBS (pH 8) and diluted to a final concentration of 20 μg/mL. Twenty microliters of phRodo/myelin/PBS was then added to pHERV-W ENV-stimulated and control cells, respectively. Upon a 3-h incubation step at 37 °C, cells were rinsed 3 times with PBS and fixed with 4% PFA. After staining with DAPI, phRodo/myelin-positive microglia were quantified. Images (20× magnification; Zeiss Axionplan 2 microscope) were captured using the same light intensity and filters for all images to be compared and were processed with Axiovision 4.2 software (Zeiss; RRID: SciRes_000111). The analysis was done using Java software (ImageJ, RRID: nif-0000-30,467/Wright Cell Imaging Facility, RRID:nif-0000-30,471). Immunopositive cells were counted in 9 randomly chosen fields per coverslip. Two coverslips were used per condition. The total number of cells per field was determined via DAPI staining. For quantification, the number of immunopositive cells was compared with the total cell number and expressed as a percentage (mean ± SEM) as previously described (67).

Primary Human Microglial Cell Culture. Adult microglia were derived from surgical resection of brain tissue from pharmacologically intractable non-malignant temporal lobe epilepsy cases, and secondary use of deidentified tissues was approved and carried out in accordance with the guidelines set by the McGill University Institutional Review Board in conjunction with the McGill University Health Centre Ethics Board under protocol ANTJ1989. Tissue provided was outside of the suspected focal site of epilepsy pathology, histopathological changes were excluded by an experienced neuropathologist, and histologically healthy specimens were included. Human microglia were isolated from this adult brain tissue using previously described protocols (69, 70). Briefly, tissue was obtained in pieces <1 mm^3 and treated with DNase (Roche) and trypsin (Thermo Fisher Scientific) for 30 min at 37 °C. Following dissociation through a nylon mesh (37 μm), the cell suspension was separated on a 30% Percoll gradient (GE Healthcare) at 31,000 × *g* for 30 min. Glial cells (oligodendroglia and microglia) were collected from underneath the myelin layer, washed, and plated at a density of 2×10^6 cells per milliliter in tissue-culture flasks. After 24 h in culture, microglia were separated by the differential adhesion properties of the cells. Microglia were grown for 4 d in flasks before gentle collection using 2 mM ethylenediaminetetraacetic acid (Sigma–Aldrich), and were then plated in minimum essential medium (MEM; Sigma–Aldrich) supplemented with 5% FBS (Wisent), 0.1% penicillin/streptomycin (Thermo Fisher Scientific), and 0.1% L-glutamine (Thermo Fisher Scientific) at a density of 1×10^6 cells per milliliter in a 6-well plate. pHERV-W ENV protein stimulation was carried out as specified for rat microglial cells. For morphology experiments, microglial F-actin was visualized with FITC-conjugated phalloidin (Sigma–Aldrich) according to the manufacturer's protocol. ELISAs of human microglial cell culture supernatants were performed after 24 h of pHERV-W ENV stimulation using TNF-α ELISA (catalog no. 555212; BD Biosciences), IL-6 ELISA (catalog no. 555220; BD Biosciences)

and IGF-1 ELISA (catalog no. DY291; R&D Systems) according to the manufacturers' protocols.

Myelinated Neuron/Glia Cocultures. Dissociated neuron/oligodendrocyte cocultures were obtained from embryonic day 16 Wistar rat cerebral cortices (Wistar rats of either sex) as previously published (67, 71). Cells were plated on 15-mm poly-D-lysine (0.1 mg/mL)–coated coverslips (65,000 cells per coverslip) and kept in myelination medium consisting of N2 and neurobasal medium (Thermo Fisher Scientific) and containing nerve growth factor (NGF) (50 ng/mL) and NT-3 (10 ng/mL) (both from R&D Systems). After 10 d in vitro (DIV10), insulin was removed and the ratio of the insulin-free N2 to neurobasal medium including B27 supplement (Thermo Fisher Scientific) was adjusted to 4:1. This myelination medium was further supplemented with 60 ng/mL tri-iodo-thyronine (Sigma–Aldrich). Final concentrations of individual N2 medium components (DMEM-F12–based, high glucose; Thermo Fisher Scientific) were as follows: insulin (10 μg/mL), transferrin (50 μg/mL), sodium selenite (5.2 ng/mL), hydrocortisone (18 ng/mL), putrescine (16 μg/mL), progesterone (6.3 ng/mL), biotin (10 ng/mL), and *N*-acetyl-L-cysteine (5 μg/mL) (all from Sigma–Aldrich); BSA (0.1%; Roth); and penicillin/streptomycin (50 units/mL; Thermo Fisher Scientific). At DIV30, cultures were supplemented with primary rat microglial cells in the presence or absence of 1,000 ng/mL recombinant pHERV-W ENV protein for another 3, 6, or 14 d. Then, coverslips were washed with PBS, fixed with 4% PFA, and processed for immunofluorescent staining. Media were changed every 72 h. PFA-fixed cocultures were blocked with PBS containing 0.5% Triton X-100 and 2% normal goat serum, and then incubated overnight in 0.1% Triton X-100 and 2% normal goat serum containing the following primary antibodies: rat anti-MBP (1/250; catalog no. MCA409S; Bio-Rad; RRID:AB_325004), rabbit anti-Iba1 (1/500; WAKO Pure Chemical Corporation; RRID:AB_839504), and mouse antineurofilament (1/1,000; catalog no. SMI-312R-500; BioLegend; RRID:AB_2314906). After 24 h, coverslips were washed with PBS and then incubated in PBS for 2 h with secondary antibodies conjugated to Alexa Fluor 488 (1/500; Thermo Fisher Scientific), Alexa Fluor 594 (1/500; Thermo Fisher Scientific), or Alexa Fluor 405 (1/500; Thermo Fisher Scientific). NFL, SYP, MBP, and TNF-α ELISAs were performed on coculture supernatants collected from DIV2 to DIV5 using rat neurofilament light polypeptide (catalog no. EKC39470; Biomatic), rat SYP (catalog no. LS-F22650; LifeSpan BioSciences, Inc.), rat MBP (catalog no. LS-F4093; LifeSpan BioSciences, Inc.), and rat TNF-α (catalog no. ab100785; Abcam) ELISA kits according to the manufacturers' protocols. All images captured on either a Zeiss Axionplan 2 microscope or a Zeiss confocal CLSM 510 microscope (Zeiss) were captured using the same light intensity and filters. Images were processed with Axiovision 4.2 software or Zen 2012 software (Zeiss). Analysis was performed using Java software (ImageJ). Immunopositive cells were counted in 9 randomly chosen fields per coverslip.

RNA Preparation, cDNA Synthesis, and Quantitative RT-PCR. Total RNA purification from cells was performed using the RNeasy procedure (Qiagen). Isolated RNA was reverse-transcribed using a high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative determination of gene expression levels was performed on a 7900HT sequence detection system (Thermo Fisher Scientific) using Power SybrGreen and TaqMan universal master mixes (Thermo Fisher Scientific) as previously described (27, 30). Primer sequences were as follows: rat iNOS (CTC AGC ACA GAG GGC TCA AAG, TGC ACC CAA ACA CCA AGG T), rat TNF-α (AGC CCT GGT ATG AGC CCA TGT A, CCG GAC TCC GTG ATG TCT AAG T), rat IL-6 (GTT GTG CAA TGG CAA TTC TGA, TCT GAC AGT GCA TCA TCG CTG), rat IL-1β (GAA ACA GCA ATG GTC GGG AC, AAG ACA CGG GTT CCA TGG TG), rat TREM2 (CCA AGG AGC CAA TCA GGA AA, GGC CAG GAG GAG AAG AAT GG), rat MerTK (TCT GAC AGA GAC CGC AGT CTT C, TGG ACA CCG TCA GTC CTT TG), rat IGF-1 (AGACGGGCATTGTGGATGA, ACATCTCCAGCCTCCTCAGATC), rat CSF-1(CGA GGT GTC GGA GCA CTG TA, TCA ACT GCT GCA AAA TCT GTA GGT) and rat FGF-2(TGG TAT GTG GCA CTG AAA CGA, CCA GGC CCC GTT TTG G). Detection of human TNF-α, human IL-6, human TREM2, human MerTK, human IGF-1 and human CSF-1 was done using TaqMan probe sets (Thermo Fisher Scientific) Hs00174128_m1, Hs00174131_m1, Hs00219132_m1, Hs01031979_m1, Hs01547656_m1, and Hs00174164_m1, respectively. Relative gene expression levels were determined according to the ΔΔcycle threshold (ΔΔCt) method (Thermo Fisher Scientific). Each sample was measured in quadruplicate; data are shown as mean values ± SEM, and the *t* test was applied to determine statistical significance (Prism 5.0c; GraphPad Software).

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1. B. D. Trapp *et al.*, Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* **338**, 278–285 (1998).
2. C. Bjartmar, B. D. Trapp, Axonal degeneration and progressive neurologic disability in multiple sclerosis. *Neurotox. Res.* **5**, 157–164 (2003).
3. T. Kuhlmann, G. Lingfeld, A. Bitsch, J. Schuchardt, W. Brück, Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* **125**, 2202–2212 (2002).
4. A. Kutzelnigg *et al.*, Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain* **128**, 2705–2712 (2005).
5. J. W. Peterson, L. Bö, S. Mörk, A. Chang, B. D. Trapp, Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Ann. Neurol.* **50**, 389–400 (2001).
6. K. A. Nave, B. D. Trapp, Axon-glial signaling and the glial support of axon function. *Annu. Rev. Neurosci.* **31**, 535–561 (2008).
7. F. Ginhoux, M. Prinz, Origin of microglia: Current concepts and past controversies. *Cold Spring Harb. Perspect. Biol.* **7**, a020537 (2015).
8. M. Kouwenhoven, N. Teleshova, V. Ozenci, R. Press, H. Link, Monocytes in multiple sclerosis: Phenotype and cytokine profile. *J. Neuroimmunol.* **112**, 197–205 (2001).
9. M. Greter, I. Lelios, A. L. Croxford, Microglia versus myeloid cell nomenclature during brain inflammation. *Front. Immunol.* **6**, 249 (2015).
10. M. L. Bennett *et al.*, New tools for studying microglia in the mouse and human CNS. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E1738–E1746 (2016).
11. J. Satoh *et al.*, TMEM119 marks a subset of microglia in the human brain. *Neuropathology* **36**, 39–49 (2016).
12. J. Correale, M. I. Gaitán, M. C. Ysrraelit, M. P. Fiol, Progressive multiple sclerosis: From pathogenic mechanisms to treatment. *Brain* **140**, 527–546 (2017).
13. C. S. Jack *et al.*, TLR signaling tailors innate immune responses in human microglia and astrocytes. *J. Immunol.* **175**, 4320–4330 (2005).
14. H. Lassmann, Mechanisms of white matter damage in multiple sclerosis. *Glia* **62**, 1816–1830 (2014).
15. P. Giannetti *et al.*, Microglia activation in multiple sclerosis black holes predicts outcome in progressive patients: An in vivo [(11)C](R)-PK11195-PET pilot study. *Neurobiol. Dis.* **65**, 203–210 (2014).
16. F. L. Heppner *et al.*, Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat. Med.* **11**, 146–152 (2005).
17. L. Airas, M. Nylund, E. Rissanen, Evaluation of microglial activation in multiple sclerosis patients using positron emission tomography. *Front. Neurol.* **9**, 181 (2018).
18. A. Lampron *et al.*, Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *J. Exp. Med.* **212**, 481–495 (2015).
19. R. Orihuela, C. A. McPherson, G. J. Harry, Microglial M1/M2 polarization and metabolic states. *Br. J. Pharmacol.* **173**, 649–665 (2016).
20. R. M. Ransohoff, A polarizing question: Do M1 and M2 microglia exist? *Nat. Neurosci.* **19**, 987–991 (2016).
21. C. Feschotte, C. Gilbert, Endogenous viruses: Insights into viral evolution and impact on host biology. *Nat. Rev. Genet.* **13**, 283–296 (2012).
22. P. Küry *et al.*, Human endogenous retroviruses in neurological diseases. *Trends Mol. Med.* **24**, 379–394 (2018).
23. H. Perron *et al.*, Endogenous retroviral genes, herpesviruses and gender in multiple sclerosis. *J. Neurol. Sci.* **286**, 65–72 (2009).
24. F. C. Hsiao *et al.*, EBV LMP-2A employs a novel mechanism to transactivate the HERV-K18 superantigen through its ITAM. *Virology* **385**, 261–266 (2009).
25. C. Nellåker *et al.*, Transactivation of elements in the human endogenous retrovirus W family by viral infection. *Retrovirology* **3**, 44 (2006).
26. H. Perron *et al.*, Human endogenous retrovirus type W envelope expression in blood and brain cells provides new insights into multiple sclerosis disease. *Mult. Scler.* **18**, 1721–1736 (2012).
27. D. Kremer *et al.*, Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. *Ann. Neurol.* **74**, 721–732 (2013).
28. J. van Horssen, S. van der Pol, P. Nijland, S. Amor, H. Perron, Human endogenous retrovirus W in brain lesions: Rationale for targeted therapy in multiple sclerosis. *Mult. Scler. Relat. Disord.* **8**, 11–18 (2016).
29. S. Sotgiu *et al.*, Multiple sclerosis-associated retrovirus and progressive disability of multiple sclerosis. *Mult. Scler.* **16**, 1248–1251 (2010).
30. D. Kremer *et al.*, The neutralizing antibody GNbAC1 abrogates HERV-W envelope protein-mediated oligodendroglial maturation blockade. *Mult. Scler.* **21**, 1200–1203 (2015).

31. A. Rolland *et al.*, The envelope protein of a human endogenous retrovirus-W family activates innate immunity through CD14/TLR4 and promotes Th1-like responses. *J. Immunol.* **176**, 7636–7644 (2006).
32. T. Touil, M. S. Deloire-Grassin, C. Vital, K. G. Petry, B. Brochet, In vivo damage of CNS myelin and axons induced by peroxynitrite. *Neuroreport* **12**, 3637–3644 (2001).
33. M. Karamita *et al.*, Therapeutic inhibition of soluble brain TNF promotes remyelination by increasing myelin phagocytosis by microglia. *JCI Insight* **2**, 87455 (2017).
34. M. S. Petrovich *et al.*, Pentoxifylline suppression of TNF-alpha mediated axonal degeneration in the rabbit optic nerve. *Neurol. Res.* **19**, 551–554 (1997).
35. Y. Kitaoka *et al.*, TNF-alpha-induced optic nerve degeneration and nuclear factor-kappaB p65. *Invest. Ophthalmol. Vis. Sci.* **47**, 1448–1457 (2006).
36. T. K. Ulland, M. Colonna, TREM2–A key player in microglial biology and Alzheimer disease. *Nat. Rev. Neurol.* **14**, 667–675 (2018).
37. L. M. Healy *et al.*, MerTK-mediated regulation of myelin phagocytosis by macrophages generated from patients with MS. *Neurol. Neuroimmunol. Neuroinflamm.* **4**, e402 (2017).
38. L. M. Healy *et al.*, MerTK is a functional regulator of myelin phagocytosis by human myeloid cells. *J. Immunol.* **196**, 3375–3384 (2016).
39. W. F. Blakemore, Regeneration and repair in multiple sclerosis: The view of experimental pathology. *J. Neurol. Sci.* **265**, 1–4 (2008).
40. M. S. Natrajan *et al.*, Retinoid X receptor activation reverses age-related deficiencies in myelin debris phagocytosis and remyelination. *Brain* **138**, 3581–3597 (2015).
41. M. S. Natrajan *et al.*, Pioglitazone regulates myelin phagocytosis and multiple sclerosis monocytes. *Ann. Clin. Transl. Neurol.* **2**, 1071–1084 (2015).
42. S. Lin *et al.*, IGF-1 protects oligodendrocyte progenitor cells and improves neurological functions following cerebral hypoxia-ischemia in the neonatal rat. *Brain Res.* **1063**, 15–26 (2005).
43. F. Jiang, T. J. Frederick, T. L. Wood, IGF-I synergizes with FGF-2 to stimulate oligodendrocyte progenitor entry into the cell cycle. *Dev. Biol.* **232**, 414–423 (2001).
44. R. L. Mozell, F. A. McMorris, Insulin-like growth factor I stimulates oligodendrocyte development and myelination in rat brain aggregate cultures. *J. Neurosci. Res.* **30**, 382–390 (1991).
45. R. Kadota *et al.*, Granulocyte colony-stimulating factor (G-CSF) protects oligodendrocyte and promotes hindlimb functional recovery after spinal cord injury in rats. *PLoS One* **7**, e50391 (2012).
46. J. Luo *et al.*, Colony-stimulating factor 1 receptor (CSF1R) signaling in injured neurons facilitates protection and survival. *J. Exp. Med.* **210**, 157–172 (2013).
47. A. M. Smith, M. Dragunow, The human side of microglia. *Trends Neurosci.* **37**, 125–135 (2014).
48. H. Lassmann, J. van Horssen, The molecular basis of neurodegeneration in multiple sclerosis. *FEBS Lett.* **585**, 3715–3723 (2011).
49. Y. You *et al.*, Demyelination precedes axonal loss in the transneuronal spread of human neurodegenerative disease. *Brain* **142**, 426–442 (2019).
50. C. Malmeström, S. Haghighi, L. Rosengren, O. Andersen, J. Lycke, Neurofilament light protein and glial fibrillary acidic protein as biological markers in MS. *Neurology* **61**, 1720–1725 (2003).
51. C. Jacobsen *et al.*, Brain atrophy and disability progression in multiple sclerosis patients: A 10-year follow-up study. *J. Neurol. Neurosurg. Psychiatry* **85**, 1109–1115 (2014).
52. T. Dziedzic *et al.*, Wallerian degeneration: A major component of early axonal pathology in multiple sclerosis. *Brain Pathol.* **20**, 976–985 (2010).
53. A. Nicot, P. V. Ratnakar, Y. Ron, C. C. Chen, S. Elkabes, Regulation of gene expression in experimental autoimmune encephalomyelitis indicates early neuronal dysfunction. *Brain* **126**, 398–412 (2003).
54. H. Neumann, I. M. Medana, J. Bauer, H. Lassmann, Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci.* **25**, 313–319 (2002).
55. M. Kostic, N. Zivkovic, I. Stojanovic, Multiple sclerosis and glutamate excitotoxicity. *Rev. Neurosci.* **24**, 71–88 (2013).
56. K. J. Smith, R. Kapoor, S. M. Hall, M. Davies, Electrically active axons degenerate when exposed to nitric oxide. *Ann. Neurol.* **49**, 470–476 (2001).
57. S. Dunzendorfer, H. K. Lee, K. Soldau, P. S. Tobias, Toll-like receptor 4 functions intracellularly in human coronary artery endothelial cells: Roles of LBP and sCD14 in mediating LPS responses. *FASEB J.* **18**, 1117–1119 (2004).
58. T. Shibata *et al.*, Intracellular TLR4/MD-2 in macrophages senses Gram-negative bacteria and induces a unique set of LPS-dependent genes. *Int. Immunol.* **23**, 503–510 (2011).
59. L. M. Haynes *et al.*, Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *J. Virol.* **75**, 10730–10737 (2001).
60. E. A. Kurt-Jones *et al.*, Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat. Immunol.* **1**, 398–401 (2000).
61. B. F. Popescu, I. Pirko, C. F. Lucchinetti, Pathology of multiple sclerosis: Where do we stand? *Continuum (Minneap Minn)* **19**, 901–921 (2013).
62. L. Bö *et al.*, Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions. *J. Neuroimmunol.* **51**, 135–146 (1994).
63. National Research Council, *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, DC, ed. 8, 2011).
64. D. Kremer *et al.*, p57kip2 is dynamically regulated in experimental autoimmune encephalomyelitis and interferes with oligodendroglial maturation. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 9087–9092 (2009).
65. K. D. McCarthy, J. de Vellis, Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* **85**, 890–902 (1980).
66. P. Göttle *et al.*, Activation of CXCR7 receptor promotes oligodendroglial cell maturation. *Ann. Neurol.* **68**, 915–924 (2010).
67. P. Göttle *et al.*, Rescuing the negative impact of human endogenous retrovirus envelope protein on oligodendroglial differentiation and myelination. *Glia* **67**, 160–170 (2019).
68. S. W. Brostoff *et al.*, The P2 protein of bovine root myelin: Isolation and some clinical and immunological properties. *J. Neurochem.* **23**, 1037–1043 (1974).
69. B. A. Durafourt, C. S. Moore, M. Blain, J. P. Antel, Isolating, culturing, and polarizing primary human adult and fetal microglia. *Methods Mol. Biol.* **1041**, 199–211 (2013).
70. A. D. Greenhalgh *et al.*, Peripherally derived macrophages modulate microglial function to reduce inflammation after CNS injury. *PLoS Biol.* **16**, e2005264 (2018).
71. P. Göttle *et al.*, Oligodendroglial maturation is dependent on intracellular protein shuttling. *J. Neurosci.* **35**, 906–919 (2015).

2.2 Neural Cell Responses Upon Exposure to Human Endogenous Retroviruses

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Abstract

Human endogenous retroviruses (HERVs) are ancient retroviral elements, which invaded the human germ line several million years ago. Subsequent retrotransposition events amplified these sequences, resulting in approximately 8% of the human genome being composed of HERV sequences today. These genetic elements, normally dormant within human genomes, can be (re)-activated by environmental factors such as infections with other viruses, leading to the expression of viral proteins and, in some instances, even to viral particle production. Several studies have shown that the expression of these retroviral elements correlates with the onset and progression of neurological diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). Further studies provided evidence on additional roles for HERVs in schizophrenia (SCZ). Since these diseases are still not well understood, HERVs might constitute a new category of pathogenic components that could significantly change our understanding of these pathologies. Moreover, knowledge about their mode of action might also help to develop novel and more powerful approaches for the treatment of these complex diseases. Therefore, the main scope of this review is a description of the current knowledge on the involvement of HERV-W and HERV-K in neurological disease specifically focusing on the effects they exert on neural cells of the central nervous system.

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Neural Cell Responses Upon Exposure to Human Endogenous Retroviruses

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Human endogenous retroviruses (HERVs) are ancient retroviral elements, which invaded the human germ line several million years ago. Subsequent retrotransposition events amplified these sequences, resulting in approximately 8% of the human genome being composed of HERV sequences today. These genetic elements, normally dormant within human genomes, can be (re)-activated by environmental factors such as infections with other viruses, leading to the expression of viral proteins and, in some instances, even to viral particle production. Several studies have shown that the expression of these retroviral elements correlates with the onset and progression of neurological diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). Further studies provided evidence on additional roles for HERVs in schizophrenia (SCZ). Since these diseases are still not well understood, HERVs might constitute a new category of pathogenic components that could significantly change our understanding of these pathologies. Moreover, knowledge about their mode of action might also help to develop novel and more powerful approaches for the treatment of these complex diseases. Therefore, the main scope of this review is a description of the current knowledge on the involvement of HERV-W and HERV-K in neurological disease specifically focusing on the effects they exert on neural cells of the central nervous system.

Keywords: human endogenous retrovirus, neurodegenerative diseases, neurons, glia, mobile genetic elements

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INVOLVEMENT OF HERVS IN NEUROLOGICAL DISEASES

Up to 8% of the human genome are of retroviral origin. These retroviral elements, termed human endogenous retroviruses (HERVs), invaded the germ line millions of years ago and have been permanently integrated into the genome of our primate ancestors (Küry et al., 2018). Following integration, retrotransposonal activity led to the amplification of these retroviral elements (Belshaw et al., 2004). While most of these retroviral genes contain intragenic deletions or nonsense mutations and are therefore presumed to be silent, some of them retained parts of their functionality and developed into enhancers of the immune defense (Grandi and Tramontano, 2018). Other genes, such as syncytin encoded by ERVWE1, a full length provirus at locus 7q21.2 on chromosome 7, were domesticated and act in placental development (Mi et al., 2000). HERV elements may normally be expressed at low levels, but environmental factors, such as hypoxia (Brutting et al., 2018), drugs (Liu et al., 2013), other viruses (Liu et al., 2017), and certain mutations (Yu et al., 2014), were shown to increase their expression. Importantly, several studies were able to show that inflammation plays a major role in HERV activation (Mameli et al., 2007; Mameli et al., 2013;

Li et al., 2015; Manghera et al., 2015; Manghera et al., 2016b; Hurst and Magiorkinis, 2017). However, current research indicates that HERVs are also associated with several neurological disorders such as multiple sclerosis (MS; Perron et al., 1989), amyotrophic lateral sclerosis (ALS; Mccormick et al., 2008), and schizophrenia (SCZ; Yolken et al., 2000), which warrants research into underlying mechanisms of activation as well as into their role in disease etiology. As underlying disease causes of many neurological conditions remain elusive, HERV-directed research might shed light on new interactions and pathological processes implicated into disease onset or progression. These entities, which are *sensu stricto* neither viruses nor physiological genes, must therefore be considered as a new category of pathogenic elements (Feschotte and Gilbert, 2012). In the present review, we will summarize what is currently known about the involvement of HERVs in neurological diseases and will specifically address functions exerted on neurons and glial cells of the central nervous system (CNS).

Of note, HERV-W has been associated, repeatedly and based on a larger number of independent studies, with MS as recently reviewed in (Dolei, 2018). This demyelinating CNS disease of unknown etiology features miscellaneous clinical symptoms such as sensory, motor, and cognitive dysfunctions. Pathophysiologically, MS is characterized by immune cell infiltration, focal inflammation, and loss of myelin sheaths, leading to white and gray matter lesions and brain atrophy (Reich et al., 2018). Axonal degeneration, observed mainly but not exclusively during progression and later disease stages (Trapp et al., 1998), is another of its hallmarks and results in irreversible deficits. Mechanistically, direct autoimmune attacks on neurons (Derfuss et al., 2010) as well as secondary effects in response to myelin loss are responsible for axonal impairment and loss. In 1989, an association between retroviral elements and MS was described based on the analysis of primary leptomeningeal cell cultures isolated from MS patients (Perron et al., 1989). While these isolated viral particles were initially termed multiple sclerosis associated retrovirus (MSRV), it was later found that MSRV belongs, in fact, to the HERV family (Dolei and Perron, 2009; Perron and Lang, 2010). Follow-up studies provided convincing evidence that activation and expression of otherwise dormant HERV-W DNA sequences and the subsequent production of the encoded envelope (ENV) protein can trigger an immune response (Perron et al., 2001; Rolland et al., 2005). Moreover, it was shown that HERV-W ENV RNA and protein levels are increased in the cerebrospinal fluid (CSF) and serum of MS patients but rarely in healthy individuals (Garson et al., 1998; Mameli et al., 2009; Perron et al., 2012). Furthermore, it was shown that HERV-W ENV activates the innate immunity, priming it against myelin proteins. Accordingly, HERV-W ENV can act as an adjuvant in a model of experimental autoimmune encephalitis (EAE), which, in turn, can be rescued by the application of an HERV-W ENV-targeted therapeutic IgG4 antibody termed GNbAC1 (EAE; Perron et al., 2013). MS histology then revealed that the HERV-W ENV protein is mainly expressed by myeloid cells (Kremer et al., 2013; Van Horssen et al., 2016). Of note, a similar correlation was observed for HERV-W and chronic inflammatory demyelinating polyneuropathy (CIDP), an inflammatory, demyelinating disease of the peripheral nervous system (PNS; Faucard et al., 2016).

Apart from roles in cancer (Grabski et al., 2019) HERV-K also appears to be involved in a subpopulation of patients with sporadic ALS. This neurodegenerative disease is characterized by the progressive loss of both cortical and spinal motor neurons (Mathis et al., 2017). Although first described in the 19th century, its pathogenesis is still poorly understood despite considerable efforts to identify causes and susceptibilities in recent decades. Elevated HERV-K reverse transcriptase (RT) activity was observed in both blood and CSF from ALS patients (Macgowan et al., 2007; Mccormick et al., 2008). So far, two loci could be identified in the 7q34 and 7q36.1 regions, leading to the expression of HERV-K elements in ALS patients (Frank et al., 2005). Initial analysis of brain autopsy tissue revealed the expression of several HERV-K transcripts in cortical and spinal neurons of ALS but not in healthy control individuals (Douville et al., 2011). Although evidence for such an involvement is increasing (Meyer et al., 2017), it is currently challenged by a recent independent study that was not able to confirm the association between elevated cortical HERV-K RNA levels and ALS (Garson et al., 2019). Despite these conflicting observations related to the detection in ALS, it must be emphasized that transgenic mice expressing the HERV-K envelope protein display progressive motor dysfunction and motor cortex volume loss (Li et al., 2015).

SCZ is a complex neuropsychiatric disorder characterized by a variety of cognitive, emotional, and perceptual disturbances. Pathophysiologically, SCZ features decreased brain volume, loss of myelin, and altered astrocyte function (Archer, 2010). In contrast to MS and ALS, both HERV-W and HERV-K have been weakly linked to SCZ based on PCR amplification from CSF and post-mortem brains as well as on protein antigenemia (Yolken et al., 2000; Karlsson et al., 2001; Frank et al., 2005; Perron et al., 2008), while another study revealed upregulation of HERV-W ENV transcripts in plasma samples of SCZ patients (Huang et al., 2011). Moreover, a new study provides evidence that, in early stages of this disease, HERV-K methylation in peripheral blood is reduced (Mak et al., 2019). Of note, these observations contradict an earlier report suggesting that HERV-W expression is reduced in SCZ patients (Weis et al., 2007). The disparity between these reports may reflect different experimental approaches or a differential use of anti-psychotic medications in SCZ patients.

MECHANISMS OF HERV ACTIVATION

It is known that silenced HERVs can be specifically activated and expressed in several neurological conditions based on complex underlying activation mechanisms. In this regard, numerous studies have established links between HERV activation and infections with viruses such as the Epstein Barr virus (EBV). In this context, EBV glycoprotein350 (EBVgp350) was found to trigger expression of HERV-W ENV in blood cells and astrocytes, possibly contributing to the onset of MS (Mameli et al., 2012;

Mameli et al., 2013). Likewise, EBV was also shown to trigger HERV-K expression (Sutkowski et al., 2001). Similar activation mechanisms were demonstrated for *Herpesviridae* HSV1 and HHV6 (Perron et al., 1993; Ruprecht et al., 2006; Brudek et al., 2007; Charvet et al., 2018), providing a possible underlying mechanism explaining the well-established epidemiological link between these viruses and the susceptibility for MS. In addition, a direct involvement of the human immunodeficiency virus (HIV) Tat protein in activating HERV-W ENV in peripheral blood mononuclear cells (PBMCs), monocyte/macrophages, and astrocytes was described (Uleri et al., 2014). Of note, in monocytes, HIV Tat inhibits the expression of syncytin-1, whereas in differentiated macrophages it is stimulated (Uleri et al., 2014). In this regard it is important to note that HERV-K plasma levels were found to positively and negatively correlate with HIV infection and antiretroviral therapy, respectively (Bowen et al., 2016). Whether HIV infection as such or antiretroviral treatment account for this observation is currently debated.

Yet, another important activator of HERV expression is the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signaling pathway, based on an earlier report demonstrating that the pro-inflammatory cytokine tumor necrosis factor (TNF) α can stimulate the, at that time so-called, ERVWE1/syncytin promoter *via* NF-κB (Mameli et al., 2007). Similarly, HERV-K expression was also shown to respond to TNFα/NF-κB signaling (Li et al., 2015; Manghera et al., 2015; Manghera et al., 2016b). Such signaling could be part of a regulatory feedback loop, taking into account that HERV long terminal repeat (LTR)-sequences act as promoters for pro-inflammatory cytokine genes (Hurst and Magiorkinis, 2017).

In human SH-SY5Y neuroblastoma cells, caffeine and aspirin were shown to induce HERV-W ENV and GAG (group-specific antigen) transcription, providing a possible link between environmental factors, drugs, and endogenous virus activation (Liu et al., 2013). Whether such exogenous triggers can also affect HERV-W induction in myeloid cells, which are highly relevant for MS (Kremer et al., 2013; Van Horssen et al., 2016), remains to be demonstrated. Regarding ALS, TAR DNA binding protein 43 (TDP43), which is involved in the sporadic form of the disease (Mackenzie and Rademakers, 2008), was found to bind to LTR sequences, leading to the expression and accumulation of HERV-K (Li et al., 2015; Manghera et al., 2016a). A further contribution to HERV-W activation in MS was proposed to be mediated *via* endoplasmic reticulum (ER) stress (Deslauriers et al., 2011). Finally, progerin, a nuclear protein involved in the accelerated aging Hutchinson–Gilford progeria syndrome, was found to strongly downregulate transcription of all classes of repetitive sequences including HERVs in dopaminergic neurons generated from induced pluripotent stem cells (Arancio, 2019). Whether corresponding lessons can be learned in light of neurodegeneration in MS or ALS needs to be shown in future. Of note, progerin was also shown to impair the nuclear factor erythroid 2–related factor 2 (Nrf2)-mediated anti-oxidative response (Kubben et al., 2016), a mechanism implicated in MS neuroprotection (Linker et al., 2011).

HERV EFFECTS EXERTED ON NEURAL CELLS

Neurons

A potential HERV impact on neurons was studied in mice using experimental overexpression of the HERV-K ENV protein, mimicking its expression in cortical and spinal neurons of ALS patients. These transgenic mice showed severe signs of neurodegeneration with progressive motor dysfunction, motor cortex volume loss, decreased synaptic activity, and spine abnormalities (Li et al., 2015). Such a phenotype implies that either endogenous damage pathways are activated or ENV protein leakage results in surface receptor activation, leading to autocrine or paracrine cell activation. CRISPR/Cas9 technology was recently used to disrupt the HERV-K ENV gene in human prostate cancer cells. By depleting ENV transcripts and proteins, this modification led to the downregulation of the above-mentioned important regulator TDP-43 (see **Figure 1**; Ibba et al., 2018). Given the formation of neurotoxic TDP-43 deposits in ALS neurons and TDP-43's implication in HERV-K activation (Douville and Nath, 2017), this study provides yet more evidence for a role of ENV proteins in neurodegeneration. This view might, however, be challenged by the observation that HERV-K ENV overexpression in neuronal cells increased their viability and prevented neurotoxicity mediated by the HIV-1 Vpr protein (Bhat et al., 2014). This study was based on the fact that HERV-K and TDP-43 constitute an important neuropathological overlap between ALS and HIV encephalitis but might not be representative for MS- or ALS-related degeneration processes. To what degree inactivation of HERV-K might also be achieved *via* epigenetic modulators such as TRIM28 remains to be shown. In neural progenitor cells, TRIM28 acts a corepressor mediating transcriptional silencing. Its deletion resulted in induction of two groups of endogenous retroviruses IAP1 and MMERVK10C (Fasching et al., 2015). Finally, in neuroblastoma cells, HERV-W ENV overexpression was reported to activate the TRPC3 channel to regulate calcium influx and to depress the SCZ relevant DISC1 protein (Chen et al., 2019). Whether this observation truly reflects cellular expression and consequences in neuropsychiatric disorders including non-transformed neuronal cells and whether it can specifically be attributed to the envelope of HERV-W remain to be studied in future.

GLIAL CELLS

Expression of HERV-W ENV has mainly been observed in myeloid cells, i.e., monocytes/macrophages and microglia in MS patient tissues, while there is scarce evidence pointing to ENV expression by astrocytes (Perron et al., 2005; Kremer et al., 2013; Van Horssen et al., 2016). As of now, it is still unclear whether there is direct astroglial expression or if astroglia only bind or internalize ENV protein. However, signs of astroglial expression have also been gathered upon activation by EBV (Mameli et al., 2012). Observations on the related syncytin-1 revealed induced astrocytic release of redox reactants, which are cytotoxic to oligodendrocytes (Antony et al., 2004), possibly acting *via* ASCT1 activation

(see **Figure 1**; Antony et al., 2007). In this regard, however, it must be stated that this study did not distinguished between the pathological HERV-W and the physiological syncytin-1. Furthermore, syncytin-1 overexpression in human microglia and astroglia was reported to activate the inflammatory marker CRP *via* TLR3 signaling (Wang et al., 2018), notably a receptor that is known to bind double-stranded RNA but is prone to nucleic acid artifacts in transfection experiments. In addition to the above-mentioned observations in neuroblastoma cells, overexpression of HERV-W ENV in human glioma cells was reported to induce expression of SCZ-linked genes encoding brain-derived neurotrophic factor (BDNF) and dopamine receptor D3 (DRD3; Huang et al., 2011), whereas the endogenous retroviral insert hsERVPRODH was found to act as a tissue-specific enhancer for the proline dehydrogenase 1 (PRODH), a candidate gene for SCZ susceptibility (Suntsova et al., 2013).

In the context of MS, stimulation of rat oligodendroglial progenitor cells (OPCs) with HERV-W ENV protein was found to impair their differentiation and to interfere with axon myelination (Kremer et al., 2013; Göttle et al., 2019). This effect is based on TLR4 activation and the subsequent induction of nitrosative stress (see **Figure 1**). The HERV-W ENV-targeted therapeutic antibody GNbAC1 was initially developed to neutralize ENV-dependent activation of immune cells, yet was also revealed to be active in rescuing oligodendroglial differentiation (Kremer et al., 2015) as well as myelination *in vitro* (Göttle et al., 2019). Of note, in relapsing MS patients, a phase 2b clinical trial using GNbAC1 has been conducted (CHANGE-MS, NCT02782858). It therefore remains to be shown to what degree clinical results reflect these preclinical findings and whether MS patients show beneficial effects on remyelination or attenuated neurodegeneration. Moreover, interfering with TLR4 surface exposition by blocking of the vascular ATPase was also found to neutralize the ENV-dependent effect on OPC differentiation and axonal myelination (Göttle et al., 2019). With regard to peripheral nervous system inflammatory damage, HERV-W ENV protein can also be detected in Schwann cells of CIDP patients. Cultured human Schwann cells exposed to or transfected with an ENV expression vector showed increased IL-6 and decreased CXCL10 transcript levels (Faucard et al., 2016), hence showing signs of altered immunocompetence in the peripheral nerve (Tzekova et al., 2014). Likewise, this activation could be neutralized *via* the GNbAC1 antibody (Faucard et al., 2016).

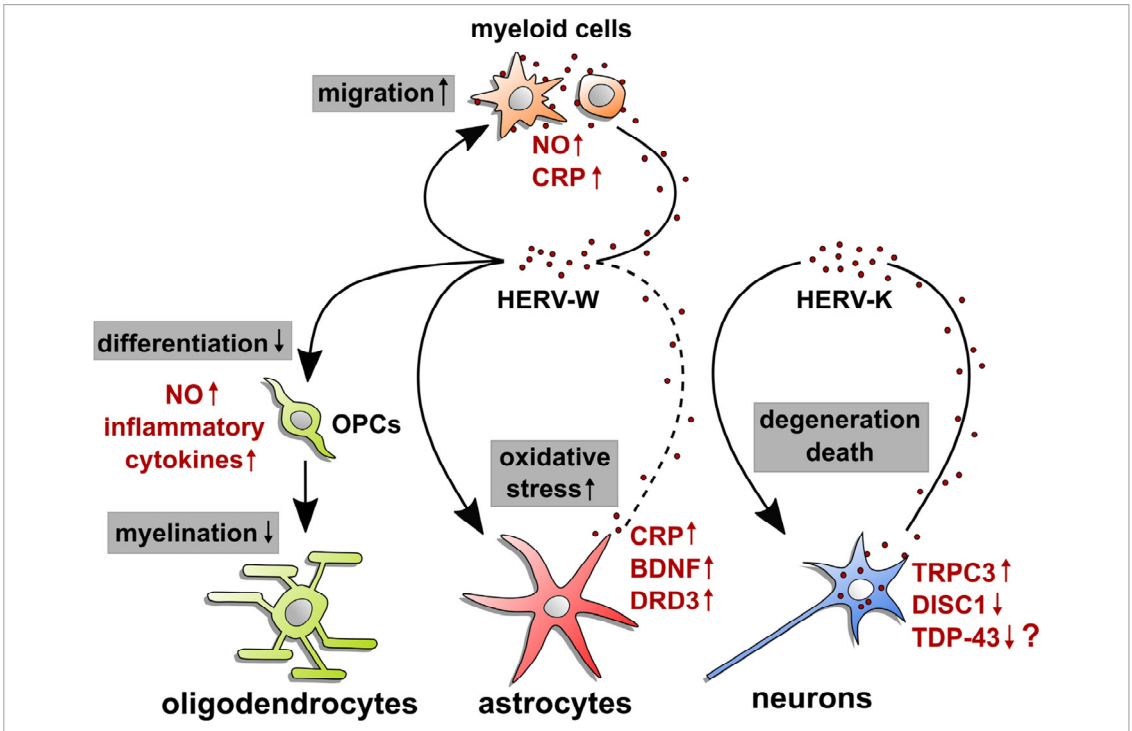

FIGURE 1 | HERV-mediated effects on neural cells. This illustration summarizes origin and observed molecular effects of HERW-W and HERV-K on cells of the central nervous system. Arrow starting points indicate cellular sources of HERV particles or proteins (red dots), whereas arrowheads point to influenced cell types. Modulated processes are shown in gray boxes, and regulated molecules are highlighted in red next to each cell type. The question mark next to TDP-43 refers to its postulated regulation in neurons. Whether microglia and astroglia respond to HERVs in an auto- and/or paracrine way and whether neurons react to internal and/or extracellular HERVs remains to be shown. OPCs: oligodendroglial progenitor cells; NO: nitric oxide; CRP: C-reactive protein; BDNF: brain-derived neurotrophic factor; DRD3: dopamine receptor D_3; TRPC3: short transient receptor potential channel 3; DISC1: disrupted in schizophrenia 1; TDP-43: TAR DNA-binding protein 43.

To what degree HERV-W ENV protein-mediated activation also modulates the capacity of Schwann cells to de- and redifferentiate and whether it could therefore affect PNS repair remains to be shown.

Although microglial cells are currently viewed as a relevant source of HERV-W ENV protein in the diseased CNS, additional autocrine/paracrine effects on these myeloid cells cannot be excluded and warrant further investigations. In this regard, it is worth mentioning that nitric oxide (NO) production and cellular migration were found to be affected in response to stimulation of rat microglia with a recombinant ENV protein (Xiao et al., 2017).

Finally, a role of HERV-W ENV in diminishing myelin repair is also important in light of the reproduced documentation on its expression in MS but also considering recent findings implying an implication in molecular mimicry. In this regard, several groups provided evidence of similarities between HERV-W ENV and myelin oligodendrocyte glycoprotein (MOG). This molecular mimicry may be an underlying mechanism leading to or fueling autoimmunity (Do Olival et al., 2013; Ramasamy et al., 2017; De Luca et al., 2019). To what degree such molecular similarities also disturb successful maturation of resident OPCs required for myelin repair needs to be investigated in future.

CONCLUSION

We here present collected evidence that endogenous retroviral elements acting either as viral particles or *via* their proteins influence neural cells in the context of degenerative CNS diseases. Once thought to be primarily involved in cell transformation (Grabski et al., 2019) and inflammation (Perron and Lang, 2010), emerging data suggests a direct role of these elements in glial and neuronal injury, which in fact goes beyond previous descriptions on the activity of a gliotoxin (Menard et al., 1998). In light of additional observations on the role of ERVs in regulating stem cell potential and fate acquisition (Gautam et al., 2017), the findings describing impacts on committed or mature cells of the CNS are probably not too surprising but warrant future investigations, even more so since neural stem cells are also involved in brain pathology and regeneration. Moreover, the currently still unmet clinical need to effectively treat neurodegeneration necessitates novel therapeutic approaches. Whether similar mechanisms also apply to activation of transposable elements implicated in, for example, chronic fatigue syndrome (CFS; Almenar-Perez et al., 2019) and to what degree currently used neutralizing antibodies can be exploited in order to prevent neural cell activation and/or neurodegeneration needs to be elucidated in the future. In this regard, it remains to be shown whether HERV-employed signaling pathways and epigenetic silencing mechanisms can be used for biomedical translation.

AUTHOR CONTRIBUTIONS

JG, DK, and PK searched the literature, interpreted manuscripts, and decided on the content of this review. JG and PK wrote the article. DK provided feedback on the manuscript draft and helped to complete the final manuscript. JG drew the figure.

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REFERENCES

Almenar-Perez, E., Ovejero, T., Sanchez-Fito, T., Espejo, J. A., Nathanson, L., and Oltra, E. (2019). Epigenetic components of myalgic encephalomyelitis/chronic fatigue syndrome uncover potential transposable element activation. *Clin. Ther.* 41, 675–698. doi: 10.1016/j.clinthera.2019.02.012

Antony, J. M., Ellestad, K. K., Hammond, R., Imaizumi, K., Mallet, F., Warren, K. G., et al. (2007). The human endogenous retrovirus envelope glycoprotein, syncytin-1, regulates neuroinflammation and its receptor expression in multiple sclerosis: a role for endoplasmic reticulum chaperones in astrocytes. *J. Immunol.* 179, 1210–1224. doi: 10.4049/jimmunol.179.2.1210

Antony, J. M., Van Marle, G., Opii, W., Butterfield, D. A., Mallet, F., Yong, V. W., et al. (2004). Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nat. Neurosci.* 7, 1088–1095. doi: 10.1038/nn1319

Arancio, W. (2019). Progerin expression induces a significant downregulation of transcription from human repetitive sequences in iPSC-derived dopaminergic neurons. *Geroscience* 41, 39–49. doi: 10.1007/s11357-018-00050-2

Archer, T. (2010). Neurodegeneration in schizophrenia. *Expert Rev. Neurother.* 10, 1131–1141. doi: 10.1586/ern.09.152

Belshaw, R., Pereira, V., Katzourakis, A., Talbot, G., Paces, J., Burt, A., et al. (2004). Long-term reinfection of the human genome by endogenous retroviruses. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4894–4899. doi: 10.1073/pnas.0307800101

Bhat, R. K., Rudnick, W., Antony, J. M., Maingat, F., Ellestad, K. K., Wheatley, B. M., et al. (2014). Human endogenous retrovirus-K(II) envelope induction protects neurons during HIV/AIDS. *PLoS One* 9, e97984. doi: 10.1371/journal.pone.0097984

Bowen, L. N., Tyagi, R., Li, W., Alfahad, T., Smith, B., Wright, M., et al. (2016). HIV-associated motor neuron disease: HERV-K activation and response to antiretroviral therapy. *Neurology* 87, 1756–1762. doi: 10.1212/WNL.0000000000003258

Brudek, T., Luhdorf, P., Christensen, T., Hansen, H. J., and Moller-Larsen, A. (2007). Activation of endogenous retrovirus reverse transcriptase in multiple sclerosis patient lymphocytes by inactivated HSV-1, HHV-6 and VZV. *J. Neuroimmunol.* 187, 147–155. doi: 10.1016/j.jneuroim.2007.04.003

Brutting, C., Narasimhan, H., Hoffmann, F., Kornhuber, M. E., Staege, M. S., and Emmer, A. (2018). Investigation of endogenous retrovirus sequences in the neighborhood of genes up-regulated in a neuroblastoma model after treatment with hypoxia-mimetic cobalt chloride. *Front. Microbiol.* 9, 287. doi: 10.3389/fmicb.2018.00287

Charvet, B., Reynaud, J. M., Gourru-Lesimple, G., Perron, H., Marche, P. N., and Horvat, B. (2018). Induction of proinflammatory multiple sclerosis-associated

retrovirus envelope protein by human herpesvirus-6A and CD46 receptor engagement. *Front. Immunol.* 9, 2803. doi: 10.3389/fimmu.2018.02803

Chen, Y., Yan, Q., Zhou, P., Li, S., and Zhu, F. (2019). HERV-W env regulates calcium influx *via* activating TRPC3 channel together with depressing DISC1 in human neuroblastoma cells. *J. Neurovirol.* 25, 101–113. doi: 10.1007/s13365-018-0692-7

De Luca, V., Martins Higa, A., Malta Romano, C., Pimenta Mambrini, G., Peroni, L. A., Trivinho-Strixino, F., et al. (2019). Cross-reactivity between myelin oligodendrocyte glycoprotein and human endogenous retrovirus W protein: nanotechnological evidence for the potential trigger of multiple sclerosis. *Micron* 120, 66–73. doi: 10.1016/j.micron.2019.02.005

Derfuss, T., Linington, C., Hohlfeld, R., and Meinl, E. (2010). Axo-glial antigens as targets in multiple sclerosis: implications for axonal and grey matter injury. *J. Mol. Med. (Berl.)* 88, 753–761. doi: 10.1007/s00109-010-0632-3

Deslauriers, A. M., Afkhami-Goli, A., Paul, A. M., Bhat, R. K., Acharjee, S., Ellestad, K. K., et al. (2011). Neuroinflammation and endoplasmic reticulum stress are coregulated by crocin to prevent demyelination and neurodegeneration. *J. Immunol.* 187, 4788–4799. doi: 10.4049/jimmunol.1004111

Do Olival, G. S., Faria, T. S., Nali, L. H., De Oliveira, A. C., Casseb, J., Vidal, J. E., et al. (2013). Genomic analysis of ERVWE2 locus in patients with multiple sclerosis: absence of genetic association but potential role of human endogenous retrovirus type W elements in molecular mimicry with myelin antigen. *Front. Microbiol.* 4, 172. doi: 10.3389/fmicb.2013.00172

Dolei, A. (2018). The aliens inside us: HERV-W endogenous retroviruses and multiple sclerosis. *Mult. Scler.* 24, 42–47. doi: 10.1177/1352458517737370

Dolei, A., and Perron, H. (2009). The multiple sclerosis-associated retrovirus and its HERV-W endogenous family: a biological interface between virology, genetics, and immunology in human physiology and disease. *J. Neurovirol.* 15, 4–13. doi: 10.1080/13550280802448451

Douville, R. N., and Nath, A. (2017). Human endogenous retrovirus-K and TDP-43 expression bridges ALS and HIV neuropathology. *Front. Microbiol.* 8, 1986. doi: 10.3389/fmicb.2017.01986

Douville, R., Liu, J., Rothstein, J., and Nath, A. (2011). Identification of active loci of a human endogenous retrovirus in neurons of patients with amyotrophic lateral sclerosis. *Ann. Neurol.* 69, 141–151. doi: 10.1002/ana.22149

Fasching, L., Kapopoulou, A., Sachdeva, R., Petri, R., Jonsson, M. E., Manne, C., et al. (2015). TRIM28 represses transcription of endogenous retroviruses in neural progenitor cells. *Cell Rep.* 10, 20–28. doi: 10.1016/j.celrep.2014.12.004

Faucard, R., Madeira, A., Gehin, N., Authier, F. J., Panaite, P. A., Lesage, C., et al. (2016). Human endogenous retrovirus and neuroinflammation in chronic inflammatory demyelinating polyradiculoneuropathy. *EBioMedicine* 6, 190–198. doi: 10.1016/j.ebiom.2016.03.001

Feschotte, C., and Gilbert, C. (2012). Endogenous viruses: insights into viral evolution and impact on host biology. *Nat. Rev. Genet.* 13, 283–296. doi: 10.1038/nrg3199

Frank, O., Giehl, M., Zheng, C., Hehlmann, R., Leib-Mosch, C., and Seifarth, W. (2005). Human endogenous retrovirus expression profiles in samples from brains of patients with schizophrenia and bipolar disorders. *J. Virol.* 79, 10890–10901. doi: 10.1128/JVI.79.17.10890-10901.2005

Garson, J. A., Tuke, P. W., Giraud, P., Paranhos-Baccala, G., and Perron, H. (1998). Detection of virion-associated MSRV-RNA in serum of patients with multiple sclerosis. *Lancet* 351, 33. doi: 10.1016/S0140-6736(98)24001-3

Garson, J. A., Usher, L., Al-Chalabi, A., Huggett, J., Day, E. F., and Mccormick, A. L. (2019). Quantitative analysis of human endogenous retrovirus-K transcripts in postmortem premotor cortex fails to confirm elevated expression of HERV-K RNA in amyotrophic lateral sclerosis. *Acta Neuropathol. Commun.* 7, 45. doi: 10.1186/s40478-019-0698-2

Gautam, P., Yu, T., and Loh, Y. H. (2017). Regulation of ERVs in pluripotent stem cells and reprogramming. *Curr. Opin. Genet. Dev.* 46, 194–201. doi: 10.1016/j.gde.2017.07.012

Göttle, P., Förster, M., Gruchot, J., Kremer, D., Hartung, H. P., Perron, H., et al. (2019). Rescuing the negative impact of human endogenous retrovirus envelope protein on oligodendroglial differentiation and myelination. *Glia* 67, 160–170. doi: 10.1002/glia.23535

Grabski, D. F., Hu, Y., Sharma, M., and Rasmussen, S. K. (2019). Close to the bedside: a systematic review of endogenous retroviruses and their impact in oncology. *J. Surg. Res.* 240, 145–155. doi: 10.1016/j.jss.2019.02.009

Grandi, N., and Tramontano, E. (2018). Human endogenous retroviruses are ancient acquired elements still shaping innate immune responses. *Front. Immunol.* 9, 2039. doi: 10.3389/fimmu.2018.02039

Huang, W., Li, S., Hu, Y., Yu, H., Luo, F., Zhang, Q., et al. (2011). Implication of the env gene of the human endogenous retrovirus W family in the expression of BDNF and DRD3 and development of recent-onset schizophrenia. *Schizophr. Bull.* 37, 988–1000. doi: 10.1093/schbul/sbp166

Hurst, T. P., and Magiorkinis, G. (2017). Epigenetic control of human endogenous retrovirus expression: focus on regulation of long-terminal repeats (LTRs). *Viruses* 9, 130. doi: 10.3390/v9060130

Ibba, G., Piu, C., Uleri, E., Serra, C., and Dolei, A. (2018). Disruption by SaCas9 endonuclease of HERV-Kenv, a retroviral gene with oncogenic and neuropathogenic potential, inhibits molecules involved in cancer and amyotrophic lateral sclerosis. *Viruses* 10, 412. doi: 10.3390/v10080412

Karlsson, H., Bachmann, S., Schroder, J., Mcarthur, J., Torrey, E. F., and Yolken, R. H. (2001). Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4634–4639. doi: 10.1073/pnas.061021998

Kremer, D., Förster, M., Schichel, T., Göttle, P., Hartung, H. P., Perron, H., et al. (2015). The neutralizing antibody GNbAC1 abrogates HERV-W envelope protein-mediated oligodendroglial maturation blockade. *Mult. Scler.* 21, 1200–1203. doi: 10.1177/1352458514560926

Kremer, D., Schichel, T., Förster, M., Tzekova, N., Bernard, C., Van Der Valk, P., et al. (2013). Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. *Ann. Neurol.* 74, 721–732. doi: 10.1002/ana.23970

Kubben, N., Zhang, W., Wang, L., Voss, T. C., Yang, J., Qu, J., et al. (2016). Repression of the antioxidant NRF2 pathway in premature aging. *Cell* 165, 1361–1374. doi: 10.1016/j.cell.2016.05.017

Küry, P., Nath, A., Creange, A., Dolei, A., Marche, P., Gold, J., et al. (2018). Human endogenous retroviruses in neurological diseases. *Trends Mol. Med.* 24, 379–394. doi: 10.1016/j.molmed.2018.02.007

Li, W., Lee, M. H., Henderson, L., Tyagi, R., Bachani, M., Steiner, J., et al. (2015). Human endogenous retrovirus-K contributes to motor neuron disease. *Sci. Transl. Med.* 7, 307ra153. doi: 10.1126/scitranslmed.aac8201

Linker, R. A., Lee, D. H., Ryan, S., Van Dam, A. M., Conrad, R., Bista, P., et al. (2011). Fumaric acid esters exert neuroprotective effects in neuroinflammation *via* activation of the Nrf2 antioxidant pathway. *Brain* 134, 678–692. doi: 10.1093/brain/awq386

Liu, C., Liu, L., Wang, X., Liu, Y., Wang, M., and Zhu, F. (2017). HBV X protein induces overexpression of HERV-W env through NF-kappaB in HepG2 cells. *Virus Genes* 53, 797–806. doi: 10.1007/s11262-017-1479-2

Liu, C. L., Chen, Y. T., Li, S., Yu, H. L., Zeng, J., Wang, X. L., et al. (2013). Activation of elements in HERV-W family by caffeine and aspirin. *Virus Genes* 47, 219–227. doi: 10.1007/s11262-013-0939-6

Macgowan, D. J., Scelsa, S. N., Imperato, T. E., Liu, K. N., Baron, P., and Polsky, B. (2007). A controlled study of reverse transcriptase in serum and CSF of HIV-negative patients with ALS. *Neurology* 68, 1944–1946. doi: 10.1212/01.wnl.0000263188.77797.99

Mackenzie, I. R., and Rademakers, R. (2008). The role of transactive response DNA-binding protein-43 in amyotrophic lateral sclerosis and frontotemporal dementia. *Curr. Opin. Neurol.* 21, 693–700. doi: 10.1097/WCO.0b013e3283168d1d

Mak, M., Samochowiec, J., Frydecka, D., Pelka-Wysiecka, J., Szmida, E., Karpinski, P., et al. (2019). First-episode schizophrenia is associated with a reduction of HERV-K methylation in peripheral blood. *Psychiatry Res.* 271, 459–463. doi: 10.1016/j.psychres.2018.12.012

Mameli, G., Astone, V., Khalili, K., Serra, C., Sawaya, B. E., and Dolei, A. (2007). Regulation of the syncytin-1 promoter in human astrocytes by multiple sclerosis-related cytokines. *Virology* 362, 120–130. doi: 10.1016/j.virol.2006.12.019

Mameli, G., Madeddu, G., Mei, A., Uleri, E., Poddighe, L., Delogu, L. G., et al. (2013). Activation of MSRV-type endogenous retroviruses during infectious mononucleosis and Epstein-Barr virus latency: the missing link with multiple sclerosis? *PLoS One* 8, e78474. doi: 10.1371/journal.pone.0078474

Mameli, G., Poddighe, L., Astone, V., Delogu, G., Arru, G., Sotgiu, S., et al. (2009). Novel reliable real-time PCR for differential detection of MSRVenv and syncytin-1 in RNA and DNA from patients with multiple sclerosis. *J. Virol. Methods* 161, 98–106. doi: 10.1016/j.jviromet.2009.05.024

Mameli, G., Poddighe, L., Mei, A., Uleri, E., Sotgiu, S., Serra, C., et al. (2012). Expression and activation by Epstein Barr virus of human endogenous retroviruses-W in blood cells and astrocytes: inference for multiple sclerosis. *PLoS One* 7, e44991. doi: 10.1371/journal.pone.0044991

Manghera, M., Ferguson-Parry, J., and Douville, R. N. (2016a). TDP-43 regulates endogenous retrovirus-K viral protein accumulation. *Neurobiol. Dis.* 94, 226–236. doi: 10.1016/j.nbd.2016.06.017

Manghera, M., Ferguson-Parry, J., Lin, R., and Douville, R. N. (2016b). NF-kappaB and IRF1 induce endogenous retrovirus K expression *via* interferon-stimulated response elements in its 5' long terminal repeat. *J. Virol.* 90, 9338–9349. doi: 10.1128/JVI.01503-16

Manghera, M., Ferguson, J., and Douville, R. (2015). ERVK polyprotein processing and reverse transcriptase expression in human cell line models of neurological disease. *Viruses* 7, 320–332. doi: 10.3390/v7010320

Mathis, S., Couratier, P., Julian, A., Vallat, J. M., Corcia, P., and Le Masson, G. (2017). Management and therapeutic perspectives in amyotrophic lateral sclerosis. *Expert Rev. Neurother.* 17, 263–276. doi: 10.1080/14737175.2016.1227705

Mccormick, A. L., Brown, R. H., Cudkowicz, M. E., Al-Chalabi, A., and Garson, J. A. (2008). Quantification of reverse transcriptase in ALS and elimination of a novel retroviral candidate. *Neurology* 70, 278–283. doi: 10.1212/01.wnl.0000297552.13219.b4

Menard, A., Amouri, R., Dobransky, T., Charriaut-Marlangue, C., Pierig, R., Cifuentes-Diaz, C., et al. (1998). A gliotoxic factor and multiple sclerosis. *J. Neurol. Sci.* 154, 209–221. doi: 10.1016/S0022-510X(97)00231-1

Meyer, T. J., Rosenkrantz, J. L., Carbone, L., and Chavez, S. L. (2017). Endogenous retroviruses: with us and against us. *Front. Chem.* 5, 23. doi: 10.3389/fchem.2017.00023

Mi, S., Lee, X., Li, X. P., Veldman, G. M., Finnerty, H., Racie, L., et al. (2000). Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* 403, 785–789. doi: 10.1038/35001608

Perron, H., and Lang, A. (2010). The human endogenous retrovirus link between genes and environment in multiple sclerosis and in multifactorial diseases associating neuroinflammation. *Clin. Rev. Allergy Immunol.* 39, 51–61. doi: 10.1007/s12016-009-8170-x

Perron, H., Dougier-Reynaud, H. L., Lomparski, C., Popa, I., Firouzi, R., Bertrand, J. B., et al. (2013). Human endogenous retrovirus protein activates innate immunity and promotes experimental allergic encephalomyelitis in mice. *PLoS One* 8, e80128. doi: 10.1371/journal.pone.0080128

Perron, H., Geny, C., Laurent, A., Mouriquand, C., Pellat, J., Perret, J., et al. (1989). Leptomeningeal cell-line from multiple-sclerosis with reverse-transcriptase activity and viral particles. *Res. Virol.* 140, 551–561. doi: 10.1016/S0923-2516(89)80141-4

Perron, H., Germi, R., Bernard, C., Garcia-Montojo, M., Deluen, C., Farinelli, L., et al. (2012). Human endogenous retrovirus type W envelope expression in blood and brain cells provides new insights into multiple sclerosis disease. *Mult. Scler.* 18, 1721–1736. doi: 10.1177/1352458512441381

Perron, H., Jouvin-Marche, E., Michel, M., Ounanian-Paraz, A., Camelo, S., Dumon, A., et al. (2001). Multiple sclerosis retrovirus particles and recombinant envelope trigger an abnormal immune response *in vitro*, by inducing polyclonal Vbeta16 T-lymphocyte activation. *Virology* 287, 321–332. doi: 10.1006/viro.2001.1045

Perron, H., Lazarini, F., Ruprecht, K., Pechoux-Longin, C., Seilhean, D., Sazdovitch, V., et al. (2005). Human endogenous retrovirus (HERV)-W ENV and GAG proteins: physiological expression in human brain and pathophysiological modulation in multiple sclerosis lesions. *J. Neurovirol.* 11, 23–33. doi: 10.1080/13550280590901741

Perron, H., Mekaoui, L., Bernard, C., Veas, F., Stefas, I., and Leboyer, M. (2008). Endogenous retrovirus type W GAG and envelope protein antigenemia in serum of schizophrenic patients. *Biol. Psychiatry* 64, 1019–1023. doi: 10.1016/j.biopsych.2008.06.028

Perron, H., Suh, M., Lalande, B., Gratacap, B., Laurent, A., Stoebner, P., et al. (1993). Herpes simplex virus ICP0 and ICP4 immediate early proteins strongly enhance expression of a retrovirus harboured by a leptomeningeal cell line from a patient with multiple sclerosis. *J. Gen. Virol.* 74 (Pt 1), 65–72. doi: 10.1099/0022-1317-74-1-65

Ramasamy, R., Joseph, B., and Whittall, T. (2017). Potential molecular mimicry between the human endogenous retrovirus W family envelope proteins and myelin proteins in multiple sclerosis. *Immunol. Lett.* 183, 79–85. doi: 10.1016/j.imlet.2017.02.003

Reich, D. S., Lucchinetti, C. F., and Calabresi, P. A. (2018). Multiple sclerosis. *N. Engl. J. Med.* 378, 169–180. doi: 10.1056/NEJMra1401483

Rolland, A., Jouvin-Marche, E., Saresella, M., Ferrante, P., Cavaretta, R., Creange, A., et al. (2005). Correlation between disease severity and *in vitro* cytokine production mediated by MSRV (multiple sclerosis associated retroviral element) envelope protein in patients with multiple sclerosis. *J. Neuroimmunol.* 160, 195–203. doi: 10.1016/j.jneuroim.2004.10.019

Ruprecht, K., Obojes, K., Wengel, V., Gronen, F., Kim, K. S., Perron, H., et al. (2006). Regulation of human endogenous retrovirus W protein expression by herpes simplex virus type 1: implications for multiple sclerosis. *J. Neurovirol.* 12, 65–71. doi: 10.1080/13550280600614973

Suntsova, M., Gogvadze, E. V., Salozhin, S., Gaifullin, N., Eroshkin, F., Dmitriev, S. E., et al. (2013). Human-specific endogenous retroviral insert serves as an enhancer for the schizophrenia-linked gene PRODH. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19472–19477. doi: 10.1073/pnas.1318172110

Sutkowski, N., Conrad, B., Thorley-Lawson, D. A., and Huber, B. T. (2001). Epstein-Barr virus transactivates the human endogenous retrovirus HERV-K18 that encodes a superantigen. *Immunity* 15, 579–589. doi: 10.1016/S1074-7613(01)00210-2

Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mork, S., and Bo, L. (1998). Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* 338, 278–285. doi: 10.1056/NEJM199801293380502

Tzekova, N., Heinen, A., and Küry, P. (2014). Molecules involved in the crosstalk between immune- and peripheral nerve Schwann cells. *J. Clin. Immunol.* 34 Suppl 1, S86–104. doi: 10.1007/s10875-014-0015-6

Uleri, E., Mei, A., Mameli, G., Poddighe, L., Serra, C., and Dolei, A. (2014). HIV Tat acts on endogenous retroviruses of the W family and this occurs *via* Toll-like receptor 4: inference for neuroAIDS. *AIDS* 28, 2659–2670. doi: 10.1097/QAD.0000000000000477

Van Horssen, J., Van Der Pol, S., Nijland, P., Amor, S., and Perron, H. (2016). Human endogenous retrovirus W in brain lesions: rationale for targeted therapy in multiple sclerosis. *Mult. Scler. Relat. Disord.* 8, 11–18. doi: 10.1016/j.msard.2016.04.006

Wang, X., Liu, Z., Wang, P., Li, S., Zeng, J., Tu, X., et al. (2018). Syncytin-1, an endogenous retroviral protein, triggers the activation of CRP *via* TLR3 signal cascade in glial cells. *Brain Behav. Immun.* 67, 324–334. doi: 10.1016/j.bbi.2017.09.009

Weis, S., Llenos, I. C., Sabunciyan, S., Dulay, J. R., Isler, L., Yolken, R., et al. (2007). Reduced expression of human endogenous retrovirus (HERV)-W GAG protein in the cingulate gyrus and hippocampus in schizophrenia, bipolar disorder, and depression. *J. Neural Transm. (Vienna)* 114, 645–655. doi: 10.1007/s00702-006-0599-y

Xiao, R., Li, S., Cao, Q., Wang, X., Yan, Q., Tu, X., et al. (2017). HIuman endogenous retrovirus W env increases nitric oxide production and enhances the migration ability of microglia by regulating the expression of inducible nitric oxide synthase. *Virol. Sin.* 32, 216–225. doi: 10.1007/s12250-017-3997-4

Yolken, R. H., Karlsson, H., Yee, F., Johnston-Wilson, N. L., and Torrey, E. F. (2000). Endogenous retroviruses and schizophrenia. *Brain Res. Rev.* 31, 193–199. doi: 10.1016/S0165-0173(99)00037-5

Yu, H., Liu, T., Zhao, Z., Chen, Y., Zeng, J., Liu, S., et al. (2014). Mutations in 3'-long terminal repeat of HERV-W family in chromosome 7 upregulate syncytin-1 expression in urothelial cell carcinoma of the bladder through interacting with c-Myb. *Oncogene* 33, 3947–3958. doi: 10.1038/onc.2013.366

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2.3 The Molecular Basis for Remyelination Failure in Multiple Sclerosis

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Abstract

Myelin sheaths in the central nervous system (CNS) insulate axons and thereby allow saltatory nerve conduction, which is a prerequisite for complex brain function. Multiple sclerosis (MS), the most common inflammatory autoimmune disease of the CNS, leads to the destruction of myelin sheaths and the myelin-producing oligodendrocytes, thus leaving behind demyelinated axons prone to injury and degeneration. Clinically, this process manifests itself in significant neurological symptoms and disability. Resident oligodendroglial precursor cells (OPCs) and neural stem cells (NSCs) are present in the adult brain, and can differentiate into mature oligodendrocytes which then remyelinate the demyelinated axons. However, for multiple reasons, in MS the regenerative capacity of these cell populations diminishes significantly over time, ultimately leading to neurodegeneration, which currently remains untreatable. In addition, microglial cells, the resident innate immune cells of the CNS, can contribute further to inflammatory and degenerative axonal damage. Here, we review the molecular factors contributing to remyelination failure in MS by inhibiting OPC and NSC differentiation or modulating microglial behavior.

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Review

The Molecular Basis for Remyelination Failure in Multiple Sclerosis

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Abstract: Myelin sheaths in the central nervous system (CNS) insulate axons and thereby allow saltatory nerve conduction, which is a prerequisite for complex brain function. Multiple sclerosis (MS), the most common inflammatory autoimmune disease of the CNS, leads to the destruction of myelin sheaths and the myelin-producing oligodendrocytes, thus leaving behind demyelinated axons prone to injury and degeneration. Clinically, this process manifests itself in significant neurological symptoms and disability. Resident oligodendroglial precursor cells (OPCs) and neural stem cells (NSCs) are present in the adult brain, and can differentiate into mature oligodendrocytes which then remyelinate the demyelinated axons. However, for multiple reasons, in MS the regenerative capacity of these cell populations diminishes significantly over time, ultimately leading to neurodegeneration, which currently remains untreatable. In addition, microglial cells, the resident innate immune cells of the CNS, can contribute further to inflammatory and degenerative axonal damage. Here, we review the molecular factors contributing to remyelination failure in MS by inhibiting OPC and NSC differentiation or modulating microglial behavior.

Keywords: multiple sclerosis; remyelination; oligodendroglial precursor cells; neural stem cells; microglia

1. Introduction

Aside from multifocal inflammation and demyelination, neurodegeneration is one of the hallmarks of multiple sclerosis (MS). It represents the key factor driving clinical disability and the diminished quality of life in this commonest autoimmune disease of the central nervous system (CNS). The relapsing subtypes of MS (RMS) have become rather well treatable with a range of drugs. By contrast, progressive MS (PMS), characterized by the steady accumulation of neurological deficits and disability, remains a therapeutic challenge. Demyelination resulting from the autoimmune damage of oligodendrocytes and a loss of myelin sheaths lies at the core of MS. Myelin sheaths, which insulate axons and guarantee safe and reliable impulse propagation, are critical for normal neural function. Their loss leads to reduced axonal integrity, which over time, results in neuronal fallout, and consequently to fixed functional deficits. Partial replacement of lost oligodendrocytes and the (re)establishment of myelin sheaths around denuded axons can occur as a result of the activation, recruitment and differentiation of resident oligodendroglial precursor cells (OPCs) and neural stem cells (NSCs). These cells can differentiate and mature into myelin-sheath forming oligodendrocytes—a repair process commonly referred to as remyelination [1]. However, remyelination remains overall inefficient, which suggests that even though OPCs and NSCs are present in the MS brain, they are prevented from effectively differentiating into new myelin-producing cells by a variety of molecular mechanisms [2–4]. Of course, to what degree a reduced activity of one or the other immature cell population is the main factor for remyelination failure in MS, remains to be shown.

One of the most important cell types involved in these mechanisms are microglia, the resident innate immune cells of the CNS which modulate particularly OPC homeostasis, but also directly contribute to axonal degeneration. In this review, we investigate which factors influence OPC and NSC differentiation, with particular attention to the role of microglia in these processes (see Table S1).

2. Oligodendroglial Precursor Cells (OPCs)

Representing 5–8% of the total cell population of the adult brain, resident OPCs are found widely distributed throughout gray and white matter, and provide a source for myelin repair following CNS injury [5–7]. However, remyelination capacity remains overall inefficient and appears to decrease with age, despite the substantial number of OPCs found in demyelinated MS lesions. Interestingly, differences in the extent of myelin regeneration can be observed between individual lesions and patients, potentially indicating heterogeneity within the OPC population and the mechanisms underlying failing myelin repair in any given patient [8]. In this regard, recent publications point to a heterogeneity within the oligodendroglial cell population based on localization [9], origin [10] and additional lineage alterations upon demyelination [11]. This may explain different responses to demyelination and perhaps even a different susceptibility to age-associated functional decline [12]. To make things more complex, recently an additional contribution to myelin repair from partially lesioned oligodendrocytes has been suggested [13,14]. This is of particular interest, as surviving mature oligodendrocytes have classically been considered as passive bystanders of remyelination. However, using two different animal models, Duncan and colleagues have now demonstrated that mature oligodendrocytes extend processes towards demyelinated axons and ensheath them, while at the same time they are connected to surviving myelin sheaths. This may point to a so far unrecognized contribution of these cells to myelin repair. In summary, understanding the reasons for OPC differentiation and/or functional maturation failure in MS will hopefully help us to develop new strategies to improve remyelination. Accordingly, during the past years the focus of MS research has shifted to the identification of therapies that promote remyelination, for instance by modulating extrinsic and intrinsic factors that either act as the inhibitors or stimulators of OPC differentiation [15–17]. Furthermore, it was found that remyelination failure correlates with age and disease duration [18,19]. This is hypothesized to result from a reduced myelin debris clearance, and a decrease of factors secreted by monocytic cells promoting OPC differentiation [20]. Notably, aging also directly restricts oligodendroglial cell differentiation, resulting from age-related intrinsic changes in the mammalian target of the rapamycin (mTOR) signaling pathway, reducing differentiation which contributes to remyelination failure [21]. With regard to regional variations, it is known that gray and white matter lesions exhibit different capacities for remyelination. This finding is based on histochemical and electron microscopic studies in mostly chronic MS brains, which showed that the efficiency and degree of remyelination in cortical gray matter lesions (GML) is significantly higher than in white matter lesions (WML) [22]. These results may be explained by data demonstrating that the number of oligodendrocytes and OPCs is more than 6-fold higher in GML than in WML [23]. At least at first glance, this is somewhat surprising, as there is evidence that more oligodendrocytes and OPCs are present in normal appearing white matter (NAWM) than in normal appearing gray matter (NAGM) [24]. However, a rationale underlying this apparent paradox might be that in gray matter, oligodendroglial recruitment is simply more efficient [23]. Yet, other possible reasons for the differences in WML/GML remyelination capacity are being discussed, and range from the potential presence of different cortical and white matter oligodendrocyte and OPC subpopulations [25–27] to differences in microenvironment. In this regard, various factors could play a detrimental role regarding WML remyelination: The specific composition of the extracellular matrix (ECM) [28], a stronger inflammatory activity and microglial density [29], as well as an increased tendency to scar formation [23]. In addition, there also seems to be a relative overexpression of various inhibitors of OPC differentiation, including, inter alia, extracellular axonal ligands, such as PSA-NCAM [30], LINGO-1 [31], Jagged [32] and Galectin-4 [33], which were all shown to directly block OPC differentiation.

Therapeutic Approaches to Promote OPC-Mediated Remyelination

Technically, one of the most effective approaches to neutralize potential inhibitors of OPC differentiation is targeted antibody therapy. An antibody that was already shown to have a good safety profile in phase 1 clinical trials for MS and amyotrophic lateral sclerosis (ALS) is ozanezumab (NCT01435993, NCT01424423). Ozanezumab is a humanized monoclonal antibody targeting the myelin-associated neurite outgrowth inhibitor NogoA. The histones of NogoA-expressing oligodendrocytes are highly acetylated, which is associated with an increased transcription of OPC differentiation inhibitors [34]. Clinically, the antibody-mediated neutralization of NogoA in a lysolecithin-induced animal model of experimental spinal cord demyelination results in enhanced remyelination and improved functional recovery [35]. Moreover, neutralization of NogoA might also be involved in enhanced synaptic plasticity and improved intrinsic repair in the adult CNS [36]. This is highly relevant, as none of the currently available MS treatments alleviates synaptic failure and network dysfunction [37]. Hence there is a need to further assess the therapeutic potential of NogoA neutralization.

Elezanumab is another humanized monoclonal antibody currently tested in phase 2 clinical trials for RMS (NCT03737851) and PMS (NCT03737812). This antibody is directed against the membrane-bound repulsive guidance molecule A (RGMa), which promotes inflammation and inhibits CNS regeneration and remyelination mainly via the multifunctional target receptor neogenin [38]. Anti-RGMa treatment leads to a decreased T cell proliferation, a decrease in pro-inflammatory interleukin production, a functional recovery in experimental autoimmune encephalomyelitis (EAE) [39] and to a prolonged conversion time to the secondary progressive disease phase [40].

Opicinumab, another monoclonal antibody, was also investigated in several clinical trials assessing its effectiveness and safety in MS patients. This antibody is directed against the Nogo-receptor interacting protein LINGO-1, which was found to negatively regulate OPC differentiation by activating RhoA and inhibiting the Akt signaling pathways [41]. The phase 2 SYNERGY trial (NCT01864148) investigated the safety and efficacy of opicinumab as an add-on therapy to intramuscular interferon beta-1a in patients with RMS. Although the primary outcome (a multicomponent improvement of function over 72 weeks) was not met, some trends emerged in subgroup analyses: Younger RMS patients with shorter disease duration and magnetic resonance imaging (MRI) features suggestive of more preserved brain tissue (i.e., bigger whole-brain and thalamic volume at baseline) had a greater therapy effect. The RENEW trial (NCT01721161) testing opicinumab in patients with acute optic neuritis (AON) did not show a significant difference in the intent-to-treat population (ITT), either. However, it could show a possible beneficial effect of opicinumab on remyelination in a pre-specified per-protocol analysis after 32 weeks of treatment, with the recovery of P100 latencies of full-field visual evoked potential (FF-VEP; [42]) as a primary outcome.

Finally, the humanized anti-pHERV-W Env antibody temelimab yielded promising results in a recently completed phase 2b study in RMS (NCT02782858) after a previous phase 2a study had already yielded encouraging results [43]. Temelimab is directed against the pathogenic envelope protein (Env) of a human endogenous retrovirus (HERV) belonging to the HERV-W family. Although the primary MRI-related endpoints of the study, with an emphasis on inflammation in the trial period from week 12 to 24, were not met, a significant dose-dependent beneficial effect on brain and thalamic atrophy, as well as on the number of T1 hypointense "black holes" could be shown in the secondary endpoints in the trial period from week 24 to 48. In addition, a potential benefit on remyelination as indicated by Magnetization Transfer Ratio (MTR) was observed. A more recent phase I study has now investigated higher doses of temelimab, and could confirm the previously shown good safety profile (NCT03574428). These results will certainly lead to further clinical studies focused on PMS. In addition to these emerging antibodies, research simultaneously focuses on the repurposing of well-established drugs regarding possible neuroregenerative effects. Accordingly, there are currently studies underway investigating simvastatin (NCT03387670), quetiapine (NCT02087631) and clemastine fumarate (NCT02040298). Simvastatin is a 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA)

reductase inhibitor commonly used for the treatment of hypercholesterolemia. Regarding its effect on remyelination there are contradictory results. While a number of studies have found that simvastatin stimulates OPC survival and differentiation [44,45], others have suggested the opposite [46]. Quetiapin on the other hand, is an atypical neuroleptic drug, which has been reported to increase OPC differentiation and myelin protein production [47]. Finally, clemastine is a histamine H1 receptor blocker, which was shown to increase OPC differentiation in disease entities such as hypoxic brain injury [48,49].

3. Neural Stem Cells (NSCs)

In the past years, growing interest has focused on endogenous and transplanted neural stem cells (NSCs) and their potential to provide oligodendrocyte replacement and to mediate myelin repair. NSCs are multipotent cells which can self-renew and differentiate into neurons and glial cells [50–52], most notably, under appropriate circumstances, also into oligodendroglia. In the adult mammalian brain, at least two distinct niches, the subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the dentate gyrus, are described to harbor NSCs. In contrast to the SVZ, SGZ-derived stem cells mainly differentiate into neurons unless they are genetically reprogrammed or trophically manipulated to produce oligodendrocytes [53–60]. In this regard, during the last years, several factors were identified which can instruct and drive oligodendrogenesis from NSCs. More importantly, it was demonstrated that SVZ NSC-derived oligodendroglial cells can contribute significantly to remyelination in several demyelination mouse models [61–65]. Of note, in some of these studies, more NSC-derived than OPC-derived newly generated oligodendrocytes were detected [64,65], pointing to the relevance of these cells regarding CNS repair. As these studies identified NSCs as a major contributing source for myelin repair, the question arises why NSC-mediated remyelination in MS is overall inefficient and even decreases over time. In this regard, several potential explanations emerged during the past years.

3.1. Aging

As already mentioned above, aging could play a role in failing remyelination. In this context it was shown that the aged SVZ suffers from a reduced number of ventricle-contacting astrocytes. These cells are the precursors for so-called transit-amplifying cells (TAPs), which are highly proliferative, and can give rise to newly differentiated oligodendrocytes. This is in line with the recent suggestion that human cellular senescence is responsible for a diminished remyelination potential in progressive MS patients [66]. Moreover, it has been repeatedly shown that in the aged brain less neurogenesis occurs [67–70], which has been explained by increased cell cycle lengths, a lower availability of growth factors and an accumulation of inhibitory factors. It is therefore conceivable that similar mechanisms apply to gliogenesis, i.e., the production of new oligodendrocytes. However, so far corresponding research has yielded contradicting results. While it was shown that neurosphere-mediated oligodendrogenesis in response to growth factors declines with age [68], several other groups found that NSC-derived oligodendrogenesis remains constant [69–71]. While an age-related decrease in trophic factor expression was described in the human SVZ by some research groups [72,73], Weissleder and colleagues reported an increase or stable expression in transcripts encoding trophic factors or their receptors, such as epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2) and Erb-B2 receptor tyrosine kinase 4 (ErbB4; [70]). On the other hand, measurements of ^{14}C incorporation into genomic DNA (a result of nuclear tests during the Cold War) demonstrated that newly generated oligodendrocytes can still be detected around the lateral wall of the adult human SVZ [74], thereby providing evidence for ongoing oligodendrogenesis in the adult.

3.2. Inflammation

In the mouse MS model, EAE inflammation was demonstrated to modulate NSC differentiation [75–77], and autoantibodies in EAE showed a strong affinity to SVZ NSCs [78]. EAE was also found to induce spontaneous apoptosis of neural stem and progenitor cells (NPCs) in vitro [76].

While several EAE studies provide evidence that NSCs can modulate chemokine levels, resulting in an impaired recruitment of immune cells to the CNS [79,80], these signaling molecules are also important for the attraction of endogenous NSCs into white matter tracts [75]. Of note, one EAE study showed that chitinase 3-like-3 (Chi3l3) activates the epidermal growth factor receptor (EGFR) on neural stem cells, which results in decreased disease severity [81]. Moreover, chemokines are important for stem cell proliferation, resulting in a lower capacity to differentiate into oligodendrocytes, and an increased differentiation into neuronal progenitors [77,82]. In contrast, transplanted NSCs were shown to participate directly in the remyelination of damaged axons [83], but also to influence OPC-based myelin regeneration after cuprizone-mediated demyelination [84]. A recent study using single cell transcriptomics on aging neural stem cell niches provided evidence of increased T cell infiltration in aged animals, which apparently impairs NSC proliferation via interferon-γ secretion [85].

3.3. Factors Involved in NSC-Based Oligodendrogenesis

A number of different factors and signaling pathways which modulate NSC-derived oligodendrogenesis have been described (summarized in [86]). The majority of the encoded proteins exert similar roles on both OPCs and NSCs. However, a few transcriptional/epigenetic regulators, such as Gli1 [87], Sirt1 [88], nuclear factor I X (NFIX; [89]), B-cell leukemia homeodomain 1 (Pbx1, [90]), prospero-related homeobox 1 gene (Prox1, [91]), drosha and nuclear factor IB (NFIB, [60]), exhibit stem cell-specific effects, and might therefore be of particular interest regarding alternative myelin repair pathways. For example, it could be shown that the negative regulatory effect on remyelination by Gli1 and Sirt1 could be partially reversed by using small molecule inhibitors (GANT61 for Gli1 and EX-527 for Sirt1, respectively). Furthermore, it was shown that nuclear factor-erythroid 2-related factor 2 (NRF2) leads to impaired differentiation and proliferation rates of hippocampal NSCs [92]. This is an interesting observation, given the fact that dimethylfumarate (DMF), a well-established and potent oral MS medication, is known to enhance NRF2 expression. Finally, FGFR3 activation was recently shown to redirect the differentiation of SVZ-derived NSCs into oligodendrocytes promoting remyelination [93]. Another point that should be considered regarding NSC-based remyelination is the heterogeneity within NSC niches. While signaling pathways involving Wnt/β-catenin, Prox1, Olig2, and Sox10 are enriched in the dorsal microdomain of the SVZ [94–97], Pbx1 can be detected throughout the entire niche [62]. p57kip2, on the other hand, appears to dominate in the lateral wall of the SVZ [86]. In contrast, NSCs located in the innermost parts of the granule cell layer of the hilus mainly give rise to neurons, but can also form new oligodendrocytes following overexpression of achaete-scute family bHLH transcription factor 1 (Ascl1, [54,58]) or the inhibition of factors such as Prox1, [97], neurofibromatosis type I (Nf1, [59]), Drosha [60], ubiquitin-specific peptidase 9, X-linked (Usp9x, [98]) or p57kip2 [55].

4. Microglia

OPC and NSC homeostasis cannot be seen in isolation from other cell populations or physiological processes. In this regard, the innate immune system, i.e., resident microglia and peripherally-derived infiltrating macrophages, have been shown to be essential for remyelination. This includes axon regeneration, the clearance of myelin debris and the release of neurotrophic factors that promote OPC differentiation [99]. Microglia are the resident innate immune cells of the CNS and play an important role in the MS disease process. On the one hand, they can adopt a pro-inflammatory phenotype, classically known as M1, contributing to inflammation and axonal damage. On the other hand, they can also take on a restorative phenotype, known as M2, which is associated with anti-inflammation, tissue

repair and phagocytosis of debris. This process, classically referred to as polarization, and as of late challenged in the scientific community [100], lies at the core of the complex role of microglia in de- and remyelination [101].

4.1. Phagocytosis of Myelin Debris

Phagocytosis of myelin debris in the MS brain is essential for the initiation of neuro-repair as myelin debris inhibits OPC differentiation and, by doing so, remyelination [102]. Among others, Fractalkine receptor (CX3CR1), which is expressed at high levels on microglia, has been identified to exert an impact on microglial phagocytic capacity. CX3CR1-deficient mice treated with cuprizone were shown to have a reduced microglial phagocytic capacity, leading to a persistent presence of myelin debris, which results in inefficient remyelination due to impaired OPC recruitment [103]. Another factor relevant for phagocytosis is triggering receptor expressed on myeloid cells (TREM2). Long-term studies of TREM2 knockout mice showed impaired myelin debris clearance, axonal dystrophy, oligodendrocyte reduction, and persistent demyelination after prolonged cuprizone treatment [104]. In addition, in the inflammatory setting of EAE, a blockade of TREM2 leads to disease progression, resulting in diffuse demyelination [105]. Other players affecting phagocytosis are the TAM family receptors MerTK and Axl. Axl knockout mice subjected to MOG-EAE feature a decreased number of activated microglia in lesions resulting in reduced myelin debris clearance [106]. Regarding MerTK, in vitro studies in human microglia which were stimulated with TGF-β revealed a strong increase in myelin ingestion based on an upregulation of MerTK and its ligands Protein S and growth arrest specific 6 (GAS6) in comparison to controls [107]. Further studies on monocyte-derived macrophages from MS patients then identified the MerTK pathway as being essential for myelin phagocytosis. MS macrophages displayed a reduced myelin uptake capacity, correlating with lower levels of MerTK and its ligands compared to healthy controls [108]. In addition to the above-mentioned factors, a recently published study has identified the pHERV-W envelope protein Env as a stimulator of microglia-associated inflammation in MS. Env reduces microglial phagocytic capacity by downregulating TREM2 and MerTK resulting in decreased myelin debris clearance. Moreover, Env was found to drive microglia-mediated axonal damage resulting in the leakage of myelin and intra-axonal indicative of axonal degeneration [109].

4.2. Microglial Stimulation of OPC Differentiation

Besides inefficient myelin debris clearance, failing paracrine stimulation of OPC differentiation by microglia is another key factor contributing to inefficient remyelination in MS. In this context, it was discovered that fibronectin aggregates accumulate in the extracellular matrix (EZM) during chronic demyelination processes and impede remyelination via inhibiting oligodendrocyte differentiation. Matrix metalloproteinases (MMPs) on the other hand, can modulate the EZM and are able to split fibronectin—as it is the case for MMP7. Microglia were identified as a major source of the MMP7 proenzyme proMMP7, which is reduced in chronic active and inactive MS lesions correlating with higher amounts of fibronectin aggregates disrupting OPC differentiation capacity [110]. In general, both M1 and M2 phenotypes are simultaneously present in MS lesions, but for successful OPC differentiation and remyelination a switch from M1 to M2 seems to be essential [111]. In this regard, the long noncoding RNA (lncRNA) GAS5 was shown to regulate microglial polarization with increased levels being found in activated ameboid microglia in MS brains. GAS5 apparently averts M2 polarization by suppressing the transcription of TRF4, via a recruitment of the polycomb repressive complex 2 (PRC2). In EAE, interference with GAS5 in microglia attenuates disease progression and promotes remyelination [112]. Beyond that, microglia are able to promote remyelination via a complex repertoire of secreted chemokines and cytokines which stimulate oligodendroglial differentiation [113]. Factors that have been identified as being supportive in this regard are, for instance, CXCL12 [114–116], semaphorin 3F [117], Activin-A [111] and Galectin-3 [118]. It is noteworthy that in chronic persistent inflammation, as it occurs in MS, microglia secrete many of these supporting factors at reduced levels, thereby contributing to ineffective remyelination [17,111,119].

5. Discussion

While the past years have seen the approval of highly potent immunomodulatory drugs which led to a paradigm shift in the treatment of RMS, therapies that stimulate myelin repair and prevent neurodegeneration are still unavailable. However, even though ultimately rather ineffective, the brain's own repair mechanisms provide us with helpful leads as to where to start the search for such therapies. Of course, in order to claim credible relevance for MS, pre-clinical studies which aim to identify promising molecules have to be conducted in a context as close as possible to the human paradigm, i.e., in human cells and/or in human tissue, which are, of course, difficult to procure. Nonetheless, the quest for myelin repair enhancement in MS has already prompted the initiation of several trials assessing the clinical effectiveness of molecules known to inhibit or stimulate OPC differentiation [16]. Of note, some of these molecules are not exclusively studied for MS, but also in patients with other CNS diseases, such as amyotrophic lateral sclerosis (ALS) or spinal cord injury (SCI) as these disorders share certain aspects of MS pathophysiology. Interestingly and probably due to their proximity to mature myelin-forming oligodendrocytes, clinical research has so far exclusively focused on molecules stimulating the differentiation of OPCs. In contrast, factors facilitating NSC differentiation or beneficially modulating microglia have not attracted an equal level of attention. This is somewhat surprising, as in some experimental animal studies more NSC-derived than OPC-derived newly generated oligodendrocytes were detected. In addition, so far there has not been a single trial investigating molecules aimed at stimulating microglia-associated phagocytosis. Yet, the plain fact that a completely new generation of potential MS medications is currently being studied remains reason enough for cautious optimism. Of note, regarding the molecular mechanisms of such therapeutic agents, there are some important caveats. First of all, potential therapeutic molecules must be cell-specific, and should not pleiotropically interfere with physiologically required pathways. As they have to work on cell populations inside the CNS, they must also be able to cross the blood-brain-barrier (BBB). While the BBB is leaky during acute MS relapses, as demonstrated by MRI-based Gadolinium enhancement, it is, to a large degree, re-established afterwards. However, microglia-mediated neurodegeneration is continuing even behind a closed BBB. In order to solve this problem, among other approaches virus-based CNS drug delivery systems have been suggested which are able to transport therapeutic molecules across an intact BBB [120]. Of course, in order to maximize tropism for (oligodendro)glia, viral systems based on JC polyomavirus (JCPyV) seem to be the most promising (Chao et al., 2018). This leads us to the next challenge, which consists in finding the right therapeutic "window of opportunity". An application of regenerative agents too late during the disease course could be fruitless, as axons might have already degenerated irreversibly. In summary, it is very likely that the MS therapy of the future will consist of a two-pronged approach, uniting established immunomodulatory treatments with remyelinating ones. Despite the aforementioned complex issues, it is very encouraging to observe the new developments in the emerging field of clinical remyelination therapy. Hopefully, we will soon have new therapeutic options at our disposal that will enable us to effectively treat both the inflammatory and neurodegenerative aspects of MS, in order not only to prevent new damage, but also to restore lost function.

Supplementary Materials: The following are available at http://www.mdpi.com/2073-4409/8/8/825/s1, Table S1. Molecules and their potential impact on remyelination in the central nervous system (CNS).

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References

1. Franklin, R.J.; Ffrench-Constant, C. Remyelination in the cns: From biology to therapy. *Nat. Rev. Neurosci.* **2008**, *9*, 839–855. [CrossRef] [PubMed]
2. Kotter, M.R.; Stadelmann, C.; Hartung, H.P. Enhancing remyelination in disease–can we wrap it up? *Brain A J. Neurol.* **2011**, *134*, 1882–1900. [CrossRef]
3. Kuhlmann, T.; Miron, V.; Cui, Q.; Wegner, C.; Antel, J.; Brück, W. Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain A J. Neurol.* **2008**, *131*, 1749–1758. [CrossRef] [PubMed]
4. Kremer, D.; Aktas, O.; Hartung, H.P.; Küry, P. The complex world of oligodendroglial differentiation inhibitors. *Ann. Neurol.* **2011**, *69*, 602–618. [CrossRef] [PubMed]
5. Hughes, E.G.; Kang, S.H.; Fukaya, M.; Bergles, D.E. Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain. *Nat. Neurosci.* **2013**, *16*, 668–676. [CrossRef] [PubMed]
6. Yeung, M.S.; Zdunek, S.; Bergmann, O.; Bernard, S.; Salehpour, M.; Alkass, K.; Perl, S.; Tisdale, J.; Possnert, G.; Brundin, L.; et al. Dynamics of oligodendrocyte generation and myelination in the human brain. *Cell* **2014**, *159*, 766–774. [CrossRef] [PubMed]
7. Young, K.M.; Psachoulia, K.; Tripathi, R.B.; Dunn, S.J.; Cossell, L.; Attwell, D.; Tohyama, K.; Richardson, W.D. Oligodendrocyte dynamics in the healthy adult cns: Evidence for myelin remodeling. *Neuron* **2013**, *77*, 873–885. [CrossRef] [PubMed]
8. Franklin, R.J.M.; Ffrench-Constant, C. Regenerating cns myelin - from mechanisms to experimental medicines. *Nat. Rev. Neurosci.* **2017**, *18*, 753–769. [CrossRef] [PubMed]
9. Marques, S.; Zeisel, A.; Codeluppi, S.; van Bruggen, D.; Mendanha Falcao, A.; Xiao, L.; Li, H.; Haring, M.; Hochgerner, H.; Romanov, R.A.; et al. Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. *Science* **2016**, *352*, 1326–1329. [CrossRef] [PubMed]
10. Vigano, F.; Mobius, W.; Gotz, M.; Dimou, L. Transplantation reveals regional differences in oligodendrocyte differentiation in the adult brain. *Nat. Neurosci.* **2013**, *16*, 1370–1372. [CrossRef]
11. Falcao, A.M.; van Bruggen, D.; Marques, S.; Meijer, M.; Jakel, S.; Agirre, E.; Samudyata; Floriddia, E.M.; Vanichkina, D.P.; Ffrench-Constant, C.; et al. Disease-specific oligodendrocyte lineage cells arise in multiple sclerosis. *Nat. Med.* **2018**, *24*, 1837–1844. [CrossRef] [PubMed]
12. Crawford, A.H.; Tripathi, R.B.; Richardson, W.D.; Franklin, R.J.M. Developmental origin of oligodendrocyte lineage cells determines response to demyelination and susceptibility to age-associated functional decline. *Cell Rep.* **2016**, *15*, 761–773. [CrossRef] [PubMed]
13. Duncan, I.D.; Radcliff, A.B.; Heidari, M.; Kidd, G.; August, B.K.; Wierenga, L.A. The adult oligodendrocyte can participate in remyelination. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E11807–E11816. [CrossRef] [PubMed]
14. Yeung, M.S.Y.; Djelloul, M.; Steiner, E.; Bernard, S.; Salehpour, M.; Possnert, G.; Brundin, L.; Frisen, J. Dynamics of oligodendrocyte generation in multiple sclerosis. *Nature* **2019**, *566*, 538–542. [CrossRef] [PubMed]
15. Kremer, D.; Göttle, P.; Hartung, H.P.; Küry, P. Pushing forward: Remyelination as the new frontier in cns diseases. *Trends Neurosci.* **2016**, *39*, 246–263. [CrossRef]
16. Küry, P.; Kremer, D.; Göttle, P. Drug repurposing for neuroregeneration in multiple sclerosis. *Neural Regen Res.* **2018**, *13*, 1366–1367. [PubMed]
17. Kremer, D.; Göttle, P.; Flores-Rivera, J.; Hartung, H.P.; Küry, P. Remyelination in multiple sclerosis: From concept to clinical trials. *Curr. Opin. Neurol.* **2019**, *32*, 378–384. [CrossRef]
18. Frischer, J.M.; Weigand, S.D.; Guo, Y.; Kale, N.; Parisi, J.E.; Pirko, I.; Mandrekar, J.; Bramow, S.; Metz, I.; Bruck, W.; et al. Clinical and pathological insights into the dynamic nature of the white matter multiple sclerosis plaque. *Ann. Neurol.* **2015**, *78*, 710–721. [CrossRef]
19. Goldschmidt, T.; Antel, J.; Konig, F.B.; Bruck, W.; Kuhlmann, T. Remyelination capacity of the ms brain decreases with disease chronicity. *Neurology* **2009**, *72*, 1914–1921. [CrossRef]
20. Ruckh, J.M.; Zhao, J.W.; Shadrach, J.L.; van Wijngaarden, P.; Rao, T.N.; Wagers, A.J.; Franklin, R.J. Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell* **2012**, *10*, 96–103. [CrossRef]
21. Neumann, B.; Baror, R.; Wijngaarden, P.v.; Franklin, R.J. Remyelination of regenerating axons. *Acta Ophthalmol.* **2017**. [CrossRef]

22. Albert, M.; Antel, J.; Bruck, W.; Stadelmann, C. Extensive cortical remyelination in patients with chronic multiple sclerosis. *Brain Pathol.* **2007**, *17*, 129–138. [CrossRef] [PubMed]
23. Chang, A.; Staugaitis, S.M.; Dutta, R.; Batt, C.E.; Easley, K.E.; Chomyk, A.M.; Yong, V.W.; Fox, R.J.; Kidd, G.J.; Trapp, B.D. Cortical remyelination: A new target for repair therapies in multiple sclerosis. *Ann. Neurol.* **2012**, *72*, 918–926. [CrossRef] [PubMed]
24. Strijbis, E.M.M.; Kooi, E.J.; van der Valk, P.; Geurts, J.J.G. Cortical remyelination is heterogeneous in multiple sclerosis. *J. Neuropathol. Exp. Neurol.* **2017**, *76*, 390–401. [CrossRef] [PubMed]
25. Noble, M.; Arhin, A.; Gass, D.; Mayer-Pröschel, M. The cortical ancestry of oligodendrocytes: Common principles and novel features. *Dev. Neurosci.* **2003**, *25*, 217–233. [CrossRef]
26. Foerster, S.; Hill, M.F.E.; Franklin, R.J.M. Diversity in the oligodendrocyte lineage: Plasticity or heterogeneity? *Glia 0* **2019**. [CrossRef] [PubMed]
27. Jäkel, S.; Agirre, E.; Mendanha Falcão, A.; van Bruggen, D.; Lee, K.W.; Knuesel, I.; Malhotra, D.; ffrench-Constant, C.; Williams, A.; Castelo-Branco, G. Altered human oligodendrocyte heterogeneity in multiple sclerosis. *Nature* **2019**, *566*, 543–547. [CrossRef]
28. Lau, L.W.; Cua, R.; Keough, M.B.; Haylock-Jacobs, S.; Yong, V.W. Pathophysiology of the brain extracellular matrix: A new target for remyelination. *Nat. Rev. Neurosci.* **2013**, *14*, 722. [CrossRef]
29. Hart, A.D.; Wyttenbach, A.; Hugh Perry, V.; Teeling, J.L. Age related changes in microglial phenotype vary between cns regions: Grey versus white matter differences. *Brainbehaviorand Immun.* **2012**, *26*, 754–765. [CrossRef]
30. Charles, P.; Hernandez, M.P.; Stankoff, B.; Aigrot, M.S.; Colin, C.; Rougon, G.; Zalc, B.; Lubetzki, C. Negative regulation of central nervous system myelination by polysialylated-neural cell adhesion molecule. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7585–7590. [CrossRef]
31. Mi, S.; Miller, R.H.; Lee, X.; Scott, M.L.; Shulag-Morskaya, S.; Shao, Z.; Chang, J.; Thill, G.; Levesque, M.; Zhang, M.; et al. Lingo-1 negatively regulates myelination by oligodendrocytes. *Nat. Neurosci.* **2005**, *8*, 745–751. [CrossRef] [PubMed]
32. Wang, S.; Sdrulla, A.D.; diSibio, G.; Bush, G.; Nofziger, D.; Hicks, C.; Weinmaster, G.; Barres, B.A. Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **1998**, *21*, 63–75. [CrossRef]
33. Stancic, M.; Slijepcevic, D.; Nomden, A.; Vos, M.J.; de Jonge, J.C.; Sikkema, A.H.; Gabius, H.J.; Hoekstra, D.; Baron, W. Galectin-4, a novel neuronal regulator of myelination. *Glia* **2012**, *60*, 919–935. [CrossRef] [PubMed]
34. Pedre, X.; Mastronardi, F.; Bruck, W.; López-Rodas, G.; Kuhlmann, T.; Casaccia, P. Changed histone acetylation patterns in normal-appearing white matter and early multiple sclerosis lesions. *J. Neurosci.* **2011**, *31*, 3435–3445. [CrossRef] [PubMed]
35. Ineichen, B.V.; Kapitza, S.; Bleul, C.; Good, N.; Plattner, P.S.; Seyedsadr, M.S.; Kaiser, J.; Schneider, M.P.; Zorner, B.; Martin, R.; et al. Nogo-a antibodies enhance axonal repair and remyelination in neuro-inflammatory and demyelinating pathology. *Acta Neuropathol.* **2017**, *134*, 423–440. [CrossRef] [PubMed]
36. Delekate, A.; Zagrebelsky, M.; Kramer, S.; Schwab, M.E.; Korte, M. Nogoa restricts synaptic plasticity in the adult hippocampus on a fast time scale. *Proc. Natl. Acad. Sci.* **2011**, *108*, 2569–2574. [CrossRef] [PubMed]
37. Di Filippo, M.; Portaccio, E.; Mancini, A.; Calabresi, P. Multiple sclerosis and cognition: Synaptic failure and network dysfunction. *Nat. Rev Neurosci.* **2018**, *19*, 599–609. [CrossRef]
38. Demicheva, E.; Cui, Y.F.; Bardwell, P.; Barghorn, S.; Kron, M.; Meyer, A.H.; Schmidt, M.; Gerlach, B.; Leddy, M.; Barlow, E.; et al. Targeting repulsive guidance molecule a to promote regeneration and neuroprotection in multiple sclerosis. *Cell Rep.* **2015**, *10*, 1887–1898. [CrossRef]
39. Muramatsu, R.; Kubo, T.; Mori, M.; Nakamura, Y.; Fujita, Y.; Akutsu, T.; Okuno, T.; Taniguchi, J.; Kumanogoh, A.; Yoshida, M.; et al. Rgma modulates t cell responses and is involved in autoimmune encephalomyelitis. *Nat. Med.* **2011**, *17*, 488–494. [CrossRef]
40. Tanabe, S.; Fujita, Y.; Ikuma, K.; Yamashita, T. Inhibiting repulsive guidance molecule-a suppresses secondary progression in mouse models of multiple sclerosis. *Cell Death Dis.* **2018**, *9*, 1061. [CrossRef]
41. Mi, S.; Pepinsky, R.B.; Cadavid, D. Blocking lingo-1 as a therapy to promote cns repair: From concept to the clinic. *CNS Drugs* **2013**, *27*, 493–503. [CrossRef]
42. Cadavid, D.; Balcer, L.; Galetta, S.; Aktas, O.; Ziemssen, T.; Vanopdenbosch, L.; Frederiksen, J.; Skeen, M.; Jaffe, G.J.; Butzkueven, H.; et al. Safety and efficacy of opicinumab in acute optic neuritis (renew): A randomised, placebo-controlled, phase 2 trial. *Lancet Neurol.* **2017**, *16*, 189–199. [CrossRef]

43. Derfuss, T.; Curtin, F.; Guebelin, C.; Bridel, C.; Rasenack, M.; Matthey, A.; Du Pasquier, R.; Schluep, M.; Desmeules, J.; Lang, A.B.; et al. A phase iia randomised clinical study of gnbac1, a humanised monoclonal antibody against the envelope protein of multiple sclerosis-associated endogenous retrovirus in multiple sclerosis patients. *Mult Scler* **2015**, *21*, 885–893. [CrossRef]
44. Paintlia, A.S.; Paintlia, M.K.; Khan, M.; Vollmer, T.; Singh, A.K.; Singh, I. Hmg-coa reductase inhibitor augments survival and differentiation of oligodendrocyte progenitors in animal model of multiple sclerosis. *Faseb J.* **2005**, *19*, 1407–1421. [CrossRef]
45. Sim, F.J.; Lang, J.K.; Ali, T.A.; Roy, N.S.; Vates, G.E.; Pilcher, W.H.; Goldman, S.A. Statin treatment of adult human glial progenitors induces ppar gamma-mediated oligodendrocytic differentiation. *Glia* **2008**, *56*, 954–962. [CrossRef]
46. Miron, V.E.; Rajasekharan, S.; Jarjour, A.A.; Zamvil, S.S.; Kennedy, T.E.; Antel, J.P. Simvastatin regulates oligodendroglial process dynamics and survival. *Glia* **2007**, *55*, 130–143. [CrossRef]
47. Xiao, L.; Xu, H.; Zhang, Y.; Wei, Z.; He, J.; Jiang, W.; Li, X.; Dyck, L.E.; Devon, R.M.; Deng, Y.; et al. Quetiapine facilitates oligodendrocyte development and prevents mice from myelin breakdown and behavioral changes. *Mol. Psychiatry* **2008**, *13*, 697–708. [CrossRef]
48. Wang, F.; Yang, Y.J.; Yang, N.; Chen, X.J.; Huang, N.X.; Zhang, J.; Wu, Y.; Liu, Z.; Gao, X.; Li, T.; et al. Enhancing oligodendrocyte myelination rescues synaptic loss and improves functional recovery after chronic hypoxia. *Neuron* **2018**, *99*, 689–701 e685. [CrossRef]
49. Cree, B.A.C.; Niu, J.; Hoi, K.K.; Zhao, C.; Caganap, S.D.; Henry, R.G.; Dao, D.Q.; Zollinger, D.R.; Mei, F.; Shen, Y.A.; et al. Clemastine rescues myelination defects and promotes functional recovery in hypoxic brain injury. *Brain* **2018**, *141*, 85–98. [CrossRef]
50. Lois, C.; Alvarez-Buylla, A. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2074–2077. [CrossRef]
51. Seri, B.; Garcia-Verdugo, J.M.; McEwen, B.S.; Alvarez-Buylla, A. Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci. Off. J. Soc. Neurosci.* **2001**, *21*, 7153–7160. [CrossRef]
52. Rivera, F.J.; Steffenhagen, C.; Kremer, D.; Kandasamy, M.; Sandner, B.; Couillard-Despres, S.; Weidner, N.; Küry, P.; Aigner, L. Deciphering the oligodendrogenic program of neural progenitors: Cell intrinsic and extrinsic regulators. *Stem Cells Dev.* **2010**, *19*, 595–606. [CrossRef]
53. Rivera, F.J.; Couillard-Despres, S.; Pedre, X.; Ploetz, S.; Caioni, M.; Lois, C.; Bogdahn, U.; Aigner, L. Mesenchymal stem cells instruct oligodendrogenic fate decision on adult neural stem cells. *Stem Cells* **2006**, *24*, 2209–2219. [CrossRef]
54. Jessberger, S.; Toni, N.; Clemenson, G.D., Jr.; Ray, J.; Gage, F.H. Directed differentiation of hippocampal stem/progenitor cells in the adult brain. *Nat. Neurosci.* **2008**, *11*, 888–893. [CrossRef]
55. Jadasz, J.J.; Rivera, F.J.; Taubert, A.; Kandasamy, M.; Sandner, B.; Weidner, N.; Aktas, O.; Hartung, H.P.; Aigner, L.; Küry, P. P57kip2 regulates glial fate decision in adult neural stem cells. *Development* **2012**, *139*, 3306–3315. [CrossRef]
56. Steffenhagen, C.; Dechant, F.X.; Oberbauer, E.; Furtner, T.; Weidner, N.; Küry, P.; Aigner, L.; Rivera, F.J. Mesenchymal stem cells prime proliferating adult neural progenitors toward an oligodendrocyte fate. *Stem Cells Dev.* **2012**, *21*, 1838–1851. [CrossRef]
57. Chetty, S.; Friedman, A.R.; Taravosh-Lahn, K.; Kirby, E.D.; Mirescu, C.; Guo, F.; Krupik, D.; Nicholas, A.; Geraghty, A.; Krishnamurthy, A.; et al. Stress and glucocorticoids promote oligodendrogenesis in the adult hippocampus. *Mol. Psychiatry* **2014**, *19*, 1275–1283. [CrossRef]
58. Braun, S.M.; Pilz, G.A.; Machado, R.A.; Moss, J.; Becher, B.; Toni, N.; Jessberger, S. Programming hippocampal neural stem/progenitor cells into oligodendrocytes enhances remyelination in the adult brain after injury. *Cell Rep.* **2015**, *11*, 1679–1685. [CrossRef] [PubMed]
59. Sun, G.J.; Zhou, Y.; Ito, S.; Bonaguidi, M.A.; Stein-O'Brien, G.; Kawasaki, N.K.; Modak, N.; Zhu, Y.; Ming, G.L.; Song, H. Latent tri-lineage potential of adult hippocampal neural stem cells revealed by nf1 inactivation. *Nat. Neurosci.* **2015**, *18*, 1722–1724. [CrossRef]
60. Rolando, C.; Erni, A.; Grison, A.; Beattie, R.; Engler, A.; Gokhale, P.J.; Milo, M.; Wegleiter, T.; Jessberger, S.; Taylor, V. Multipotency of adult hippocampal nscs in vivo is restricted by drosha/nfib. *Cell Stem Cell* **2016**, *19*, 653–662. [CrossRef]

61. Menn, B.; Garcia-Verdugo, J.M.; Yaschine, C.; Gonzalez-Perez, O.; Rowitch, D.; Alvarez-Buylla, A. Origin of oligodendrocytes in the subventricular zone of the adult brain. *J. Neurosci. Off. J. Soc. Neurosci.* **2006**, *26*, 7907–7918. [CrossRef] [PubMed]
62. Aguirre, A.; Dupree, J.L.; Mangin, J.M.; Gallo, V. A functional role for egfr signaling in myelination and remyelination. *Nat. Neurosci.* **2007**, *10*, 990–1002. [CrossRef] [PubMed]
63. Mecha, M.; Feliu, A.; Carrillo-Salinas, F.J.; Mestre, L.; Guaza, C. Mobilization of progenitors in the subventricular zone to undergo oligodendrogenesis in the theiler's virus model of multiple sclerosis: Implications for remyelination at lesions sites. *Exp. Neurol.* **2013**, *250*, 348–352. [CrossRef] [PubMed]
64. Xing, Y.L.; Roth, P.T.; Stratton, J.A.; Chuang, B.H.; Danne, J.; Ellis, S.L.; Ng, S.W.; Kilpatrick, T.J.; Merson, T.D. Adult neural precursor cells from the subventricular zone contribute significantly to oligodendrocyte regeneration and remyelination. *J. Neurosci. Off. J. Soc. Neurosci.* **2014**, *34*, 14128–14146. [CrossRef] [PubMed]
65. Brousse, B.; Magalon, K.; Durbec, P.; Cayre, M. Region and dynamic specificities of adult neural stem cells and oligodendrocyte precursors in myelin regeneration in the mouse brain. *Biol. Open* **2015**, *4*, 980–992. [CrossRef]
66. Nicaise, A.M.; Wagstaff, L.J.; Willis, C.M.; Paisie, C.; Chandok, H.; Robson, P.; Fossati, V.; Williams, A.; Crocker, S.J. Cellular senescence in progenitor cells contributes to diminished remyelination potential in progressive multiple sclerosis. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 9030–9039. [CrossRef] [PubMed]
67. Kuhn, H.G.; Dickinson-Anson, H.; Gage, F.H. Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. *J. Neurosci. Off. J. Soc. Neurosci.* **1996**, *16*, 2027–2033. [CrossRef]
68. Bouab, M.; Paliouras, G.N.; Aumont, A.; Forest-Berard, K.; Fernandes, K.J. Aging of the subventricular zone neural stem cell niche: Evidence for quiescence-associated changes between early and mid-adulthood. *Neuroscience* **2011**, *173*, 135–149. [CrossRef]
69. Capilla-Gonzalez, V.; Cebrian-Silla, A.; Guerrero-Cazares, H.; Garcia-Verdugo, J.M.; Quinones-Hinojosa, A. The generation of oligodendroglial cells is preserved in the rostral migratory stream during aging. *Front. Cell. Neurosci.* **2013**, *7*, 147. [CrossRef]
70. Weissleder, C.; Fung, S.J.; Wong, M.W.; Barry, G.; Double, K.L.; Halliday, G.M.; Webster, M.J.; Weickert, C.S. Decline in proliferation and immature neuron markers in the human subependymal zone during aging: Relationship to egf- and fgf-related transcripts. *Front. Aging Neurosci.* **2016**, *8*, 274. [CrossRef]
71. Bergmann, O.; Liebl, J.; Bernard, S.; Alkass, K.; Yeung, M.S.; Steier, P.; Kutschera, W.; Johnson, L.; Landen, M.; Druid, H.; et al. The age of olfactory bulb neurons in humans. *Neuron* **2012**, *74*, 634–639. [CrossRef] [PubMed]
72. Weickert, C.S.; Webster, M.J.; Colvin, S.M.; Herman, M.M.; Hyde, T.M.; Weinberger, D.R.; Kleinman, J.E. Localization of epidermal growth factor receptors and putative neuroblasts in human subependymal zone. *J. Comp. Neurol.* **2000**, *423*, 359–372. [CrossRef]
73. Chong, V.Z.; Webster, M.J.; Rothmond, D.A.; Weickert, C.S. Specific developmental reductions in subventricular zone erbb1 and erbb4 mrna in the human brain. *Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci.* **2008**, *26*, 791–803. [CrossRef] [PubMed]
74. Ernst, A.; Alkass, K.; Bernard, S.; Salehpour, M.; Perl, S.; Tisdale, J.; Possnert, G.; Druid, H.; Frisen, J. Neurogenesis in the striatum of the adult human brain. *Cell* **2014**, *156*, 1072–1083. [CrossRef] [PubMed]
75. Cohen, M.E.; Fainstein, N.; Lavon, I.; Ben-Hur, T. Signaling through three chemokine receptors triggers the migration of transplanted neural precursor cells in a model of multiple sclerosis. *Stem Cell Res.* **2014**, *13*, 227–239. [CrossRef] [PubMed]
76. Sajad, M.; Zargan, J.; Sharma, J.; Chawla, R.; Arora, R.; Umar, S.; Khan, H.A. Increased spontaneous apoptosis of rat primary neurospheres in vitro after experimental autoimmune encephalomyelitis. *Neurochem. Res.* **2011**, *36*, 1017–1026. [CrossRef] [PubMed]
77. Arvidsson, L.; Covacu, R.; Estrada, C.P.; Sankavaram, S.R.; Svensson, M.; Brundin, L. Long-distance effects of inflammation on differentiation of adult spinal cord neural stem/progenitor cells. *J. Neuroimmunol.* **2015**, *288*, 47–55. [CrossRef]
78. Kesidou, E.; Touloumi, O.; Lagoudaki, R.; Nousiopoulou, E.; Theotokis, P.; Poulatsidou, K.N.; Boziki, M.; Kofidou, E.; Delivanoglou, N.; Minti, F.; et al. Humoral response in experimental autoimmune encephalomyelitis targets neural precursor cells in the central nervous system of naive rodents. *J. Neuroinflammation* **2017**, *14*, 227. [CrossRef]

79. De Feo, D.; Merlini, A.; Brambilla, E.; Ottoboni, L.; Laterza, C.; Menon, R.; Srinivasan, S.; Farina, C.; Garcia Manteiga, J.M.; Butti, E.; et al. Neural precursor cell-secreted tgf-beta2 redirects inflammatory monocyte-derived cells in cns autoimmunity. *J. Clin. Investig.* **2017**, *127*, 3937–3953. [CrossRef]
80. Ravanidis, S.; Poulatsidou, K.N.; Lagoudaki, R.; Touloumi, O.; Polyzoidou, E.; Lourbopoulos, A.; Nousiopoulou, E.; Theotokis, P.; Kesidou, E.; Tsalikakis, D.; et al. Subcutaneous transplantation of neural precursor cells in experimental autoimmune encephalomyelitis reduces chemotactic signals in the central nervous system. *Stem Cells Transl. Med.* **2015**, *4*, 1450–1462. [CrossRef]
81. Starossom, S.C.; Campo Garcia, J.; Woelfle, T.; Romero-Suarez, S.; Olah, M.; Watanabe, F.; Cao, L.; Yeste, A.; Tukker, J.J.; Quintana, F.J.; et al. Chi3l3 induces oligodendrogenesis in an experimental model of autoimmune neuroinflammation. *Nat. Commun.* **2019**, *10*, 217. [CrossRef]
82. Hagman, S.; Makinen, A.; Yla-Outinen, L.; Huhtala, H.; Elovaara, I.; Narkilahti, S. Effects of inflammatory cytokines ifn-gamma, tnf-alpha and il-6 on the viability and functionality of human pluripotent stem cell-derived neural cells. *J. Neuroimmunol.* **2019**, *331*, 36–45. [CrossRef]
83. Greenberg, M.L.; Weinger, J.G.; Matheu, M.P.; Carbajal, K.S.; Parker, I.; Macklin, W.B.; Lane, T.E.; Cahalan, M.D. Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E2349–E2355. [CrossRef]
84. Einstein, O.; Friedman-Levi, Y.; Grigoriadis, N.; Ben-Hur, T. Transplanted neural precursors enhance host brain-derived myelin regeneration. *J. Neurosci. Off. J. Soc. Neurosci.* **2009**, *29*, 15694–15702. [CrossRef]
85. Dulken, B.W.; Buckley, M.T.; Navarro Negredo, P.; Saligrama, N.; Cayrol, R.; Leeman, D.S.; George, B.M.; Boutet, S.C.; Hebestreit, K.; Pluvinage, J.V.; et al. Single-cell analysis reveals t cell infiltration in old neurogenic niches. *Nature* **2019**. [CrossRef]
86. Akkermann, R.; Beyer, F.; Küry, P. Heterogeneous populations of neural stem cells contribute to myelin repair. *Neural Regen. Res.* **2017**, *12*, 509–517.
87. Samanta, J.; Grund, E.M.; Silva, H.M.; Lafaille, J.J.; Fishell, G.; Salzer, J.L. Inhibition of gli1 mobilizes endogenous neural stem cells for remyelination. *Nature* **2015**, *526*, 448–452. [CrossRef]
88. Rafalski, V.A.; Ho, P.P.; Brett, J.O.; Ucar, D.; Dugas, J.C.; Pollina, E.A.; Chow, L.M.; Ibrahim, A.; Baker, S.J.; Barres, B.A.; et al. Expansion of oligodendrocyte progenitor cells following sirt1 inactivation in the adult brain. *Nat. Cell Biol.* **2013**, *15*, 614–624. [CrossRef]
89. Zhou, B.; Osinski, J.M.; Mateo, J.L.; Martynoga, B.; Sim, F.J.; Campbell, C.E.; Guillemot, F.; Piper, M.; Gronostajski, R.M. Loss of nfix transcription factor biases postnatal neural stem/progenitor cells toward oligodendrogenesis. *Stem Cells Dev.* **2015**, *24*, 2114–2126. [CrossRef]
90. Grebbin, B.M.; Hau, A.C.; Gross, A.; Anders-Maurer, M.; Schramm, J.; Koss, M.; Wille, C.; Mittelbronn, M.; Selleri, L.; Schulte, D. Pbx1 is required for adult subventricular zone neurogenesis. *Development* **2016**, *143*, 2281–2291. [CrossRef]
91. Karalay, O.; Doberauer, K.; Vadodaria, K.C.; Knobloch, M.; Berti, L.; Miquelajauregui, A.; Schwark, M.; Jagasia, R.; Taketo, M.M.; Tarabykin, V.; et al. Prospero-related homeobox 1 gene (prox1) is regulated by canonical wnt signaling and has a stage-specific role in adult hippocampal neurogenesis. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 5807–5812. [CrossRef]
92. Robledinos-Anton, N.; Rojo, A.I.; Ferreiro, E.; Nunez, A.; Krause, K.H.; Jaquet, V.; Cuadrado, A. Transcription factor nrf2 controls the fate of neural stem cells in the subgranular zone of the hippocampus. *Redox Biol.* **2017**, *13*, 393–401. [CrossRef]
93. Kang, W.; Nguyen, K.C.Q.; Hebert, J.M. Transient redirection of svz stem cells to oligodendrogenesis by fgfr3 activation promotes remyelination. *Stem Cell Rep.* **2019**, *12*, 1223–1231. [CrossRef]
94. Ortega, F.; Gascon, S.; Masserdotti, G.; Deshpande, A.; Simon, C.; Fischer, J.; Dimou, L.; Chichung Lie, D.; Schroeder, T.; Berninger, B. Oligodendrogliogenic and neurogenic adult subependymal zone neural stem cells constitute distinct lineages and exhibit differential responsiveness to wnt signalling. *Nat. Cell Biol.* **2013**, *15*, 602–613. [CrossRef]
95. Azim, K.; Fischer, B.; Hurtado-Chong, A.; Draganova, K.; Cantu, C.; Zemke, M.; Sommer, L.; Butt, A.; Raineteau, O. Persistent wnt/beta-catenin signaling determines dorsalization of the postnatal subventricular zone and neural stem cell specification into oligodendrocytes and glutamatergic neurons. *Stem Cells* **2014**, *32*, 1301–1312. [CrossRef]
96. Azim, K.; Rivera, A.; Raineteau, O.; Butt, A.M. Gsk3beta regulates oligodendrogenesis in the dorsal microdomain of the subventricular zone via wnt-beta-catenin signaling. *Glia* **2014**, *62*, 778–779. [CrossRef]

97. Bunk, E.C.; Ertaylan, G.; Ortega, F.; Pavlou, M.A.; Gonzalez Cano, L.; Stergiopoulos, A.; Safaiyan, S.; Vols, S.; van Cann, M.; Politis, P.K.; et al. Prox1 is required for oligodendrocyte cell identity in adult neural stem cells of the subventricular zone. *Stem Cells* **2016**, *34*, 2115–2129. [CrossRef]
98. Oishi, S.; Zalucki, O.; Premarathne, S.; Wood, S.A.; Piper, M. Usp9x deletion elevates the density of oligodendrocytes within the postnatal dentate gyrus. *Neurogenesis* **2016**, *3*, e1235524. [CrossRef]
99. Rawji, K.S.; Mishra, M.K.; Yong, V.W. Regenerative capacity of macrophages for remyelination. *Front. Cell Dev. Biol.* **2016**, *4*, 47. [CrossRef]
100. Ransohoff, R.M. A polarizing question: Do m1 and m2 microglia exist? *Nat. Neurosci.* **2016**, *19*, 987–991. [CrossRef]
101. Tang, Y.; Le, W. Differential roles of m1 and m2 microglia in neurodegenerative diseases. *Mol. Neurobiol.* **2016**, *53*, 1181–1194. [CrossRef] [PubMed]
102. Kotter, M.R.; Li, W.W.; Zhao, C.; Franklin, R.J. Myelin impairs cns remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J. Neurosci. Off. J. Soc. Neurosci.* **2006**, *26*, 328–332. [CrossRef] [PubMed]
103. Lampron, A.; Larochelle, A.; Laflamme, N.; Prefontaine, P.; Plante, M.M.; Sanchez, M.G.; Yong, V.W.; Stys, P.K.; Tremblay, M.E.; Rivest, S. Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *J. Exp. Med.* **2015**, *212*, 481–495. [CrossRef] [PubMed]
104. Poliani, P.L.; Wang, Y.; Fontana, E.; Robinette, M.L.; Yamanishi, Y.; Gilfillan, S.; Colonna, M. Trem2 sustains microglial expansion during aging and response to demyelination. *J. Clin. Investig.* **2015**, *125*, 2161–2170. [CrossRef] [PubMed]
105. Piccio, L.; Buonsanti, C.; Mariani, M.; Cella, M.; Gilfillan, S.; Cross, A.H.; Colonna, M.; Panina-Bordignon, P. Blockade of trem-2 exacerbates experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* **2007**, *37*, 1290–1301. [CrossRef] [PubMed]
106. Weinger, J.G.; Brosnan, C.F.; Loudig, O.; Goldberg, M.F.; Macian, F.; Arnett, H.A.; Prieto, A.L.; Tsiperson, V.; Shafit-Zagardo, B. Loss of the receptor tyrosine kinase axl leads to enhanced inflammation in the cns and delayed removal of myelin debris during experimental autoimmune encephalomyelitis. *J. Neuroinflammation* **2011**, *8*, 49. [CrossRef] [PubMed]
107. Healy, L.M.; Perron, G.; Won, S.Y.; Michell-Robinson, M.A.; Rezk, A.; Ludwin, S.K.; Moore, C.S.; Hall, J.A.; Bar-Or, A.; Antel, J.P. Mertk is a functional regulator of myelin phagocytosis by human myeloid cells. *J. Immunol.* **2016**, *196*, 3375–3384. [CrossRef]
108. Healy, L.M.; Jang, J.H.; Won, S.Y.; Lin, Y.H.; Touil, H.; Aljarallah, S.; Bar-Or, A.; Antel, J.P. Mertk-mediated regulation of myelin phagocytosis by macrophages generated from patients with ms. *Neurol. (R) Neuroimmunol. Neuroinflammation* **2017**, *4*, e402. [CrossRef]
109. Kremer, D.; Gruchot, J.; Weyers, V.; Oldemeier, L.; Gottle, P.; Healy, L.; Ho Jang, J.; Kang, T.X.Y.; Volsko, C.; Dutta, R.; et al. Pherv-w envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* **2019**. [CrossRef]
110. Wang, P.; Gorter, R.P.; de Jonge, J.C.; Nazmuddin, M.; Zhao, C.; Amor, S.; Hoekstra, D.; Baron, W. Mmp7 cleaves remyelination-impairing fibronectin aggregates and its expression is reduced in chronic multiple sclerosis lesions. *Glia* **2018**, *66*, 1625–1643. [CrossRef]
111. Miron, V.E.; Boyd, A.; Zhao, J.W.; Yuen, T.J.; Ruckh, J.M.; Shadrach, J.L.; van Wijngaarden, P.; Wagers, A.J.; Williams, A.; Franklin, R.J.M.; et al. M2 microglia and macrophages drive oligodendrocyte differentiation during cns remyelination. *Nat. Neurosci.* **2013**, *16*, 1211–1218. [CrossRef]
112. Sun, D.; Yu, Z.; Fang, X.; Liu, M.; Pu, Y.; Shao, Q.; Wang, D.; Zhao, X.; Huang, A.; Xiang, Z.; et al. Lncrna gas5 inhibits microglial m2 polarization and exacerbates demyelination. *Embo Rep.* **2017**, *18*, 1801–1816. [CrossRef] [PubMed]
113. Olah, M.; Amor, S.; Brouwer, N.; Vinet, J.; Eggen, B.; Biber, K.; Boddeke, H.W. Identification of a microglia phenotype supportive of remyelination. *Glia* **2012**, *60*, 306–321. [CrossRef] [PubMed]
114. Patel, J.R.; McCandless, E.E.; Dorsey, D.; Klein, R.S. Cxcr4 promotes differentiation of oligodendrocyte progenitors and remyelination. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11062–11067. [CrossRef] [PubMed]
115. Kremer, D.; Cui, Q.L.; Göttle, P.; Kuhlmann, T.; Hartung, H.P.; Antel, J.; Küry, P. Cxcr7 is involved in human oligodendroglial precursor cell maturation. *Plos ONE* **2016**, *11*, e0146503. [CrossRef] [PubMed]
116. Göttle, P.; Kremer, D.; Jander, S.; Odemis, V.; Engele, J.; Hartung, H.P.; Küry, P. Activation of cxcr7 receptor promotes oligodendroglial cell maturation. *Ann. Neurol.* **2010**, *68*, 915–924. [CrossRef]

117. Piaton, G.; Aigrot, M.S.; Williams, A.; Moyon, S.; Tepavcevic, V.; Moutkine, I.; Gras, J.; Matho, K.S.; Schmitt, A.; Soellner, H.; et al. Class 3 semaphorins influence oligodendrocyte precursor recruitment and remyelination in adult central nervous system. *Brain A J. Neurol.* **2011**, *134*, 1156–1167. [CrossRef] [PubMed]
118. Pasquini, L.A.; Millet, V.; Hoyos, H.C.; Giannoni, J.P.; Croci, D.O.; Marder, M.; Liu, F.T.; Rabinovich, G.A.; Pasquini, J.M. Galectin-3 drives oligodendrocyte differentiation to control myelin integrity and function. *Cell Death Differ.* **2011**, *18*, 1746–1756. [CrossRef]
119. Thomas, L.; Pasquini, L.A. Galectin-3-mediated glial crosstalk drives oligodendrocyte differentiation and (re)myelination. *Front. Cell. Neurosci.* **2018**, *12*, 297. [CrossRef]
120. McCall, R.L.; Cacaccio, J.; Wrabel, E.; Schwartz, M.E.; Coleman, T.P.; Sirianni, R.W. Pathogen-inspired drug delivery to the central nervous system. *Tissue Barriers* **2014**, *2*, e944449. [CrossRef]

2.4 Human endogenous retroviruses: ammunition for myeloid cells in neurodegenerative diseases?

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PERSPECTIVE

Human endogenous retroviruses: ammunition for myeloid cells in neurodegenerative diseases?

Activation of myeloid cells by human endogenous retroviral entities: While the exact causes of neurological diseases such as multiple sclerosis (MS) or amyotrophic lateral sclerosis (ALS) are still elusive, there is evidence of a new category of pathogenic elements called human endogenous retroviruses (HERVs) which seem to contribute to their evolution and progression by exerting inflammatory and degenerative effects (Küry et al., 2018). HERVs are ancient retroviral elements which account for up to 8% of the human genome and it is known that environmental factors can trigger their (re-)expression (Küry et al., 2018). The resulting production of viral particles and/or proteins, especially from members of the HERV-W and HERV-K family, is strongly correlated with the onset and progression of neurological diseases, such as MS and ALS (Küry et al., 2018).

In ALS, HERV-K expression is mainly found in neurons (Li et al., 2015), whereas in MS increased HERV-W RNA and protein levels have been confirmed for microglia and macrophages (Mameli et al., 2007). In this context, a correlation between induced gliotoxicity, reverse transcriptase activity and HERV-W expression in macrophage cell culture supernatants derived from MS patients had previously been documented (Menard et al., 1997). Subsequent studies were able to detect HERV-W envelope (ENV) protein expression in myeloid cells (i.e., microglia and macrophages) in areas of active demyelination as well as at the rims of chronic active lesions (van Horssen et al., 2016; Kremer et al., 2019). In contrast, only few HERV-W ENV-positive astroglial and lymphoid cells could be detected (van Horssen et al., 2016). Similar to MS, expression of the multicopy HERV-W family in schizophrenia was detected in peripheral blood mononuclear cells (Perron et al., 2012). Against this backdrop, we will focus this perspective on HERV-mediated effects on myeloid cells.

Myeloid cells are part of the innate immune system and can be divided into different subpopulations. On the one hand, there are monocytes circulating in the bloodstream. On the other hand, macrophages are tissue-localized cells, which can be found in virtually every organ. Both monocytes and macrophages originate from hematopoietic stem cells in the bone marrow. In contrast to that, the tissue-localized myeloid cells of the central nervous system, the so-called microglial cells originate from embryonic yolk sac progenitors.

Early functional studies demonstrated that the HERV-W ENV protein can activate the receptors Toll-like receptor 4 (**Figure 1**) and cluster of differentiation 14 (**Figure 1**) on human monocytes leading to the production of pro-inflammatory cytokines (Rolland et al., 2006). In addition, the ENV protein was shown to stimulate dendritic cells, a related myeloid cell type, to promote T helper cell type 1 differentiation (Rolland et al., 2006). A similar activation of dendritic cells and a triggering role in experimental autoimmune encephalomyelitis in mice was then corroborated in a follow up *in vivo* study (Perron et al., 2013). In addition to that, microglial cells also respond strongly to HERV-W ENV protein exposure. As recently demonstrated, ENV induces the expression of pro-inflammatory cytokines and of nitric oxide in these cells while it reduces anti-inflammatory and neuroprotective parameters (Kremer et al., 2019). Furthermore, ENV protein drives microglial cells to physically interact with myelinated axons and to induce leakage of intra-axonal and myelin proteins. These observations suggest an entirely novel axon damage mechanism, which could explain the tight axonal phenotypes of myeloid cells found in chronic active MS lesions (Kremer et al., 2019). In addition, they also provide a biomedical rationale for the anti-degenerative effects observed in a recent phase 2b clinical trial which tested the HERV-W ENV-neu-

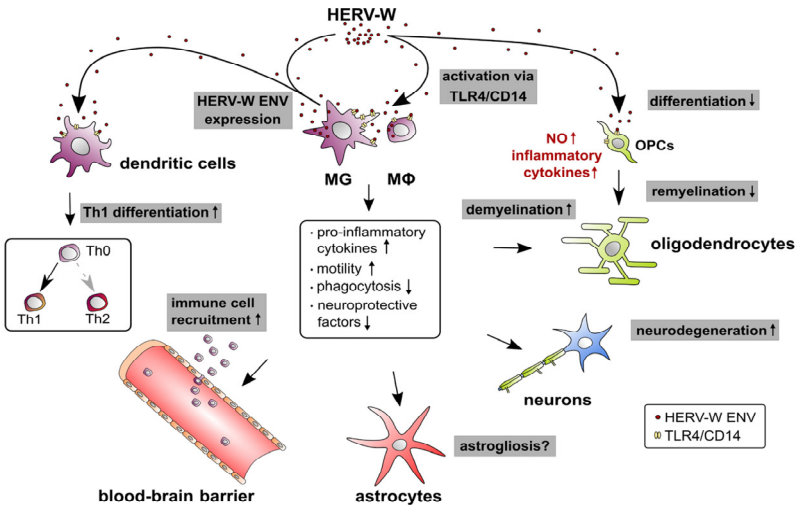

Figure 1 HERV-W mediated effects on myeloid cells.
This illustration summarizes the origin and observed molecular effects of HERV-W on myeloid cells and how it affects neural cells of the central nervous system. Arrow starting points indicate the cellular sources of HERV-W particles or proteins (red dots) while arrowheads point to the influences on different cell types. TLR4/CD14 receptors are marked in yellow. Modulated processes are shown in grey boxes, regulated myeloid molecules and processes are shown in the central panel and regulated molecules in non-myeloid cells are shown in red. Whether microglia and macrophages respond to HERV-W in an auto- and/or paracrine way remains to be shown. CD14: Cluster of differentiation 14; HERV-W: human endogenous retroviruse-W; MG: microglia; M ϕ: macrophage; NO: nitric oxide; OPCs: oligodendroglial progenitor cells; Th: T-helper cell; TLR4: Toll-like receptor 4.

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tralizing antibody temelimab in MS patients (CHANGE-MS, NCT02782858 followed by ANGEL-MS NCT03239860). In light of these recent developments, it is worth mentioning that a previous study already reported increased nitric oxide production in response to ENV overexpression in a microglial cell line and that cell migration was enhanced (Xiao et al., 2017).

In summary, the current available data point to a preferred activation of HERVs in myeloid cells such as monocytes/macrophages and microglial cells leading to endothelial and oligodendroglial stress reactions which may contribute to disease pathology and impaired regeneration (Küry et al., 2018). On the other hand, additional autocrine/paracrine effects of HERVs on the polarization and phenotype of myeloid cells have been reported recently. As there are several specialized macrophage populations present at central nervous system interfaces such as the dura mater, the leptomeninges, the perivascular space and the choroid plexus (Kierdorf et al., 2019), it will be of interest to analyze their specific reactions to ENV production or stimulation. All the more, as HERV-W was initially discovered in a leptomeningeal cell line derived from a MS patient (Perron et al., 1989). Successful treatment of neurodegeneration is still an unmet clinical need particularly in progressive MS, so future studies are required to better understand associated myeloid phenotypes and their functional roles. Ultimately, from a therapeutic standpoint the goal is to identify new means to modulate and control HERV activation and expression.

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References

Kierdorf K, Masuda T, Jordao MJC, Prinz M (2019) Macrophages at CNS interfaces: ontogeny and function in health and disease. Nat Rev Neurosci 20:547-562.

Kremer D, Gruchot J, Weyers V, Oldemeier L, Göttle P, Healy L, Ho Jang J, Kang TXY, Volsko C, Dutta R, Trapp BD, Perron H, Hartung HP, Küry P (2019) pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. Proc Natl Acad Sci U S A 116:15216-15225.

Küry P, Nath A, Creange A, Dolei A, Marche P, Gold J, Giovannoni G, Hartung HP, Perron H (2018) Human endogenous retroviruses in neurological diseases. Trends Mol Med 24:379-394.

Li W, Lee MH, Henderson L, Tyagi R, Bachani M, Steiner J, Campanac E, Hoffman DA, von Geldern G, Johnson K, Maric D, Morris HD, Lentz M, Pak K, Mammen A, Ostrow L, Rothstein J, Nath A (2015) Human endogenous retrovirus-K contributes to motor neuron disease. Sci Transl Med 7:307ra153.

Mameli G, Astone V, Arru G, Marconi S, Lovato L, Serra C, Sotgiu S, Bonetti B, Dolei A (2007) Brains and peripheral blood mononuclear cells of multiple sclerosis (MS) patients hyperexpress MS-associated retrovirus/HERV-W endogenous retrovirus, but not Human herpesvirus 6. J Gen Virol 88:264-274.

Menard A, Amouri R, Michel M, Marcel F, Brouillet A, Belliveau J, Geny C, Deforges L, Malcus-Vocanson C, Armstrong M, Lyon-Caen O, Mandrand B, Dobransky T, Rieger F, Perron H (1997) Gliotoxicity, reverse transcriptase activity and retroviral RNA in monocyte/macrophage culture supernatants from patients with multiple sclerosis. FEBS Lett 413:477-485.

Perron H, Geny C, Laurent A, Mouriquand C, Pellat J, Perret J, Seigneurin JM (1989) Leptomeningeal cell line from multiple sclerosis with reverse transcriptase activity and viral particles. Res Virol 140:551-561.

Perron H, Dougier-Reynaud HL, Lomparski C, Popa I, Firouzi R, Bertrand JB, Marusic S, Portoukalian J, Jouvin-Marche E, Villiers CL, Touraine JL, Marche PN (2013) Human endogenous retrovirus protein activates innate immunity and promotes experimental allergic encephalomyelitis in mice. PLoS One 8:e80128.

Perron H, Hamdani N, Faucard R, Lajnef M, Jamain S, Daban-Huard C, Sarrazin S, LeGuen E, Houenou J, Delavest M, Moins-Teisserenc H, Bengoufa D, Yolken R, Madeira A, Garcia-Montojo M, Gehin N, Burgelin I, Ollagnier G, Bernard C, Dumaine A, et al. (2012) Molecular characteristics of Human Endogenous Retrovirus type-W in schizophrenia and bipolar disorder. Transl Psychiatry 2:e201.

Rolland A, Jouvin-Marche E, Viret C, Faure M, Perron H, Marche PN (2006) The envelope protein of a human endogenous retrovirus-W family activates innate immunity through CD14/TLR4 and promotes Th1-like responses. J Immunol 176:7636-7644.

van Horssen J, van der Pol S, Nijland P, Amor S, Perron H (2016) Human endogenous retrovirus W in brain lesions: Rationale for targeted therapy in multiple sclerosis. Mult Scler Relat Disord 8:11-18.

Xiao R, Li S, Cao Q, Wang X, Yan Q, Tu X, Zhu Y, Zhu F (2017) Human endogenous retrovirus W env increases nitric oxide production and enhances the migration ability of microglia by regulating the expression of inducible nitric oxide synthase. Virol Sin 32:216-225.

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2.5 Translational value of choroid plexus imaging for tracking neuroinflammation in mice and humans

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Abstract

Neuroinflammation is a pathophysiological hallmark of multiple sclerosis and has a close mechanistic link to neurodegeneration. Although this link is potentially targetable, robust translatable models to reliably quantify and track neuroinflammation in both mice and humans are lacking. The choroid plexus (ChP) plays a pivotal role in regulating the trafficking of immune cells from the brain parenchyma into the cerebrospinal fluid (CSF) and has recently attracted attention as a key structure in the initiation of inflammatory brain responses. In a translational framework, we here address the integrity and multidimensional characteristics of the ChP under inflammatory conditions and question whether ChP volumes could act as an interspecies marker of neuroinflammation that closely interrelates with functional impairment. Therefore, we explore ChP characteristics in neuroinflammation in patients with multiple sclerosis and in two experimental mouse models, cuprizone diet-related demyelination and experimental autoimmune encephalomyelitis. We demonstrate that ChP enlargement-reconstructed from MRI-is highly associated with acute disease activity, both in the studied mouse models and in humans. A close dependency of ChP integrity and molecular signatures of neuroinflammation is shown in the performed transcriptomic analyses. Moreover, pharmacological modulation of the blood-CSF barrier with natalizumab prevents an increase of the ChP volume. ChP enlargement is strongly linked to emerging functional impairment as depicted in the mouse models and in multiple sclerosis patients. Our findings identify ChP characteristics as robust and translatable hallmarks of acute and ongoing neuroinflammatory activity in mice and humans that could serve as a promising interspecies marker for translational and reverse-translational approaches.

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Contribution on experimental design, realization and publication

Planning and realization of animal experiments (CPZ and EAE). Establishment and realization of tissue isolation for transcriptome and histological analysis. Establishment and performance of histological analysis of the diseased choroid plexus.

Translational value of choroid plexus imaging for tracking neuroinflammation in mice and humans

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Neuroinflammation is a pathophysiological hallmark of multiple sclerosis and has a close mechanistic link to neurodegeneration. Although this link is potentially targetable, robust translatable models to reliably quantify and track neuroinflammation in both mice and humans are lacking. The choroid plexus (ChP) plays a pivotal role in regulating the trafficking of immune cells from the brain parenchyma into the cerebrospinal fluid (CSF) and has recently attracted attention as a key structure in the initiation of inflammatory brain responses. In a translational framework, we here address the integrity and multidimensional characteristics of the ChP under inflammatory conditions and question whether ChP volumes could act as an interspecies marker of neuroinflammation that closely interrelates with functional impairment. Therefore, we explore ChP characteristics in neuroinflammation in patients with multiple sclerosis and in two experimental mouse models, cuprizone diet-related demyelination and experimental autoimmune encephalomyelitis. We demonstrate that ChP enlargement—reconstructed from MRI—is highly associated with acute disease activity, both in the studied mouse models and in humans. A close dependency of ChP integrity and molecular signatures of neuroinflammation is shown in the performed transcriptomic analyses. Moreover, pharmacological modulation of the blood–CSF barrier with natalizumab prevents an increase of the ChP volume. ChP enlargement is strongly linked to emerging functional impairment as depicted in the mouse models and in multiple sclerosis patients. Our findings identify ChP characteristics as robust and translatable hallmarks of acute and ongoing neuroinflammatory activity in mice and humans that could serve as a promising interspecies marker for translational and reverse-translational approaches.

multiple sclerosis | choroid plexus | neuroinflammation | disease activity

The choroid plexus (ChP), which extends along the floor of the lateral ventricles and the roof of the third and fourth ventricles (1), is a highly vascularized tissue that represents one key structure between the blood and the cerebrospinal fluid (CSF). The ChP plays an essential role in CSF production and brain waste clearance pathways, including the recently discovered glymphatic transport of CSF along the periarterial spaces (2–4). Additional functions of the ChP include maintenance of the neural parenchyma via secretion of neurotrophic factors and modulation of solute and immune cell trafficking across epithelial cells (5). Moreover, the ChP is involved in neuronal repair and restoration of function via gene expression and regulation of immune cell content (6–8).

Taken together, the blood–CSF barrier (BCSFB) in the ChP and the blood–brain barrier (BBB) regulate the entry of cells and solutes into the central nervous system (CNS). When these barriers and other regulatory mechanisms collapse, immune cells enter the CNS and may initiate neuroinflammatory diseases such as multiple sclerosis (MS), leading to demyelination and neuroaxonal degeneration. In line with this, inflammation and disruption of the ChP tissue architecture was detected in MS (9, 10). In postmortem studies, patients with MS showed an increased number of antigen-presenting cells in ChP stroma, as well as an infiltration of leukocytes from the periphery, a disruption of tight junctions in the ChP epithelium, and an endothelial overexpression of adhesion molecules involved in lymphocyte migration. Thus, the ChP can be considered one of the gateways for the migration of inflammatory cells into the brain, which indicates its structural importance in the pathogenesis of CNS diseases (11). These observations on a microscale potentially disrupt the morphology of the ChP also on a macroscale (12, 13). Despite the evidence available from neuropathological studies, we still lack an in vivo demonstration that the ChP is involved in the inflammatory process characterizing MS.

The cuprizone mouse model is a valuable tool to assess demyelination dynamics, since it induces early oligodendrocyte apoptosis, followed by astroglial and microglial activation and

Significance

Neuroinflammation is a hallmark of multiple sclerosis and is linked to neurodegeneration. This study provides pathophysiological insights into the cross-dependency between neuroinflammation and choroid plexus characteristics in both mice and humans. Our work relates an enlargement of choroid plexus volume to ongoing neuroinflammation and emerging clinical disability in two large cohorts of multiple sclerosis patients as well as in two mouse models, the cuprizone diet-related demyelination and the experimental autoimmune encephalomyelitis. Choroid plexus characterization as measured by high-resolution MRI thus represents a reliable and translatable interspecies marker for the quantification of neuroinflammation and disease trajectories that is strongly associated with functional outcomes.

Author contributions: G.G.-E., S.G.M., and S.G. designed research; V.F., G.G.-E., D.C., P.A., J.G., M.D., C.H., T.M., S.B., A.R., M.C., J.K., L.W., C.F., H.L., T.R., M.M., and S.G. performed research; V.F., G.G.-E., D.C., P.A., P.K., M.O., T.R., and M.M. analyzed data; and V.F. and G.G.-E. wrote the paper.

The authors declare no competing interest.

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demyelination (14, 15). Although minor damage to the BBB has been described in this model (16), T and B cells are considered to play only a minor role during cuprizone-induced demyelination (17, 18). Moreover, immunohistochemistry has identified the presence of neuroinflammatory processes with anatomical affinity, particularly in the lining of the ChP (19). Thus, with the cuprizone model, two main aspects related to the MS pathology can be investigated: first, mechanisms underlying innate immune cell-driven myelin and axonal degeneration, and second, remyelination of the demyelinated axons (18).

On the other hand, experimental autoimmune encephalomyelitis (EAE)—the classic animal model of MS—is characterized by T cell and monocyte infiltration in the CNS associated with local inflammation and primary demyelination. In EAE, effector T cells enter the CSF from the leptomeninges or the ChP across activated vessels before spreading to the CNS tissues (20–23), suggesting that the ChP may be one of the initial sites of T cell entrance into the brain. In fact, it has been shown that the very late antigen-4 (VLA-4) and the melanoma cell adhesion molecule (MCAM) are important for transendothelial migration of T helper (Th)17 cells. VLA-4 plays a key role by mediating the initial rolling and adhesion steps of transmigration through interaction with its receptor, vascular cell adhesion molecule-1 (VCAM-1), expressed on endothelial cells upon inflammation (24). Targeting VLA-4 restricts most encephalitogenic T cells from migrating into the CNS (25), while blockade of MCAM more specifically inhibits Th17 cell migration into the CNS via the ChP endothelium (26).

Studies in mouse models mimicking aging in humans have provided further evidence that the ChP is involved in age-related immune cell recruitment, glial activation, and cognitive functioning (27, 28). These findings emphasize the impact of immune homeostasis within the ChP in shaping the brain's structural integrity and the clinical phenotype. However, while our understanding of the role of the ChP in brain homeostasis is growing, much less is known about its role in MS despite the intersection between ChP function and the neuroimmune axis. We hypothesized that inflammation through immune cell infiltration leads to an altered volume of the ChP and that MRI-derived ChP assessment provides a structural interspecies biomarker of inflammatory disease activity. Hence, this study aimed to investigate how ChP morphology is linked to disease activity in humans with MS and two experimental mouse models of CNS demyelination.

In this translational study, we identified an association between the ChP volumes as derived from high-field MRI, with clinical disability in a large cohort of MS patients over 3 y of annual follow-up. We validated our findings in a second cohort of treatment-naive MS patients at disease onset and after 4 y. Ultimately, we investigated the relationship between ChP volumes and CSF markers of BBB dysfunction and the effects of pharmacological inhibition of immune cell migration into the CNS on ChP volume changes. Moreover, we acquired MRI in the cuprizone-diet mouse model to determine whether ChP morphometry is related to demyelination and remyelination processes, depicting MS-like pathophysiology. Likewise, imaging in the EAE model served to evaluate the relationship of ChP morphology and functional impairment in mice. To evaluate the possible molecular mechanisms underlying ChP alterations, we further conducted immunohistochemistry and transcriptomic analyses from ChP tissue on both EAE and cuprizone models, which allow us to bridge the gap between ChP integrity and neuroinflammation.

Results

ChP Enlargement Is Associated with Disease Activity in MS. Bilateral ChP volumes were found to be enlarged in 330 MS patients when compared to healthy controls (HC) (Fig. 1*A* and *SI Appendix*, Figs. S1 and S2; n (HC) = 57; t = 7, $P < 0.001$). Larger ChP in MS patients were associated with the severity of clinical disability (assessed via Expanded Disability Status Scale [EDSS] scores) after adjusting for sex, age, disease duration, and intracranial volume (Fig. 2*A*) at baseline (standardized beta [β] = 0.170; $P = 0.004$) and after 4 y (β = 0.210; $P < 0.001$). These associations were also seen during the yearly follow-ups in between (*SI Appendix*, Fig. S3*A*). In addition, enlarged ChP volume was related with cortical thinning (Fig. 3*C*; $r = -0.328$, $P < 0.001$). Decreased cognitive performance measured with the Symbol Digit Modalities Test (SDMT), recognized as being particularly sensitive to the slowed processing of information that is commonly seen in MS, was also associated with ChP enlargement (Fig. 3*F*; $r = -0.375$; $P < 0.001$) after adjusting for sex, age, and intracranial volume.

Within the main MS cohort, a further comparison between patients with no evidence of disease activity (NEDA) and patients with disease activity (EDA) over a period of 2 y was conducted. Here, patients with EDA displayed larger ChP volumes at baseline compared to patients with NEDA (Mann–Whitney U test; $P < 0.001$). In comparison with healthy controls, both MS patients with NEDA and EDA showed significantly larger ChP volumes (Fig. 3*A*). Based on the hypothesis that ChP enlargement may mirror neuroinflammatory activity, we also assessed ChP morphometry with regard to demyelinating lesion load (high versus low T2-weighted hyperintense lesion volumes based on the median value). Our results demonstrated enlarged ChP volumes in patients with higher lesion load, while preserved ChP morphometry was observed in patients with a low degree of hyperintense lesions on T2-weighted images (Fig. 3*B*; Mann–Whitney U test; $P < 0.001$).

Finally, within the statistical framework, we applied structural equation modeling (SEM) to assess whether MRI-derived volumetric measurements of ChP represent a better biomarker related to EDSS scores in comparison to T1 contrast-enhancing lesions or new or enlarging T2 lesions. SEM identified the ChP volume as the strongest prognostic factor for EDSS scores (standardized coefficient [s] = 0.71, $P = 0.0005$). T1 contrast-enhancing lesions (s = 0.45, $P = 0.357$), as well as new or enlarging T2 lesions (s = 0.35, $P = 0.245$), did not reach significance in the designed predictive model.

ChP Enlargement in MS Is Confirmed in an Independent Validation Cohort. In the replication cohort of 235 treatment-naive MS patients at disease onset, enlarged ChP volume was concordantly associated with increased clinical disability after adjusting for sex, age, disease duration, and intracranial volume at baseline (β = 0.157; $P = 0.020$) and 4-y follow-up ($r = 0.289$, $P = 0.007$) (Fig. 2*B*). These positive associations were also seen after the 1- and 2-y follow-ups (*SI Appendix*, Fig. S3*B*). Consistently, as within the main cohort, in these patients, ChP enlargement was inversely associated with cortical thinning (Fig. 3*D*; $r = -0.277$, $P = 0.011$) after adjusting for sex, age, and intracranial volume.

ChP Enlargement Associates with Albumin in the CSF. Breakdown of the BBB is commonly seen in MS, yet it is not specific to the disease. Given the high concentration of albumin in plasma, it readily passes from the circulation into the CNS during BBB disruption and, thus, the albumin quotient (plasma to CSF) is used as an indicator of BBB dysfunction. Associations between enlarged ChP volumes were attested (*SI Appendix*, Table S3) with CSF albumin (Fig. 3*E*; $r = 0.281$, $P = 0.018$) and albumin quotient ($r = 0.292$, $P = 0.014$) but not for serum albumin ($r = 0.053$, $P = 0.662$). Notably, no associations between ChP volume and lymphocyte subsets (CD4+, CD8+, CD19+, and CD56+ cells) in the peripheral blood were found in patients (*SI Appendix*, Fig. S5 *A–D* and Table S4).

High-Efficacy Treatment Prevents ChP Enlargement. Untreated MS patients had larger ChP volumes at follow-up compared to baseline (*SI Appendix*, Fig. S4; $t = 0.011$, $P = 0.011$). MS patients under dimethyl fumarate (DMF) treatment had larger ChP volumes at follow-up compared to baseline (*SI Appendix*, Fig. S4; $t = 2.74$, $P = 0.0078$),

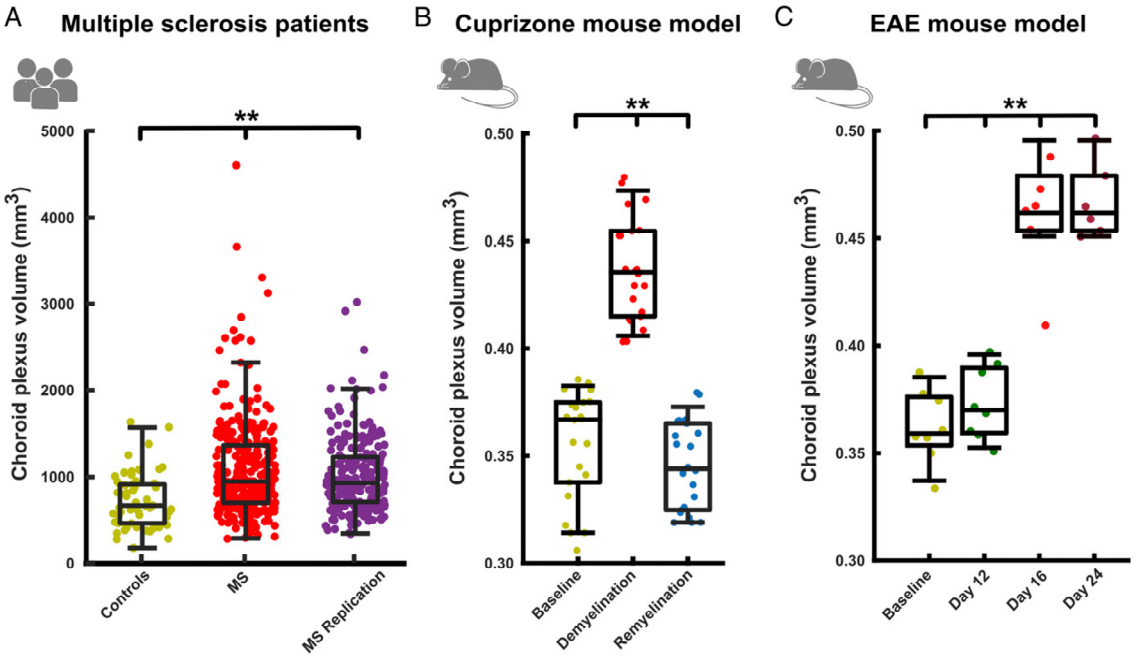

Fig. 1. Volumetric differences in the choroid plexus in human and mouse models. Box plots depicting choroid plexus volumes as derived from individual MRI. (*A*) In humans, enlarged volumes in MS patients with respect to HCs are evidenced. In mice, choroid plexus volumes showed a similar enlargement during the course of (*B*) the cuprizone-induced demyelination and (*C*) with increased disability during the course of the EAE mouse model. $^{**}P < 0.01$.

whereas patients under natalizumab (NAT) therapy showed stable ChP volumes at follow-up (*SI Appendix*, Fig. S4; $t = 1.26$, $P > 0.05$).

Cuprizone Mouse Model: ChP Volume Changes under De- and Remyelination. In our translational approach, we utilized a preclinical mouse model allowing the evaluation of demyelination and remyelination, which served to verify the volumetric changes of the ChP and its functional correlates (*SI Appendix*, Figs. S1 and S2). This animal model served to study consequences of myelin loss under transient pathological conditions. The comparison of the ChP volumes between untreated mice at baseline and after 2 wk of cuprizone diet depicted larger ChP volumes at the maximum of demyelination (Fig. 1*B*; $t = 4.578$, $P = 0.00023$). Subsequently, when myelin synthesis was reenabled by reintroduction of normal food after 6 wk, the mice presented significantly decreased ChP volume in comparison to the demyelination phase ($t = -3.567$, $P = 0.0048$). ChP morphology after remyelination induction was similar to that of the initially untreated period ($t = 1.897$, $P = 0.46$).

The open field (OF) test was applied in order to evaluate basal exploratory behavior via the distance traveled. Animals tested in the OF at baseline showed no significant correlation between the corresponding ChP volume and the distance traveled (*SI Appendix*, Fig. S6*A*; $r = 0.402$, $P = 0.063$). After 6 wk of cuprizone diet—at the peak of induced demyelination—the animals were measured again, and we observed a significant positive association between distance traveled and the ChP volume (*SI Appendix*, Fig. S6*B*; $r = 0.497$, $P = 0.018$). After diet withdrawal, the animals were again rescanned, and the traveled distance was measured. There was still a significant association between the anxiety-like behavior and the plexus volume at this time point (*SI Appendix*, Fig. S6*C*; $r = 0.652$, $P < 0.001$).

EAE Mouse Model: ChP Volume Changes under Autoimmune Inflammation. In addition to the intoxication model with cuprizone-induced demyelination, we employed the classic inflammatory EAE model to confirm our findings from the MS patient cohort in a second mouse model (*SI Appendix*, Fig. S1). Here, the administration of myelin-derived peptides causes an immune reaction against specific antigenic myelin proteins. In the EAE model, the peak of demyelination was reached 10 to 15 d after injection and was primarily confined to the spinal cord, although a certain degree of demyelination was also detected in the optic nerve, cerebral cortex, and cerebellum. Mice were scanned at baseline, day 12, day 16, and day 24, and ChP volumes were determined (Fig. 1*C*). ChP volume was significantly larger at day 24 ($t = 5.9$; $P = 0.00078$), day 16 ($t = 6.8$; $P = 0.00045$), and day 12 ($t = 3.4$; $P = 0.038$) in comparison to baseline.

We linked the ChP volume with the functional impairment in mice measured with the EAE score, a measure of disease severity characterizing motor deficits. We found positive associations between ChP morphology and the EAE severity scores at day 12 (*SI Appendix*, Fig. S6*D*; $r = 0.682$, $P = 0.023$), day 16 (*SI Appendix*, Fig. S6*E*; $r = 0.591$, $P = 0.042$), and day 24 (*SI Appendix*, Fig. S6*F*; $r = 0.664$, $P = 0.031$).

Enhanced Microglia Activation in the ChP at the Peak of the Disease in Both Mouse Models. In the cuprizone-diet mice (Fig. 4 *A–D*), the immunohistochemistry on ChP sections showed a significantly higher number of Iba1+ and Clec7a+ cells, as markers of microglial infiltration and activation, at the peak of demyelination in comparison to untreated mice ($P < 0.001$). A total of 4 wk after cuprizone diet withdrawal, the number of Iba1+ cells was comparable to naïve levels. Similarly, the number of CD3+ cells was significantly higher at the peak of demyelination ($P < 0.01$) but comparable to naïve mice 4 wk after diet withdrawal.

In EAE mice (Fig. 4 *E–H*), we found that the number of ChP Iba1+ cells was significantly higher at the peak of disease at day 14

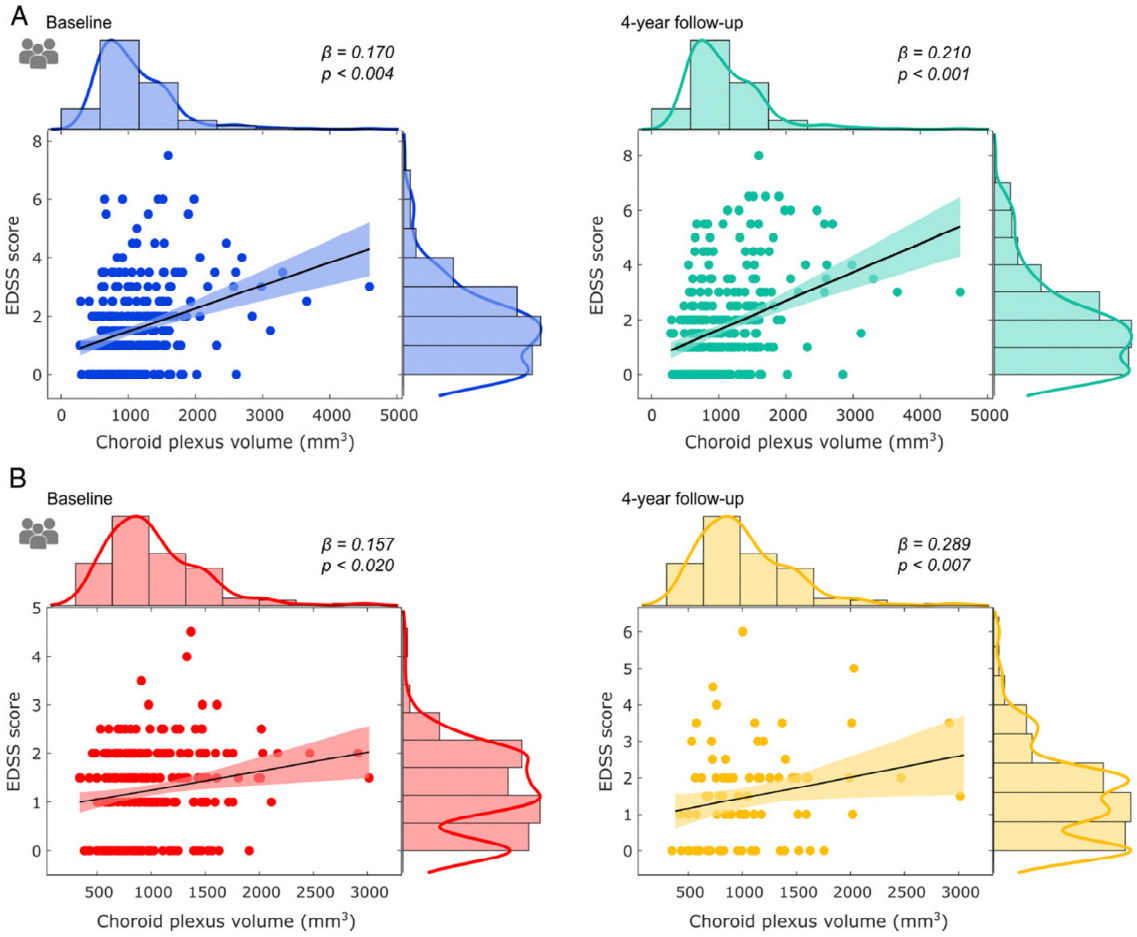

Fig. 2. Associations between clinical disability and choroid plexus enlargement in MS patients. Scatter plots depicting the association between choroid plexus volume and clinical disability, as measured with EDSS, in the study cohort (*A*), at baseline (blue) and at 4-y follow-up (green). In addition, the scatter plots derived from (*B*) the replication cohort are provided for baseline (red) and 4-y follow-up (yellow).

($P < 0.01$) and in the remission phase at day 21 ($P < 0.05$) after immunization in comparison to the naïve mice. In line with this, we observed a higher number of CD3+ cells in comparison to naïve mice 14 ($P < 0.01$) and 21 ($P < 0.05$) d after immunization.

ChP Volumes Relate to Glial Activity. Through immunohistochemistry, we observed positive associations between the number of cortical glial fibrillary acidic protein (GFAP+) positive cells, which is a specific marker for astrocyte content, and the ChP volume at baseline (Fig. 5*A*; $r = 0.560$, $P < 0.001$), full demyelination (Fig. 5*B*; $r = 0.630$, $P < 0.001$), and full remyelination (Fig. 5*C*; $r = 0.570$, $P < 0.001$). This observation supports the link between astrocyte activity and MRI-measured microstructural integrity in the gray matter regions. In addition, we were able to link the ChP volume to the ionized calcium-binding adapter molecule 1 (Iba1), a marker of microglia. At baseline, the association between ChP volume and Iba1 showed only a trend toward significance (Fig. 5*D*; $r = 0.380$, $P = 0.080$), whereas we found significant associations during full demyelination (Fig. 5*E*; $r = 0.600$, $P < 0.001$) and full remyelination (Fig. 5*F*; $r = 0.560$, $P < 0.001$).

ChP Transcriptomics Depict Neuroinflammation. The transcriptomic analyses from isolated ChP at peak of disease (cuprizone demyclination and EAE) showed that in comparison to basclinc, gcnc expression in the ChP of both models revealed a shared signature of differentially up-regulated functional pathways primary related to neuroinflammation and cell-to-cell interactions, particularly a gene signature for genes associated with the regulation of leukocyte (T cell) adhesion, differentiation, and activation (Fig. 6).

Discussion

Over the last years, high-field structural MRI has become a powerful tool in deciphering and predicting specific brain pathology patterns (29, 30) and disease courses in inflammatory brain diseases such as MS (31). In particular, advances in brain imaging acquisition and postprocessing have improved the field and made tremendous contributions to our understanding of evolving neuroinflammation and neurodegeneration (32). The strength of MRI comes from its ability to provide quantifiable markers with high spatial accuracy that can trace disease trajectories both for research and clinical studies, focusing on both focal and widespread damage in the CNS (33).

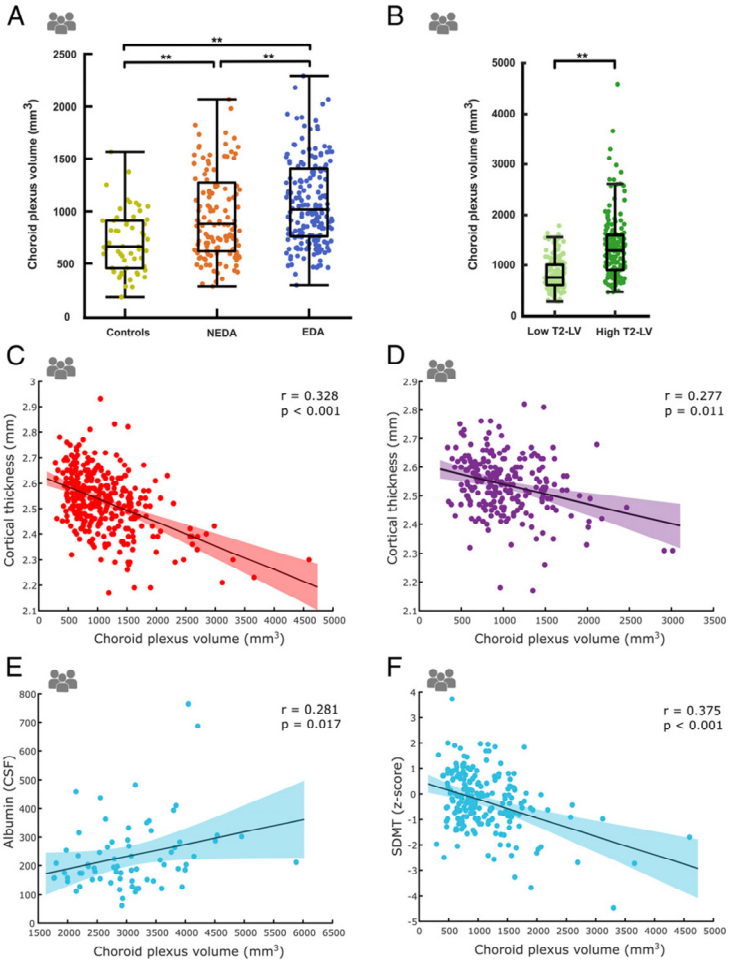

Fig. 3. Volumetric differences and associations of the choroid plexus with clinical, MRI, CSF, and cognitive measures. Choroid plexus volumes, derived from individual MRI in humans, depicting enlarged volumes in MS patients with EDA in comparison to MS patients with NEDA, each in comparison to healthy individuals' choroid plexus volume (*A*). Choroid plexus volumes depicting enlarged volumes in MS patients with high T2 lesion load in the MRI in comparison to patients with low T2 lesion load (*B*). Scatter plots depicting the association between choroid plexus volume and cortical thickness in the study cohort (C) and the replication cohort (*D*). Scatter plots depicting the association between choroid plexus volume and albumin in the CSF (*E*). Scatter plots depicting the association between choroid plexus volume and cognitive performance in the study cohort, as measured with the Symbol Digit Modalities Test (*F*). $^{**}P < 0.01$.

The current study identified an early association between enlargement of the ChP and disease severity in two large cohorts of MS patients, including both treatment-naïve patients and patients under immunomodulatory treatment. Predictive causal modeling through SEM confirmed that the ChP volumes were not only significant determinants that influence EDSS development in MS over time but also outperform conventional MRI biomarkers like T1 contrast-enhancing lesions and new T2 lesions. The translational analyses from two experimental in vivo animal models of acute neuroinflammation and de- and remyelination allow the establishment of volumetric ChP measurements as structural markers of neuroinflammation in mice. Thus, in both species, humans and mice, larger ChP volumes represent reproducible imaging surrogates of functional impairment, thus, suggesting that ChP can be used as a target in studies evaluating disease progression or therapy outcomes.

The underlying mechanisms leading to ChP enlargement with neuroinflammation appear to be complex and related to the interaction between the peripheral immune system and ChP stroma and epithelium cells. As shown in postmortem brain studies, the ChP of MS patients is characterized by a high proportion of MHC class II receptor T lymphocytes, which are indicative of active antigen presentation (10) by granulocyte and CD8+ T cell infiltration in the ChP stroma (34), and by overexpression of lymphocyte adhesion molecules by ChP endothelial cells (9). The presence of high levels of CD68+ activated macrophages in the ChP stroma and increased expression of VCAM-1 in the ChP vasculature (10) suggest an induction of endothelial immune proliferation and migration that might contribute to ChP enlargement. Accordingly, transcriptomic analyses on both EAE and cuprizone-diet models showed the involvement of up-regulated functional pathways involving T cell adhesion, differentiation, and activation as well as

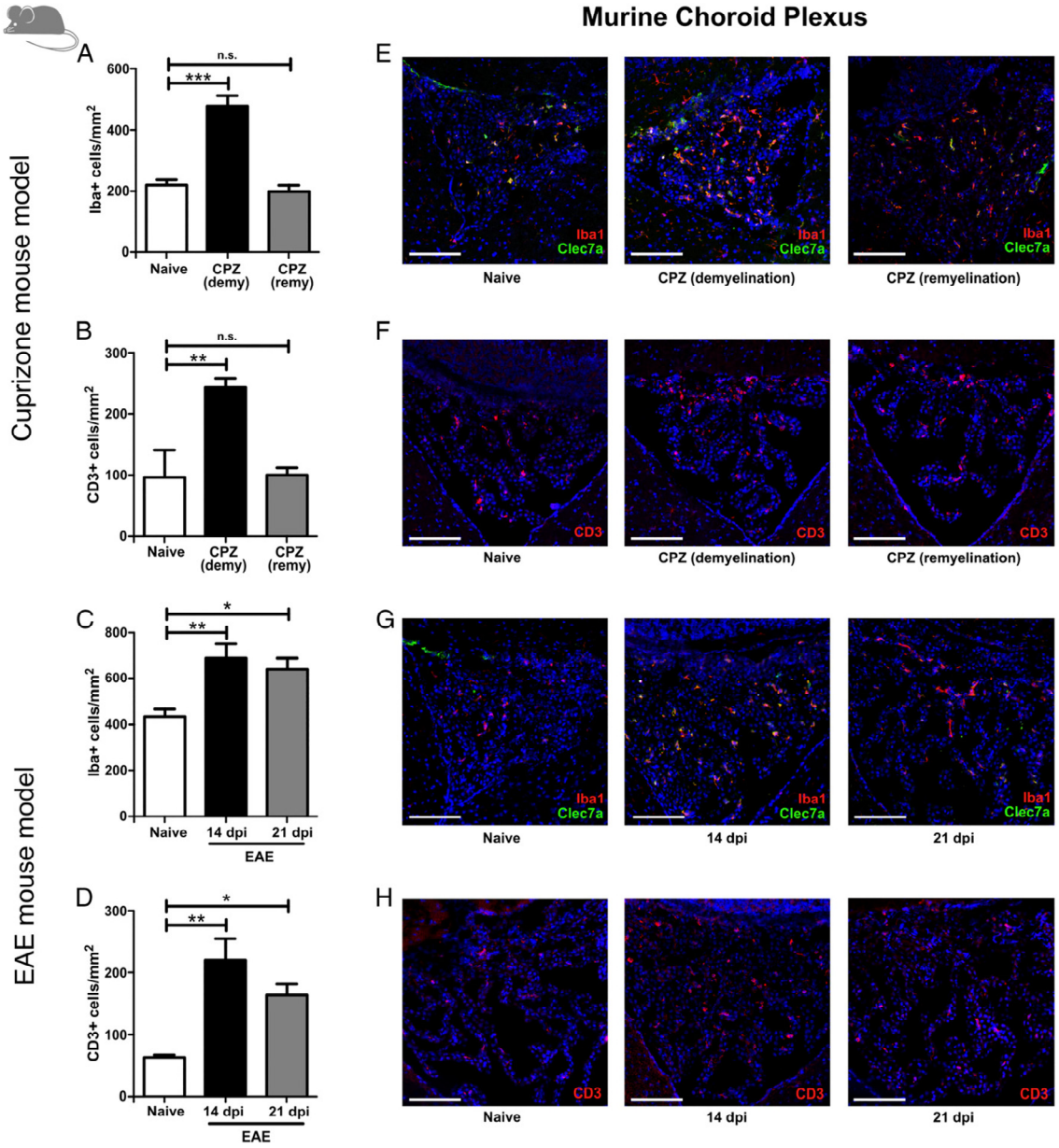

Fig. 4. Increased numbers of activated microglia and infiltrating T cells in the murine choroid plexus. Numbers of Iba1+ and CD3+ cells within the choroid plexus at different stages of the (*A* and *B*) cuprizone and (*C* and *D*) EAE mouse models were analyzed by immunohistochemistry. Representative images of the fluorescent stainings of the (*E* and *F*) cuprizone mice and (*G* and *H*) EAE mice depicting Iba1 (red)/Clec7a (green) positive activated microglia as well as CD3 (red) positive T cells. Nuclei were always counterstained with DAPI (blue). Statistical analysis was performed using ANOVA with Dunnett's post hoc test compared to naïve mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (Scale bar: 100 μm.)

negative regulation of neuroimmune system during demyelination, in which VCAM-1 had a leading role, providing a direct link between enlarged ChP and neuroinflammation.

In the cuprizone model, ChP volume increase occurred during the demyelination phase as a transient enlargement; the ChP volume returned to baseline values after cuprizone diet withdrawal (remyelination). Although the cuprizone model does not require leukocyte migration into the CNS for the induction of demyelination (35, 36), the immune response to demyelination triggers the activation of CNS-resident cells (astrocytes and microglia) and the transmigration through the brain barriers (BBB and BCSFB) of peripheral immune cells (macrophages) into the areas of demyelination

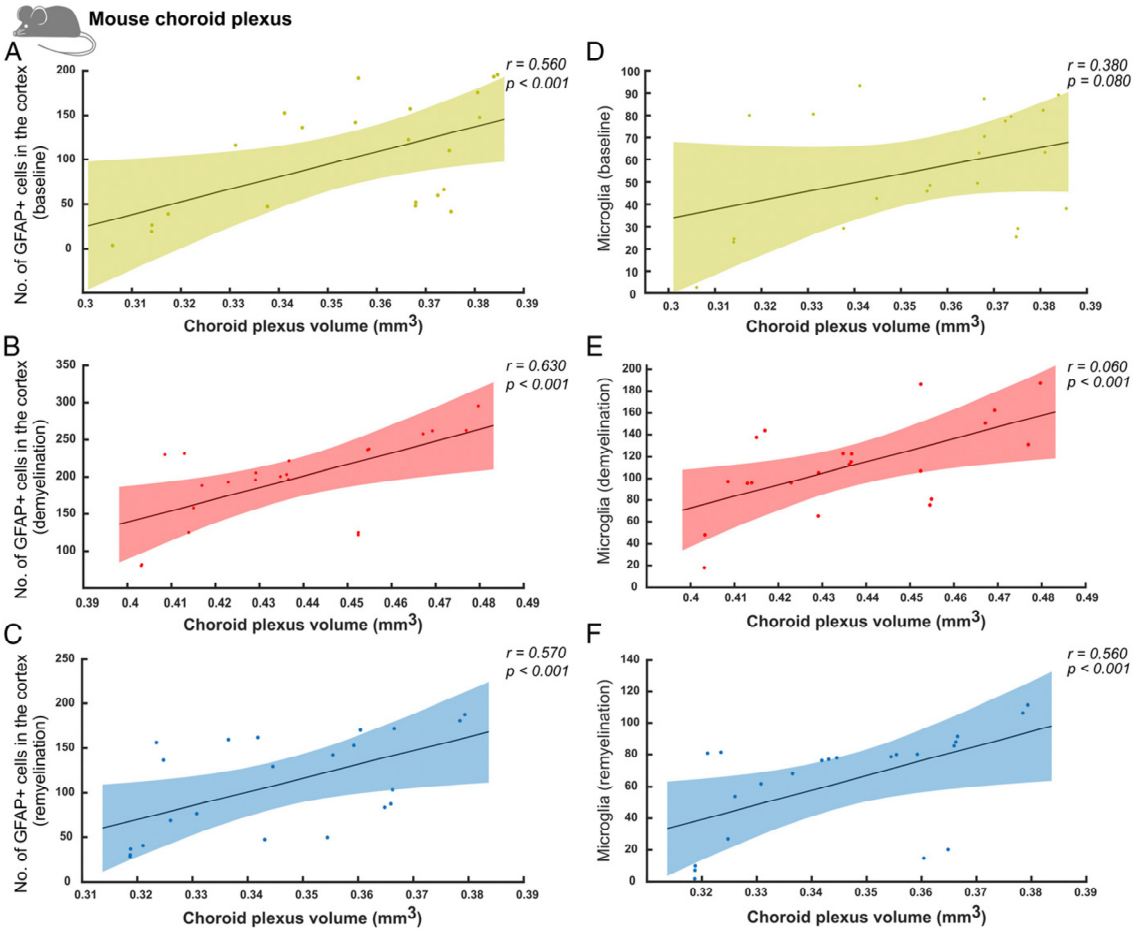

Fig. 5. Associations between choroid plexus volume and astrocyte and microglia counts in relation to the degree of myelination. Scatter plots in the left column illustrate the association between the number of GFAP+ cells in the cortex and the MRI-derived choroid plexus volume at baseline (*A*), demyelination (*B*), and remyelination (C) in the cuprizone mouse model. Right column scatter plots illustrate the association between the number of microglia cells in the cortex and the MRI-derived choroid plexus volume at baseline (*D*), demyelination (*E*), and remyelination (*F*) in the cuprizone mouse model.

(18, 37). In fact, the BBB permeability is increased at the peak of demyelination in the cuprizone model (16, 36). Astrocytes, which are involved in regulating endothelial function via their end feet (38), create a local inflammatory milieu that likely participates in destabilizing the BCSFB integrity mediated by down-regulation of tight junction proteins (16). Moreover, infiltrating cells from the periphery are not only detected in the cuprizone-induced lesions but also in regions distant from the areas of demyelination, including the ChP, leptomeninges, and perivascular regions (37, 39, 40). Thus, one explanation for ChP enlargement in the cuprizone model could be the secondary infiltration of immune cells from the periphery migrating through the ChP epithelium into the CNS in response to the stimuli derived from the cuprizone-damaged tissue. This hypothesis is in accordance with the up-regulation of genes in the ChP involved in cell adhesion and immune receptor activity in response to neuroinflammation in the cuprizone model.

Another mechanistic view of the depicted transcriptomic results could relate ChP enlargement to microglia activation, oxidative damage, and mitochondrial injury leading to hypoxia as a part of the cascade of events that lead to tissue injury (41, 42). Accordingly, in the cuprizone model, demyelination was evidenced to be accompanied by an overexpression of genes involved in oxidative and adenosine triphosphate (ATP)–dependent metabolic processes related to mitochondrial function. Thus, an overarching mechanism common to both EAE and cuprizone models could be related to mitochondrial energy failure within the ChP, most likely as a consequence of oxidative injury [possibly in the epithelial cells (41)] leading to a barrier dysfunction and ChP enlargement. A coexistence of those mechanisms with an immune response coinitiation, with ChP endothelium recruitment of T lymphocytes (which are normally confined to the CSF) causing an initial wave of Th17 cells (T cells mediating autoimmune demyelination) that enter the ChP stroma, is likely (21).

Furthermore, our results show an association between ChP enlargement and elevated CSF albumin and albumin quotient. ChP epithelial cells have a high cellular specificity for albumin transfer from the blood into the ventricular CSF (43). However, CSF albumin levels are dependent on the rate of albumin influx from distinct sources (e.g., transport from blood to CSF in the ChP, BBB leakage, and potential synthesis within the CNS) (44–46) as well as

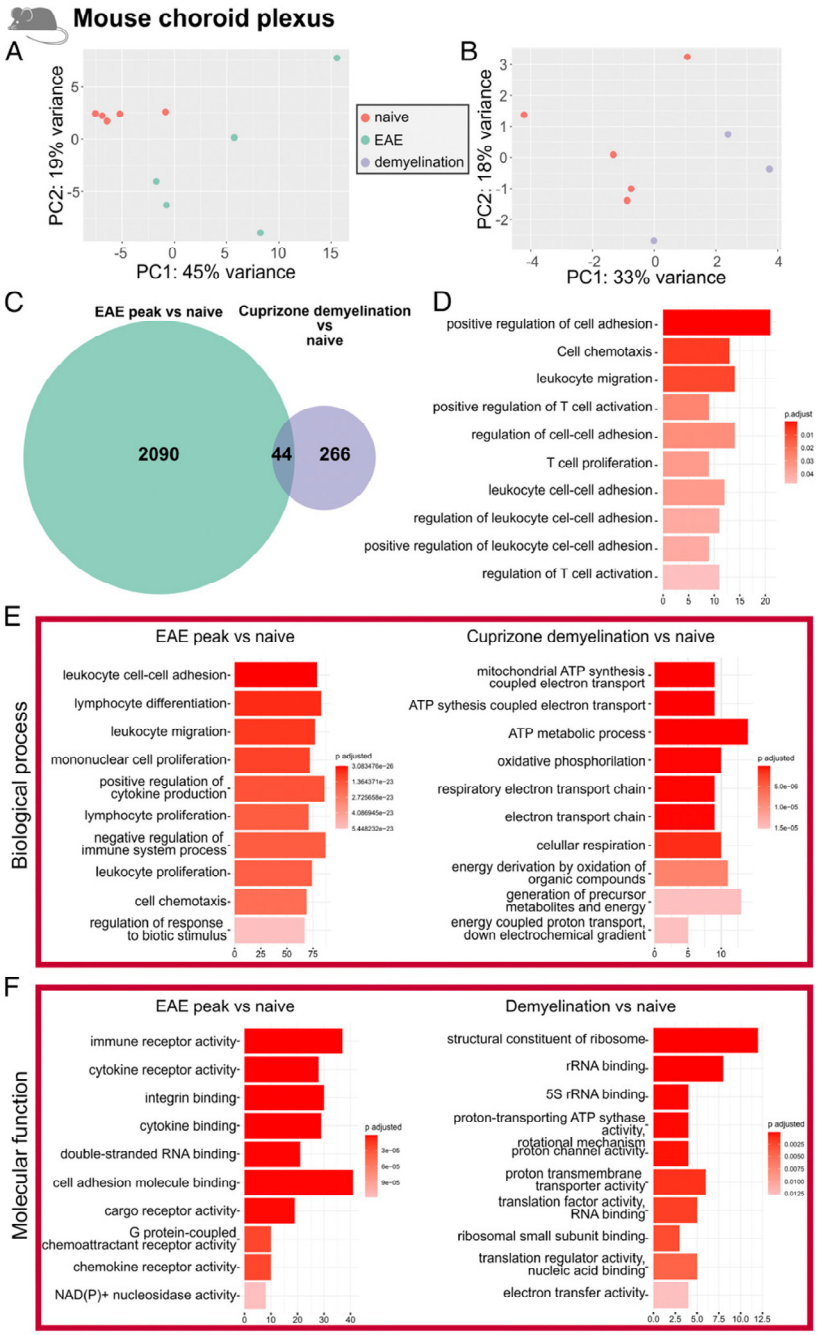

Fig. 6. Choroid plexus transcriptomics from the RNA sequencing. In *A*, the principal component analyses (PCA) show a good separation between the EAE peak ($n = 5$) and the naive EAE group ($n = 5$). The PCA analyses in the cuprizone model (*B*) show a very good cluster separation between the demyelination group ($n = 3$) and the naive group ($n = 5$). In *C*, the Venn diagram shows the differential expressed genes for the comparison between the EAE peak versus naive ($n = 2,090$) and cuprizone-diet induced demyelination versus naive ($n = 266$), and their shared gene expression ($n = 44$). In *D*, the common top 10 up-regulated biological processes pathways both in the EAE peak and the cuprizone demyelination groups are shown. In *E*, the up-regulated biological processes pathways for the two group comparisons, namely peak EAE versus naive and the cuprizone demyelination versus naive are shown, followed by the molecular function pathways in *F*, respectively. The up-regulated functional pathways shown here are all *P* adjusted < 0.05.

the rate of efflux (e.g., turnover of CSF) (47). During neuroinflammation, cellular alterations within the ChP (10) and reduced expression of tight junctions between the epithelial cells (48, 49) increase the permeability of the BCSFB for plasma constituents, including albumin. This influx might enlarge the volume of the cellular components of the ChP due to the interaction of colloid osmotic pressure and hydrostatic pressure on transmembrane water transport. However, changes in albumin transfer through the ChP, BBB leakage, and potentially increased CNS synthesis of albumin by microglia could all affect the CSF albumin concentration, suggesting that different pathways contribute to the observed association of albumin levels with ChP volume.

A recent study using brain tissue samples from patients with progressive MS proposed an important role of the ChP in immune homeostasis and indicated the occurrence of mild inflammatory processes within the ChP (34). The authors suggested that the ChP is only marginally involved in immune cell migration into the CNS in the neurodegeneration-dominated phase of the disease (34). However, we suggest that the early immune permeability of homeostasis response led to CNS tissue injury. In line with our observed relationship between enlarged ChP volumes and disability, previous studies suggested that in the EAE mouse model, inflammation in the ChP and CSF precedes the formation of brain and spinal cord inflammatory infiltrates as well as the development of demyelinating white matter lesions (50, 51). Moreover, the phenomenon that parenchymal CSF circulation is altered in EAE has received increasing attention in light of the recognition of the glymphatic system within the brain (4). The CSF flows from the ChP through the ventricles, enters the brain parenchyma via the perivascular spaces along arteries, and exits along the perivenous spaces to the cervical lymphatics, the arachnoid granulations, and the meningeal lymphatics (2, 52). Recently, an enlargement of ventricle volumes was demonstrated in EAE, which resolved upon clinical remission (53). This study showed how inflammatory processes at the ChP result in expansion of the cerebral ventricles and thus has implications for our findings. The transient ventricle enlargement shown by Millward et al. might be the consequence of an impaired CSF elimination associated with meningeal inflammation. However, these inflammatory processes likely interfere with the normal function of the ChP leading to altered CSF composition or CSF overproduction, as recently demonstrated in inflammation (54) that might drive both ventricle and ChP enlargement. Hence, the increased volume of the ChP could result from the concurrent activation of increased proliferation of ChP cells due to CSF hypersecretion (55), invasion of the ChP by nonresident immune cells (21), and possibly also edema (56).

Whereas in untreated and DMF-treated MS patients the ChP enlarges over time, we did not observe a significant ChP enlargement in NAT-treated MS patients. This finding offers two explanations. First, NAT is highly effective in the suppression of neuroinflammation and revealed a significantly better efficacy in comparison to untreated MS patients (57) as well as to DMF-treated MS patients (58). As NAT reduces inflammation more effectively, ChP volume reaches a plateau and ceases to further increase. Second, and related, the mode of action itself may also be responsible for the observed ChP volume stability in NAT-treated MS patients. NAT blocks the α4-β1 integrin on mononuclear leukocytes and decreases the binding to VCAM-1 (59, 60); thereby, NAT impedes transendothelial migration and the infiltration of leukocytes into the CNS (61). Leukocytes, in turn, have been reported to accumulate in the ChP by passing through the intercellular spaces between the ChP epithelial cells by the paracellular route (62).

Whether the ChP is a more sensitive and earlier marker of MS disease severity than other imaging markers and whether ChP enlargement is a consequence or an immediate cause of MS pathophysiology remains to be clarified. In MS, focal inflammation is known to be associated with widespread white matter pathology and brain topological reorganization (63, 64) as well as gray matter pathology (46, 65). Here, we extend previous observations by focusing on one of the key barriers, the BCSFB, compared to established surrogate markers of neuroinflammation in MS.

This study is not without limitations. First, the delineation of the ChP may not entirely capture its intricate structure. While algorithms for better delineating the ChP based on in vivo MRI data are under development (66), these commonly require complex computations or specialized acquisitions that are not available in clinical settings (67). However, manual delineation is very time consuming, and given the large amount of data, it becomes error prone. For this reason, we decided to employ an accurate automatic ChP parcellation technique, which has been widely utilized (68, 69) and is reliable compared to manual delineation (70). A second limitation is that the neuroinflammatory correlates of ChP were measured from routine peripheral and CSF samples. Despite the high spatial resolution in MRI, other imaging modalities, such as positron emission tomography, can visualize molecular characteristics in real time, and physiological parameters can be quantified in active disease processes within the ChP (71, 72). To overcome this disadvantage of structural MRI, we used a translational approach, which included cellular and imaging characterization allowing a direct comparison and replication of the presented findings.

Overall, this study provides evidence that from the early disease stages, volumetric alterations in the ChP occur in response to neuroinflammatory processes. Furthermore, enlarged ChP volume in MS patients is related to disease severity. This supports its crucial role in the regulation of the neuroimmune axis, which is related to brain homeostasis and interaction with the peripheral immune and inflammatory systems. The identification of enlarged ChP is a promising marker for an improved understanding and monitoring of disease pathology in MS. Larger volumes of the ChP can assist to identify patients at high risk for increased disease activity, who may benefit from early treatment.

Methods and Materials

Participants. Patients included in the main analyses and HCs were recruited in the Department of Neurology at the University Medical Center of the Johannes Gutenberg University Mainz in Germany. Patients with an initial diagnosis of either clinically isolated syndrome (CIS) (73) or relapsing-remitting MS (RRMS, according to 2010 McDonald criteria) (74) were prospectively recruited. After satisfying the study's inclusion criteria (75), patients were comprehensively examined and observed on an annual basis (for a 4-y follow-up period) according to a standardized assessment plan outlined elsewhere (76). Baseline 3T MRI datasets and 4-y clinical follow-up data were available for 330 patients, which were finally included into the analysis.

From these 330 patients, we further evaluated data of 71 patients (mean age ± SD 31.2 ± 9.4 y, 25 males) for whom albumin content in CSF and serum was available, 42 patients on DMF treatment (34.5 ± 9.0 y; 14 males) for whom immune cell subsets (CD4+, CD8+, CD19+, and CD56+ cells) data were available, and 36 patients (30.6 ± 8.1 y; 14 males) on NAT treatment.

Patients in the replication cohort were diagnosed according to the revised McDonald criteria 2010 (74). The following exclusion criteria were applied for all patients: any preexisting medical condition known to be associated with brain pathology; pregnancy; previous or current addiction to substances; relapses or systemic therapy with steroids (intravenous, intrathecal, or oral) within the month before the MRI examination; or a history of additional neurological or psychiatric disorders. Baseline 3T MRI datasets and 4-y clinical follow-up data were available for all 235 patients, which were finally included into the analysis.

The control group of 57 healthy individuals was randomly selected from our database from subjects without a neurological or a systemic immunological disease, who underwent a 1-y MRI assessment. Subjects were 18 y of age or older, in good general health, and were cognitively intact (i.e., able to understand the procedures and requirements and give informed consent).

Clinical Assessment. Each patient was clinically assessed by an experienced neurologist, and the EDSS score was determined at disease onset (study entrance), annually for 2 y, and after 4 y. The SDMT, a paper and pencil task, was used to measure cognitive processing speed (77).

Clinical Characteristics of the Participants. At baseline, within the main cohort of 330 patients (*SI Appendix*, Table S1), 63 (19%) patients were diagnosed with CIS and 267 (81%) patients already had definite RRMS; 232 females (70%)/98 males (30%); mean age 36.1 ± 10.8 y; median EDSS 1.5 (0 to 7.5). The mean disease duration was 38.3 ± 52.6 mo. EDSS scores significantly changed over the 3 y of follow-up ($P < 0.001$; nonparametric Friedman test).

Within the replication cohort (*SI Appendix*, Table S2), which was a treatment-naïve cohort, out of 235 patients (175 females [74%]/60 males [26%]; mean age 33.4 ± 9.6 y; median EDSS 1.5 [0 to 4.5]), 87 (37%) were diagnosed with CIS and 148 (63%) with RRMS. The mean disease duration was 6.5 ± 48.1 mo. The replication cohort included a subgroup of 87 patients with a clinical follow-up of 4 y, in whom the EDSS scores did not significantly change over the 4 y of follow-up ($P = 0.312$; nonparametric Mann–Whitney U test).

MRI Preprocessing and Parcellation. Detailed acquisition parameters are found in *SI Appendix, Supplementary Materials and Methods*. For all participants, automated parcellation of ChP in the lateral ventricles was performed from T1-w images using FreeSurfer (version 6.0; https://surfer.nmr.mgh.harvard.edu/). Technical details of the volume-based subcortical parcellation pipeline in FreeSurfer have been described elsewhere (78, 79). Briefly, a probabilistic atlas, built by manual labeling on a training dataset normalized to the MNI305 space resulting in a point-to-point correspondence between all training subjects, is used as parcellation prior for all brain regions. This atlas provides the probability of each brain region to belong to a given voxel, the probability of each brain region given the classification of neighboring voxels (neighborhood function), and the probability distribution function of voxel intensities, modeled as a normal distribution, for each brain region at each voxel. Then, newly introduced images are parceled by normalizing the new image to the common space and incorporating the subject-specific voxel intensities to find the optimal parcellation that maximizes the probability of observing the input data.

To ensure that the results including ChP volumes are not driven by other confounding factors, we also extracted ventricular volume (by combining left and right lateral ventricles) as well as total intracranial volume. The ventricular volume was further used as independent variable in other analyses.

Following brain ChP and ventricle parcellation by tiling the boundary of white matter mass, an initial white surface is created for each cerebral hemisphere, which is further refined following intensity gradients of the white matter and gray matter to generate the final gray–white surface. This surface is then extended to follow the intensity gradient of gray matter and CSF, leading to the creation of the pial surface. Finally, cortical thickness at each surface vertex is computed as the average distance from each vertex in the gray–white surface to the corresponding point in the pial surface (78).

Flow Cytometry. In the DMF group, lymphocyte subset counts of CD4+, CD8+, CD19+, and CD56+ cells were quantified with flow cytometry. Blood samples were collected from each patient at baseline and after follow-up. Fresh blood samples initially drawn into the ethylenediamine tetraacetic acid–containing tubes were then transferred to 5-mL fluorescence-activated cell sorting tubes and washed twice before erythrocytes were lysed with lysing solution at room temperature (RT). The cells were exposed to corresponding fluorochrome-conjugated monoclonal antibodies against CD4, CD8, CD19, and CD56. The absolute values of lymphocyte subsets were determined with TruCount beads (BD Biosciences).

Animal Models. For the model on demyelination and remyelination, experiments were performed on C57BL6J mice ($N = 10$; females, 9 wk old at the beginning of treatment, Envigo). All efforts were made to minimize stress for the animals in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines (80). Food and water were available ad libitum. Cuprizone [bis(cyclohexylidenehydrazide)] was mixed with rodent pellet chow (0.2%). This compound is toxic for mature oligodendrocytes because it interferes with their internal mitochondrial metabolism and induces full demyelination after 6 wk of diet. Mice were measured at three time points: 1) baseline (before cuprizone diet), 2) after 6 wk of cuprizone diet (model of demyelination), and 3) after 6 wk of normal food reintroduction (model of remyelination). MRI was acquired at the three time points using a 9.4-Tesla small animal scanner with a mouse brain surface coil (Bio-Spec 94/20; Bruker BioSpin MRI GmbH). Mice were first anesthetized in a warmed Plexiglas box with 5% isoflurane (Baxter) in 1 L/min O_2. Isoflurane dosage was reduced to 1 to 1.5% in 1 L/min O_2/compressed air 30/70 vol% for positioning in the animal cradle and subsequent scanning. Stable physiology was controlled by continuous monitoring of body temperature via a rectal temperature probe (36.5 ± 0.5 °C) and by respiration rate (80 to 100 breaths/minute).

For the second animal model, EAE was induced by subcutaneous injection of 200 mg MOG peptide (Myelin Oligodendrocyte Glycoprotein Peptide Fragment 35 to 55; Charité) emulsified in complete Freund's adjuvant (Sigma-Aldrich) containing 200 mg *Mycobacterium tuberculosis* H37RA (Difco). Pertussis toxin (400 ng; Enzo Life Sciences) in 200 mL phosphate-buffered saline (PBS) was injected intraperitoneally at the day of immunization and 2 d later. Disease severity was scored daily in an anonymized fashion by two independent investigators using a scale from 0 to 5 (EAE score) as described elsewhere (81). In the EAE model, the peak of demyelination is reached after 10 to 15 d from the injection, primarily confined to the spinal cord, although a certain degree of demyelination is also detected in the optic nerve, cerebral cortex, and cerebellum. Mice were scanned at baseline, day 12, day 16, and day 24, and ChP volumes were determined with the aid of the Brain Extraction Toolkit.

Tissue Processing and Immunohistochemistry. EAE- and cuprizone-treated animals were deeply anesthetized with isoflurane and transcardially perfused with 20 mL cold PBS followed by 20 mL 4% paraformaldehyde. Brains were then removed and postfixed in the same fixative for 1 d at 4 °C, followed by 24 to 48 h cryoprotective dehydration in 30% sucrose at 4 °C. Afterward, brains were embedded in Tissue-Tek (Sakura Finetek Europe), frozen, and stored at −30 °C until preparation of 12-µm sections using a cryostat (Leica CM30510S). Sections were also stored at −30 °C.

For immunohistochemistry, sections of the ChP were thawed and left to dry for at least 15 min at RT. Before blocking, sections were rehydrated for 5 min in distilled water, transferred to −20 °C acetone (5 min), and washed in 1× Tris-buffered saline (TBS) (pH 7.6) and 1× TBS-T (TBS containing 0.02% Triton) for 5 min each. Blocking was performed with 3% normal goat serum (NGS) and 10% biotin-free bovine serum albumin (BSA; in TBS-T) for 30 min at RT, followed by application of the following antibodies (in 3% NGS and 10% BSA in TBS) and incubation overnight: rabbit anti-Iba1 (Wako Chemicals), rat anti-Clec7a (Dectin1; In Vivogen), and rabbit anti-CD3 (DAKO). Sections were washed two times for 5 min in TBS and incubated with DAPI and the species-appropriate fluorochrome-conjugated secondary antibody (1:500 in PBS) for 30 min at RT: goat anti-rabbit Alexa 594 (Thermo Fisher) and goat anti-rat Alexa 488 (Thermo Fisher). Images were taken using a Zeiss CLSM microscope 510 (CLSM 510, Zeiss); microglial infiltration and activation as well as T cell infiltration was quantified using the ImageJ BioVoxxel software (82) by an investigator anonymized to the experimental groups.

Images of slices containing the neocortex were collected from both hemispheres. A maximum of 11 slices per mouse were analyzed and considered as technical replicates for analysis of the cortex. For GFAP staining, images were acquired using 20- and 40-fold objectives and analyzed by counting the number of diaminobenzidine-positive cells per square millimeter.

Functional Testing in the Cuprizone Mouse Model. Behavioral responses to a novel environment were measured in an OF apparatus at baseline, demyelination, and remyelination. Animals were tested in the OF arena (35 × 40 × 40 cm), where, during the test, the mice were allowed to move freely around and explore the environment. In this study, the distance traveled was taken as a read-out of behavioral abnormalities (83) and expressed as a metric measurement (in centimeters), with longer distances indicating cognitive interference or anxiety-like behavior in the cuprizone diet.

RNA Sequencing and Analysis. Brains from naïve (baseline) as well as from EAE (days 14 and 21) and cuprizone-treated C57/Bl6 mice (peak demyelination and peak remyelination) were used to isolate ChP tissue. ChP tissue was manually dissected from the lateral, third, and fourth ventricles using an illuminated stereo microscope, as described elsewhere (84). Tissue from single mice was then immediately enzymatically digested in 300 µL Hank's balanced salt solution (HBSS) (Gibco; Catalog No. 14025-092) containing collagenase and dispase (Merck; Catalog No. 11097113001; concentration: 0.1 mg/mL) for 30 min at 37 °C on an orbital shaker. Subsequently, tissue was homogenized through a cell strainer (70-µm pore size) by an insulin syringe and washed with 600 µL HBSS solution. The sample was centrifuged at RT at 500 × g for 5 min. Supernatant was discarded, and the cell pellet was resuspended in 350 µL RLT buffer. For RNA isolation, Quiagen RNeasy Micro Kit (Catalog No. 74004) was used according to the manufacturer's instructions. Quality and amount of RNA were verified by NanoDrop and Bioanalyzer RNA 6,000 nano Kit (Agilent). Samples with RNA integrity number values >6.5 were used for RNA sequencing. NEBNext ribosomal RNA depletion was performed followed by NEBNext directional Ultra RNA II Library preparation and sequencing on NextSeq500 (Illumina) platform (75 cycles, high output version 2 kit). Raw sequencing data were filtered with the fastp program (85) to eliminate low-quality reads. Additionally, the parameters -g -x -p have been set for polyG

tail trimming, polyX tail trimming, and overrepresented sequence analysis. The quality of the trimmed data has been assured with fastqc. Afterward, the data were aligned to the latest reference genome (GRCm39 for mouse) using the long-read Spliced Transcript Alignment to a Reference aligner. Low-quality alignments have been filtered out using samtools, and the remaining high-quality alignments have been quantified using StringTie. The statistical analysis of the gene counts and principal component analyses were carried out with DESeq2 in R. The gene ontology analysis has been performed with clusterProfiler. The Venn diagram has been created with VennDiagram. The databases org.Hs.eg.db and org.Mm.eg.db have been used in R for annotating purposes.

Statistics. Statistical analyses were conducted in SPSS (version 23: IBM Corporation). First, the Shapiro–Wilk test was performed for the demographic and clinical variables to assess the distribution normality. Continuous and ordinal variables were compared using Student's *t* test and Mann–Whitney *U* test, respectively. Categorical variables were compared using a Pearson's χ^2 test.

The nonparametric Friedman test was used to explore longitudinal differences between the EDSS at the four time points in the study cohort. The Wilcoxon signed-rank test was used to test for differences between the EDSS at two time points in the replication cohort. Unless otherwise indicated, values are expressed as mean ± SD. ChP volumes were compared with the EDSS scores by multiple linear regression adjusting for age, sex, disease duration, and intracranial volume. Results from the regression model are given with standardized beta coefficients (β) and the corresponding *P* value; *P* values <0.05 were considered statistically significant. Lastly, an SEM was applied on MRI-derived markers (T1 contrast-enhancing lesions and new and/or enlarging T2 lesions) and the ChP volume to determine the marker that best predicted EDSS in our MS cohort (*SI Appendix, Supplementary Materials and Methods*). SEM was adjusted for sample size using the Root Mean Square Error of Approximation index, which improves precision without increasing bias (86).

Study Approval. For human participants, the study was approved by the local medical ethics committee of the State Medical Association (approval number 837.543.11 [8085]). Written informed consent in accordance with the Declaration of Helsinki was obtained from all subjects before participation. For animal experiments, the study was conducted in accordance with guidelines of local German authorities (Landesamt für Natur, Umwelt und Verbraucherschutz identification number: 84-02.04.2015.A585).

Data Availability. The data that support the findings of this study are included in this published article (and its *SI Appendix*), but restrictions apply to the availability of these data, which were used under license for the current study and so are not publicly available. Data are, however, available from the authors upon reasonable request.

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1. D. Talhada *et al.*, The choroid plexus: Simple structure, complex functions. *J. Neurosci. Res.* **98**, 751–753 (2020).
2. A. Louveau *et al.*, Structural and functional features of central nervous system lymphatic vessels. *Nature* **523**, 337–341 (2015).
3. J. Kipnis, Multifaceted interactions between adaptive immunity and the central nervous system. *Science* **353**, 766–771 (2016).
4. J. J. Iliff *et al.*, A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. *Sci. Transl. Med.* **4**, 147ra111 (2012).
5. E. E. Benarroch, Choroid plexus–CSF system: Recent developments and clinical correlations. *Neurology* **86**, 286–296 (2016).
6. K. Baruch, M. Schwartz, CNS-specific T cells shape brain function via the choroid plexus. *Brain Behav. Immun.* **34**, 11–16 (2013).
7. F. Marques *et al.*, The choroid plexus in health and in disease: Dialogues into and out of the brain. *Neurobiol. Dis.* **107**, 32–40 (2017).
8. R. Spector, S. Robert Snodgrass, C. E. Johanson, A balanced view of the cerebrospinal fluid composition and functions: Focus on adult humans. *Exp. Neurol.* **273**, 57–68 (2015).
9. B. Engelhardt, K. Wolburg-Buchholz, H. Wolburg, Involvement of the choroid plexus in central nervous system inflammation. *Microsc. Res. Tech.* **52**, 112–129 (2001).
10. M. Vercellino *et al.*, Involvement of the choroid plexus in multiple sclerosis autoimmune inflammation: A neuropathological study. *J. Neuroimmunol.* **199**, 133–141 (2008).
11. E. H. Wilson, W. Weninger, C. A. Hunter, Trafficking of immune cells in the central nervous system. *J. Clin. Invest.* **120**, 1368–1379 (2010).
12. S. Kant, E. G. Stopa, C. E. Johanson, A. Baird, G. D. Silverberg, Choroid plexus genes for CSF production and brain homeostasis are altered in Alzheimer's disease. *Fluids Barriers CNS* **15**, 34 (2018).
13. E. Perez-Gracia, R. Blanco, M. Carmona, E. Carro, I. Ferrer, Oxidative stress damage and oxidative stress responses in the choroid plexus in Alzheimer's disease. *Acta Neuropathol.* **118**, 497–504 (2009).
14. D. Ciolac, S. A. Groppa, G. Gonzalez-Escamilla, "Translational characterization of the glia role in multiple sclerosis" in *Translational Methods for Multiple Sclerosis Research*, S. G. Meuth, S. Groppa, Eds. (Springer, 2021), pp. 61–76.
15. F. M. Glaser, T. Ruck, "Translational animal models for MS and related neuroimmunological disorders" in *Translational Methods for Multiple Sclerosis Research*, S. G. Meuth, S. Groppa, Eds. (Springer, 2021), pp. 13–27.
16. S. A. Berghoff *et al.*, Blood-brain barrier hyperpermeability precedes demyelination in the cuprizone model. *Acta Neuropathol. Commun.* **5**, 94 (2017).
17. Y. Wolf *et al.*, Microglial MHC class II is dispensable for experimental autoimmune encephalomyelitis and cuprizone-induced demyelination. *Eur. J. Immunol.* **48**, 1308–1318 (2018).
18. J. Zhan *et al.*, The cuprizone model: Dos and do nots. *Cells* **9**, 843 (2020).
19. A. Nack *et al.*, Expression of translocator protein and [18F]-GE180 ligand uptake in multiple sclerosis animal models. *Cells* **8**, 94 (2019).
20. A. Reboldi *et al.*, C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat. Immunol.* **10**, 514–523 (2009).
21. P. Kivisäkk *et al.*, Human cerebrospinal fluid central memory CD4+ T cells: Evidence for trafficking through choroid plexus and meninges via P-selectin. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 8389–8394 (2003).
22. B. Engelhardt, R. M. Ransohoff, The ins and outs of T-lymphocyte trafficking to the CNS: Anatomical sites and molecular mechanisms. *Trends Immunol.* **26**, 485–495 (2005).
23. I. Bartholomäus *et al.*, Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* **462**, 94–98 (2009).
24. O. Steiner *et al.*, Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood-brain barrier endothelium. *J. Immunol.* **185**, 4846–4855 (2010).
25. T. A. Yednock *et al.*, Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* **356**, 63–66 (1992).
26. J. Breuer *et al.*, Blockade of MCAM/CD146 impedes CNS infiltration of T cells over the choroid plexus. *J. Neuroinflammation* **15**, 236 (2018).
27. K. Baruch *et al.*, Aging-induced type I interferon response at the choroid plexus negatively affects brain function. *Science* **346**, 89–93 (2014).
28. K. Baruch *et al.*, CNS-specific immunity at the choroid plexus shifts toward destructive Th2 inflammation in brain aging. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 2264–2269 (2013).
29. M. Calabrese *et al.*, Regional distribution and evolution of gray matter damage in different populations of multiple sclerosis patients. *PLoS One* **10**, e0135428 (2015).
30. M. D. Steenwijk *et al.*, Cortical atrophy patterns in multiple sclerosis are non-random and clinically relevant. *Brain* **139**, 115–126 (2016).
31. J. Krämer *et al.*, Imaging in mice and men: Pathophysiological insights into multiple sclerosis from conventional and advanced MRI techniques. *Prog. Neurobiol.* **182**, 101663 (2019).
32. O. Ciccarelli *et al.*, Pathogenesis of multiple sclerosis: Insights from molecular and metabolic imaging. *Lancet Neurol.* **13**, 807–822 (2014).
33. R. Cortese, S. Collorone, O. Ciccarelli, A. T. Toosy, Advances in brain imaging in multiple sclerosis. *Ther. Adv. Neurol. Disord.* **12**, 1756286419859722 (2019).
34. S. Rodríguez-Lorenzo *et al.*, Inflammation of the choroid plexus in progressive multiple sclerosis: Accumulation of granulocytes and T cells. *Acta Neuropathol. Commun.* **8**, 9 (2020).
35. A. V. Caprariello *et al.*, Biochemically altered myelin triggers autoimmune demyelination. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 5528–5533 (2018).
36. J. Praet, C. Guglielmetti, Z. Berneman, A. Van der Linden, P. Ponsaerts, Cellular and molecular neuropathology of the cuprizone mouse model: Clinical relevance for multiple sclerosis. *Neurosci. Biobehav. Rev.* **47**, 485–505 (2014).
37. V. Yakimov *et al.*, Continuous cuprizone intoxication allows active experimental autoimmune encephalomyelitis induction in C57BL/6 mice. *Histochem. Cell Biol.* **152**, 119–131 (2019).
38. Y. Guo *et al.*, Pathogenic implications of cerebrospinal fluid barrier pathology in neuromyelitis optica. *Acta Neuropathol.* **133**, 597–612 (2017).
39. E. J. McMahon, K. Suzuki, G. K. Matsushima, Peripheral macrophage recruitment in cuprizone-induced CNS demyelination despite an intact blood-brain barrier. *J. Neuroimmunol.* **130**, 32–45 (2002).
40. C. Reinbach *et al.*, CD44 expression in the cuprizone model. *Brain Res.* **1745**, 146950 (2020).
41. G. R. Campbell *et al.*, Clonally expanded mitochondrial DNA deletions within the choroid plexus in multiple sclerosis. *Acta Neuropathol.* **124**, 209–220 (2012).

42. D. H. Mahad, B. D. Trapp, H. Lassmann, Pathological mechanisms in progressive multiple sclerosis. *Lancet Neurol.* **14**, 183–193 (2015).
43. S. A. Liddelow *et al.*, Cellular specificity of the blood-CSF barrier for albumin transfer across the choroid plexus epithelium. *PLoS One* **9**, e106592 (2014).
44. S.-M. Ahn *et al.*, Human microglial cells synthesize albumin in brain. *PLoS One* **3**, e2829 (2008).
45. T. Uher *et al.*, Increased albumin quotient (QAlb) in patients after first clinical event suggestive of multiple sclerosis is associated with development of brain atrophy and greater disability 48 months later. *Mult. Scler.* **22**, 770–781 (2016).
46. J. Kroth *et al.*, Increased cerebrospinal fluid albumin and immunoglobulin A fractions forecast cortical atrophy and longitudinal functional deterioration in relapsing-remitting multiple sclerosis. *Mult. Scler.* **25**, 338–343 (2019).
47. S. M. LeVine, Albumin and multiple sclerosis. *BMC Neurol.* **16**, 47 (2016).
48. H. Wolburg, K. Wolburg-Buchholz, B. Engelhardt, "Involvement of tight junctions during transendothelial migration of mononuclear cells in experimental autoimmune encephalomyelitis" in *Neuroinflammation in Stroke*, U. Dirnagl, B. Elger, Eds. (Ernst Schering Research Foundation Workshop, Springer, 2004), vol. 47, pp. 17–38.
49. G. L. Suidan, J. R. Mcdole, Y. Chen, I. Pirko, A. J. Johnson, Induction of blood brain barrier tight junction protein alterations by CD8 T cells. *PLoS One* **3**, e3037 (2008).
50. D. A. Brown, P. E. Sawchenko, Time course and distribution of inflammatory and neurodegenerative events suggest structural bases for the pathogenesis of experimental autoimmune encephalomyelitis. *J. Comp. Neurol.* **502**, 236–260 (2007).
51. C. Schmitt, N. Strazielle, J. F. Ghersi-Egea, Brain leukocyte infiltration initiated by peripheral inflammation or experimental autoimmune encephalomyelitis occurs through pathways connected to the CSF filled compartments of the forebrain and midbrain. *J. Neuroinflammation* **9**, 187 (2012).
52. J. J. Iliff *et al.*, Brain-wide pathway for waste clearance captured by contrast-enhanced MRI. *J. Clin. Invest.* **123**, 1299–1309 (2013).
53. J. M. Millward *et al.*, Transient enlargement of brain ventricles during relapsing-remitting multiple sclerosis and experimental autoimmune encephalomyelitis. *JCI Insight* **5**, e140040 (2020).
54. J. K. Karimy *et al.*, Inflammation-dependent cerebrospinal fluid hypersecretion by the choroid plexus epithelium in posthemorrhagic hydrocephalus. *Nat. Med.* **23**, 997–1003 (2017).
55. B. Z. Barkho, E. S. Monuki, Proliferation of cultured mouse choroid plexus epithelial cells. *PLoS One* **10**, e0121738 (2015).
56. E. Cardia *et al.*, Morphological modifications of the choroid plexus in a rodent model of acute ventriculitis induced by gram-negative liquoral sepsis. Possible implications in the pathophysiology of hypersecretory hydrocephalus. *Childs Nerv. Syst.* **11**, 511–516 (1995).
57. C. H. Polman *et al.*; AFFIRM Investigators, A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl. J. Med.* **354**, 899–910 (2006).
58. B. L. Vollmer *et al.*, Natalizumab versus fingolimod and dimethyl fumarate in multiple sclerosis treatment. *Ann. Clin. Transl. Neurol.* **6**, 252–262 (2018).
59. A. Mathias *et al.*, Impaired T-cell migration to the CNS under fingolimod and dimethyl fumarate. *Neurol. Neuroimmunol. Neuroinflamm.* **4**, e401 (2017).
60. M. Skarica, C. Eckstein, K. A. Whartenby, P. A. Calabresi, Novel mechanisms of immune modulation of natalizumab in multiple sclerosis patients. *J. Neuroimmunol.* **235**, 70–76 (2011).
61. R. R. Lobb, M. E. Hemler, The pathophysiologic role of alpha 4 integrins in vivo. *J. Clin. Invest.* **94**, 1722–1728 (1994).
62. C. Kaur, G. Rathnasamy, E. A. Ling, The choroid plexus in healthy and diseased brain. *J. Neuropathol. Exp. Neurol.* **75**, 198–213 (2016).
63. V. Fleischer *et al.*, Graph theoretical framework of brain networks in multiple sclerosis: A review of concepts. *Neuroscience* **403**, 35–53 (2019).
64. M. Muthuraman *et al.*, Covarying patterns of white matter lesions and cortical atrophy predict progression in early MS. *Neurol. Neuroimmunol. Neuroinflamm.* **7**, e681 (2020).
65. R. Magliozzi *et al.*, The CSF profile linked to cortical damage predicts multiple sclerosis activity. *Ann. Neurol.* **88**, 562–573 (2020).
66. E. Tadayon *et al.*; Alzheimer's Disease Neuroimaging Initiative, Improving choroid plexus segmentation in the healthy and diseased brain: Relevance for Tau-PET imaging in dementia. *J. Alzheimers Dis.* **74**, 1057–1068 (2020).
67. B. Alicioglu, G. Yilmaz, O. Tosun, N. Bulakbasi, Diffusion-weighted magnetic resonance imaging in the assessment of choroid plexus aging. *Neuroradiol. J.* **30**, 490–495 (2017).
68. H. Murck *et al.*, Ventricular volume, white matter alterations and outcome of major depression and their relationship to endocrine parameters – A pilot study. *World J. Biol. Psychiatry* **22**, 104–118 (2021).
69. G. Zhou, J. Hotta, M. K. Lehtinen, N. Forss, R. Hari, Enlargement of choroid plexus in complex regional pain syndrome. *Sci. Rep.* **5**, 14329 (2015).
70. P. Lizano *et al.*, Association of choroid plexus enlargement with cognitive, inflammatory, and structural phenotypes across the psychosis spectrum. *Am. J. Psychiatry* **176**, 564–572 (2019).
71. C. M. Lee *et al.*, 18F-flortaucipir binding in choroid plexus: Related to race and hippocampus signal. *J. Alzheimers Dis.* **62**, 1691–1702 (2018).
72. S.L. Baker, T.M. Harrison, A. Maass, R. La Joie, W.J. Jagust, Effect of off-target binding on (18)F-flortaucipir variability in healthy controls across the life span. *J. Nucl. Med.* **60**, 1444–1451 (2019).
73. F. Barkhof *et al.*, Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. *Brain* **120**, 2059–2069 (1997).
74. C. H. Polman *et al.*, Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann. Neurol.* **69**, 292–302 (2011).
75. A. Johnen *et al.*, Can we predict cognitive decline after initial diagnosis of multiple sclerosis? Results from the German National early MS cohort (KKNMS). *J. Neurol.* **266**, 386–397 (2019).
76. O. von Bismarck *et al.*, Treatment choices and neuropsychological symptoms of a large cohort of early MS. *Neurol. Neuroimmunol. Neuroinflamm.* **5**, e446 (2018).
77. L. Strober *et al.*; Multiple Sclerosis Outcome Assessments Consortium (MSOAC), Symbol digit modalities test: A valid clinical trial endpoint for measuring cognition in multiple sclerosis. *Mult. Scler.* **25**, 1781–1790 (2019).
78. B. Fischl, FreeSurfer. *Neuroimage* **62**, 774–781 (2012).
79. R. S. Desikan *et al.*, An automated labeling system for subdividing the human cerebral cortex on MRI scans into gyral based regions of interest. *Neuroimage* **31**, 968–980 (2006).
80. C. Kilkenny, W. Browne, I. C. Cuthill, M. Emerson, D. G. Altman; NC3Rs Reporting Guidelines Working Group, Animal research: Reporting in vivo experiments: The ARRIVE guidelines. *Br. J. Pharmacol.* **160**, 1577–1579 (2010).
81. S. Bittner *et al.*, Endothelial TWIK-related potassium channel-1 (TREK1) regulates immune-cell trafficking into the CNS. *Nat. Med.* **19**, 1161–1165 (2013).
82. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
83. M. Cerina *et al.*, Myelination- and immune-mediated MR-based brain network correlates. *J. Neuroinflammation* **17**, 1–16 (2020).
84. T. R. Menheniott, M. Charalambous, A. Ward, Derivation of primary choroid plexus epithelial cells from the mouse. *Methods Mol. Biol.* **633**, 207–220 (2010).
85. S. Chen, Y. Zhou, Y. Chen, J. Gu, fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**, i884–i890 (2018).
86. K. Kelley, K. Lai, Accuracy in parameter estimation for the root mean square error of approximation: Sample size planning for narrow confidence intervals. *Multivariate Behav. Res.* **46**, 1–32 (2011).

2.6 Siponimod Modulates the Reaction of Microglial Cells to Pro-Inflammatory Stimulation

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Abstract

Siponimod (Mayzent®), a sphingosine 1-phosphate receptor (S1PR) modulator which prevents lymphocyte egress from lymphoid tissues, is approved for the treatment of relapsing-remitting and active secondary progressive multiple sclerosis. It can cross the blood–brain barrier (BBB) and selectively binds to S1PR1 and S1PR5 expressed by several cell populations of the central nervous system (CNS) including microglia. In multiple sclerosis, microglia are a key CNS cell population moving back and forth in a continuum of beneficial and deleterious states. On the one hand, they can contribute to neurorepair by clearing myelin debris, which is a prerequisite for remyelination and neuroprotection. On the other hand, they also participate in autoimmune inflammation and axonal degeneration by producing pro-inflammatory cytokines and molecules. In this study, we demonstrate that siponimod can modulate the microglial reaction to lipopolysaccharide-induced pro-inflammatory activation.

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Communication

Siponimod Modulates the Reaction of Microglial Cells to Pro-Inflammatory Stimulation

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Abstract: Siponimod (Mayzent®), a sphingosine 1-phosphate receptor (S1PR) modulator which prevents lymphocyte egress from lymphoid tissues, is approved for the treatment of relapsing-remitting and active secondary progressive multiple sclerosis. It can cross the blood–brain barrier (BBB) and selectively binds to S1PR1 and S1PR5 expressed by several cell populations of the central nervous system (CNS) including microglia. In multiple sclerosis, microglia are a key CNS cell population moving back and forth in a continuum of beneficial and deleterious states. On the one hand, they can contribute to neurorepair by clearing myelin debris, which is a prerequisite for remyelination and neuroprotection. On the other hand, they also participate in autoimmune inflammation and axonal degeneration by producing pro-inflammatory cytokines and molecules. In this study, we demonstrate that siponimod can modulate the microglial reaction to lipopolysaccharide-induced pro-inflammatory activation.

Keywords: sphingosine 1-phosphate receptor signalling; multiple sclerosis; neurodegeneration; modulation; polarization

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1. Introduction

Myelin sheaths in the human central nervous system (CNS) stabilize, trophically support and electrically insulate axons but are destroyed in demyelinating diseases such as multiple sclerosis (MS). As a result, saltatory signal conduction is interrupted and axonal damage occurs manifesting itself in various clinical symptoms [1]. Siponimod (BAF312), an orally administered sphingosine-1-phophate receptor (S1PR) modulator binding selectively to S1PR1 and S1PR5, reduces relapse rate and inflammatory disease activity in relapsing remitting MS (RRMS) [2]. In addition, it was shown to slow down disease progression and brain atrophy in secondary progressive MS (SPMS) [3] pointing to potential anti-neurodegenerative properties. Furthermore, several studies have demonstrated that siponimod reduces myelin loss in organotypic slice cultures [4] and prevents synaptic loss in the MS animal model myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalitis (MOG-EAE) [5]. While the exact underlying mechanisms are still unclear, microglia (MG) might be highly relevant in this context [6]. MG are glial cells in the CNS, play a key role in MS-associated inflammatory processes [7] and express both S1PR1 and S1PR5 [8]. While they can adopt an anti-inflammatory phenotype in which they clear myelin debris and promote regeneration, i.e., exert neuroprotective effects [8,9], they are also able to produce pro-inflammatory cytokines in response to MS-related CNS

injury [10,11]. Thereby the inflammatory process is upheld by cytokine-mediated activation of other immune cells ultimately leading to myelin destruction and subsequent neurodegeneration. Of note, MG can switch back and forth between pro- and anti-inflammatory phenotypes based on the underlying disease pathology—a process called polarization. Taken together, it is therefore conceivable that via a modulation of MG polarization siponimod could potentially exert effects on ongoing neurodegenerative processes in MS.

2. Results

2.1. Siponimod Modulates Microglial Morphology and Actin Filament Organization

In order to better understand the effects of siponimod on brain-resident cells in the context of MS, we investigated whether siponimod can modulate the behaviour of primary rat microglia in an inflammatory milieu. To this end, we used lipopolysaccharide (LPS) as a pro-inflammatory cue. We stimulated cultured primary rat microglia with either 10/50 µM siponimod or 100 ng/mL LPS and compared their reaction to a simultaneous stimulation with both reagents. As respective controls, we used DMSO for siponimod and ddH_2O for LPS, either alone or in combination (Figure 1). With regard to the seemingly high siponimod concentrations used in our experiments performed in 10% foetal calf serum-containing medium, several studies have demonstrated > 99.9% protein binding for siponimod [12]. Moreover, other studies have shown that the 10 and 50 µM (total) drug concentrations used by us are indeed observed in the CNS of EAE-mice receiving efficacious treatment with siponimod [13]. In greater detail, Bigaud and colleagues showed that feeding mice with a diet containing 100 mg per kg pellet results in total brain siponimod levels exceeding 10 µM. Using Iba1 staining for visualization we found that LPS stimulation increases the number of microglia (Figure 1A,B blue bars) compared to control stimulated cells (Figure 1B white bars). At the higher concentration of 50 µM but not at 10 µM, siponimod treatment (Figure 1B green bars) also resulted in an increase in cell number compared to control cells (Figure 1B white bars). However, co-stimulation with LPS (Figure 1B red bars) resulted in a significant cell number reduction compared to LPS-stimulated cells (Figure 1B blue bars).

It has been well-described previously that activated microglia change their morphology and cytoskeletal actin organization [14]. Therefore, we quantified the size of microglial cells, confirming that LPS induces microglia to significantly increase their mean cell area (Figure 1A,C blue bars) in comparison to control cells (Figure 1C white bars). At the higher concentration of 50 µM, siponimod alone slightly altered cell morphology in comparison to control cells (Figure 1C green bars). Most importantly, however, we observed that co-stimulation of microglia with siponimod at a concentration of 50 µM and LPS significantly reduced the LPS-induced increase in mean cell area (Figure 1C red bar).

In order to evaluate actin cytoskeleton organization, we used Alexa488 coupled phalloidin to specifically stain filamentous and non-filamentous forms of actin (Figure 1D). As expected, control cells displayed a homogenous distribution of mostly non-filamentous forms of actin. In contrast, LPS-stimulated microglia featured strong filamentous forms of actin. However, microglia stimulated with 50 µM siponimod alone no longer showed any filamentous actin at all and when co-stimulated with LPS the actin organization was again homogenously distributed and only very few cells showed filamentous actin.

2.2. Siponimod Modulates iNOS Protein Expression

Nitric oxide (NO) is a free radical found at high concentrations in inflammatory multiple sclerosis (MS) lesions. This is based on an increased expression of inducible form of nitric oxide synthase (iNOS) in cells such as microglia, myeloid cells and astrocytes. NO plays a role in the disruption of the blood–brain barrier (BBB), oligodendrocyte injury, demyelination, axonal degeneration, mitochondrial dysfunction and impairment of axonal conduction [15,16]. We therefore sought to investigate whether siponimod may also modulate iNOS expression (Figure 2). Using the same stimulation scheme as delineated above, we found that LPS alone lead to a significant increase in iNOS protein expression (Figure 2A blue columns, Figure 2B) in comparison to controls (Figure 2A white bars, Figure 2B). How-

ever, at both concentrations of 10 and 50 µM siponimod significantly reduced LPS-induced microglial iNOS expression at protein level (Figure 2A red columns, Figure 2B).

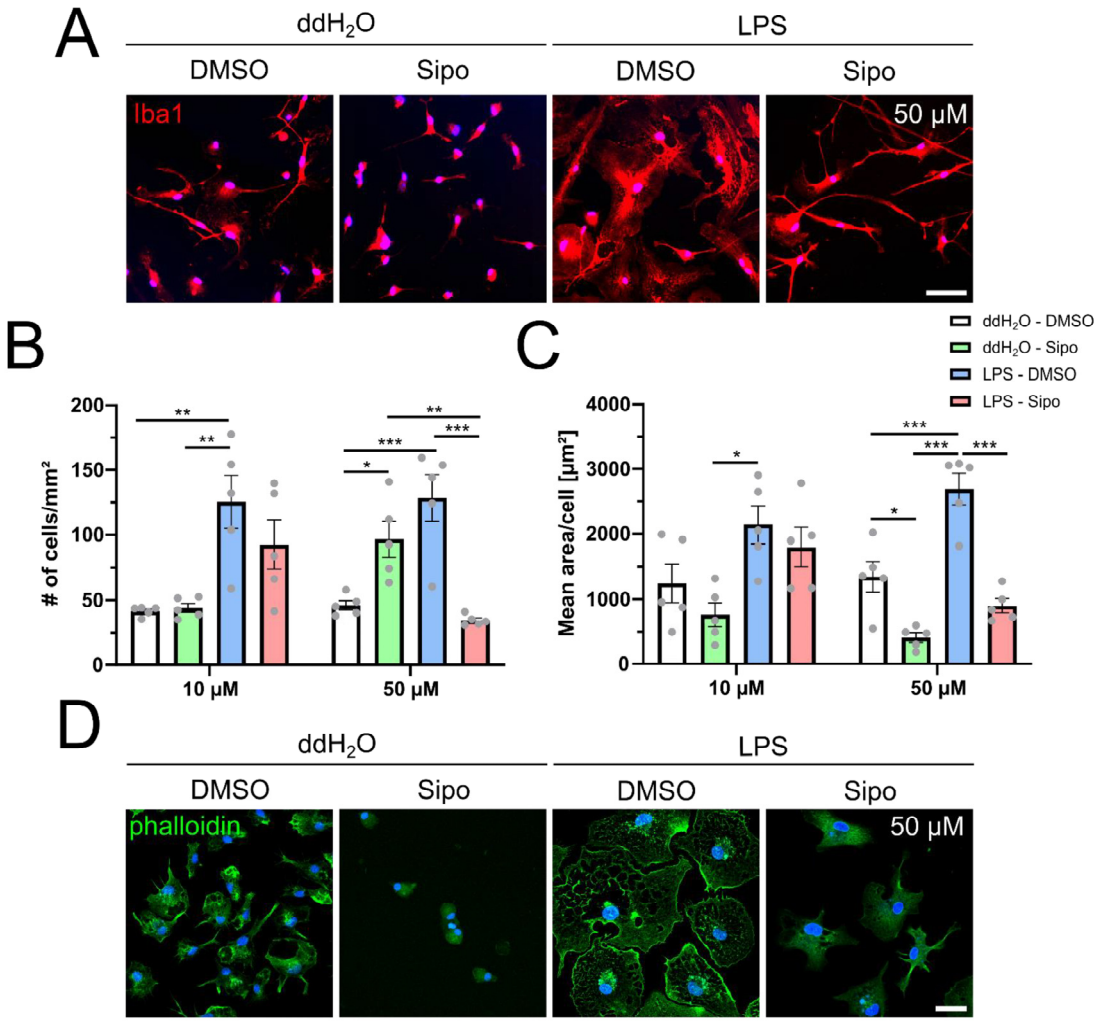

Figure 1. Siponimod modulates morphology and actin cytoskeleton organization of primary rat microglia in an inflammatory milieu. (**A**) Representative images of +/− 50 µM siponimod +/− LPS stimulated microglia after 3 days stained against Iba1 and DAPI. (**B**) Quantification of microglial cell numbers +/− 10/50 µM siponimod +/− LPS after 3 days. (**C**) Quantification of the mean cell area of microglial cells by dividing the total area of Iba1 staining by the cell counts from microglia stimulated for 3 days with +/− 10/50 µM siponimod +/− LPS. (**D**) Evaluation of cytoskeletal organization via Alexa488-coupled phalloidin of 3 days stimulated microglia (+/− 50 µM siponimod, +/− LPS). Data are presented as mean values ± SEM. Grey dots represent individual data points. Significance was assessed by 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Graph-Pad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). The experimental groups were considered significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar: (**A**) 50 µm, (**D**) 25 µm.

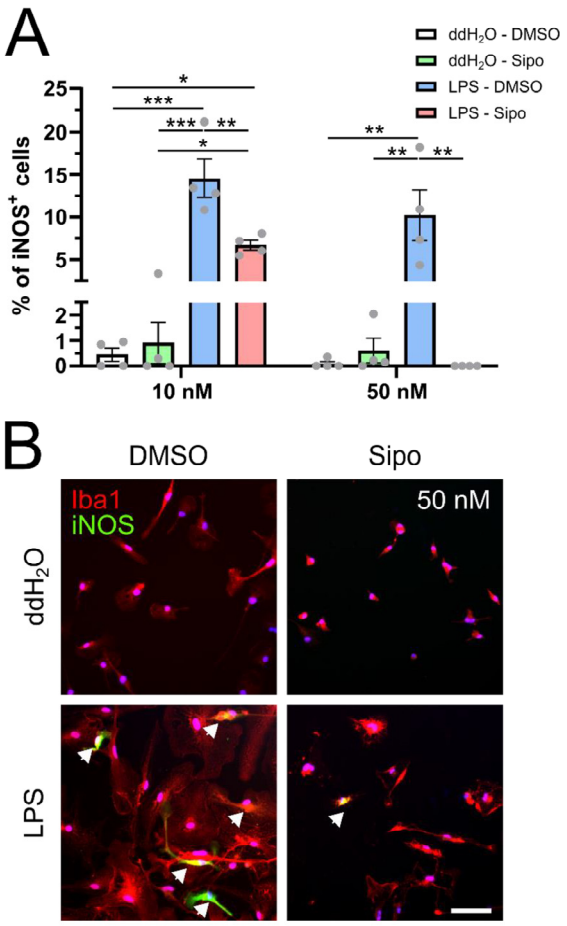

Figure 2. Siponimod reduces microglial iNOS protein expression in an inflammatory milieu. (**A**) Quantification of the percentage of iNOS positive microglia after 3 days of stimulation with +/− 10/50 µM siponimod +/− LPS. (**B**) Representative images of Iba1, iNOS co staining after stimulation for 3 days with +/− 50 µM siponimod +/− LPS. Data are presented as mean values ± SEM. Grey dots represent individual data points and white arrows point to iNOS positive microglia. Significance was assessed by 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Graph-Pad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). The experimental groups were considered significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar: 50 µm.

2.3. Siponimod Modulates Microglial Cytokine Gene Expression

Using the same stimulation scheme, we next investigated if siponimod also modulates microglial cyto- and chemokine gene expression patterns relevant in the course of MS (Figure 3). We found that in parallel to our morphology experiments (see Figure 1), LPS induced microglia to significantly increase the expression of the pro-inflammatory factors, tumor necrosis factor-α (Tnf/TNFα; Figure 3A blue bar) and interleukin-1β (Il1b/IL-1β; Figure 3E blue bar) in comparison to control cells (Figure 3A,E white bars). Siponimod at a concentration of 50 µM alone did not alter microglial gene expression in comparison to control cells (Figure 3A,E green bars). However, siponimod significantly reduced the LPS-induced increase in TNFα and IL-1β (Figure 3A,E red bars) which we corroborated at protein levels (Figure 3B,F red bars). Increased TNFα production can be found in

active MS lesions, sera and the cerebrospinal fluid (CSF) of MS patients. It contributes to neuronal death as well as axonal damage and also correlates with the severity and progression of the disease [8,17–19]. IL-1β is a pro-inflammatory cytokine which induces excitotoxic neurodegeneration. In addition, there is an association between IL-1β CSF levels and disability progression in RRMS patients [20]. We also found that siponimod prevented an LPS-induced induction of interferon-β (Ifnb/IFNβ, Figure 2C red bar) and of interleukin-10 (Il10/IL-10, Figure 2D red bar) in comparison to control-stimulated cells. In addition, the secretion of IL-10 and IFNβ proteins was significantly decreased upon siponimod stimulation (Figure 3D,H red bars) compared to LPS alone (Figure 3D,H blue bars). IL-10 is a potent anti-inflammatory cytokine that is able to suppress the synthesis of pro-inflammatory cytokines such as IFNγ [21–23]. IFNβ possesses both pro- and anti-inflammatory properties and was the first substance to be used clinically as a drug against RRMS [24]. Of note, non-polarized microglial cells exhibited either very low or below-detection-limit levels of corresponding secreted cytokines.

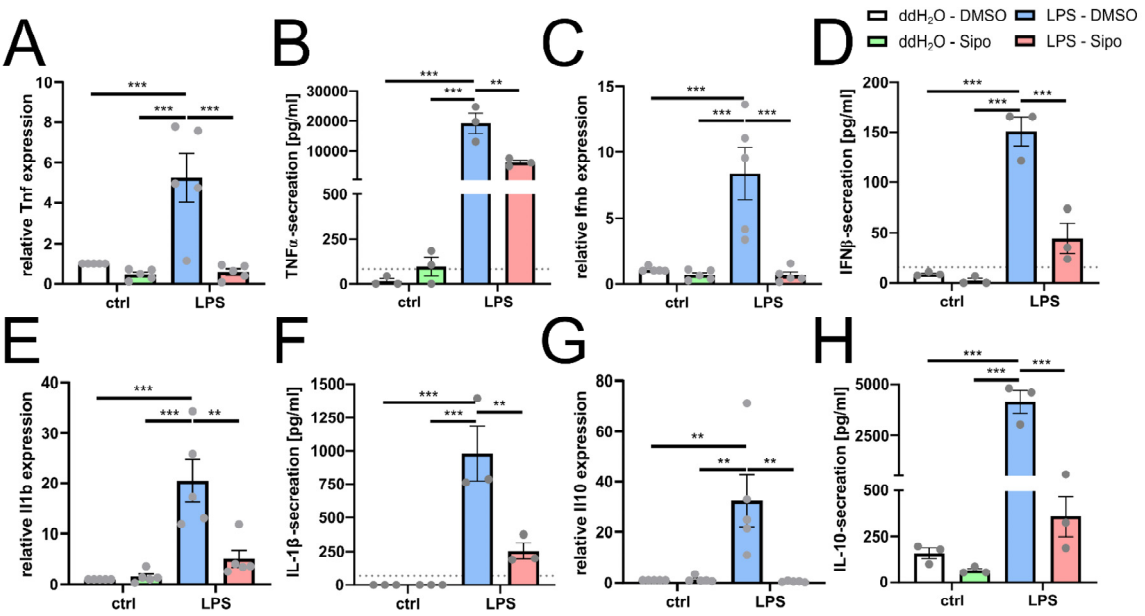

Figure 3. Siponimod modulates microglial cytokine expression and secretion in an inflammatory milieu. (**A**,**C**,**E**,**G**) quantitative RT-PCR analysis of Tnf (**A**), Ifnb (**C**), Il1b (**E**) and Il10 (**G**) gene expression after stimulation for 1 day with +/− 50 µM siponimod +/− LPS. (**B**,**D**,**F**,**H**) Quantification of TNF-α (**B**), IFNβ (**D**), IL-1β (**F**) and IL-10 (**H**) protein concentration in the cell culture medium of microglial cells stimulated for 3 days with +/− 50 µM siponimod +/− LPS using respective quantitative sandwich ELISA assays. Grey-dotted lines indicate the lowest standard of the ELISA kit: TNFα = 82.3 pg/mL, IFNβ = 15.63 pg/mL, IL-1β = 68.59 pg/mL and IL-10 = 8.23 pg/mL. Data are presented as mean values ± SEM. Grey dots represent individual data points. Significance of gene expression analysis as well as ELISA was assessed by 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Graph-Pad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). The experimental groups were considered significantly different at ** $p < 0.01$, *** $p < 0.001$.

2.4. Siponimod Modulates Immunological Signature in Pro-Inflammatory Triggered Microglial Cells

Since we found that siponimod exerted an anti-inflammatory effect on LPS-stimulated microglial cells, bulk RNA sequencing was performed to further describe the resulting microglial gene expression signature. Using the same scheme as described above, we com-

pared the transcriptomes of cells stimulated with the combination of LPS and siponimod with cells stimulated with LPS alone. In order to assess significantly dysregulated genes, plotting the $\log_2$-fold-change (FC) against the $\log_{10}$-adjusted *p*-value (false discovery rate, FDR, Figure 4A) was performed. This resulted in the identification of Clec5a, Fst, Cd9, Clec4d, Cdkn1c and Cd34 as the genes most significantly upregulated in the presence of LPS and siponimod compared to LPS stimulation alone. Furthermore, Cxcl11, Angptl4, Cd38 and Fscn1 were identified as most significantly downregulated. Using a fold-change difference of ± 1.5 and adjusted *p*-value ≤ 0.05 to determine differentially expressed genes (DEGs) in the siponimod and LPS-treated group compared to the LPS alone group, we identified 3425 DEGs (Figure 4B), of which 1658 were significantly upregulated and 1767 were significantly downregulated. To further characterize this microglial signature, we performed gene enrichment analysis (GO Biol. Processes, Figure 4C,D), identifying "response to unfolded protein", "response to endoplasmatic reticulum stress" and "response to starvation" as the most significant pathways related to upregulated DEGs. On the other hand, downregulated DEGs were enriched in "positive regulation of immune response", "leukocyte activation", "regulation of cytokine production" and "innate immune response". This demonstrated that the immunological pathways activated by LPS in microglia were downregulated in response to siponimod. The top 20 downregulated genes of each of these four biological processes are summarized in Tables 1–4. Since cytokines are the main mediators of inflammation, we furthermore wanted to investigate potentially modified cytokine signatures (Figure 4E). This showed that the majority of cytokines were downregulated by siponimod in LPS-treated microglia with the most notable genes being Ifnb1, Ita, Tnfsf14, Il16, Cxcl13 and Cxcl10. In contrast, we found that Faslg, Il1rn and Tnfsf18 were significantly upregulated.

Table 1. Positive regulation of immune response.

Gene Symbol	$\log_2$ Fold Change	*p* Adjusted
Lta	−4.42	2.07×10^{-6}
Cd38	−4.04	4.58×10^{-24}
C3ar1	−2.69	8.27×10^{-10}
Pycard	−2.69	8.97×10^{-8}
Card11	−2.59	1.60×10^{-12}
Tifa	−2.53	3.81×10^{-13}
Cd180	−2.47	1.37×10^{-6}
Nlrp3	−2.10	2.02×10^{-13}
Tfrc	−1.91	1.31×10^{-6}
Lat2	−1.82	3.68×10^{-11}
Slamf1	−1.76	5.45×10^{-6}
RT1-N3	−1.76	8.05×10^{-8}
Cmklr1	−1.74	2.19×10^{-5}
Ctsh	−1.72	4.42×10^{-6}
Cd81	−1.71	1.79×10^{-5}
Dhx58	−1.65	4.29×10^{-6}
Ada	−1.60	3.02×10^{-6}
Xrcc5	−1.59	1.21×10^{-6}
Lacc1	−1.57	3.02×10^{-6}
Trex1	−1.57	1.51×10^{-6}
Nectin2	−1.52	2.44×10^{-5}
Cyrib	−1.36	5.69×10^{-6}
Tlr6	−1.32	3.37×10^{-6}
Nod1	−1.29	8.86×10^{-6}
Ifi35	−1.25	7.32×10^{-6}

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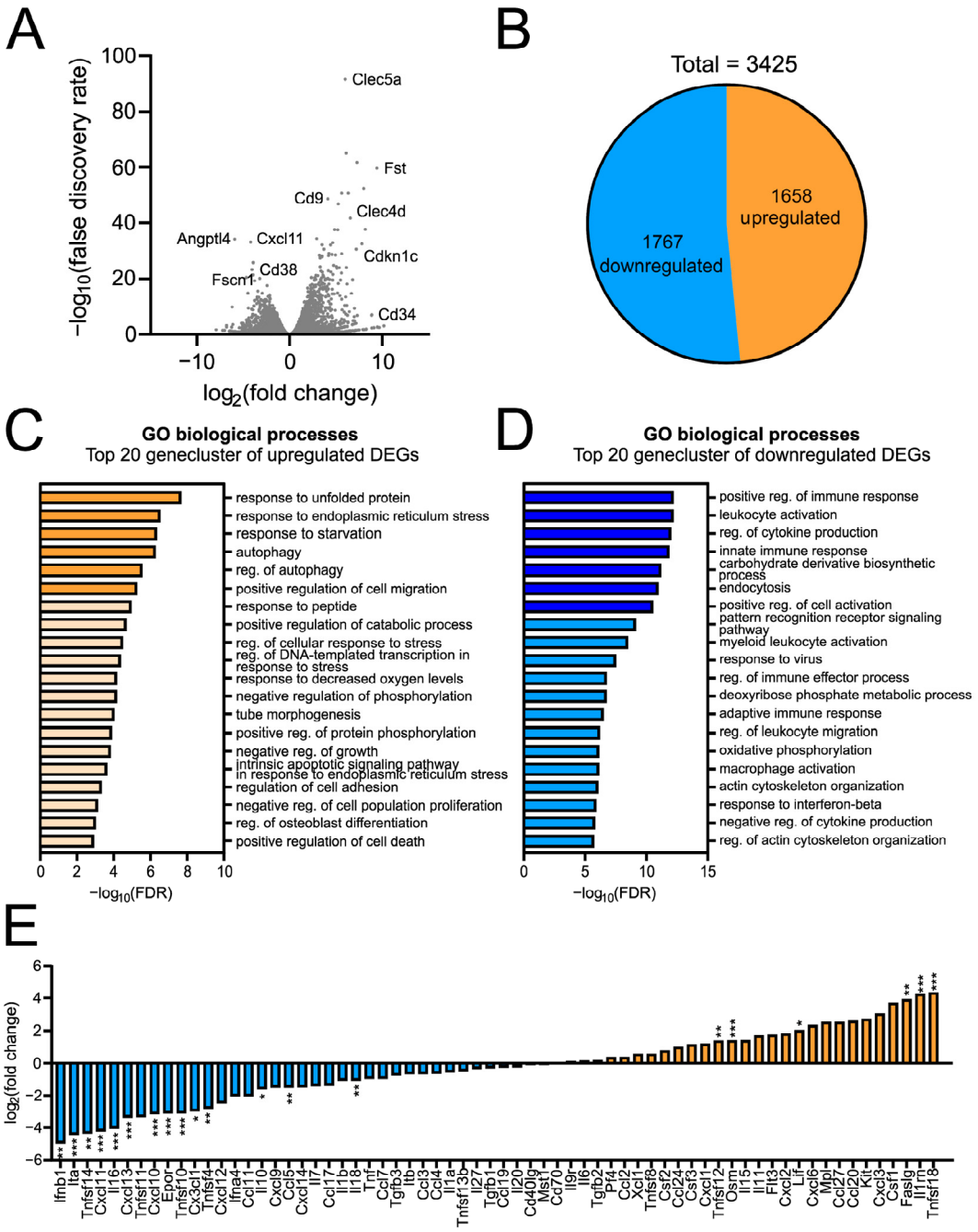

Figure 4. Siponimod alters the immunological signature of LPS-stimulated microglia. Volcano plots showing $\log_2$(fold-change) against $\log_{10}$(false discovery rate) for the comparisons of LPS/siponimod costimulated versus LPS-stimulated microglia (**A**). Identification of 3425 DEGs (fold change of ±1.5 and an FDR adjusted *p*-value of ≤0.05), of which 1658 were up- and 1767 were downregulated (**B**). Gene Set Enrichment and Pathway analysis of up- (**C**) and downregulated (**D**) DEGs in order of their

$\log_{10}$(FDR) according to the Benjamin and Hochberg adjustment. Cytokine signature of LPS-siponimod co-stimulated compared to LPS alone (**E**). Significance of transcriptome analysis was assessed by the RNA-Seq tool (version 2.6). The experimental groups were considered significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2. Leukocyte activation.

Gene Symbol	$\log_2$ Fold Change	p Adjusted
Il21r	−3.66	1.46×10^{-7}
P2ry12	−3.44	4.64×10^{-7}
Cxcl13	−3.35	7.65×10^{-7}
Fgl2	−3.20	4.82×10^{-11}
Cxcl10	−3.14	1.62×10^{-10}
Cd244	−2.94	1.36×10^{-15}
Slamf9	−2.88	2.15×10^{-7}
Tnfrsf11a	−2.81	1.94×10^{-7}
Klf2	−2.81	2.27×10^{-7}
Pycard	−2.69	8.97×10^{-8}
Anxa3	−2.60	2.16×10^{-7}
Card11	−2.59	1.60×10^{-12}
Clec4a3	−2.54	7.55×10^{-7}
Cd180	−2.47	1.37×10^{-6}
Trpm2	−2.43	6.88×10^{-13}
Sh3pxd2a	−2.37	1.25×10^{-7}
Actb	−2.33	5.90×10^{-8}
Il6r	−2.30	5.13×10^{-10}
Lrrk1	−2.09	1.13×10^{-6}
Tfrc	−1.91	1.31×10^{-6}
Axl	−1.86	5.20×10^{-8}
Lat2	−1.82	3.68×10^{-11}
Ubash3b	−1.61	4.55×10^{-8}
Xrcc5	−1.59	1.21×10^{-6}
Prdx2	−1.35	1.84×10^{-7}

Table 3. Regulation of cytokine production.

Gene Symbol	$\log_2$ Fold Change	p Adjusted
Cxcl11	−4.20	9.69×10^{-34}
Il16	−4.05	5.43×10^{-7}
P2ry12	−3.44	4.64×10^{-7}
Cxcl13	−3.35	7.65×10^{-7}
Siglec8	−3.28	1.76×10^{-9}
Fgl2	−3.20	4.82×10^{-11}
Cxcl10	−3.14	1.62×10^{-10}
Cd244	−2.94	1.36×10^{-15}
Tnfrsf11a	−2.81	1.94×10^{-7}
Klf2	−2.81	2.27×10^{-7}
Samhd1	−2.72	2.29×10^{-7}
C3ar1	−2.69	8.27×10^{-10}
Pycard	−2.69	8.97×10^{-8}
Card11	−2.59	1.60×10^{-12}
Clec4a3	−2.54	7.55×10^{-7}
Dagla	−2.32	2.04×10^{-7}
Il6r	−2.30	5.13×10^{-10}
Cxcl17	−2.28	8.05×10^{-8}
Nlrp3	−2.10	2.02×10^{-13}
Ezr	−1.91	1.30×10^{-7}
Ccr5	−1.90	1.45×10^{-7}

Table 3. *Cont.*

Gene Symbol	$\log_2$ Fold Change	*p* Adjusted
Axl	−1.86	5.20×10^{-8}
Ctnnbip1	−1.77	1.13×10^{-6}
Nt5e	−1.48	2.00×10^{-7}
Prdx2	−1.35	1.84×10^{-7}

Table 4. Innate immune response.

Gene Symbol	$\log_2$ Fold Change	*p* Adjusted
Cx3cr1	−3.68	2.18×10^{-6}
Wfdc21	−3.22	6.08×10^{-10}
Evl	−3.17	1.72×10^{-11}
Cxcl10	−3.14	1.62×10^{-10}
Clec4a1	−2.78	6.22×10^{-7}
Pycard	−2.69	8.97×10^{-8}
Ccl12	−2.59	1.23×10^{-6}
Clec4a3	−2.54	7.55×10^{-7}
Tifa	−2.53	3.81×10^{-13}
Mrc1	−2.43	4.99×10^{-10}
Gbp4	−2.34	4.46×10^{-9}
Fes	−2.33	1.35×10^{-6}
Ly86	−2.10	2.17×10^{-5}
Nlrp3	−2.10	2.02×10^{-13}
Lrrk1	−2.09	1.13×10^{-6}
Aif1	−1.90	5.24×10^{-6}
Coro1a	−1.73	4.18×10^{-5}
Dhx58	−1.65	4.29×10^{-6}
Sla	−1.59	5.03×10^{-6}
Trim25	−1.58	1.22×10^{-6}
Trex1	−1.57	1.51×10^{-6}
Tmem106a	−1.43	1.22×10^{-5}
Tlr6	−1.32	3.37×10^{-6}
Nod1	−1.29	8.86×10^{-6}
Ifi35	−1.25	7.32×10^{-6}

3. Discussion

There is conclusive evidence that siponimod exerts beneficial effects on different aspects of EAE, an established animal model for MS. It decreases disease severity, the degree of demyelination and improves cortical network functionality [4,5]. However, the exact cellular mechanisms underlying these effects remain largely elusive. The same is true for siponimod's effects on disease progression and brain atrophy in secondary progressing (SP)MS [3]. In this regard, it is important to mention that fingolimod, another S1PR modulator, has already been shown to modulate microglial activation [25,26] which could be further corroborated by PET-CT imaging of MS lesions [27]. Given its more specific receptor profile in comparison to fingolimod, it is of great interest to investigate whether siponimod exerts similar functions.

Our findings now show that in an inflammatory milieu, siponimod (i) protects microglia from adopting activated cytoskeletal architecture and morphologies, (ii) reduces iNOS protein expression, (iii) modulates microglial cytokine expression and (iv) downregulates microglial immunological pathways. These results point to specific effects of siponimod on microglial cells. So far, studies investigating the role of microglia in this context solely used immortalized cell lines. In contrast, this is now the first study to provide data generated in primary rat microglial cells.

Our results indicate that siponimod prevents TNFα upregulation in an inflammatory milieu. TNFα is a pleiotropic pro-inflammatory cytokine which is, amongst other glial cells,

produced by microglia [28]. It potentiates glutamate excitotoxicity [29], increases neuroinflammatory responses [30], impairs oligodendroglial differentiation [31] and even induces oligodendrocyte cell death [32]. In MS, TNFα is present in active lesions and its level in the cerebrospinal fluid (CSF) is correlated with disease severity and progression [19]. However, despite beneficial effects of a neutralization of TNFα in animal models its inhibition in the clinical context has resulted in increased disease activity and lesion load progression [33]. This underlines the limited transferability of results generated in in vitro/animal models to the human paradigm. Another cytokine the upregulation of which was reduced by siponimod under inflammatory conditions was IL-1β. This cytokine is present in MS lesions [34,35] and the CSF of MS patients [36–38] where its levels are correlated with the number and volume of MS lesions [38]. In EAE, IL-1β is mostly produced by myeloid cells infiltrating the CNS [34] and contributes to leukocyte recruitment and BBB disruption [35].

Interestingly, we also found that siponimod prevented an LPS-induced induction of interferon-β. This is of particular interest as this molecule was the first to be used as an MS drug even though its exact mode of action is not yet fully understood. In general, the beneficial clinical treatment effect is considered to be related to several overlapping mechanisms in the peripheral immune system. This includes the down-regulation of the major histocompatibility complex (MHC) class II expression present on the antigen-presenting cells (i.e., dendritic cells, Langerhans cells and B-cell lymphocytes), the induction of T-cell production of interleukin 10 (IL-10), which shifts the balance toward the anti-inflammatory T helper (Th)-2 cells and the inhibition of T-cell migration [39]. At the same time, only a few studies have investigated the direct impact of interferon-β on brain-resident cells [40,41]. However, these studies were performed exclusively in transgenic animal models and partly contradict each other so that the role of interferon-β is still not entirely clarified.

Finally, siponimod also prevented LPS-induced upregulation of IL-10. IL-10 is a potent anti-inflammatory cytokine which is, among other cell types, expressed by microglia [42,43]. It decreases the release of TNFα, IL-1β, IL-6, IL-8, IL-12 and IL-23, ameliorates the course of EAE, reduces the proliferation of TH1 and TH2 cells, decreases antigen presentation of monocytes and macrophages and has the capacity to act in a neuroprotective manner [17,22]. In MS, IL-10 secretion is decreased prior to relapse and increased during remission. However, no clinical studies investigating the potential benefit of IL-10 for MS have been conducted so far.

The analysis of the transcriptome of LPS/siponimod co-stimulated microglia vs. LPS only stimulated microglia revealed follistatin (Fst), cyclin dependent kinase inhibitor 1c (Cdkn1c), Cd9 and Cd34, as well as Cxcl11, Cd38 and fascin-1 (Fscn1) among the most significantly differentially expressed genes, all of which are strongly associated with microglial polarization states in different injuries and diseases [44–50]. This further underlines that siponimod stimulation leads to a specific change in the pattern of microglial gene regulation which cannot be assigned to a "classic" M1 or M2 polarization and appears to be more complex. However, the fact that the top 4 cluster of the gene enrichment analysis related to the 1767 downregulated genes were all associated with immunological functions indicates that it has significant immunomodulatory properties. However, besides the association of downregulated DEGs with immunological functions, we found that siponimod modulates additional intriguing clusters of biological processes. For instance, the upregulated DEGs display enrichments in the response to unfolded protein as well as endoplasmic reticulum (ER) stress which is in line with previous findings linking sphingosine-1-phosphate metabolism to these processes [51]. In this regard, mainly ceramide, one of sphingosine-1-phosphate's precursors, was found to specifically induce ER stress via the CD95-PERK signalling pathway leading to an increase in unfolded protein [52]. Taken together, this finding suggests that although S1PR signalling is inhibited by siponimod treatment, sphingosine-1-phosphate metabolism remains intact. Furthermore, autophagy, another pathway enriched upon siponimod treatment, is thought to be one of the key regulators of innate immune responses [53]. Apart from that, we found downreg-

ulated DEG clusters in endocytosis, one of the key mechanisms of S1PR signalling and clusters in actin cytoskeletal organisation, which we gauge to be in line with our finding that siponimod modulates microglial morphology as shown in Figure 1D.

As stated above, the interplay of different cytokines is an important factor in MS. Therefore, our observation that siponimod creates a completely new cytokine signature in pro-inflammatory microglia is of great interest even though it cannot be characterized within the framework of M1/M2 polarization. In this regard, recent RNA sequencing (RNASeq) and genome-wide association studies (GWAS) suggest the existence of several subgroups of disease-associated microglia (DAMs) [54–57]. All these DAMs, which were first described in neurodegenerative diseases such as Alzheimer's disease, show unique transcriptional and functional signatures.

Regarding the limitations of our study, we are aware that the use of bacterial endotoxin as a pro-inflammatory stimulus is debatable. Even though LPS is the most commonly used molecule in this context [58,59] recent studies suggest that in CNS damage cytokines such as interferon γ (IFNγ) and TNFα may be also appropriate [36,60]. For instance, LPS was found to evoke higher pro-inflammatory gene expression but also increased several anti-inflammatory genes which is in line with our finding of an increased IL-10 and IFNβ expression. However, identical to our study, these results were generated in rat microglia so that the translation to the human paradigm is still pending. Furthermore, in this study we only examined parallel stimulation with LPS and siponimod. We selected this approach to generate first insights into the effects of siponimod on a single CNS cell type in a pro-inflammatory milieu to which the RNASeq data additionally contributed. In future studies, it will therefore be of interest to investigate to what degree siponimod (pre)treatment can be protective or whether a delayed application exerts similar rescue effects.

In general, even though we show a direct effect of siponimod on CNS-resident cells, this medication certainly exerts its most profound effects on pro-inflammatory peripheral immune cells. It effectively prevents them from leaving lymphoid tissues and thereby averts CNS immune cell infiltration. However, it is known from post mortem histopathology that microglia, that were primed by invading peripheral immune cells during initial disease relapses, create a milieu of smouldering inflammation behind a closed blood–brain barrier (BBB). As a result, siponimod might exert a beneficial effect on these microglia-mediated processes which are probably not even visible on conventional MRI, let alone clinically. This might also explain why siponimod is effective in active secondary progressive MS. In contrast and correspondingly, it has no effect in primary progressive MS where neurodegeneration is predominant. In conclusion, it is conceivable that via a modulation of microglial behaviour siponimod modulates neuroinflammatory processes in MS. Future studies will have to further define the exact microglial subtype associated with siponimod stimulation and its impact in MS.

4. Materials and Methods

4.1. Primary Rat Microglial Cell Culture

All animal use complies with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The Institutional Review Board (IRB) of the ZETT (Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben) at the Heinrich Heine University Düsseldorf has approved all animal procedures under licences O69/11. Briefly, dissociated P1 Wister rat cortices were cultured on T-75 cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, UK) substituted with 10% foetal calf serum (FCS; Capricorn Scientific, Palo Alto, CA, USA) and 4 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 50 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) as previously described [61]. After 10 days, flasks were shaken at 180 rpm/min at 37 °C for 2 h and microglia-containing supernatants were collected. Afterwards, cell suspensions were centrifuged for 5 min at 300× *g* at 4 °C, supernatants were discarded and the pellet was resuspended in 10 mL and

plated onto bacterial dishes and kept in the incubator (37 °C, 5% CO_2 and 90% humidity) allowing for cell attachment to the surface. Culture flasks were again loaded with fresh DMEM medium and shaken for another 22 h at 37 °C in order to increase the final cell yield. Afterwards, supernatants were again transferred to bacterial dishes to allow for attachment. Microglia-containing bacterial dishes from the first and second shaking steps were checked for viability via bright-field microscopy, medium was discarded and cells rinsed with Dulbecco's Phosphate Buffered Saline (D-PBS). Microglia were dislodged by accutase (Thermo Fisher Scientific, Darmstadt, Germany), which was stopped by FCS-containing DMEM medium. Microglial cell suspensions were then centrifuged for 5 min at 300× *g* at 4 °C and cell-free supernatants were discarded. Afterwards, cell pellets were resuspended in 80 µL MACS buffer containing 0.5% BSA in D-PBS and 20 µL of anti-rat CD11b/c microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) were added for 15 min at 2–8 °C to allow for binding. Cells were then washed adding 2 mL of MACS buffer and spun down for 5 min at 300× *g* at 4 °C. Supernatants were again discarded and pellets were resuspended in 500 µL MACS buffer and subjected to MACS-sorting according to the manufacturer's protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). The resulting cell suspension was again spun down for 5 min at 300× *g* at 4 °C, pellets were resuspended in 1 mL DMEM and cell viability and numbers were quantified using trypan blue staining. Average cell purities as assessed by Iba1-positivity were consistently around 98%. Microglia were seeded on 8-well Lab-Tek chamber slides for immunocytochemistry or 24-well plates for the analysis of mRNA transcripts at different concentrations in microglia medium (10% FCS, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin in DMEM). For stimulation experiments, siponimod (BAF312; kindly provided by Novartis) was solved at a concentration of 50 mM in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) was solved in ddH_2O at a concentration of 1 mg/mL. Both reagents were aliquoted in appropriate amounts and stored at −80 °C. Each aliquot was thawed once and discarded afterwards. One day after microglial isolation, cells were stimulated with 10 or 50 µM siponimod or similar amounts of DMSO with or without 100 ng/mL LPS in microglia media. After 1 and 3 days, respectively, cell cultures were either fixated for follow up immune-cytochemistry or lysed for RNA preparation, cDNA synthesis and qPCR analysis.

4.2. Immunocytochemistry

For immunocytochemistry, microglia were fixed for 10 min with 4% paraformaldehyde (PFA), D-PBS washed, blocked for 45 min using 10% normal donkey serum (NDS; Sigma-Aldrich, St. Louis, MO, USA) and 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in D-PBS. Afterwards, cells were incubated at 4 °C overnight with primary antibody solution containing, 10% NDS and 0,1% Triton X-100 in D-PBS with rabbit anti-Iba1 (1/500, WAKO Pure Chemical Corporation, Osaka, Japan; RRID: AB_839504) and goat anti-iNOS (1/250; Abcam, Cambridge, UK, RRID: AB_301857). Following D-PBS washes secondary donkey anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 594 (1/500; Thermo Fisher Scientific, Darmstadt, Germany) were added for 2 h at room temperature. Nuclei were stained in parallel with 4′, 6-diamidino-2-phenylindole (DAPI; 20 ng/mL, Roche, Basel, Switzerland). Cells were mounted using Citifluor (Citifluor, London, UK) and images were captured on an Axioplan 2 microscope (Zeiss, Jena, Germany) using the same exposure times and light intensities. Phalloidin (Phalloidin CruzFluor 488 Conjugate, Santa Cruz Biotechnology, Dallas, TX, USA) staining was performed according to the manufacturers protocol. Briefly, 1000× phalloidin was diluted 1:1000 together with DAPI (20 ng/mL) in PBS. Prefixed cells were incubated with this solution for 1 h at room temperature. Afterwards, cells were washed 4 times with PCR and mounted using Citifluor (Citifluor, London, UK). The analysis of immune-positive cells was performed on 7 images per well and 2 wells per treatment and replicate, leading to 14 analysed images per treatment and replicate. On average, each image contained ~30 cells resulting in more than 2000 cells per condition being analysed. The quantification was performed using ImageJ software

(National Institute of Health (NIH), Bethesda, MD, USA). For the analysis of the mean area per cell, merged images were uploaded in ImageJ software, scale bars were set according to microscope settings and the channels were split. The number of cells was assessed by creating a binary image with a threshold to the DAPI channel (80, 255), applying water shedding and analysing all particles with a size of 150–3000 pixels and a circularity of 0.4–1.00. The total area of Iba1-stainings was analysed again by applying a threshold (30, 255), creating a binary image and measuring the total area of Iba1-positive staining. Afterwards, a ratio of the total area of Iba1-positive staining and the total number of cells was calculated. iNOS positive microglia were quantified manually, using the ImageJ tool "cell-counter".

4.3. RNA Preparation, cDNA Synthesis and Quantitative Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

RNA preparation, cDNA synthesis and quantitative RT-PCR were performed as recently described [61]. Briefly, total RNA purification from cells was performed using the RNeasy procedure (Qiagen, Hilden, Germany). Isolated RNA was afterwards reverse transcribed using the high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Darmstadt, Germany). Quantitative determination of gene expression levels was performed on a 7900HT sequence detection system (Thermo Fisher Scientific) using Power SybrGreen PCR master mix (Thermo Fisher Scientific) [62,63]. Following primer sequences were generated via PrimerExpress 2.0 software (Applied Biosystems/Thermo Fisher Scientific, Darmstadt, Germany), as well as tested and determined: Gapdh forward: GAA CGG GAA GCT CAC TGG C, Gapdh reverse: GCA TGT CAG ATC CAC AAC GG, Il1b forward: GAA ACA GCA ATG GTC GGG AC, Il1b reverse: AAG ACA CGG GTT CCA TGG TG, Il10 forward: CCC AGA AAT CAA GGA GCA TTT G, Il10 reverse: CAG CTG TAT CCA GAG GGT CTT CA, Ifnb1 forward: TGG AAG GCT CAA CCT CAG CTA, Ifnb1 reverse: GGG TGC ATC ACC TCC ATA GG, Tnf forward: AGC CC TGG TAT GAG CCC ATG TA, Tnf reverse: CCG GAC TCC GTG ATG TCT AAG T. Gapdh, which proved to be the most accurate and stable normalization gene among a number of others, such as Hprt1, Odc and Tbp, was used as reference gene. Relative gene expression levels were determined according to the ΔΔCt method (Thermo Fisher Scientific) and each sample was measured in duplicate.

4.4. Bulk RNA Sequencing

To generate RNASeq data, RNASeq libraries were prepared from DNase digested total RNA samples quantified by Qubit RNA HS Assay (Thermo Fisher Scientific) and capillary electrophoresis using the Fragment Analyzer and the "Total RNA Standard Sensitivity Assay" (Agilent Technologies, Inc. Santa Clara, CA, USA). All samples in this study showed high quality RNA Quality Numbers (RQN; mean = 9.8). The library preparation was performed according to the manufacturer's protocol using the 'VAHTS™ Universal RNA-Seq Library Prep Kit for Illumina® V6 with mRNA capture module'. Briefly, 150 ng total RNA were used for mRNA capturing, fragmentation, the synthesis of cDNA, adapter ligation and library amplification. Bead purified libraries were normalized and finally sequenced on the HiSeq 3000/4000 system (Illumina Inc. San Diego, CA, USA) with a read setup of SR 1 × 150 bp. The bcl2fastq tool (v2.20.0.422) was used to convert the bcl files to fastq files as well for adapter trimming and demultiplexing. Data analyses on fastq files were conducted with CLC Genomics Workbench (version 22.0.2, QIAGEN, Venlo. NL). The reads of all probes were adapter trimmed (Illumina TruSeq) and quality trimmed (using the default parameters: bases below Q13 were trimmed from the end of the reads, ambiguous nucleotides maximal 2). Mapping was performed against the Rattus norvegicus (mRatBN7.2.106; 5 July 2022) genome sequence. After grouping of samples (three biological replicates each) according to their respective experimental condition, the statistical differential expression was determined using the Differential Expression for RNA-Seq tool (version 2.6, CLC Genomics Workbench). The resulting p values were corrected for multiple testing by FDR and differentially expressed genes (DEGs) were filtered setting

a threshold at the FDR adjusted p-value of 0.05 and a fold-change of ± 1.5. The Gene Set Enrichment and Pathway analysis of differentially up- and downregulated genes was performed using Metascape platform using default parameters (*R. norvegicus*; 1 August 2022).

4.5. ELISA

To assess the microglial secretion of TNFα, IL-1β, IL-10 and IFNβ, the culture medium was harvested, spun down at 1000$\times$ g for 5 min at 4 °C, frozen on dry ice and stored at −80 °C. On the day of analysis, all reagents were thawed and adjusted to RT before the culture medium was measured in duplets using the following colorimetric sandwich ELISA kits according the manufacturers protocol: rat TNF alpha ELISA Kit (ab100785, Abcam), rat IL-1 beta ELISA kit (ab100768, abcam), rat IL-10 ELISA Kit (ab100764, abcam), Rat IFN-beta ELISA Kit (NBP3-06753, Novus Biologicals). After the generation of a 4-parameter logistic standard curve using Graph Pad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA), total protein concentrations were calculated. Protein levels below the detection limits of the used ELISAs were set to 0.

4.6. Statistical Analysis

Data are presented as mean values ± standard error of the mean (SEM). All data passed the Shapiro–Wilk test for normality. Therefore, significance was assessed either by 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test or by an unpaired students t-test both using Graph-Pad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). The experimental groups were considered significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n represents the number of independent experiments.

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Informed Consent Statement: Not applicable.

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References

1. Trapp, B.D.; Peterson, J.; Ransohoff, R.M.; Rudick, R.; Mork, S.; Bo, L. Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* **1998**, *338*, 278–285. [CrossRef] [PubMed]
2. Kappos, L.; Li, D.K.; Stuve, O.; Hartung, H.P.; Freedman, M.S.; Hemmer, B.; Rieckmann, P.; Montalban, X.; Ziemssen, T.; Hunter, B.; et al. Safety and Efficacy of Siponimod (BAF312) in Patients With Relapsing-Remitting Multiple Sclerosis: Dose-Blinded, Randomized Extension of the Phase 2 BOLD Study. *JAMA Neurol.* **2016**, *73*, 1089–1098. [CrossRef]
3. Kappos, L.; Bar-Or, A.; Cree, B.A.C.; Fox, R.J.; Giovannoni, G.; Gold, R.; Vermersch, P.; Arnold, D.L.; Arnould, S.; Scherz, T.; et al. Siponimod versus placebo in secondary progressive multiple sclerosis (EXPAND): A double-blind, randomised, phase 3 study. *Lancet* **2018**, *391*, 1263–1273. [CrossRef]
4. O'Sullivan, C.; Schubart, A.; Mir, A.K.; Dev, K.K. The dual S1PR1/S1PR5 drug BAF312 (Siponimod) attenuates demyelination in organotypic slice cultures. *J. Neuroinflamm.* **2016**, *13*, 31. [CrossRef] [PubMed]
5. Gentile, A.; Musella, A.; Bullitta, S.; Fresegna, D.; De Vito, F.; Fantozzi, R.; Piras, E.; Gargano, F.; Borsellino, G.; Battistini, L.; et al. Siponimod (BAF312) prevents synaptic neurodegeneration in experimental multiple sclerosis. *J. Neuroinflamm.* **2016**, *13*, 207. [CrossRef] [PubMed]
6. Kipp, M. Does Siponimod Exert Direct Effects in the Central Nervous System? *Cells* **2020**, *9*, 1771. [CrossRef] [PubMed]
7. Voet, S.; Prinz, M.; van Loo, G. Microglia in Central Nervous System Inflammation and Multiple Sclerosis Pathology. *Trends Mol. Med.* **2019**, *25*, 112–123. [CrossRef] [PubMed]
8. Karamita, M.; Barnum, C.; Mobius, W.; Tansey, M.G.; Szymkowski, D.E.; Lassmann, H.; Probert, L. Therapeutic inhibition of soluble brain TNF promotes remyelination by increasing myelin phagocytosis by microglia. *JCI Insight* **2017**, *2*, e87455. [CrossRef]
9. Tham, C.S.; Lin, F.F.; Rao, T.S.; Yu, N.; Webb, M. Microglial activation state and lysophospholipid acid receptor expression. *Int. J. Dev. Neurosci.* **2003**, *21*, 431–443. [CrossRef]
10. Orihuela, R.; McPherson, C.A.; Harry, G.J. Microglial M1/M2 polarization and metabolic states. *Br. J. Pharmacol.* **2016**, *173*, 649–665. [CrossRef]
11. Jack, C.S.; Arbour, N.; Manusow, J.; Montgrain, V.; Blain, M.; McCrea, E.; Shapiro, A.; Antel, J.P. TLR signaling tailors innate immune responses in human microglia and astrocytes. *J. Immunol.* **2005**, *175*, 4320–4330. [CrossRef] [PubMed]
12. Gardin, A.; Dodman, A.; Kalluri, S.; Neelakantham, S.; Tan, X.; Legangneux, E.; Shakeri-Nejad, K. Pharmacokinetics, safety, and tolerability of siponimod (BAF312) in subjects with severe renal impairment: A single-dose, open-label, parallel-group study. *Int. J. Clin. Pharmacol. Ther.* **2017**, *55*, 54–65. [CrossRef] [PubMed]
13. Bigaud, M.; Rudolph, B.; Briard, E.; Beerli, C.; Hofmann, A.; Hermes, E.; Muellershausen, F.; Schubart, A.; Gardin, A. Siponimod (BAF312) penetrates, distributes, and acts in the central nervous system: Preclinical insights. *Mult. Scler. J.-Exp. Transl. Clin.* **2021**, *7*, 20552173211049168. [CrossRef] [PubMed]
14. Abd-El-Basset, E.; Fedoroff, S. Effect of bacterial wall lipopolysaccharide (LPS) on morphology, motility, and cytoskeletal organization of microglia in cultures. *J. Neurosci. Res.* **1995**, *41*, 222–237. [CrossRef] [PubMed]
15. Smith, K.J.; Lassmann, H. The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* **2002**, *1*, 232–241. [CrossRef]
16. Tang, X.; Lan, M.; Zhang, M.; Yao, Z. Effect of nitric oxide to axonal degeneration in multiple sclerosis via downregulating monocarboxylate transporter 1 in oligodendrocytes. *Nitric Oxide* **2017**, *67*, 75–80. [CrossRef]
17. Gobel, K.; Ruck, T.; Meuth, S.G. Cytokine signaling in multiple sclerosis: Lost in translation. *Mult. Scler.* **2018**, *24*, 432–439. [CrossRef]
18. Tolosa, L.; Caraballo-Miralles, V.; Olmos, G.; Llado, J. TNF-alpha potentiates glutamate-induced spinal cord motoneuron death via NF-kappaB. *Mol. Cell. Neurosci.* **2011**, *46*, 176–186. [CrossRef] [PubMed]
19. Sharief, M.K.; Hentges, R. Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis. *N. Engl. J. Med.* **1991**, *325*, 467–472. [CrossRef] [PubMed]
20. Rossi, S.; Motta, C.; Studer, V.; Macchiarulo, G.; Volpe, E.; Barbieri, F.; Ruocco, G.; Buttari, F.; Finardi, A.; Mancino, R.; et al. Interleukin-1beta causes excitotoxic neurodegeneration and multiple sclerosis disease progression by activating the apoptotic protein p53. *Mol. Neurodegener.* **2014**, *9*, 56. [CrossRef]
21. Imitola, J.; Chitnis, T.; Khoury, S.J. Cytokines in multiple sclerosis: From bench to bedside. *Pharmacol. Ther.* **2005**, *106*, 163–177. [CrossRef]
22. Kwilasz, A.J.; Grace, P.M.; Serbedzija, P.; Maier, S.F.; Watkins, L.R. The therapeutic potential of interleukin-10 in neuroimmune diseases. *Neuropharmacology* **2015**, *96*, 55–69. [CrossRef] [PubMed]
23. Cua, D.J.; Hutchins, B.; LaFace, D.M.; Stohlman, S.A.; Coffman, R.L. Central nervous system expression of IL-10 inhibits autoimmune encephalomyelitis. *J. Immunol.* **2001**, *166*, 602–608. [CrossRef] [PubMed]
24. Bolivar, S.; Anfossi, R.; Humeres, C.; Vivar, R.; Boza, P.; Munoz, C.; Pardo-Jimenez, V.; Olivares-Silva, F.; Diaz-Araya, G. IFN-beta Plays Both Pro- and Anti-inflammatory Roles in the Rat Cardiac Fibroblast Through Differential STAT Protein Activation. *Front. Pharmacol.* **2018**, *9*, 1368. [CrossRef] [PubMed]
25. Noda, H.; Takeuchi, H.; Mizuno, T.; Suzumura, A. Fingolimod phosphate promotes the neuroprotective effects of microglia. *J. Neuroimmunol.* **2013**, *256*, 13–18. [CrossRef] [PubMed]

26. Jackson, S.J.; Giovannoni, G.; Baker, D. Fingolimod modulates microglial activation to augment markers of remyelination. *J. Neuroinflamm.* **2011**, *8*, 76. [CrossRef]
27. Sucksdorff, M.; Rissanen, E.; Tuisku, J.; Nuutinen, S.; Paavilainen, T.; Rokka, J.; Rinne, J.; Airas, L. Evaluation of the Effect of Fingolimod Treatment on Microglial Activation Using Serial PET Imaging in Multiple Sclerosis. *J. Nucl. Med.* **2017**, *58*, 1646–1651. [CrossRef]
28. Raffaele, S.; Lombardi, M.; Verderio, C.; Fumagalli, M. TNF Production and Release from Microglia via Extracellular Vesicles: Impact on Brain Functions. *Cells* **2020**, *9*, 2145. [CrossRef] [PubMed]
29. Zou, J.Y.; Crews, F.T. TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: Neuroprotection by NF kappa B inhibition. *Brain Res.* **2005**, *1034*, 11–24. [CrossRef]
30. Kollias, G.; Douni, E.; Kassiotis, G.; Kontoyiannis, D. The function of tumour necrosis factor and receptors in models of multi-organ inflammation, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. *Ann. Rheum. Dis.* **1999**, *58* (Suppl. S1), I32–I39. [CrossRef]
31. Bonora, M.; De Marchi, E.; Patergnani, S.; Suski, J.M.; Celsi, F.; Bononi, A.; Giorgi, C.; Marchi, S.; Rimessi, A.; Duszynski, J.; et al. Tumor necrosis factor-alpha impairs oligodendroglial differentiation through a mitochondria-dependent process. *Cell Death Differ.* **2014**, *21*, 1198–1208. [CrossRef] [PubMed]
32. Jurewicz, A.; Matysiak, M.; Tybor, K.; Selmaj, K. TNF-induced death of adult human oligodendrocytes is mediated by c-jun NH2-terminal kinase-3. *Brain* **2003**, *126*, 1358–1370. [CrossRef] [PubMed]
33. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. TNF neutralization in MS: Results of a randomized, placebo-controlled multicenter study. *Neurology* **1999**, *53*, 457–465. [CrossRef]
34. Ronchi, F.; Basso, C.; Preite, S.; Reboldi, A.; Baumjohann, D.; Perlini, L.; Lanzavecchia, A.; Sallusto, F. Experimental priming of encephalitogenic Th1/Th17 cells requires pertussis toxin-driven IL-1beta production by myeloid cells. *Nat. Commun.* **2016**, *7*, 11541. [CrossRef]
35. Argaw, A.T.; Zhang, Y.; Snyder, B.J.; Zhao, M.L.; Kopp, N.; Lee, S.C.; Raine, C.S.; Brosnan, C.F.; John, G.R. IL-1beta regulates blood-brain barrier permeability via reactivation of the hypoxia-angiogenesis program. *J. Immunol.* **2006**, *177*, 5574–5584. [CrossRef]
36. Hauser, S.L.; Doolittle, T.H.; Lincoln, R.; Brown, R.H.; Dinarello, C.A. Cytokine accumulations in CSF of multiple sclerosis patients: Frequent detection of interleukin-1 and tumor necrosis factor but not interleukin-6. *Neurology* **1990**, *40*, 1735–1739. [CrossRef]
37. Dujmovic, I.; Pekmezovic, T.; Obrenovic, R.; Nikolic, A.; Spasic, M.; Mostarica Stojkovic, M.; Drulovic, J. Cerebrospinal fluid and serum uric acid levels in patients with multiple sclerosis. *Clin. Chem. Lab. Med.* **2009**, *47*, 848–853. [CrossRef]
38. Lin, C.C.; Edelson, B.T. New Insights into the Role of IL-1beta in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis. *J. Immunol.* **2017**, *198*, 4553–4560. [CrossRef]
39. Jakimovski, D.; Kolb, C.; Ramanathan, M.; Zivadinov, R.; Weinstock-Guttman, B. Interferon beta for Multiple Sclerosis. *Cold Spring Harb. Perspect. Med.* **2018**, *8*, a032003. [CrossRef]
40. Guo, B.; Chang, E.Y.; Cheng, G. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. *J. Clin. Investig.* **2008**, *118*, 1680–1690. [CrossRef]
41. Prinz, M.; Schmidt, H.; Mildner, A.; Knobeloch, K.P.; Hanisch, U.K.; Raasch, J.; Merkler, D.; Detje, C.; Gutcher, I.; Mages, J.; et al. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. *Immunity* **2008**, *28*, 675–686. [CrossRef] [PubMed]
42. Kettenmann, H.; Hanisch, U.K.; Noda, M.; Verkhratsky, A. Physiology of microglia. *Physiol. Rev.* **2011**, *91*, 461–553. [CrossRef]
43. Ledeboer, A.; Breve, J.J.; Wierinckx, A.; van der Jagt, S.; Bristow, A.F.; Leysen, J.E.; Tilders, F.J.; Van Dam, A.M. Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglial and microglial cells. *Eur. J. Neurosci.* **2002**, *16*, 1175–1185. [CrossRef] [PubMed]
44. Holloway, R.K.; Ireland, G.; Sullivan, G.; Becher, J.C.; Smith, C.; Boardman, J.P.; Gressens, P.; Miron, V.E. Microglial inflammasome activation drives developmental white matter injury. *Glia* **2021**, *69*, 1268–1280. [CrossRef] [PubMed]
45. Rice, R.A.; Pham, J.; Lee, R.J.; Najafi, A.R.; West, B.L.; Green, K.N. Microglial repopulation resolves inflammation and promotes brain recovery after injury. *Glia* **2017**, *65*, 931–944. [CrossRef]
46. Clayton, K.; Delpech, J.C.; Herron, S.; Iwahara, N.; Ericsson, M.; Saito, T.; Saido, T.C.; Ikezu, S.; Ikezu, T. Plaque associated microglia hyper-secrete extracellular vesicles and accelerate tau propagation in a humanized APP mouse model. *Mol. Neurodegener.* **2021**, *16*, 18. [CrossRef]
47. Kovacs, M.; Trias, E.; Varela, V.; Ibarburu, S.; Beckman, J.S.; Moura, I.C.; Hermine, O.; King, P.H.; Si, Y.; Kwon, Y.; et al. CD34 Identifies a Subset of Proliferating Microglial Cells Associated with Degenerating Motor Neurons in ALS. *Int. J. Mol. Sci.* **2019**, *20*, 3880. [CrossRef]
48. Wu, X.B.; He, L.N.; Jiang, B.C.; Shi, H.; Bai, X.Q.; Zhang, W.W.; Gao, Y.J. Spinal CXCL9 and CXCL11 are not involved in neuropathic pain despite an upregulation in the spinal cord following spinal nerve injury. *Mol. Pain* **2018**, *14*, 1744806918777401. [CrossRef]
49. Mayo, L.; Jacob-Hirsch, J.; Amariglio, N.; Rechavi, G.; Moutin, M.J.; Lund, F.E.; Stein, R. Dual role of CD38 in microglial activation and activation-induced cell death. *J. Immunol.* **2008**, *181*, 92–103. [CrossRef]
50. Yu, S.; Cheng, L.; Tian, D.; Li, Z.; Yao, F.; Luo, Y.; Liu, Y.; Zhu, Z.; Zheng, M.; Jing, J. Fascin-1 is Highly Expressed Specifically in Microglia After Spinal Cord Injury and Regulates Microglial Migration. *Front. Pharmacol.* **2021**, *12*, 729524. [CrossRef]

51. Park, W.J.; Park, J.W. The role of sphingolipids in endoplasmic reticulum stress. *FEBS Lett.* **2020**, *594*, 3632–3651. [CrossRef] [PubMed]
52. Park, J.J.; Lee, J.H.; Li, Q.; Diaz, K.; Chang, Y.T.; Chung, S.K. Divergent syntheses of all stereoisomers of phytosphingosine and their use in the construction of a ceramide library. *Bioorganic Chem.* **2008**, *36*, 220–228. [CrossRef] [PubMed]
53. Plaza-Zabala, A.; Sierra-Torre, V.; Sierra, A. Assessing Autophagy in Microglia: A Two-Step Model to Determine Autophagosome Formation, Degradation, and Net Turnover. *Front. Immunol.* **2020**, *11*, 620602. [CrossRef] [PubMed]
54. Keren-Shaul, H.; Spinrad, A.; Weiner, A.; Matcovitch-Natan, O.; Dvir-Szternfeld, R.; Ulland, T.K.; 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*, 1276–1290.e17. [CrossRef]
55. Deczkowska, A.; Keren-Shaul, H.; Weiner, A.; Colonna, M.; Schwartz, M.; Amit, I. Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell* **2018**, *173*, 1073–1081. [CrossRef] [PubMed]
56. Tay, T.L.; Sagar; Dautzenberg, J.; Grun, D.; Prinz, M. Unique microglia recovery population revealed by single-cell RNAseq following neurodegeneration. *Acta Neuropathol. Commun.* **2018**, *6*, 87. [CrossRef]
57. Pulido-Salgado, M.; Vidal-Taboada, J.M.; Barriga, G.G.; Sola, C.; Saura, J. RNA-Seq transcriptomic profiling of primary murine microglia treated with LPS or LPS + IFNgamma. *Sci. Rep.* **2018**, *8*, 16096. [CrossRef]
58. Chen, Z.; Jalabi, W.; Shpargel, K.B.; Farabaugh, K.T.; Dutta, R.; Yin, X.; Kidd, G.J.; Bergmann, C.C.; Stohlman, S.A.; Trapp, B.D. Lipopolysaccharide-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4. *J. Neurosci.* **2012**, *32*, 11706–11715. [CrossRef]
59. Ye, X.; Zhu, M.; Che, X.; Wang, H.; Liang, X.J.; Wu, C.; Xue, X.; Yang, J. Lipopolysaccharide induces neuroinflammation in microglia by activating the MTOR pathway and downregulating Vps34 to inhibit autophagosome formation. *J. Neuroinflamm.* **2020**, *17*, 18. [CrossRef]
60. Lively, S.; Schlichter, L.C. Microglia Responses to Pro-inflammatory Stimuli (LPS, IFNgamma+TNFalpha) and Reprogramming by Resolving Cytokines (IL-4, IL-10). *Front. Cell. Neurosci.* **2018**, *12*, 215. [CrossRef]
61. Kremer, D.; Gruchot, J.; Weyers, V.; Oldemeier, L.; Göttle, P.; Healy, L.; Ho Jang, J.; Kang, T.X.Y.; Volsko, C.; Dutta, R.; et al. pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 15216–15225. [CrossRef] [PubMed]
62. Kremer, D.; Schichel, T.; Forster, M.; Tzekova, N.; Bernard, C.; van der Valk, P.; van Horssen, J.; Hartung, H.P.; Perron, H.; Küry, P. Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. *Ann. Neurol.* **2013**, *74*, 721–732. [CrossRef] [PubMed]
63. Kremer, D.; Forster, M.; Schichel, T.; Göttle, P.; Hartung, H.P.; Perron, H.; Küry, P. The neutralizing antibody GNbAC1 abrogates HERV-W envelope protein-mediated oligodendroglial maturation blockade. *Mult. Scler.* **2015**, *21*, 1200–1203. [CrossRef] [PubMed]

2.7 Interplay between activation of endogenous retroviruses and inflammation as common pathogenic mechanism in neurological and psychiatric disorders

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Abstract

Human endogenous retroviruses (ERVs) are ancestorial retroviral elements that were integrated into our genome through germline infections and insertions during evolution. They have repeatedly been implicated in the aetiology and pathophysiology of numerous human disorders, particularly in those that affect the central nervous system. In addition to the known association of ERVs with multiple sclerosis and amyotrophic lateral sclerosis, a growing number of studies links the induction and expression of these retroviral elements with the onset and severity of neurodevelopmental and psychiatric disorders. Although these disorders differ in terms of overall disease pathology and causalities, a certain degree of (subclinical) chronic inflammation can be identified in all of them. Based on these commonalities, we discuss the bidirectional relationship between ERV expression and inflammation and highlight that numerous entry points to this reciprocal sequence of events exist, including initial infections with ERV-activating pathogens, exposure to non-infectious inflammatory stimuli, and conditions in which epigenetic silencing of ERV elements is disrupted.

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Interplay between activation of endogenous retroviruses and inflammation as common pathogenic mechanism in neurological and psychiatric disorders

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ABSTRACT

Human endogenous retroviruses (ERVs) are ancestorial retroviral elements that were integrated into our genome through germline infections and insertions during evolution. They have repeatedly been implicated in the aetiology and pathophysiology of numerous human disorders, particularly in those that affect the central nervous system. In addition to the known association of ERVs with multiple sclerosis and amyotrophic lateral sclerosis, a growing number of studies links the induction and expression of these retroviral elements with the onset and severity of neurodevelopmental and psychiatric disorders. Although these disorders differ in terms of overall disease pathology and causalities, a certain degree of (subclinical) chronic inflammation can be identified in all of them. Based on these commonalities, we discuss the bidirectional relationship between ERV expression and inflammation and highlight that numerous entry points to this reciprocal sequence of events exist, including initial infections with ERV-activating pathogens, exposure to non-infectious inflammatory stimuli, and conditions in which epigenetic silencing of ERV elements is disrupted.

1. Introduction

Endogenous retroviruses (ERVs) are inherited genetic elements derived from exogenous retroviral infections occurring throughout the evolution of the genome. In general, ERVs belong to a retrotransposon subgroup of mobile genomic elements and comprise 5–8 % of the human genome (Lander et al., 2001). In other mammalian genomes, ERVs are similarly abundant and comprise, for example, approximately 10 % of the mouse genome (Stocking & Kozak, 2008). It is thought that multiple independent infectious events generated a unique genomic ERV content in different species, with additional genetic recombination leading to more than 100.000 ERV loci known in humans with extensive interindividual variations (Nellaker et al., 2012; Thomas et al., 2018).

ERVs are traditionally classified into three classes (I, II and III), based on relatedness to the exogenous *Gammaretrovirus*, *Betaretrovirus* and *Spumaretrovirus*, respectively. Within this classification individual ERV lineages are referred to as "families", and comprise groups of ERVs that are assumed to derive from a single germline invasion event (Gifford et al., 2018).

While human ERVs are often regarded as genomic parasites, their ancestorial embedding in our genomes suggests a certain degree of domestication and symbiosis (Küry et al., 2018). An illustrative example of positive evolutionary selection are syncytin 1 and 2, which represent envelope (Env) genes of the ERVW-1 and ERVFRD-1, respectively. The encoded proteins play an important role in placentogenesis and might also be involved in foetal-maternal immune tolerance (Xiang & Liang, 2021). A similar functional domestication emerged for ERV-encoded group specific antigens (GAGs), some of which pertain to key processes of memory consolidation in the mammalian brain, including long-term potentiation and long-term depression (Pastuzyn et al., 2018).

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Whereas most human ERVs (HERVs) appear to be inherently inactivated, multiple studies revealed activation of some of these elements in neurodevelopmental disorders such as autism spectrum disorder (ASD) or attention deficit hyperactivity disorder (ADHD), psychiatric disorders such as schizophrenia (SZ) and bipolar disorder (BD), and neuroinflammatory/neurodegenerative disorders such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). While all these clinical conditions differ in terms of overall disease pathology and causalities, a common feature is that they are all associated with different degrees of (subclinical) chronic inflammation. Based on these commonalities, we aim at discussing the potential relationships between ERV activation and expression along the induction of inflammatory processes, thereby examining whether a direct sequence of events can be deduced.

2. Human endogenous retroviruses as a central element of neurodevelopmental, psychiatric and neuroinflammatory disorders

2.1. Neurodevelopmental disorders

Autism spectrum disorder (ASD) was one of the first neurodevelopmental disorders in which abnormal retroviral activity was identified. It is a pervasive developmental disorder, affecting 1 in every 160 children worldwide, characterized by three main core symptoms, namely impairments in social interaction, deficits in verbal and nonverbal communication, and presence of restricted and repetitive behaviours (Santos et al., 2022). It was shown to involve immunological dysfunction with several ASD risk genes encoding components of the immune system (Meltzer & Van de Water, 2017). Signs of microglia activation and increased production of inflammatory cytokines and chemokines, including interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-12p40, tumor necrosis factor-α (TNF-α) and chemokine C—C motif ligand (CCL)-2, in the brain parenchyma and cerebral spinal fluid (CSF) were described (Onore et al., 2012). Furthermore, elevated levels of pro-inflammatory cytokines in plasma were identified in medication-free ASD patients from the ages of 2 to 5 compared to age-matched, normally developing and healthy control children and to children with other developmental disabilities (Ashwood et al., 2011). The overproduction of peripheral cytokines in ASD children was further associated with impaired communication skills and aberrant behaviours (Ashwood et al., 2011).

Env gene expression of HERV-H, HERV-K, HERV-W and HEMO (different subtypes of human ERVs) was investigated in peripheral blood mononuclear cells (PBMCs) from ASD children, their parents and from corresponding healthy controls. ASD patients showed significantly higher HERV-H Env transcript levels as compared to healthy children (Balestrieri et al., 2019). Intriguingly, PBMCs from mothers of ASD children also showed significantly higher levels of HERV-H Env transcripts in comparison to mothers of the control group (Balestrieri et al., 2019). Similar findings were obtained for HERV-K and HEMO Env gene expression. On the contrary, the transcriptional activity of HERV-W Env was significantly lower in ASD children as compared to healthy controls, but significantly higher in their mothers and fathers compared to the corresponding control group (Balestrieri et al., 2019).

Abnormally high expression levels of ERV components were also reported in two distinct mouse models of ASD (Cipriani et al., 2018b). The first mouse model involved BTBR T + tf/J inbred mice, which corresponds to an idiopathic ASD model capturing several ASD-related behavioural traits, including impairments in social interaction, communication, and cognitive flexibility, as well as high levels of repetitive behaviours. The second model was based on CD-1 outbred mice which were prenatally treated with the anticonvulsant and histone deacetylase inhibitor valproic acid (VPA). These animals show ASD-like behavioural alterations, including early motor hyperactivity, social deficits, and cognitive impairments. Both models showed consistently increased transcriptional activity of several ERV families in whole embryos, as well as in postnatal blood and brain samples (Cipriani et al., 2018b). Moreover, expression levels of pro-inflammatory cytokines and toll like receptors (TLRs) were also significantly elevated after prenatal VPA treatment (Cipriani et al., 2018b).

ERVs also seem to be implicated in attention deficit hyperactivity disorder (ADHD), a neurodevelopmental condition usually detected before the onset of late adolescence or early adulthood. ADHD is characterized to a varying degree by difficulties in maintaining sustained attention and executive functions, motor hyperactivity, and impulsivity (American Psychiatric Association, 2013). As the majority of children with ADHD have a high prevalence of allergic diseases, it is likely that immune- and inflammation responses are involved in the aetiology of this disorder (Tsai et al., 2013). When PBMCs of 30 subjects with ADHD and 30 healthy controls were analysed, increased expression of HERV-H was found in PBMCs of ADHD subjects, with Env transcript levels correlating positively with inattention and hyperactivity (Balestrieri et al., 2014). Furthermore, drug-naive ADHD patients showed a reduction in HERV-H Env mRNA levels in response to administration of methylphenidate, a commonly used drug for treating ADHD symptoms (Cipriani et al., 2018a).

2.2. Psychiatric disorders

Abnormal ERV expression was also identified in schizophrenia (SZ), a major psychiatric disorder affecting up to 1 % of the world's population (Charlson et al., 2018). It is characterized by varying degrees of cognitive impairments, emotional aberrations, and behavioural anomalies, which together undermine basic processes of perception, reasoning, and judgment. Typically, the onset of full-blown SZ is in early adulthood and includes a myriad of symptoms. These symptoms can be referred to as positive symptoms (e.g. visual and/or auditory hallucinations, delusions, paranoia, psychomotor agitation), negative symptoms (e.g. social withdrawal, apathy, deficits in motivation and reward-related functions), and cognitive symptoms (e.g. deficits in executive functioning, working memory, and attention) (Owen et al., 2016).

Increasing evidence suggests that the immune system is involved in the pathogenesis and pathophysiology of SZ. Support for this notion includes epidemiological findings of increased risk of SZ following early-life exposure to infectious pathogens or inflammatory stimuli (Brown & Meyer, 2018), along with post-mortem and imaging studies demonstrating glial anomalies and increased expression of cytokines and other mediators of inflammation in the brain and periphery in people with SZ (Miller et al., 2011; Trepanier et al., 2016). Noticeable inflammatory abnormalities, however, are evident only in a subgroup of SZ cases (Fillman et al., 2013; Fillman et al., 2016; Purves-Tyson et al., 2021) and may predict poorer clinical outcomes and treatment responses (Hoang et al., 2022; Mondelli et al., 2015).

Furthermore, it was recently demonstrated that patients with SZ or bipolar disorder (BD) can be stratified into subgroups with differing inflammatory and clinical profiles based on HERV-W Env protein antigenemia and cytokines (Tamouza et al., 2021). In this study, two main clusters of patients were identified which were best predicted by the presence or absence of the HERV-W Env protein. HERV-W expression was associated with increased serum levels of inflammatory cytokines and higher childhood maltreatment scores. Furthermore, patients with SZ expressing the HERV-W Env protein showed more manic symptoms and higher daily chlorpromazine equivalents. These findings add to a previous study identifying retroviral polymerase gene sequences of the HERV-W family in the CSF of 29 % of individuals with recent-onset SZ or schizoaffective disorder (Karlsson et al., 2001). Transcripts from HERV-W family genes were also found to be upregulated in the frontal cortex of brains from individuals with SZ (Karlsson et al., 2001). A more recent publication found that patients with first-episode psychosis displayed lower levels DNA methylation at HERV-K loci, whereas chronic patients with SZ did not differ from matched controls with regards to HERV-H methylation (Mak et al., 2019). In addition, it was found that HERV-K

methylation levels correlated positively with the chlorpromazine equivalents, indicating that antipsychotic medications may contribute to the normalization of aberrant HERV-H methylation patterns along the clinical course of schizophrenia (Mak et al., 2019).

Abnormal ERV expression was found in BD, a heterogeneous psychiatric disorder characterized by fluctuating symptoms involving episodes of mania and depression and intermittent periods of euthymia. The number of episodes and duration of each state varies markedly between individuals. Manic episodes typically include a reduced need for sleep, increased energy, rapid speech, increased libido, reckless behaviour, grandiose thoughts, and elevations in mood. In severe episodes, psychotic symptoms such as delusions and hallucinations may also be present. While the precise etiopathology of BD is still ill-defined (Harrison et al., 2018), several studies indicate that it involves changes in the innate and adaptive immune system including inflammation. For example, patients with BD often show increased serum concentrations of interleukins and C-reactive protein (CRP), a protein which rises in response to inflammation. Moreover, inflammatory alterations have been detected in the brain parenchyma of patients with BD (Harrison et al., 2018). Similarly to SZ, HERV-W transcripts and proteins were repeatedly found to be elevated in the blood, CSF and brains of patients with BD (Li et al., 2019; Perron et al., 2012; Tamouza et al., 2021). Intriguingly, a recent study showed that patients with BD who were positive for HERV-W Env protein had increased serum levels of IL-1β and an earlier disease onset as compared to patients were negative for HERV-W Env protein (Tamouza et al., 2021), suggesting that differential HERV-W activity may define distinct subgroups in bipolar disorder.

2.3. Neurological disorders

Human ERVs have long been speculated to be involved in the aetiology and pathophysiology of neurological disorders, especially in multiple sclerosis (MS) (Perron et al., 1989). MS is characterized by a primary attack to oligodendrocytes, the myelinating glial cells of the central nervous system (CNS), and by the subsequent demyelination of axons and axonal degeneration. These neurodegenerative processes eventually result in irreversible loss of sensory, motor, and cognitive functions. Infiltrating lymphocytes, monocytes/macrophages, activated astrocytes and microglia represent some of the key pathological features of MS, whereas their contribution varies between acute, relapsing/remitting and chronic, progressive disease stages (Matthews, 2019). Nevertheless, despite the plethora of studies conducted over the last decades, the precise aetiology of MS remains elusive.

Since the initial discovery of retroviral elements in the leptomenigeal cells of MS patients (Perron et al., 1989), MS has been repeatedly associated with human retroviral elements in general, and with the HERV-W Env gene in particular. These associations further involved pro-inflammatory effects on innate and adaptive immune cells, impacts on endothelial cells of the blood–brain-barrier (BBB), impaired regenerative responses of remyelinating oligodendroglial precursor cells, as well as activation and polarisation of microglial towards an axon-damaging phenotype (Küry et al., 2018). Hence, there is converging evidence suggesting a broad impact of ERV activation and expression in the development and progression of MS. A pathological involvement of the HERV-W Env protein in the aetiology of MS has recently been confirmed by a clinical trial, in which a therapeutic Env-neutralizing antibody was administered to MS patients. The clinical outcome revealed reduced brain atrophy rates as well as stabilized radiological markers of white matter integrity thus an impact on neurodegeneration as well as on repair activities (Hartung et al., 2022).

Following the thread of neurological disease associated with the expression of human ERVs, we find amyotrophic lateral sclerosis (ALS) with an estimated prevalence of 4 to 8 cases per 100.000 persons (Longinetti & Fang, 2019). This disease is characterized by a progressive loss of cortical and spinal motor neurons, resulting in motor dysfunction and motor cortex volume loss (Li et al., 2015a; Mathis et al., 2017). On the immunological aspect, ALS involves microglia and astrocyte activation as well as T cell infiltration and inflammatory cytokine overproduction (Liu and Wang, 2017).

A study conducted among 23 ALS patients and 21 patients suffering from other neurological diseases serving as controls, found the presence of reverse transcriptase (RT) activity in the sera of 56 % of the ALS patients versus in 19 % of the control group (MacGowan et al., 2007). It was furthermore observed that non-symptomatic blood relatives of ALS patients also had increased RT activity in comparison to the ALS patient's spouses, which served as control group (Steele et al., 2005). These findings thus support the notion that the RT proteins derive from an endogenous origin rather than from an exogenous viral infection. Furthermore, all major genetic components of the HERV-K genome, i.e., Gag, Pol and Env, were found to be elevated in post-mortem brain samples from ALS patients (Li et al., 2015b). While the detection and involvement of HERV-K in ALS is still debated, studies on cultured neurons showed that both, the entire genome and the Env gene caused a similar decrease in cell numbers and retraction of neurites in a dose-dependent manner. Likewise, transgenic overexpression of the HERV-K Env protein in motor neurons provided strong evidence that this leads to pathological hallmarks of ALS, including progressive motor dysfunction and motor cortex volume loss (Li et al., 2015b). Moreover, 24 weeks of antiretroviral therapy on 29 ALS patients resulted in a progressive decrease of copy numbers of HML-2 (subtype of HERV-K) and the majority of participants (82 %) revealed to be "responders". Among the responders, respiratory function and motor neuron dysfunction was ameliorated, indicating a slower progression of the disease. This outcome supports a possible role for HERV-K in the clinical course of the disease (Garcia-Montojo et al., 2021).

Taking together, an intricate interaction between endogenous retroviral elements and inflammation-related immune responses appear to play a role in the onset and/or progression of these neurological conditions. However, it is currently unclear to what degree endogenous retroviruses act in a unified way in all these disorder and whether inflammation acts as a trigger of ERV activation or, the other way round, whether immune processes are specifically initiated and supported by ERVs. These questions are addresses in the subsequent section, where the relationship between epigenetic control mechanisms, ERV expression and inflammation are discussed in more detail.

3. (Re-)Awakening of endogenous retroviruses

Under physiological conditions, the majority of endogenous retroviruses, particularly those with primarily pathological functions, are thought to be in a dormant state and suppressed by molecular mechanisms of epigenetic silencing. Upon certain events, however, such epigenetic repression can break down, leading to a (re)activation of these retroviral entities, possibly initiating disease onsets and/or or accelerating disease progression. We therefore aim at summarizing the current knowledge regarding the epigenetic mechanisms that maintain repression and facilitate activation of pathological ERVs.

Based on the current state of evidence, it appears that various epigenetic processes are relevant for controlling ERV expression, including localisation of proviruses in the heterochromatin, blocking long terminal repeat (LTR) access, CpG methylation and histone deacetylation. The predominant view is that these epigenetic processes assure overall ERV silencing, whereas small leakages at transcription levels can still occur (Leung & Lorincz, 2012).

Most CpG islands are found to be methylated throughout the human genome, including those encompassing ERVs (CGIs) (Deaton et al., 2011). In this regard, a genome-wide microarray approach identified human ERV families to be heavily methylated in healthy tissues (Szpakowski et al., 2009). Furthermore, differences in silencing modes were associated with the evolutionary age of ERV insertions. Indeed, evolutionary "young" LTRs are CpG rich and amenable to DNA methylation, whereas the expression of evolutionary "old" ERVs appear to be

controlled mostly via histone modifications (Ohtani et al., 2018). This distinction was confirmed by a study showing a correlation between HERV-K (HML-2) 5' LTR methylation and transcriptional suppression of its provirus in the Tera-1 cell line (Lavie et al., 2005).

Histone acetylation blocks the positive charges on lysine residues, which destabilises chromatin and favours transcriptional activation. Thus, histone deacetylation represents another epigenetic silencing mechanism with relevance to ERVs (Bannister & Kouzarides, 2011). Acetylation of lysine residues in histones is catalysed by histone acetyltransferases (HATs) and counteracted by histone deacetylases (HDACs). As the use of HDAC inhibitors alone did not significantly induce ERV expression in humans (HERV-K [HML-2], HERV-W, HERV-FRD) in cell lines with the dormant HIV-1 virus or primary T cells infected with HIV-1 (Hurst et al., 2016), it is unlikely that histone deacetylation alone may be the primary epigenetic mechanism underling ERV repression, but it nevertheless may act in combination with CpG methylation to do so. This hypothesis is supported by the observation that the combination of the HDAC inhibitor trichostatin A and the DNA methylation inhibitor 5′-azacytidine increased HERV-Fc1 expression in human embryonic kidney cells, whereas trichostatin A alone did not (Laska et al., 2012).

In addition to histone acetylation, histone methylation appears to be another epigenetic mechanism relevant for controlling ERV expression. In support of this notion, an enrichment of repressive histone marks such as trimethylation of histone 3 lysine 9 (H3K9) or H4K20 was described for mouse ERVs (Day et al., 2010; Mikkelsen et al., 2007) and for HERV-K (Campos-Sánchez et al., 2016). Furthermore, during early embryonic development, Krüppel-associated box zinc finger proteins (KRAB-ZFP) are critical for establishing and maintaining histone methylation and heterochromatin formation. Human KRAB-ZFP binding sites are highly concentrated within transposons, mainly retrotransposons, including human ERVs. Most of these transposable elements have lost their transposable potential, indicating that the KRAB-ZFP has silenced them by curbing them in heterochromatin (Imbeault et al., 2017; Thomas & Schneider, 2011). Of note, LTR-containing retrotransposons seemed to have co-evolved with KRAB-ZFP genes, as the integration of each family of human ERVs coincided with a new KRAB-ZFP (Lukic et al., 2014; Thomas & Schneider, 2011). Binding of KRAB-ZFP to chromatin leads to the recruitment of other proteins via the KRAB domain, forming larger protein complexes that modify histones (Thomas & Schneider, 2011). This includes the scaffold protein TRIM28/KAP1, DNA methyltransferases (DNMT)-1 and DNMT3a/b, as well as the histone lysine methyltransferase SETDB1. The latter was found to be critical for global repression of ERVs, as supported by findings in SETDB1 knock-out mice showing increased ERV expression in B-lymphocytes as compared to wild-type mice (Collins et al., 2015).

Finally, nucleosome positioning has been hypothesised by some authors to regulate ERV transcription (Fuchs et al., 2011). The binding of transcription factors, specificity protein (Sp)1 and Sp3 Sp1 to the LTR, would free the transcription starting sites from nucleosomes, allowing the genetic expression. HERV-K (HML-2) sequences were described to lack the classical TATA box element of common RNA polymerase II promoters, necessary for transcriptional initiation. These authors found that HERV-K LTR sequences contain alternative transcription starting sites. They showed that Sp1 and Sp3 have three binding sites within the LTRs of HERV-K proviruses and when knocked down, the promoter activity was significantly reduced (Fuchs et al., 2011).

Thus, while various molecular mechanisms mediating epigenetic silencing of ERVs have been identified, there are also several processes that can re-awake these retroviral elements from their dormant state. For example, ultraviolet (UV) radiation exposure, which is known to be associated with epigenetic modifications such as alterations in DNA methylation, DNA methyltransferase activities and histone acetylation, was shown to lead to transcriptional activation of the HERV-K pol gene as well as to enhance expression of Env protein in melanoma cells (Schanab et al., 2011). Likewise, several nutritional factors have been shown to affect human ERV repression. One example is B-carotene, which was shown to increase DNA methylation of HERV-W (Bollati et al., 2014). Furthermore, vitamin C was identified to be an important cofactor for DNA methyltransferase inhibitors (DNMTis) in treating neoplasia. Combined application of DNMTi and vitamin C resulted in diminished ERV DNA methylation and subsequent increases in ERV expression (Liu et al., 2016).

In addition, certain drugs are known to modulate the silencing of ERVs by acting on epigenetic regulators. HDAC inhibitors such as valproic acid (VPA) have extensively been used in the treatment of neurological and psychiatric disorders, and in this context, an upregulation of several class I and class II human ERV elements by VPA in a dose-dependent manner was described in brain cell lines (Diem et al., 2012). Moreover, upregulation of HERV-W and ERV9 transcription was detected in post-mortem brains of schizophrenic patients that were undergoing chronic VPA treatment (Diem et al., 2012). The HDAC inhibitor vorinostat was also found to modulate ERV expression. Elements belonging to the ERV-L and HERV-9 families were found to be predominantly down- and upregulated, respectively in $CD4^+$ T cells, after vorinostat treatment (White et al., 2018). Furthermore, treatment with the DNMTi 5-aza-deoxycytidine was found to increase HERV-E mRNA expression in CD4 + T cells in patients with systemic lupus erythematosus (Wu et al., 2015). Altered DNA methylation has also been associated in human leukaemia cell lines and hematopoietic stem cells upon decitabine and hydroquinone exposure, resulting in elevated ERV expression (Conti et al., 2016).

A couple of recent publications have also shed light onto a different mechanism involving the histone variant H3.3 for the (re)awakening of ERVs. They showed that the loss of the histone variant H3.3 leads to a reduction of suppressing H3K9me3 marks at ERV elements. This in turn would open up binding sites for the interferon regulatory factor family of transcription factors (Guo et al., 2022). Furthermore, another publication described that the H3.3 chaperone death-associated protein 6 (Daxx), alpha-thalassaemia X-linked mental retardation (Atrx) lead to derepression of ERVs via histone acetylation and/or methylation (Gerber et al., 2021).

Of particular interest is the capability of viruses to change the epigenetic landscape of host cells to ensure proper replication (summarized in (Tsai & Cullen, 2020)). Importantly, some viral infections represent a risk factor in the development of certain HERV-associated diseases, including for example Epstein bar virus (EBV) and human *Herpesviridae* (HHV)-6 and herpes simplex viruses (HSV)-1 in MS (Römer, 2021). Thus, viral exposures could be one of the environmental factors linking altered ERV activity/expression to neurological and psychiatric disorders. In support of this notion, exposure of B cells to EBV was found to cause a genome-wide activation of LTR sequences (Leung et al., 2018) in these cells. The EBV-mediated activation of LTRs further coincided with local DNA hypomethylation (Leung et al., 2018). Moreover, EBV infection is thought to change host epigenetics on the long-term, thereby counteracting the immune reaction and further unlocking endogenous retroviral elements (Buschle & Hammerschmidt, 2020). Similar observations were made upon infection of primary fibroblast cells with influenza A virus, which led to the transactivation of the Env gene in the HERV-W locus ERVWE1 (Li et al., 2014). This induction was likely triggered by an in infection-mediated decrease in the repressive histone mark H3K9me3 as well as by lowered SETDB1 expression (Li et al., 2014).

Taken together, there is strong evidence that endogenous retroviral elements need to be epigenetically unlocked before they can be activated. Several environmental factors, including infections, nutrition, and certain drugs are thought to play key roles in the process of epigenetic unlocking. However, these processes are only partially understood to date, such that additional longitudinal studies are warranted to decipher the temporal sequences of molecular events acting on inserted viral elements and leading to their release – prior or concomitant to disease development.

4. Activation of ERV expression

While epigenetic de-repression of endogenous retroviral elements itself can already induce mild expression levels, epigenetic mechanisms alone do not explain why ERVs are highly expressed in certain disorders. Thus, abnormally high ERV expression in some pathological contexts is likely to be the result of intricate interactions between epigenetic de-repression and other factors. As discussed below, various environmental factors, such as microorganisms, nutrients, and stress, as well as intrinsic components, such as cytokines and hormones, have been identified to act on ERV expression. For example, human ERV expression is known to be modifiable by hormones, both under physiological and pathological conditions. In females, basal HERV-K fluctuates as a function of the menstrual cycle, suggesting a regulation of HERV-K by the sex hormones progesterone and estradiol (Mueller et al., 2018). These findings are corroborated by the recent publication showing that progesterone and estradiol synergistically activate HERV-K involving binding of progesterone receptor and the octamer-binding transcription factor 4 (OCT4) to HERV-K LTRs (Nguyen et al., 2019).

While in the context of ALS strong evidence support the TDP43 protein as activator of HERV-K expression (Li et al., 2015b) there is also converging evidence supporting a direct impact of systemic inflammation on ERV expression, likely also via acting on flanking LTR sequences (Kovalskaya et al., 2006). LTR sequences feature strong gene regulatory sequences and contain many binding sites for transcription factors, including sites for the pro-inflammatory nuclear factor kappa light chain enhancer of activated B-cells (NF-κB) (Manghera & Douville, 2013; Thompson et al., 2016). Given that NF-κB regulates various aspects of innate and adaptive immunity and can be activated by numerous pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IFNγ, binding of NF-κB to flanking LTR sequences may readily provide a direct mode of action of how inflammation can drive human ERV transcription (Liu and Wang, 2017). In this context, IFNγ was shown to induce the expression of the HERV-K Gag-Pro-Pol polyprotein as well as of the reverse transcriptase in human astrocytes and neurons (Manghera et al., 2015). In the same cells, however, TNFα was found to induce HERV-K transcription through interferon regulatory factor-1 (IRF1) and NF-κB binding to the interferon-stimulated response elements (ISRE) (Manghera et al., 2016). Similarly, exposure to TNFα, IFNγ, IL-6 or IL-1 was shown to boost the activity of the ERVWE1/syncytin promoter via NF-κB in human U-87MG astrocytes (Mameli et al., 2007). Moreover, TNFα also appears to shift the open reading frame of the HERV-K Env gene, thereby giving rise to the conotoxin-like protein (CTXLP). This protein is similar to the neurotoxic conotoxin protein of marine snails and, more importantly bears similarities to the human immunodeficiency virus (HIV) tat protein. CTXLP can act in a positive feedback loop via binding to ISREs within the HERV-K promoter, thereby further stimulating its expression. CTXLP was also demonstrated to enhance nuclear NF-κB p65 expression, which then tunes into HERV-K transcription (Curzio et al., 2020).

Of note, a recent transcriptome study supports the notion that inflammation leads to ERV induction in humans, as revealed by correlations between the expression of various endogenous retroviruses with different injuries such burn, trauma and septic shock (Mommert et al., 2020; Tabone et al., 2018). Whereas none of these injuries are primarily associated with the common risk factors for developing neurological disorders, all of them are associated with a strong inflammatory reaction and a concomitant upregulation of at least five different human ERVs. Additional evidence supporting a primary role of inflammation in stimulating ERV transcription can be obtained from a clinical investigation using PBMCs derived from ADHD children. As outlined above, children with ADHD who were treated with methylphenidate displayed decreased HERV-H expression in PBMCs (Cipriani et al., 2018a). Notably, ex vivo induction of HERV-H in drug-naïve PBMCs was then shown to occur in response to a T cell activation cocktail, containing IL-2 and phytohemagglutinin, but was not observed in PBMCs from drug-treated ADHD children and healthy controls (Cipriani et al., 2018a).

Besides inflammatory responses, infections with viruses, the intestinal microbiota, and protozoans can also modulate ERV expression (Küry et al., 2018). Indeed, numerous viral infections, including HIV-1, the *Herpesviridae* HSV-1, HHV6 and EBV as well as SARS-CoV2, have repeatedly shown to directly induce the transcription of endogenous retroviral elements (summarized in (Küry et al., 2018), see also (Balestrieri et al., 2021)).

One of these mentioned viral infections is the EBV infection. We previously mentioned that EBV is likely to overcome the epigenetic barriers and change the host epigenetic landscape on the long term, resulting in the evasion of the immune system. *In vitro* studies showed EBV glycoprotein 350 (EBVgp350) induction of the HERV-W Env expression in astrocytes, B cells and monocytes of MS patients. This process showed to be NF-κB signalling dependent (Mameli et al., 2012). This finding was further corroborated by a clinical study showing increased HERV-W expression in patients with EBV dependent mononucleosis (Mameli et al., 2013). Recent reports have reinforced the causal involvement of EBV in MS aetiology (Bjornevik et al., 2022; Lanz et al., 2022), additionally supporting an active participation of HERV-W in the development and progression of MS. Similar effects are shown with respect to HERV-K18. EBV latent membrane proteins 1 and 2A, but also EBV itself, through interaction with its cellular receptor complement receptor 2 (CD21) induce HERV-K18 expression in resting B lymphocytes (Hsiao et al., 2006; Sutkowski et al., 2001).

Following human herpes virus (HHV) 6A infection, HERV-W Env expression is induced through the transmembrane glycoprotein CD46, while no induction was observed upon exposure to HHV6B or the measles virus vaccine strain (Charvet et al., 2018). On the other hand, both subtypes HHV6A and HHV6B were found to activate HERV-K18 in B cells and PBMCs, respectively (Tai et al., 2009; Turcanova et al., 2009). In this context, a recent publication discusses accumulating evidence supporting the view that EBV, HHV6 and HERV-W can influence each other, eventually leading to dysregulation of the immune response (summarized in (Meier et al., 2021)).

Although it has been proposed that ERV-inducing viruses act via increasing the affinity of transcription factors to LTR binding sites (Manghera & Douville, 2013), it is important to point out that the NF-κB signalling pathway can also be directly activated by viral infections (Santoro et al., 2003). Hence, a direct induction of ERV expression by viral infections is indistinguishable from an indirect activation through inflammatory i.e., NF-κB signalling pathways in terms of its end product. Furthermore, most of available studies were correlative in nature, and therefore, they fall short in answering the question whether infectious agents exert direct effects on ERV expression, or whether these effects are indirectly mediated by pro-inflammatory pathways and/or epigenetic unlocking processes discussed above. Indeed, because systemic inflammation and viral infections share similar signalling pathways, it is difficult to distinguish temporally between those two ERV effectors. Moreover, none of the available studies ascertained the epigenetic *status quo*. Therefore, for ERV activation to occur in response to infection and/or inflammation, it remains unknown whether prior epigenetic de-repression is a necessary step in order to turn cells susceptible to ERV responses.

It is also worth considering that the activation of ERVs could also be beneficial in some conditions. A certain degree of ERV expression could potentially contribute to inherent host defence mechanism by inducing resistance against superinfections (Villarreal, 2011). Indeed, such beneficial effects of ERVs may arise because endogenous and exogenous entities reveal high similarities in their protein and nucleic acid sequences (Grandi & Tramontano, 2017) and/or because ERV proteins might interact with the same receptors as the exogenous viral proteins (Spencer et al., 2003). This might also explain why ERV sequences have survived the evolutionary purge.

5. Induction of inflammation-related processes by ERVs

While inflammation is one of the factors that can stimulate ERV expression, endogenous retroviral proteins can induce inflammatory responses in different cell types as well. Hence, ERVs themselves appear to have pro-inflammatory effects. One of the first studies supporting this hypothesis demonstrated that the HERV-W Env protein is capable of activating the TLR4 pathway in human monocytes (Rolland et al., 2006). The authors also revealed that dendritic cells were similarly triggered by HERV-W Env protein, leading to the promotion of Th1 differentiation. Additional evidence for the involvement of TLR4 signalling after HERV-W Env protein exposure was then provided using genetically modified HEK-Blue cells (Charvet et al., 2018). A similar pro-inflammatory polarisation was also shown for primary human and rat microglia, which displayed elevated pro-inflammatory cytokine and chemokine production as well as nitric oxide levels after exposure to HERV-W Env protein. This polarisation was further associated with an axon-damaging microglial phenotype (Kremer et al., 2019). Moreover, Env protein activated microglial cells were also shown to mediate synaptic NMDA receptor dispersal – a molecular process associated with psychosis (Johansson et al., 2020).

Oligodendroglial precursors are cells with generally low immunocompetence (Kremer et al., 2010) yet they are critically involved in the MS pathology. They can provide replacement of lost oligodendrocytes and myelin sheaths – cells and structures that represent primary targets of the autoimmune reaction in MS – hence they represent one of the few regeneration conferring cells of the adult CNS. HERV-W Env protein stimulation of TLR4 was shown to promote nitrosative stress generation in these glial cells, leading to an impaired differentiation reaction and reduced axonal myelination. It was therefore suggested that endogenous retrovirus activation is restricting naturally occurring repair activities (Kremer et al., 2013). Furthermore, HERV-W Env inhibition by its neutralizing GNbAC1/Temelimab antibody, as well as TLR4 blockage by different pharmaceutical TLR4 inhibitors reduced the Env-mediated effects, suggesting that they were indeed dependent on Env and TLR4 (Göttle et al., 2019; Göttle et al., 2021; Kremer et al., 2015). Of note, an involvement of HERV-W Env in microglial axon damage as well in constraining myelin regeneration was later supported indirectly by the clinical examination of the anti-Env antibody GNbAC1/Temelimab (Hartung et al., 2022), adding up to the numerous effects of HERV-W in the context of MS the observation of an inflammatory response in endothelial cells upon stimulation with HERV-W Env protein. Furthermore, a weakening of the BBB was suggested, as intercellular adhesion molecule (ICAM)-1 was induced, a major mediator of leukocyte adhesion to endothelial cells which is also associated with BBB permeability (Abadier et al., 2015; Duperray et al., 2015).

Another study showed that HERV-W Env overexpression in a human glioma cell line increases the TNFα/IL-10 ratio, in a TLR4 dependent manner. Furthermore, this study describes that myeloid differentiation primary response–88 s (MyD88s) mRNA levels (a splice variant of MyD88 acting as a downstream negative regulator of TLR4) were decreased by HERV-W Env. When MyD88s was overexpressed however, the inflammatory pathway stimulated by HERV-Env was down-regulated, counteracting the HERV-W initial effect (Xiang & Liang, 2021).

The expression of HERV-W Env protein showed to correlate with cytokine levels in PBMCs derived from patients suffering from chronic inflammatory demyelinating polyradiculoneuropathy (CIPD) – an autoimmune condition of the peripheral nervous system (PNS). Likewise, human Schwann cells exposed to or transfected with HERV-W Env presented increased IL-6 and CXCL10 expression levels (Faucard et al., 2016), supporting that a pro-inflammatory response of immune- and neural cells to HERV-W Env is not restricted to the CNS. Beside the well-described interaction with TLR4, it is proposed that human ERVs also interact with and activate other receptors such TLR2, MFSD2, ASCT1/Slc1a4, ASCT2/Slc1a5 and MCT-1/Slc16a1 (Antony et al., 2007; Blanco-Melo et al., 2017; Blond et al., 2000; Esnault et al., 2008; Reuven et al., 2014). All of them are thought to be involved in pro-inflammatory responses but functional analyses related to potential interactions with ERVs are mostly missing.

In the context of HERV-K, it was recently described that the HERV-K encoded deoxyuridine triphosphate nucleotidohydrolase (dUTPase) is expressed in circulating monocytes and macrophages of patients with pulmonary arterial hypertension. Furthermore, HERV-K dUTPase was shown to induce the expression of the pro-inflammatory cytokine IL-6 in pulmonary arterial endothelial cells (Saito et al., 2017), which was proposed to be also dependent on TLR4 as well as on melanoma cell adhesion molecule signalling pathways (Otsuki et al., 2021). Of note, as the human leukocyte antigen (HLA) cluster represents one of the main genetic risk factors for the development of autoimmune diseases, the observation that HERV-K9 elements are located in the proximity as a result of the so-called hitchhiking effect (Kulski et al., 2008), is of further interest. Likewise, the HLA-8.1 ancestral haplotype, which is known to be protective against schizophrenia, was not found to contain the HERV-K element as compared to other pro-inflammatory ancestral haplotypes (Stewart et al, 2004), providing another link between retroviral elements and inflammation in psychiatric disorders.

Immune dysregulation and the modulated immune cell polarization present yet other mechanisms through which endogenous retroviruses can foster a pro-inflammatory environment. In this context, Superantigens (Sag) are known as inflammatory triggers that can stimulate much larger numbers of T cells than ordinary antigens. On that account, they become of specific interest in the context of autoimmune diseases such as MS. A large number of studies indeed describe HERV-K18 dependent Sag effects (Hsiao et al., 2006; Tai et al., 2009). Although not in that detail, similar effects are described for other ERVs such as HERV-Fc1, mouse mammary tumor virus (MMTV) and HERV-W (Gröger et al., 2020; Perron et al., 2001; Xu et al., 1996). In the context of SAGs, it was shown that viral HERV-W particles isolated from MS derived cells or via application of recombinant HERV-W Env protein, can induce polyclonal Vβ16 *T*-lymphocyte activation (Perron et al., 2001). Similar effects are described for HERV-K18, as it was shown that HERV-K18 Sags induce Vβ7 T cell activation (Stauffer et al., 2001).

More general evidence for a functional implication of ERVs in immune dysregulation can be deduced from observations on the HERV-W Env protein acting as an adjuvant and thereby activating CNS autoinflammation (experimental autoimmune encephalomyelitis). In this context, a direct involvement of the encoded Env protein was shown, given the observed rescue effect upon application of the neutralising antibody termed GNbAC1/Temelimab (Perron et al., 2013).

An indirect scenario is suggested, upon the discovery of the epigenetic de-repression of IFNγ, a Th1 related gene. This gene becomes transcriptionally active once its endogenous retroviral neighbour becomes transcriptionally active too. This transactivation leads to changes in the expression profiles of differentiated Th2 cells, rendering them transcriptionally similar to Th1 cells (Adoue et al., 2019). Of note, in physiological conditions upon Th2 differentiation, Th1 related genes become epigenetically silenced and vice versa (Sanders, 2006). Similar effects were identified by a transcriptome study, showing an activation of the HERV-neighbouring gene CD55 in monocytes and neutrophils of patients with various injuries such as burn, trauma and septic shock (Mommert et al., 2020; Tabone et al., 2018). CD55 encodes a glycoprotein involved in the regulation of the complement cascade, suggesting a modulatory role of human ERVs in the immune response during an ongoing inflammatory process.

Beyond traditional, mainly MS-related inflammatory scenarios, emerging observations in the context of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) have further corroborated the concept of ERVs fostering inflammatory processes. HERV-W was found to be specifically induced in a cohort of 30 COVID-19 patients, with peripheral HERV-W Env protein levels even exceeding increased levels, which have been previously observed in MS patients (Balestrieri et al.,

2021). Interestingly, as opposed to myeloid cells being main producers of HERV-W Env in MS, lymphocytes were identified to express HERV-W Env in these COVID-19 patients. In parallel, using an *ex vivo* healthy donor PBMC stimulation approach, a temporal correlation between inflammatory markers and the ERV expression was established. This study revealed that the induction of Env expression by SARS-CoV-2 spike protein occurs prior to the expression of IL-6 (3 h and 24 h, respectively), with IL-6 representing a key marker of the inflammatory response, which eventually can amount to cytokine storms. Hence, it was concluded that HERV-W induction might indeed contribute to critical, overshooting immune reactions and therefore lead to more severe disease courses in COVID-19 patients. Likewise, such a scenario might also account for chronic low inflammation in sub-acute patients suffering from long-term consequences of COVID-19 (Balestrieri et al., 2021).

6. Concluding remarks

Although endogenous retroviral elements have long been detected and described in health and disease, ERV research is still at infancy when it comes to the evaluation of their precise etiopathological role in in neurological, neurodevelopmental, neurodegenerative, or psychiatric disorders. The presence of abnormal ERV expression in multiple brain disorders suggests that abnormal activation of endogenous retroviral elements may reflect a common mechanism for shared pathologies, including (but possibly not limited to) inflammation. Recent studies aiming at neutralizing ERV proteins in pathological contexts such as MS provide initial evidence that ERVs are not simply incidental phenomena, but instead they are pathologically relevant. The current view is that ERVs can trigger inflammatory processes through multiple pathways of the innate and adaptive arms of the immune system. At the same time, inflammatory signals may drive the (re-)activation and/or maintain the expression of ERVs, leading to a sequence of reciprocal cause and effect (Fig. 1). Based on the current state of research, it is likely that the numerous entry points to this reciprocal sequence of events exist, including initial infections with ERV-activating pathogens, exposure to non-infectious inflammatory stimuli such as trauma or burn, and conditions in which epigenetic silencing of ERV elements are disrupted. With regards to the latter, epigenetic factors may be crucial for determining the susceptibility towards developing ERV-associated pathologies, and therefore, determining epigenetic factors interacting with endogenous retroviral elements should become a research priority. In addition, more longitudinal and mechanistic studies are warranted in order to further corroborate the pathological relevance of ERV expression in neurological and psychiatric disorders.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

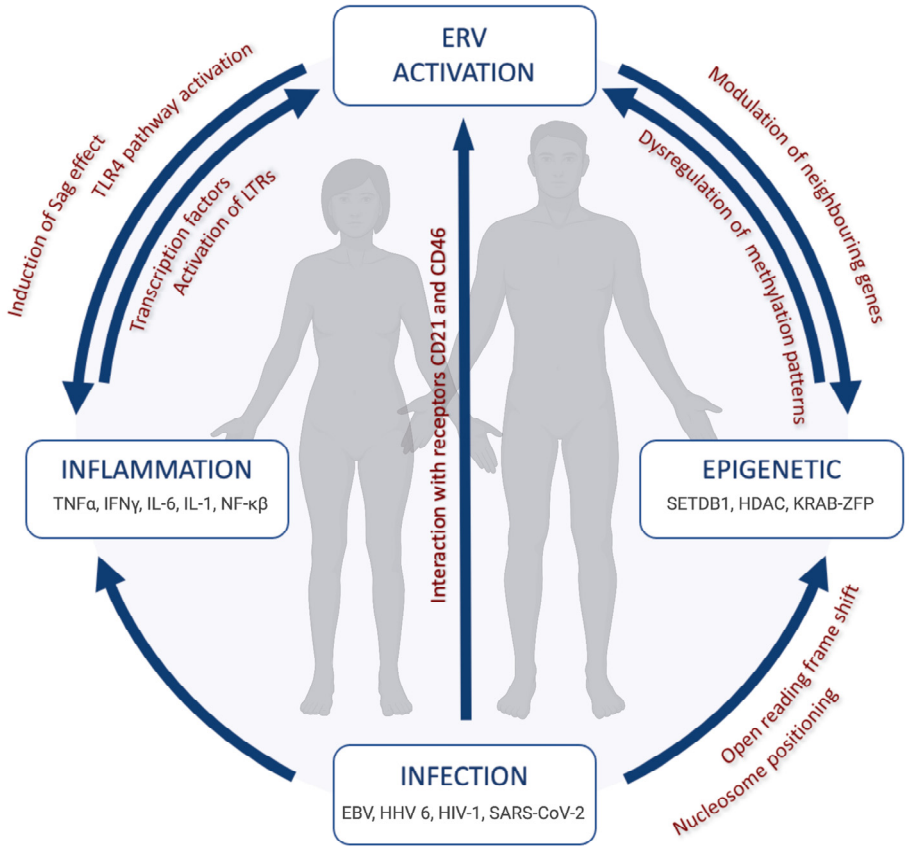

Fig. 1. Relationship between ERV activation, inflammation, infection and epigenetic processes. Multiple processes such as inflammation, epigenetic unlocking as well as exposure to certain infections can lead to the activation of ERVs. Upon ERV activation, feedback signals can amplify inflammatory processes and alter epigenetic programs. The figure summarizes some of the molecular factors and processes involved in each of these relationships.

Data availability

No data was used for the research described in the article.

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References

Abadier, M., Haghayegh Jahromi, N., Cardoso Alves, L., Boscacci, R., Vestweber, D., Barnum, S., Deutsch, U., Engelhardt, B., Lyck, R., 2015. Cell surface levels of endothelial ICAM-1 influence the transcellular or paracellular T-cell diapedesis across the blood-brain barrier. Eur. J. Immunol. 45 (4), 1043–1058.

Adoue, V., Binet, B., Malbec, A., Fourquet, J., Romagnoli, P., van Meerwijk, J.P.M., Joffre, O.P., 2019. The histone methyltransferase SETDB1 controls T helper cell lineage integrity by repressing endogenous retroviruses. Immunity 50 (3), 629–644. https://doi.org/10.1016/j.immuni.2019.01.003.

Antony, J.M., Ellestad, K.K., Hammond, R., Imaizumi, K., Mallet, F., Warren, K.G., Power, C., 2007. The human endogenous retrovirus envelope glycoprotein, syncytin-1, regulates neuroinflammation and its receptor expression in multiple sclerosis: a role for endoplasmic reticulum chaperones in astrocytes. J. Immunol. 179 (2), 1210–1224. https://doi.org/10.4049/jimmunol.179.2.1210.

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I., Van de Water, J., 2011. Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. Brain Behav. Immun. 25 (1), 40–45. https://doi.org/10.1016/j.bbi.2010.08.003.

American Psychiatric Association, D. (2013). Diagnostic and statistical manual of mental disorders: DSM-5 (Vol. 5): American psychiatric association Washington, DC.

Balestrieri, E., Pitzianti, M., Matteucci, C., D'Agati, E., Sorrentino, R., Baratta, A., Caterina, R., Zenobi, R., Curatolo, P., Garaci, E., Sinibaldi-Vallebona, P., Pasini, A., 2014. Human endogenous retroviruses and ADHD. World J. Biol. Psychiatry 15 (6), 499–504. https://doi.org/10.3109/15622975.2013.862345.

Balestrieri, E., Matteucci, C., Cipriani, C., Grelli, S., Ricceri, L., Calamandrei, G., Vallebona, P.S., 2019. Endogenous retroviruses activity as a molecular signature of neurodevelopmental disorders. Int. J. Mol. Sci. 20 (23) https://doi.org/10.3390/ijms20236050.

Balestrieri, E., Minutolo, A., Petrone, V., Fanelli, M., Iannetta, M., Malagnino, V., Zordan, M., Vitale, P., Charvet, B., Horvat, B., Bernardini, S., Garaci, E., di Francesco, P., Sinibaldi Vallebona, P., Sarmati, L., Grelli, S., Andreoni, M., Perron, H., Matteucci, C., 2021. Evidence of the pathogenic HERV-W envelope expression in T lymphocytes in association with the respiratory outcome of COVID-19 patients. EBioMedicine 66, 103341.

Bannister, A.J., Kouzarides, T., 2011. Regulation of chromatin by histone modifications. Cell Res. 21 (3), 381–395. https://doi.org/10.1038/cr.2011.22.

Bjornevik, K., Cortese, M., Healy, B.C., Kuhle, J., Mina, M.J., Leng, Y., Elledge, S.J., Niebuhr, D.W., Scher, A.I., Munger, K.L., Ascherio, A., 2022. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. Science 375 (6578), 296–301. https://doi.org/10.1126/science.abj8222.

Blanco-Melo, D., Gifford, R.J., Bieniasz, P.D., 2017. Co-option of an endogenous retrovirus envelope for host defense in hominid ancestors. Elife 6. https://doi.org/10.7554/eLife.22519.

Blond, J.-L., Lavillette, D., Cheynet, V., Bouton, O., Oriol, G., Chapel-Fernandes, S., Mandrand, B., Mallet, F., Cosset, F.-L., 2000. An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor. J. Virol. 74 (7), 3321–3329. https://doi.org/10.1128/jvi.74.7.3321-3329.2000.

Bollati, V., Favero, C., Albetti, B., Tarantini, L., Moroni, A., Byun, H.-M., Motta, V., Conti, D., Tirelli, A., Vigna, L., Bertazzi, P., Pesatori, A., 2014. Nutrients intake is associated with DNA methylation of candidate inflammatory genes in a population of obese subjects. Nutrients 6 (10), 4625–4639. https://doi.org/10.3390/nu6104625.

Brown, A.S., Meyer, U., 2018. Maternal immune activation and neuropsychiatric illness: a translational research perspective. Am. J. Psychiatry 175 (11), 1073–1083. https://doi.org/10.1176/appi.ajp.2018.17121311.

Buschle, A., Hammerschmidt, W., 2020. Epigenetic lifestyle of Epstein-Barr virus. Semin. Immunopathol. 42 (2), 131–142. https://doi.org/10.1007/s00281-020-00792-2.

Campos-Sánchez, R., Cremona, M.A., Pini, A., Chiaromonte, F., Makova, K.D., Kosakovsky Pond, S.L., 2016. Integration and fixation preferences of human and mouse endogenous retroviruses uncovered with functional data analysis. PLoS Comput. Biol. 12 (6) https://doi.org/10.1371/journal.pcbi.1004956.

Charlson, F.J., Ferrari, A.J., Santomauro, D.F., Diminic, S., Stockings, E., Scott, J.G., McGrath, J.J., Whiteford, H.A., 2018. Global epidemiology and burden of schizophrenia: findings from the global burden of disease study 2016. Schizophr. Bull. 44 (6), 1195–1203. https://doi.org/10.1093/schbul/sby058.

Charvet, B., Reynaud, J.M., Gourru-Lesimple, G., Perron, H., Marche, P.N., Horvat, B., 2018. Induction of proinflammatory multiple sclerosis-associated retrovirus envelope protein by human herpesvirus-6A and CD46 receptor engagement. Front. Immunol. 9, 2803. https://doi.org/10.3389/fimmu.2018.02803.

Cipriani, C., Pitzianti, M.B., Matteucci, C., D'Agati, E., Miele, M.T., Rapaccini, V., Grelli, S., Curatolo, P., Sinibaldi-Vallebona, P., Pasini, A., Balestrieri, E., 2018a. The decrease in human endogenous retrovirus-H activity runs in parallel with improvement in ADHD symptoms in patients undergoing methylphenidate therapy. Int. J. Mol. Sci. 19 (11), 3286.

Cipriani, C., Ricceri, L., Matteucci, C., De Felice, A., Tartaglione, A.M., Argaw-Denboba, A., Pica, F., Grelli, S., Calamandrei, G., Sinibaldi Vallebona, P., Balestrieri, E., 2018b. High expression of Endogenous Retroviruses from intrauterine life to adulthood in two mouse models of Autism Spectrum Disorders. Sci. Rep. 8 (1) https://doi.org/10.1038/s41598-017-19035-w.

Collins, P.L., Kyle, K.E., Egawa, T., Shinkai, Y., Oltz, E.M., 2015. The histone methyltransferase SETDB1 represses endogenous and exogenous retroviruses in B lymphocytes. Proc. Natl. Acad. Sci. USA 112 (27), 8367–8372. https://doi.org/10.1073/pnas.1422187112.

Conti, A., Rota, F., Ragni, E., Favero, C., Motta, V., Lazzari, L., Bollati, V., Fustinoni, S., Dieci, G., 2016. Hydroquinone induces DNA hypomethylation-independent overexpression of retroelements in human leukemia and hematopoietic stem cells. Biochem. Biophys. Res. Commun. 474 (4), 691–695. https://doi.org/10.1016/j.bbrc.2016.05.010.

Curzio, D.D., Gurm, M., Turnbull, M., Nadeau, M.-J., Meek, B., Rempel, J.D., Fineblit, S., Jonasson, M., Hebert, S., Ferguson-Parry, J., Douville, R.N., 2020. Pro-inflammatory signaling upregulates a neurotoxic conotoxin-like protein encrypted within human endogenous retrovirus-K. Cells 9 (7), 1584. https://doi.org/10.3390/cells9071584.

Day, D.S., Luquette, L.J., Park, P.J., Kharchenko, P.V., 2010. Estimating enrichment of repetitive elements from high-throughput sequence data. Genome Biol. 11 (6), R69. https://doi.org/10.1186/gb-2010-11-6-r69.

Deaton, A.M., Webb, S., Kerr, A.R., Illingworth, R.S., Guy, J., Andrews, R., Bird, A., 2011. Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res. 21 (7), 1074–1086. https://doi.org/10.1101/gr.118703.110.

Diem, O., Schäffner, M., Seifarth, W., Leib-Mösch, C., Hashimoto, K., 2012. Influence of antipsychotic drugs on human endogenous retrovirus (HERV) transcription in brain cells. PLoS One 7 (1), e30054. https://doi.org/10.1371/journal.pone.0030054.

Duperray, A., Barbe, D., Raguenez, G., Weksler, B.B., Romero, I.A., Couraud, P.-O., Perron, H., Marche, P.N., 2015. Inflammatory response of endothelial cells to a human endogenous retrovirus associated with multiple sclerosis is mediated by TLR4. Int. Immunol. 27 (11), 545–553. https://doi.org/10.1093/intimm/dxv025.

Esnault, C., Priet, S., Ribet, D., Vernochet, C., Bruls, T., Lavialle, C., Weissenbach, J., Heidmann, T., 2008. A placenta-specific receptor for the fusogenic, endogenous retrovirus-derived, human syncytin-2. Proc. Natl. Acad. Sci. USA 105 (45), 17532–17537. https://doi.org/10.1073/pnas.0807413105.

Faucard, R., Madeira, A., Gehin, N., Authier, F.-J., Panaite, P.-A., Lesage, C., Burgelin, I., Bertel, M., Bernard, C., Curtin, F., Lang, A.B., Steck, A.J., Perron, H., Kuntzer, T., Créange, A., 2016. Human endogenous retrovirus and neuroinflammation in chronic inflammatory demyelinating polyradiculoneuropathy. EBioMedicine 6, 190–198. https://doi.org/10.1016/j.ebiom.2016.03.001.

Fillman, S.G., Cloonan, N., Catts, V.S., Miller, L.C., Wong, J., McCrossin, T., Cairns, M., Weickert, C.S., 2013. Increased inflammatory markers identified in the dorsolateral prefrontal cortex of individuals with schizophrenia. Mol. Psychiatry 18 (2), 206–214. https://doi.org/10.1038/mp.2012.110.

Fillman, S.G., Weickert, T.W., Lenroot, R.K., Catts, S.V., Bruggemann, J.M., Catts, V.S., Weickert, C.S., 2016. Elevated peripheral cytokines characterize a subgroup of people with schizophrenia displaying poor verbal fluency and reduced Broca's area volume. Mol. Psychiatry 21 (8), 1090–1098. https://doi.org/10.1038/mp.2015.90.

Fuchs, N.V., Kraft, M., Tondera, C., Hanschmann, K.M., Lower, J., Lower, R., 2011. Expression of the human endogenous retrovirus (HERV) group HML-2/HERV-K does not depend on canonical promoter elements but is regulated by transcription factors Sp1 and Sp3. J. Virol. 85 (7), 3436–3448. https://doi.org/10.1128/JVI.02539-10.

Garcia-Montojo, M., Fathi, S., Norato, G., Smith, B.R., Rowe, D.B., Kiernan, M.C., Vucic, S., Mathers, S., van Eijk, R.P.A., Santamaria, U., Rogers, M.-L., Malaspina, A., Lombardi, V., Mehta, P.R., Westeneng, H.-J., van den Berg, L.H., Al-Chalabi, A., Gold, J., Nath, A., 2021. Inhibition of HERV-K (HML-2) in amyotrophic lateral sclerosis patients on antiretroviral therapy. J. Neurol. Sci. 423, 117358. https://doi.org/10.1016/j.jns.2021.117358.

Gerber, J.P., Russ, J., Chandrasekar, V., Offermann, N., Lee, H.-M., Spear, S., Guzzi, N., Maida, S., Pattabiraman, S., Zhang, R., Kayvanjoo, A.H., Datta, P., Kasturiarachchi, J., Sposito, T., Izotova, N., Händler, K., Adams, P.D., Marafioti, T., Enver, T., Wenzel, J., Beyer, M., Mass, E., Bellodi, C., Schultze, J.L., Capasso, M., Nimmo, R., Salomoni, P., 2021. Aberrant chromatin landscape following loss of the H3.3 chaperone Daxx in haematopoietic precursors leads to Pu.1-mediated neutrophilia and inflammation. Nat. Cell Biol. 23 (12), 1224–1239. https://doi.org/10.1038/s41556-021-00774-y.

Gifford, R.J., Blomberg, J., Coffin, J.M., Fan, H., Heidmann, T., Mayer, J., Stoye, J., Tristem, M., Johnson, W.E., 2018. Nomenclature for endogenous retrovirus (ERV) loci. Retrovirology 15 (1), 59. https://doi.org/10.1186/s12977-018-0442-1.

Göttle, P., Forster, M., Gruchot, J., Kremer, D., Hartung, H.P., Perron, H., Küry, P., 2019. Rescuing the negative impact of human endogenous retrovirus envelope protein on oligodendroglial differentiation and myelination. Glia 67 (1), 160–170. https://doi.org/10.1002/glia.23535.

Göttle, P., Schichel, K., Reiche, L., Werner, L., Zink, A., Prigione, A., Küry, P., 2021. TLR4 associated signaling disrupters as a new means to overcome HERV-W envelope-mediated myelination deficits. Front. Cell. Neurosci. 15, 777542 https://doi.org/10.3389/fncel.2021.777542.

Grandi, N., Tramontano, E., 2017. Type W Human Endogenous Retrovirus (HERV-W) integrations and their mobilization by L1 Machinery: contribution to the human transcriptome and impact on the host physiopathology. Viruses 9 (7). https://doi.org/10.3390/v9070162.

Gröger, V., Wieland, L., Naumann, M., Meinecke, A.-C., Meinhardt, B., Rossner, S., Ihling, C., Emmer, A., Staege, M.S., Cynis, H., 2020. Formation of HERV-K and HERV-Fc1 envelope family members is suppressed on transcriptional and translational level. Int. J. Mol. Sci. 21 (21), 7855.

Guo, P., Liu, Y., Geng, F., Daman, A.W., Liu, X., Zhong, L., Ravishankar, A., Lis, R., Barcia Durán, J.G., Itkin, T., Tang, F., Zhang, T., Xiang, J., Shido, K., Ding, B.-S., Wen, D., Josefowicz, S.Z., Rafii, S., 2022. Histone variant H3.3 maintains adult haematopoietic stem cell homeostasis by enforcing chromatin adaptability. Nat. Cell Biol. 24 (1), 99–111. https://doi.org/10.1038/s41556-021-00795-7.

Harrison, P.J., Geddes, J.R., Tunbridge, E.M., 2018. The emerging neurobiology of bipolar disorder. Trends Neurosci. 41 (1), 18–30. https://doi.org/10.1016/j.tins.2017.10.006.

Hartung, H.-P., Derfuss, T., Cree, B.AC., Sormani, M.P., Selmaj, K., Stutters, J., Prados, F., MacManus, D., Schneble, H.-M., Lambert, E., Porchet, H., Glanzman, R., Warne, D., Curtin, F., Kornmann, G., Buffet, B., Kremer, D., Küry, P., Leppert, D., Rückle, T., Barkhof, F., 2022. Efficacy and safety of temelimab in multiple sclerosis: results of a randomized phase 2b and extension study. Mult Scler 28 (3), 429–440. https://doi.org/10.1177/13524585211024997.

Hoang, D., Xu, Y., Lutz, O., Bannai, D., Zeng, V., Bishop, J.R., Lizano, P., 2022. Inflammatory subtypes in antipsychotic-naive first-episode schizophrenia are associated with altered brain morphology and topological organization. Brain Behav. Immun. 100, 297–308. https://doi.org/10.1016/j.bbi.2021.11.019.

Hsiao, F.C., Lin, M., Tai, A., Chen, G., Huber, B.T., 2006. Cutting edge: Epstein-Barr virus transactivates the HERV-K18 superantigen by docking to the human complement receptor 2 (CD21) on primary B cells. J. Immunol. 177 (4), 2056–2060. https://doi.org/10.4049/jimmunol.177.4.2056.

Hurst, T., Pace, M., Katzourakis, A., Phillips, R., Klenerman, P., Frater, J., Magiorkinis, G., 2016. Human endogenous retrovirus (HERV) expression is not induced by treatment with the histone deacetylase (HDAC) inhibitors in cellular models of HIV-1 latency. Retrovirology 13, 10. https://doi.org/10.1186/s12977-016-0242-4.

Imbeault, M., Helleboid, P.Y., Trono, D., 2017. KRAB zinc-finger proteins contribute to the evolution of gene regulatory networks. Nature 543 (7646), 550–554. https://doi.org/10.1038/nature21683.

Johansson, E.M., Bouchet, D., Tamouza, R., Ellul, P., Morr, A.S., Avignone, E., Germi, R., Leboyer, M., Perron, H., Groc, L., 2020. Human endogenous retroviral protein triggers deficit in glutamate synapse maturation and behaviors associated with psychosis. Sci. Adv. 6 (29), eabc0708. https://doi.org/10.1126/sciadv.abc0708.

Karlsson, H., Bachmann, S., Schroder, J., McArthur, J., Torrey, E.F., Yolken, R.H., 2001. Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia. Proc. Natl. Acad. Sci. USA 98 (8), 4634–4639. https://doi.org/10.1073/pnas.061021998.

Kovalskaya, E., Buzdin, A., Gogvadze, E., Vinogradova, T., Sverdlov, E., 2006. Functional human endogenous retroviral LTR transcription start sites are located between the R and U5 regions. Virology 346 (2), 373–378. https://doi.org/10.1016/j.virol.2005.11.007.

Kremer, D., Aktas, O., Hartung, H.P., Küry, P., 2010. Immunobiology of the oligodendrocyte. In: Armati, P., Mathey, E. (Eds.), The Biology of Oligodendrocytes. Cambridge University Press, Cambridge, pp. 115–136.

Kremer, D., Schichel, T., Förster, M., Tzekova, N., Bernard, C., van der Valk, P., van Horssen, J., Hartung, H.-P., Perron, H., Küry, P., 2013. Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. Ann. Neurol. 74 (5), 721–732.

Kremer, D., Forster, M., Schichel, T., Göttle, P., Hartung, H.P., Perron, H., Küry, P., 2015. The neutralizing antibody GNbAC1 abrogates HERV-W envelope protein-mediated oligodendroglial maturation blockade. Mult. Scler. 21 (9), 1200–1203. https://doi.org/10.1177/1352458514560926.

Kremer, D., Gruchot, J., Weyers, V., Oldemeier, L., Göttle, P., Healy, L., Ho Jang, J., Kang, Y., Xu, T., Volsko, C., Dutta, R., Trapp, B.D., Perron, H., Hartung, H.-P., Küry, P., 2019. pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. Proc. Natl. Acad. Sci. USA 116 (30), 15216–15225. https://doi.org/10.1073/pnas.1901283116.

Kulski, J.K., Shigenari, A., Shiina, T., Ota, M., Hosomichi, K., James, I., Inoko, H., 2008. Human endogenous retrovirus (HERVK9) structural polymorphism with haplotypic HLA-A allelic associations. Genetics 180 (1), 445–457. https://doi.org/10.1534/genetics.108.090340.

Küry, P., Nath, A., Créange, A., Dolei, A., Marche, P., Gold, J., Giovannoni, G., Hartung, H.-P., Perron, H., 2018. Human endogenous retroviruses in neurological diseases. Trends Mol. Med. 24 (4), 379–394. https://doi.org/10.1016/j.molmed.2018.02.007.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L., Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.-F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E., Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, JoAnn, Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blöcker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.-C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G.R., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., Lancet, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F.A., Stupka, E., Szustakowki, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.-P., Yeh, R.-F., Collins, F., Guyer, M.S., Peterson, J., Felsenfeld, A., Wetterstrand, K.A., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Patrinos, A., Morgan, M.J., 2001. Initial sequencing and analysis of the human genome. Nature 409 (6822), 860–921. https://doi.org/10.1038/35057062.

Lanz, T.V., Brewer, R.C., Ho, P.P., Moon, J.-S., Jude, K.M., Fernandez, D., Fernandes, R.A., Gomez, A.M., Nadj, G.-S., Bartley, C.M., Schubert, R.D., Hawes, I.A., Vazquez, S.E., Iyer, M., Zuchero, J.B., Teegen, B., Dunn, J.E., Lock, C.B., Kipp, L.B., Cotham, V.C., Ueberheide, B.M., Aftab, B.T., Anderson, M.S., DeRisi, J.L., Wilson, M.R., Bashford-Rogers, R.J.M., Platten, M., Garcia, K.C., Steinman, L., Robinson, W.H., 2022. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. Nature 603 (7900), 321–327. https://doi.org/10.1038/s41586-022-04432-7.

Laska, M.J., Brudek, T., Nissen, K.K., Christensen, T., Moller-Larsen, A., Petersen, T., Nexo, B.A., 2012. Expression of HERV-Fc1, a human endogenous retrovirus, is increased in patients with active multiple sclerosis. J. Virol. 86 (7), 3713–3722. https://doi.org/10.1128/JVI.06723-11.

Lavie, L., Kitova, M., Maldener, E., Meese, E., Mayer, J., 2005. CpG methylation directly regulates transcriptional activity of the human endogenous retrovirus family HERV-K(HML-2). J. Virol. 79 (2), 876–883. https://doi.org/10.1128/JVI.79.2.876-883.2005.

Leung, D.C., Lorincz, M.C., 2012. Silencing of endogenous retroviruses: when and why do histone marks predominate? Trends Biochem. Sci. 37 (4), 127–133. https://doi.org/10.1016/j.tibs.2011.11.006.

Leung, A., Trac, C., Kato, H., Costello, K.R., Chen, Z., Natarajan, R., Schones, D.E., 2018. LTRs activated by Epstein-Barr virus-induced transformation of B cells alter the transcriptome. Genome Res. 28 (12), 1791–1798. https://doi.org/10.1101/gr.233585.117.

Li, H., Chen, Y., Li, Y., Yin, B.o., Tang, W., Yu, X., Huang, W., Geng, D., Zhang, B., 2015a. Altered cortical activation during action observation in amyotrophic lateral sclerosis patients: a parametric functional MRI study. Eur. Radiol. 25 (9), 2584–2592. https://doi.org/10.1007/s00330-015-3671-x.

Li, W., Lee, M.-H., Henderson, L., Tyagi, R., Bachani, M., Steiner, J., Campanac, E., Hoffman, D.A., von Geldern, G., Johnson, K., Maric, D., Morris, H.D., Lentz, M., Pak, K., Mammen, A., Ostrow, L., Rothstein, J., Nath, A., 2015b. Human endogenous retrovirus-K contributes to motor neuron disease. Sci. Transl. Med. 7 (307) https://doi.org/10.1126/scitranslmed.aac8201.

Li, F., Nellåker, C., Sabunciyan, S., Yolken, R.H., Jones-Brando, L., Johansson, A.-S., Owe-Larsson, B., Karlsson, H., Caughey, B.W., 2014. Transcriptional derepression of the ERVWE1 locus following influenza A virus infection. J. Virol. 88 (8), 4328–4337. https://doi.org/10.1128/JVI.03628-13.

Li, F., Sabunciyan, S., Yolken, R.H., Lee, D., Kim, S., Karlsson, H., Ruprecht, K., 2019. Transcription of human endogenous retroviruses in human brain by RNA-seq analysis. PLoS One 14 (1). https://doi.org/10.1371/journal.pone.0207353.

Liu, M., Ohtani, H., Zhou, W., Ørskov, A.D., Charlet, J., Zhang, Y.W., Shen, H., Baylin, S.B., Liang, G., Grønbæk, K., Jones, P.A., 2016. Vitamin C increases viral mimicry induced by 5-aza-2'-deoxycytidine. Proc. Natl. Acad. Sci. USA 113 (37), 10238–10244. https://doi.org/10.1073/pnas.1612262113.

Liu, J., Wang, F., 2017. Role of neuroinflammation in amyotrophic lateral sclerosis: cellular mechanisms and therapeutic implications. Front. Immunol. 8, 1005. https://doi.org/10.3389/fimmu.2017.01005.

Longinetti, E., Fang, F., 2019. Epidemiology of amyotrophic lateral sclerosis: an update of recent literature. Curr. Opin. Neurol. 32 (5), 771–776. https://doi.org/10.1097/WCO.0000000000000730.

Lukic, S., Nicolas, J.C., Levine, A.J., 2014. The diversity of zinc-finger genes on human chromosome 19 provides an evolutionary mechanism for defense against inherited

endogenous retroviruses. Cell Death Differ. 21 (3), 381–387. https://doi.org/10.1038/cdd.2013.150.
MacGowan, D.J., Scelsa, S.N., Imperato, T.E., Liu, K.N., Baron, P., Polsky, B., 2007. A controlled study of reverse transcriptase in serum and CSF of HIV-negative patients with ALS. Neurology 68 (22), 1944–1946. https://doi.org/10.1212/01.wnl.0000263188.77797.99.
Mak, M., Samochowiec, J., Frydecka, D., Pełka-Wysiecka, J., Szmida, E., Karpiński, P., Sąsiadek, M.M., Piotrowski, P., Samochowiec, A., Misiak, B., 2019. First-episode schizophrenia is associated with a reduction of HERV-K methylation in peripheral blood. Psychiatry Res. 271, 459–463. https://doi.org/10.1016/j.psychres.2018.12.012.
Mameli, G., Astone, V., Khalili, K., Serra, C., Sawaya, B.E., Dolei, A., 2007. Regulation of the syncytin-1 promoter in human astrocytes by multiple sclerosis-related cytokines. Virology 362 (1), 120–130. https://doi.org/10.1016/j.virol.2006.12.019.
Mameli, G., Poddighe, L., Mei, A., Uleri, E., Sotgiu, S., Serra, C., Manetti, R., Dolei, A., Villoslada, P., 2012. Expression and activation by Epstein Barr virus of human endogenous retroviruses-W in blood cells and astrocytes: inference for multiple sclerosis. PLoS One 7 (9), e44991. https://doi.org/10.1371/journal.pone.0044991.
Mameli, G., Madeddu, G., Mei, A., Uleri, E., Poddighe, L., Delogu, L.G., Maida, I., Babudieri, S., Serra, C., Manetti, R., Mura, M.S., Dolei, A., Stewart, J.P., 2013. Activation of MSRV-type endogenous retroviruses during infectious mononucleosis and Epstein-Barr virus latency: the missing link with multiple sclerosis? PLoS One 8 (11), e78474. https://doi.org/10.1371/journal.pone.0078474.
Manghera, M., Douville, R.N., 2013. Endogenous retrovirus-K promoter: a landing strip for inflammatory transcription factors? Retrovirology 10, 16. https://doi.org/10.1186/1742-4690-10-16.
Manghera, M., Ferguson, J., Douville, R., 2015. ERVK polyprotein processing and reverse transcriptase expression in human cell line models of neurological disease. Viruses 7 (1), 320–332. https://doi.org/10.3390/v7010320.
Manghera, M., Ferguson-Parry, J., Lin, R., Douville, R.N., 2016. NF-kappaB and IRF1 Induce Endogenous Retrovirus K Expression via Interferon-Stimulated Response Elements in Its 5' Long Terminal Repeat. J. Virol. 90 (20), 9338–9349. https://doi.org/10.1128/JVI.01503-16.
Mathis, S., Couratier, P., Julian, A., Corcia, P., Le Masson, G., 2017. Current view and perspectives in amyotrophic lateral sclerosis. Neural Regen Res 12 (2), 181–184. https://doi.org/10.4103/1673-5374.200794.
Matthews, P.M., 2019. Chronic inflammation in multiple sclerosis - seeing what was always there. Nat. Rev. Neurol. 15 (10), 582–593. https://doi.org/10.1038/s41582-019-0240-y.
Meier, U.C., Cipian, R.C., Karimi, A., Ramasamy, R., Middeldorp, J.M., 2021. Cumulative roles for epstein-barr virus, human endogenous retroviruses, and human herpes virus-6 in driving an inflammatory cascade underlying MS pathogenesis. Front. Immunol. 12, 757302 https://doi.org/10.3389/fimmu.2021.757302.
Meltzer, A., Van de Water, J., 2017. The role of the immune system in autism spectrum disorder. Neuropsychopharmacology 42 (1), 284–298. https://doi.org/10.1038/npp.2016.158.
Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T.-K., Koche, R.P., Lee, W., Mendenhall, E., O'Donovan, A., Presser, A., Russ, C., Xie, X., Meissner, A., Wernig, M., Jaenisch, R., Nusbaum, C., Lander, E.S., Bernstein, B.E., 2007. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448 (7153), 553–560. https://doi.org/10.1038/nature06008.
Miller, B.J., Buckley, P., Seabolt, W., Mellor, A., Kirkpatrick, B., 2011. Meta-analysis of cytokine alterations in schizophrenia: clinical status and antipsychotic effects. Biol. Psychiatry 70 (7), 663–671. https://doi.org/10.1016/j.biopsych.2011.04.013.
Mommert, M., Tabone, O., Guichard, A., Oriol, G., Cerrato, E., Denizot, M., Cheynet, V., Pachot, A., Lepape, A., Monneret, G., Venet, F., Brengel-Pesce, K., Textoris, J., Mallet, F., 2020. Dynamic LTR retrotransposon transcriptome landscape in septic shock patients. Crit. Care 24 (1). https://doi.org/10.1186/s13054-020-2788-8.
Mondelli, V., Ciufolini, S., Belvederi Murri, M., Bonaccorso, S., Di Forti, M., Giordano, A., Marques, T.R., Zunszain, P.A., Morgan, C., Murray, R.M., Pariante, C.M., Dazzan, P., 2015. Cortisol and inflammatory biomarkers predict poor treatment response in first episode psychosis. Schizophr. Bull. 41 (5), 1162–1170. https://doi.org/10.1093/schbul/sbv028.
Mueller, O., Moore, D.W., Giovannucci, J., Etter, A.R., Peterson, E.M., Mudge, A., Liu, Y., 2018. Expression of human endogenous retroviruses in peripheral leukocytes during the menstrual cycle suggests coordinated hormonal regulation. AIDS Res. Hum. Retroviruses 34 (11), 909–911. https://doi.org/10.1089/AID.2018.0059.
Nellaker, C., Keane, T.M., Yalcin, B., Wong, K., Agam, A., Belgard, T.G., Flint, J., Adams, D.J., Frankel, W.N., Ponting, C.P., 2012. The genomic landscape shaped by selection on transposable elements across 18 mouse strains. Genome Biol. 13 (6), R45. https://doi.org/10.1186/gb-2012-13-6-r45.
Nguyen, T.D., Davis, J., Eugenio, R.A., Liu, Y., 2019. Female sex hormones activate human endogenous retrovirus type K through the OCT4 transcription factor in T47D breast cancer cells. AIDS Res. Hum. Retroviruses 35 (3), 348–356. https://doi.org/10.1089/AID.2018.0173.
Ohtani, H., Liu, M., Zhou, W., Liang, G., Jones, P.A., 2018. Switching roles for DNA and histone methylation depend on evolutionary ages of human endogenous retroviruses. Genome Res. 28 (8), 1147–1157. https://doi.org/10.1101/gr.234229.118.
Onore, C., Careaga, M., Ashwood, P., 2012. The role of immune dysfunction in the pathophysiology of autism. Brain Behav. Immun. 26 (3), 383–392. https://doi.org/10.1016/j.bbi.2011.08.007.
Otsuki, S., Saito, T., Taylor, S., Li, D., Moonen, J.-R., Marciano, D.P., Harper, R.L., Cao, A., Wang, L., Ariza, M.E., Rabinovitch, M., 2021. Monocyte-released HERV-K dUTPase engages TLR4 and MCAM causing endothelial mesenchymal transition. JCI Insight. https://doi.org/10.1172/jci.insight.146416.
Owen, M.J., Sawa, A., Mortensen, P.B., 2016. Schizophrenia. Lancet 388 (10039), 86–97. https://doi.org/10.1016/S0140-6736(15)01121-6.
Pastuzyn, E.D., Day, C.E., Kearns, R.B., Kyrke-Smith, M., Taibi, A.V., McCormick, J., Yoder, N., Belnap, D.M., Erlendsson, S., Morado, D.R., Briggs, J.A.G., Feschotte, C., Shepherd, J.D., 2018. The neuronal gene arc encodes a repurposed retrotransposon gag protein that mediates intercellular RNA transfer. Cell 173 (1), 275. https://doi.org/10.1016/j.cell.2018.03.024.
Perron, H., Geny, C., Laurent, A., Mouriquand, C., Pellat, J., Perret, J., Seigneurin, J.M., 1989. Leptomeningeal cell line from multiple sclerosis with reverse transcriptase activity and viral particles. Res. Virol. 140 (6), 551–561. https://doi.org/10.1016/s0923-2516(89)80141-4.
Perron, H., Jouvin-Marche, E., Michel, M., Ounanian-Paraz, A., Camelo, S., Dumon, A., Lafon, M., 2001. Multiple sclerosis retrovirus particles and recombinant envelope trigger an abnormal immune response in vitro, by inducing polyclonal Vbeta16 T-lymphocyte activation. Virology 287 (2), 321–332. https://doi.org/10.1006/viro.2001.1045.
Perron, H., Hamdani, N., Faucard, R., Lajnef, M., Jamain, S., Daban-Huard, C., Sarrazin, S., LeGuen, E., Houenou, J., Delavest, M., Moins-Teisserenc, H., Bengoufa, D., Yolken, R., Madeira, A., Garcia-Montojo, M., Gehin, N., Burgelin, I., Ollagnier, G., Bernard, C., Dumaine, A., Henrion, A., Gombert, A., Le Dudal, K., Charron, D., Krishnamoorthy, R., Tamouza, R., Leboyer, M., 2012. Molecular characteristics of Human Endogenous Retrovirus type-W in schizophrenia and bipolar disorder. Transl. Psychiatry 2 (12). https://doi.org/10.1038/tp.2012.125.
Perron, H., Dougier-Reynaud, H.-L., Lomparski, C., Popa, I., Firouzi, R., Bertrand, J.-B., Marusic, S., Portoukalian, J., Jouvin-Marche, E., Villiers, C.L., Touraine, J.-L., Marche, P.N., Villoslada, P., 2013. Human endogenous retrovirus protein activates innate immunity and promotes experimental allergic encephalomyelitis in mice. PLoS One 8 (12), e80128. https://doi.org/10.1371/journal.pone.0080128.
Purves-Tyson, T.D., Weber-Stadlbauer, U., Richetto, J., Rothmond, D.A., Labouesse, M. A., Polesel, M., Robinson, K., Shannon Weickert, C., Meyer, U., 2021. Increased levels of midbrain immune-related transcripts in schizophrenia and in murine offspring after maternal immune activation. Mol. Psychiatry 26 (3), 849–863. https://doi.org/10.1038/s41380-019-0434-0.
Reuven, E.M., Ali, M., Rotem, E., Schwarzter, R., Gramatica, A., Futerman, A.H., Shai, Y., Douek, D.C., 2014. The HIV-1 envelope transmembrane domain binds TLR2 through a distinct dimerization motif and inhibits TLR2-mediated responses. PLoS Pathog. 10 (8), e1004248. https://doi.org/10.1371/journal.ppat.1004248.
Rolland, A., Jouvin-Marche, E., Viret, C., Faure, M., Perron, H., Marche, P.N., 2006. The envelope protein of a human endogenous retrovirus-W family activates innate immunity through CD14/TLR4 and promotes Th1-like responses. J. Immunol. 176 (12), 7636–7644. https://doi.org/10.4049/jimmunol.176.12.7636.
Römer, C., 2021. Viruses and endogenous retroviruses as roots for neuroinflammation and neurodegenerative diseases. Front. Neurosci. 15, 648629 https://doi.org/10.3389/fnins.2021.648629.
Saito, T., Miyagawa, K., Chen, S.-Y., Tamosiuniene, R., Wang, L., Sharpe, O., Samayoa, E., Harada, D., Moonen, J.-R., Cao, A., Chen, P.-I., Hennigs, J.K., Gu, M., Li, C.G., Leib, R.D., Li, D., Adams, C.M., del Rosario, P.A., Bill, M., Haddad, F., Montoya, J.G., Robinson, W.H., Fantl, W.J., Nolan, G.P., Zamanian, R.T., Nicolls, M. R., Chiu, C.Y., Ariza, M.E., Rabinovitch, M., 2017. Upregulation of human endogenous retrovirus-K is linked to immunity and inflammation in pulmonary arterial hypertension. Circulation 136 (20), 1920–1935. https://doi.org/10.1161/CIRCULATIONAHA.117.027589.
Sanders, V.M., 2006. Epigenetic regulation of Th1 and Th2 cell development. Brain Behav. Immun. 20 (4), 317–324. https://doi.org/10.1016/j.bbi.2005.08.005.
Santoro, M.G., Rossi, A., Amici, C., 2003. NF-kappaB and virus infection: who controls whom. EMBO J. 22 (11), 2552–2560. https://doi.org/10.1093/emboj/cdg267.
Santos, S., Ferreira, H., Martins, J., Gonçalves, J., Castelo-Branco, M., 2022. Male sex bias in early and late onset neurodevelopmental disorders: shared aspects and differences in Autism Spectrum Disorder, Attention Deficit/hyperactivity Disorder, and Schizophrenia. Neurosci. Biobehav. Rev. 135, 104577 https://doi.org/10.1016/j.neubiorev.2022.104577.
Schanab, O., Humer, J., Gleiss, A., Mikula, M., Sturlan, S., Grunt, S., Okamoto, I., Muster, T., Pehamberger, H., Waltenberger, A., 2011. Expression of human endogenous retrovirus K is stimulated by ultraviolet radiation in melanoma. Pigment Cell Melanoma Res 24 (4), 656–665. https://doi.org/10.1111/j.1755-148X.2011.00860.x.
Spencer, T.E., Mura, M., Gray, C.A., Griebel, P.J., Palmarini, M., 2003. Receptor usage and fetal expression of ovine endogenous betaretroviruses: implications for coevolution of endogenous and exogenous retroviruses. J. Virol. 77 (1), 749–753. https://doi.org/10.1128/jvi.77.1.749-753.2003.
Stauffer, Y., Marguerat, S., Meylan, F., Ucla, C., Sutkowski, N., Huber, B., Conrad, B., 2001. Interferon-alpha-induced endogenous superantigen. a model linking environment and autoimmunity. Immunity 15 (4), 591–601. https://doi.org/10.1016/s1074-7613(01)00212-6.
Steele, A.J., Al-Chalabi, A., Ferrante, K., Cudkowicz, M.E., Brown Jr., R.H., Garson, J.A., 2005. Detection of serum reverse transcriptase activity in patients with ALS and unaffected blood relatives. Neurology 64 (3), 454–458. https://doi.org/10.1212/01.WNL.0000150899.76130.71.
Stewart, C.A., Horton, R., Allcock, R.J.N., Ashurst, J.L., Atrazhev, A.M., Coggill, P., Dunham, I., Forbes, S., Halls, K., Howson, J.M.M., Humphray, S.J., Hunt, S., Mungall, A.J., Osoegawa, K., Palmer, S., Roberts, A.N., Rogers, J., Sims, S., Wang, Y. u., Wilming, L.G., Elliott, J.F., de Jong, P.J., Sawcer, S., Todd, J.A., Trowsdale, J., Beck, S., 2004. Complete MHC haplotype sequencing for common disease gene mapping. Genome Res. 14 (6), 1176–1187. https://doi.org/10.1101/gr.2188104.

Stocking, C., Kozak, C.A., 2008. Murine endogenous retroviruses. Cell. Mol. Life Sci. 65 (21), 3383–3398. https://doi.org/10.1007/s00018-008-8497-0.
Sutkowski, N., Conrad, B., Thorley-Lawson, D.A., Huber, B.T., 2001. Epstein-Barr virus transactivates the human endogenous retrovirus HERV-K18 that encodes a superantigen. Immunity 15 (4), 579–589. https://doi.org/10.1016/s1074-7613(01)00210-2.
Szpakowski, S., Sun, X., Lage, J.M., Dyer, A., Rubinstein, J., Kowalski, D., Sasaki, C., Costa, J., Lizardi, P.M., 2009. Loss of epigenetic silencing in tumors preferentially affects primate-specific retroelements. Gene 448 (2), 151–167. https://doi.org/10.1016/j.gene.2009.08.006.
Tabone, O., Mommert, M., Jourdan, C., Cerrato, E., Legrand, M., Lepape, A., Allaouchiche, B., Rimmelé, T., Pachot, A., Monneret, G., Venet, F., Mallet, F., Textoris, J., 2018. Endogenous retroviruses transcriptional modulation after severe infection. Trauma and Burn. Front Immunol 9. https://doi.org/10.3389/fimmu.2018.03091.
Tai, A.K., Luka, J., Ablashi, D., Huber, B.T., 2009. HHV-6A infection induces expression of HERV-K18-encoded superantigen. J. Clin. Virol. 46 (1), 47–48. https://doi.org/10.1016/j.jcv.2009.05.019.
Tamouza, R., Meyer, U., Foiselle, M., Richard, J.-R., Wu, C.-L., Boukouaci, W., Le Corvoisier, P., Barrau, C., Lucas, A., Perron, H., Leboyer, M., 2021. Identification of inflammatory subgroups of schizophrenia and bipolar disorder patients with HERV-W ENV antigenemia by unsupervised cluster analysis. Transl. Psychiatry 11 (1). https://doi.org/10.1038/s41398-021-01499-0.
Thomas, J., Perron, H., Feschotte, C., 2018. Variation in proviral content among human genomes mediated by LTR recombination. Mob DNA 9, 36. https://doi.org/10.1186/s13100-018-0142-3.
Thomas, J.H., Schneider, S., 2011. Coevolution of retroelements and tandem zinc finger genes. Genome Res. 21 (11), 1800–1812. https://doi.org/10.1101/gr.121749.111.
Thompson, P.J., Macfarlan, T.S., Lorincz, M.C., 2016. Long terminal repeats: from parasitic elements to building blocks of the transcriptional regulatory repertoire. Mol. Cell 62 (5), 766–776. https://doi.org/10.1016/j.molcel.2016.03.029.
Trepanier, M.O., Hopperton, K.E., Mizrahi, R., Mechawar, N., Bazinet, R.P., 2016. Postmortem evidence of cerebral inflammation in schizophrenia: a systematic review. Mol. Psychiatry 21 (8), 1009–1026. https://doi.org/10.1038/mp.2016.90.
Tsai, J.D., Chang, S.N., Mou, C.H., Sung, F.C., Lue, K.H., 2013. Association between atopic diseases and attention-deficit/hyperactivity disorder in childhood: a population-based case-control study. Ann. Epidemiol. 23 (4), 185–188. https://doi.org/10.1016/j.annepidem.2012.12.015.
Tsai, K., Cullen, B.R., 2020. Epigenetic and epitranscriptomic regulation of viral replication. Nat. Rev. Microbiol. 18 (10), 559–570. https://doi.org/10.1038/s41579-020-0382-3.
Turcanova, V.L., Bundgaard, B., Hollsberg, P., 2009. Human herpesvirus-6B induces expression of the human endogenous retrovirus K18-encoded superantigen. J. Clin. Virol. 46 (1), 15–19. https://doi.org/10.1016/j.jcv.2009.05.015.
Villarreal, L.P., 2011. Viral ancestors of antiviral systems. Viruses 3 (10), 1933–1958. https://doi.org/10.3390/v3101933.
White, C.H., Beliakova-Bethell, N., Lada, S.M., Breen, M.S., Hurst, T.P., Spina, C.A., Richman, D.D., Frater, J., Magiorkinis, G., Woelk, C.H., 2018. Transcriptional modulation of human endogenous retroviruses in primary CD4+ T cells following vorinostat treatment. Front. Immunol. 9, 603. https://doi.org/10.3389/fimmu.2018.00603.
Wu, Z., Mei, X., Zhao, D., Sun, Y., Song, J., Pan, W., Shi, W., 2015. DNA methylation modulates HERV-E expression in CD4+ T cells from systemic lupus erythematosus patients. J. Dermatol. Sci. 77 (2), 110–116. https://doi.org/10.1016/j.jdermsci.2014.12.004.
Xiang, Y., Liang, H., 2021. The regulation and functions of endogenous retrovirus in embryo development and stem cell differentiation. Stem Cells Int 2021, 6660936. https://doi.org/10.1155/2021/6660936.
Xu, L., Wrona, T.J., Dudley, J.P., 1996. Exogenous mouse mammary tumor virus (MMTV) infection induces endogenous MMTV sag expression. Virology 215 (2), 113–123. https://doi.org/10.1006/viro.1996.0014.

2.8 Transgenic expression of the HERV-W envelope protein leads to polarized glial cell populations and a neurodegenerative environment

Joel Gruchot, Isabel Lewen, Michael Dietrich, Laura Reiche, Mustafa Sindi, Christina Hecker, Felisa Herrero, Benjamin Charvet, Ulrike Weber-Stadlbauer, Hans-Peter Hartung, Philipp Albrecht, Hervé Perron, Urs Meyer and Patrick Küry

Abstract

The human endogenous retrovirus type W (HERV-W) has been identified and repeatedly confirmed as human-specific pathogenic entity affecting many cell types in multiple sclerosis (MS). Our recent contributions revealed the encoded envelope (ENV) protein to disturb myelin repair by interfering with successful oligodendroglial precursor differentiation and by polarizing microglial cells towards an axon-damage phenotype. Indirect proof of ENV's anti-regenerative and degenerative activities has been gathered recently in clinical trials using a neutralizing anti-ENV therapeutic antibody. Yet direct proof of its mode of action can only be presented here based on transgenic ENV expression in mice. Upon demyelination we observed myelin repair deficits, neurotoxic microglial and astroglial cells and increased axon degeneration traits. Experimental autoimmune encephalomyelitis activity progressed faster in mutant- as compared to wildtype litter mates equally accompanied by activated glial cells. This study therefore provides for the first time direct evidence on HERV-W ENV's contribution to the overall negative impact of this activated viral entity in MS.

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Establishment, execution and analysis of an *ex vivo* co-culture model via qPCR and immunocytochemistry. Planning and realization of animal experiments (CPZ and EAE). Establishment and realization of tissue isolation for qPCR and histological analysis. Establishment and realization of histological analysis of the diseased corpus callosum and spinal cord. Conceptualization and preparation of a manuscript including figure design.

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Main Manuscript for

Transgenic expression of the HERV-W envelope protein leads to polarized glial cell populations and a neurodegenerative environment

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Classification: Biological Sciences; Neuroscience

Keywords: endogenous retrovirus; multiple sclerosis; neurodegeneration; myelin repair; glia

This PDF file includes:

Main Text
Figures 1 to 6

Abstract

The human endogenous retrovirus type W (HERV-W) has been identified and repeatedly confirmed as human-specific pathogenic entity affecting many cell types in multiple sclerosis (MS). Our recent contributions revealed the encoded envelope (ENV) protein to disturb myelin repair by interfering with oligodendroglial precursor differentiation and by polarizing microglial cells towards an axon-damage phenotype. Indirect proof of ENV's anti-regenerative and degenerative activities has been gathered recently in clinical trials using a neutralizing anti-ENV therapeutic antibody. Yet direct proof of its mode of action can only be presented here based on transgenic ENV expression in mice. Upon demyelination we observed myelin repair deficits, neurotoxic microglia and astroglia and increased axon degeneration. Experimental autoimmune encephalomyelitis activity progressed faster in mutant mice equally accompanied by activated glial cells. This study therefore provides for the first time direct evidence on HERV-W ENV's contribution to the overall negative impact of this activated viral entity in MS.

Significance Statement

Although neurodegeneration is a hallmark of multiple sclerosis (MS), its progression is still not fully understood. However, it is the major factor leading to clinical disability that still cannot be addressed therapeutically. With the here presented study, we provide the first direct evidence that HERV-W ENV expression results in multiple glial cell deteriorations and accompanied neuropathology *in vivo*. This data therefore suggest that activation of this endogenous retroviral element is indeed causally contributing to MS. Our findings will therefore help understanding the molecular and cellular processes being modulated by the currently clinically tested anti-HERV-W neutralization strategy and will support the development of this approach into clinical therapy.

Main Text

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) of still unknown etiology. This disease is primarily characterized by peripheral immune cell infiltration, focal inflammation, as well as the loss of oligodendrocytes and myelin sheaths leading to white and gray matter lesions. During disease progression immune cell infiltration ceases and neurodegeneration predominates leading to irreversible sensory-, motor-, and cognitive deficits (1). Besides peripheral immune cells, brain resident microglial- and astroglial cells are also involved in the disease process particularly in progressive stages as they were shown to adapt neurotoxic- and damage associated profiles and phenotypes (2).
In 1989 an association between retroviral elements and MS was described based on the analysis of primary leptomeningeal cell cultures isolated from MS patients (3). While initially termed multiple sclerosis associated retrovirus (MSRV), it was later found to belong to the family of human endogenous retroviruses (HERVs) and referred to as HERV type W (4). Subsequent studies on HERV-W provided convincing evidence that activation and expression of this otherwise dormant sequences and the subsequent production of the encoded envelope (ENV) protein can exacerbate the immune response (5-7). It was then shown that HERV-W ENV RNA and protein levels are

increased in the cerebrospinal fluid (CSF) and serum of MS patients (8-10). In MS brains HERV-W ENV protein was found as acellular deposits and to be mainly expressed by myeloid cells whereas a few HERV-W ENV-positive astroglial- and lymphoid cells could also be detected (11, 12). Herpesviridae have been shown to activate dormant HERVs among them also the Epstein Barr Virus (EBV) (13, 14) which was recently suggested to be a leading cause for MS (15), thus further corroborating a functional implication of HERV-W in the disease process.
Apart from immune- and endothelial cell activation (5, 7, 16), our own research provided strong evidence that also myelin repair is impaired by HERV-W ENV (17, 18). Furthermore, a role in polarization of microglial cells towards a demyelination and neurodegeneration related phenotype was reported (11). Yet, owing to its human specific origin these studies relied on histological assessments combined with functional experiments done ex vivo. Nevertheless, an implication of HERV-W in neurodegeneration and white matter repair was further supported by clinical trials on a HERV-W ENV neutralizing antibody termed Temelimab as a significant reduction in brain atrophy levels as well as improved myelin integrity were observed in treated MS patients but also relied upon an indirect analysis, i.e. magnetic resonance imaging(19).
We here, for the first time, report a role of HERV-W ENV in MS related disease processes based on the analysis of a novel viable transgenic mouse line mimicking endogenous HERV-W ENV activity in the diseased CNS. Apart from exacerbated autoimmune activities, a strong negative impact on oligodendrogenesis and remyelination along with neurotoxic microglial and astroglial cell populations were observed.

Results

Transgenic HERV-W ENV expression fosters demyelination and reduces remyelination
In our previous studies the HERV-W envelope (ENV) protein was shown to affect glial cells (18, 20) and in MS ENV expression was described by myeloid, astroglial- and lymphoid cells, leading to acellular deposits of shed protein (12, 20). Such an expression pattern likely results from long-term activation scenarios but in light of the still missing information on HERV-W genomic integration sites and expression-driving sequences (20, 21) reveals to be difficult to mimic. In order to analyze the effects of endogenous ENV expression in a functional in vivo context, we used a previously established mouse model (22), in which the HERV-W ENV transgene (pV14; GenBank accession number: AF331500.1) is expressed under the control of the ubiquitous CAG promotor but is additionally regulated by its 3' long terminal repeat sequence (LTR). To confirm ENV transcript and protein expression in the mouse brain quantitative RT-PCR as well as automated western blot techniques were used (Fig. 1A-B). Based on the reported hexameric extracellular appearance of the ENV protein and the resulting unique solubility and antigenic characteristics, particularly when using human-specific antibodies in a mouse background (23), the analysis via automated Simple Western technology revealed to be the essential. To analyze the effects of transgenic ENV expression on remyelination, we applied the well-established cuprizone model of demyelination (24-26). Transgenic and wildtype littermate mice (hemizygote males and homozygote females) were fed for 7 weeks with 0.2% cuprizone (CPZ) chow and subsequently switched to control diet to induce remyelination (Fig. 1C). Histological analysis was carried out at time points 5 and 7 weeks of demyelination as well as at 1, 2 and 3 weeks during remyelination. Luxol fast blue (LFB) staining of the caudal corpus callosum revealed that transgenic ENV protein increases/accelerates cuprizone mediated demyelination and further impedes remyelination of this brain structure (Fig. 1D,E). Moreover, anti-amyloid precursor protein (APP) staining revealed increased densities of APP-positive spheroids in transgenic corpus callosum (Fig. 1F,G), suggesting enhanced neurodegeneration in transgenic mice.

Transgenic HERV-W ENV expression affects oligodendroglial cell differentiation
To analyze the observed changes in myelin integrity of CAG-Env mice in greater details, we analyzed a number of different oligodendroglial differentiation markers. In contrast to wildtype animals, transgenic CAG-Env mice displayed significantly reduced numbers of platelet-derived growth factor receptor-α (Pdgfrα)-positive oligodendroglial precursor cells (OPCs) in the course of

cuprizone treatment (Fig. 2A-B). This is most likely resulting from fewer proliferating OPCs in the early phases of CPZ treatment (5 weeks CPZ) as revealed by anti-Pdgfrα/Ki67 costaining (Fig. 2F-G). To further analyze oligodendroglial differentiation, SRY-Box transcription factor 10 (Sox10) and adenomatous-polyposis-coli (APC) staining was performed. Sox10-positive/APC-negative cells correspond to differentiating OPCs and were found to be significantly reduced in numbers in CAG-Env mice compared to wildtype animals throughout de- and remyelination (Fig. 2A,C). The number of Sox10/APC double-positive maturing oligodendrocytes was also found to be impaired in transgenic brains particularly upon CPZ withdrawal hence in the remyelination phase (Fig. 2A,D). Furthermore, densities of early (re)myelinating oligodendrocytes marked by their expression of the breast carcinoma amplified sequence 1 (Bcas1) protein (27), were also significantly reduced in transgenic tissues at all stages analyzed. Of note, no differences in related to myelin and oligodendroglial parameters were observed in control (untreated) wildtype and transgenic mice (Fig. 1E,F; Fig. 2A-E). These observations clearly demonstrate that transgenic expression of the HERV-W ENV protein indeed affects oligodendroglial differentiation in vivo at multiple levels leading to impaired myelination - thereby corroborating our previous ex vivo findings (17, 18).

Impaired de- and remyelination is characterized by HERV-W ENV driven activation of microglial cells

We previously reported that the HERV-W ENV protein polarizes microglial cells ex vivo towards an axon damaging phenotype (11). Current advances in characterizing microglial activation at molecular levels throughout a number of CNS diseases and models lead to the description of disease-associated microglial gene signatures and markers. A few of these disease-associated markers were used to study microglial activation in the CAG-Env transgenic mouse model. C-type lectin domain family 7 member A (Clec7a) was described as highly upregulated gene associated with different neurodegenerative diseases (28-30), whereas, complement C1q subcomponent subunit A (C1qa) was described as a key signaling protein of disease-associated microglia leading to the neurotoxic activation of astrocytes (31). Cluster of differentiation 74 (Cd74) was found to be induced in activated microglia also associated with neurodegenerative diseases and aging (28, 32, 33). We performed gene expression analysis of extracted corpus callosum tissue and observed that all three genes (Clec7a, Cd74 and C1qa) were expressed at similar levels in untreated mice but significantly induced during the course of CPZ induced demyelination and remyelination in transgenic CAG-Env mice as compared to wildtype animals (Fig. 3A-C; as revealed by calculating area under the curve (AUC) values). Immunohistochemical staining against the ionized calcium-binding adapter molecule 1 (Iba1), Clec7a and Cd74 proteins was performed next (Fig. 3D). Analyzing the degree of Iba1-positive areas in the corpus callosum already indicated, that transgenic animals experienced a stronger microglial activation as compared to wildtype mice especially at earlier time-points (5 and 7 weeks of cuprizone treatment; Fig. 3D-E). Unchallenged wildtype and CAG-Env mice displayed no differences in Iba1-positive areas as well as in Iba1/Clec7a or Iba1/Cd74 double-positive areas (Fig. 3D-I). At all stages of cuprizone-treatment, numbers of Clec7a-positive microglial cells were significantly increased in transgenic mice (Fig. 3D) resulting in increased Iba1/Clec7a double-positive areas (Fig. 3F). When normalized against total Iba1-positive areas (Fig. 3G), the proportion of Clec7a-positive, disease-associated microglia was significantly elevated upon transgene expression. While peaking slightly later the analysis of Iba1/Cd74 double-positive cells further confirmed the activated microglial phenotype in transgenic mice under cuprizone application (Fig. 3D,H,I).

HERV-W ENV protein driven activation of astrocytes

Besides microglial activation, astroglial cell polarization is of further interest in the context of neurodegenerative diseases. Yet direct HERV-W ENV dependent effects exerted on astroglia have not been reported despite the fact that they express toll-like receptor 4 (TLR4) (34), one of the receptors for HERV-W ENV (7). However, several markers for activated and/or neurotoxic astrocytes including the complement cascade have recently been described as being induced in CNS pathologies. Complement component 3 (C3) and especially its cleaved form C3d was described in astrocytes from MS patients but also in different MS models (31, 35). In contrast to

the general activation marker C3d, lipocalin-2 (Lcn2) was assigned as neurotoxic astrocyte marker, as the secreted protein can induce neuronal death (31, 36). In order to gain first insights into astroglial activation, C3 and Lcn2 gene expression analysis of isolated corpus callosum tissue was performed.
C3 expression was significantly increased in CAG-Env corpus callosum tissue upon CPZ treatment, whereas control (unchallenged) animals displayed no difference in transcript levels between wildtype and transgenic mice (Fig. 4A). Lcn2 displayed a different expression profile with a strong peak of expression in transgenic corpus callosum tissue after 5 weeks of demyelination whereas in wildtype litter mates only a mild increase in expression peaking at 2 weeks post cuprizone feeding was observed (Fig. 4B). Again, control animals showed no difference in Lcn2 expression. To corroborate this observation at protein level, glial fibrillary acidic protein (Gfap)-positive astrocytes were evaluated for their expression of C3d and Lcn2 proteins by immunohistochemistry (Fig. 4C). As Gfap itself is often already described as an activation marker for astrocytes in vivo, Gfap-positive areas were quantified upon CPZ treatment. This revealed that in transgenic mice astroglial activation was increased in both phases, under CPZ treatment and during remyelination (Fig. 4D). Analyzing C3d/Gfap double-positive areas then demonstrated a significant elevation of C3d-positive astrocytes in the corpus callosum of transgenic animals versus wildtype mice (Fig. 4E). However, the proportion of C3d-positive astrocytes normalized to all Gfap-positive astrocytes remained unchanged (Fig. 4F). Since Lcn2 expression was found to be particularly limited to the active demyelination phase (Fig. 4B), quantification of Gfap/C3d/Lcn2 triple-positive cells was performed and confirmed a strong induction of neurotoxic astrocytes during cuprizone treatment (Fig. 4G). However, in contrast to C3d the proportion of Lcn2-positive astrocytes over all activated astrocytes changed with significantly elevated cell densities after 5 and 7 weeks of cuprizone treatment (Fig. 4H). Naïve (control) animals, again, showed no significant differences in the occurrence of Gfap, Gfap/C3d double-positive as well as Gfap/C3d/Lcn2 triple-positive cells (Fig. 4C-H). These observations suggest that transgene expression generally increases astroglial activation levels and specifically boosts the presence of neurotoxic phenotypes.

HERV-W dependent activation of microglia and astrocytes ex vivo
It has previously been demonstrated that astrocytes can be activated via TLR4 receptor signaling (34) but also in response to microglial cytokines (31). In the HERV-W context we already described that microglial cells express and secrete pro-inflammatory cytokines such as tumor necrosis factor-α (TNF α), interleukin (IL)-1β and IL-6 (11) as well as the above described C1qa (Fig. 3A). We therefore examined to what degree observed astrocyte reactions, were directly elicited by the ENV protein or mediated by ENV activated microglia (11). To this end we isolated microglial- and astroglial cells from rat primary mixed glial cultures using magnetic-activated-cell-sorting (MACS) and combined these two cell types preventing direct cell contacts but enabling exchange of cytokines and signaling peptides (Fig. 5A). These cultures were then stimulated for 24 h with 1 µg/ml recombinant HERV-W ENV protein. Anti-Gfap, anti-Iba1 immunocytochemistry revealed >98% pure astrocytes and that no microglial cells were able to cross the barrier of the cell culture insert (Fig. 5B-C). Since HERV-W ENV activated microglia displayed a strong induction of inducible nitric oxide synthases (iNOS) expression (11) cell culture inserts were stained using anti-Iba1 and anti-iNOS antibodies confirming microglial activation as well as stable cell densities (Fig. 5D-F). To analyze the activation status of astroglial cells transcript levels of anti-inflammatory S100 calcium-binding protein A10 (S100a10) as well as of pro-inflammatory serpin family G member 1 (Serping1), C3d and Lcn2 were quantified. S100a10 expression was significantly downregulated upon HERV-W ENV treatment regardless of whether microglia were present or not (Fig. 5G) whereas Serping1, C3d and Lcn2 transcript levels were induced (Fig. H-J). Although all these pro-inflammatory genes were already induced by the ENV protein alone, their expression was further boosted by the presence of ENV- activated microglial cells. These findings were corroborated at protein levels with

Gfap-positive astrocytes expressing C3d and Lcn2 proteins in response to ENV exposure and being amplified in presence of microglial cells (Fig. 5K-M).

Transgenic HERV-W ENV expression leads to an aggravated EAE course

In order to analyze the effects of transgenic HERV-W ENV expression in the context of neuroinflammation, we applied myelin oligodendrocyte glycoprotein fragment 33-55 (MOG33-55) peptide-induced experimental autoimmune encephalomyelitis (EAE, Fig. 6A) and analyzed inflamed lumbar spinal cords. By analyzing the clinical score of diseased mice, a significant worsening of symptoms could be observed in CAG-Env mice as compared to wildtype litter mates particularly in the period between 18 to 20 days post induction (dpi) and thereafter (Fig. 6B,D). Animals were then perfused at 20 dpi and the lumbar spinal cords were analyzed in terms of lesion formation (anti-MBP immunohistochemistry, Fig. 6C). No differences regarding lesion numbers of were observed between transgenic and wildtype mice (Fig. 6E), but relative lesion sizes were significantly increased in response to the transgene expression (Fig. 6F). Further immunohistological characterization of lesions then revealed that also Iba1-positive areas were significantly elevated in CAG-Env mice compared to wildtype animals (Fig. 6G,I). Likewise, when assessing Clec7a positivity an even more pronounced increase in areas harboring neurotoxic microglia and macrophages was observed (Fig. 6G,J). Interestingly, in transgenic mice Clec7a-positive myeloid cells were also found outside lesion cores, indicating that neuroinflammation was less focally restricted in mutant mice (Fig. 6G, arrowheads). Moreover, astroglial activation was examined via Gfap-, C3d- and Lcn2 expression (Fig. 6H). Quantification revealed a significant rise in relative Gfap-positive areas in the transgenic background (Fig. 6K). This was accompanied by increased areas featuring neurotoxic astrocytes (Gfap/C3d/Lcn2 triple-positive area; Fig. 6M), whereas areas with only activated astrocytes - Gfap/C3d-positive cells that lack Lcn2 expression – were slightly reduced (Fig. 6L).

Discussion

Activation and expression of this endogenous retroviral element has only been described in a few pathological instances such as in MS, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), type-1 diabetes (T1D) as well as in neurodevelopmental disorders (22, 37, 38). In addition, most recent evidence points to a strong activation in some Covid19 patients (39, 40). While initially discovered in MS patient-derived leptomeningeal cells (3), most information regarding impact and functions of HERV-W and its envelope protein (ENV) relates to this autoimmune disease with immune-, endothelial-, oligodendroglial-, and microglial cells being implicated (38, 41, 42). However, data on the functional role of this human specific pathogenic entity have so far only been gathered from histopathological observations on autopsy material combined with ex vivo primary cell- and tissue-based functional assays. Yet a direct proof of concept on the cellular responses evoked by the expression of the HERV-W ENV protein in vivo was missing so far.

We here describe for the first time strong reactions of the three glial cell types in response to transgenic HERV-W ENV expression, most notably only under MS related pathological conditions (EAE and CPZ), with microglial- and astroglial phenotypes in accordance with recent descriptions of disease-associated glial signatures (30, 31, 43, 44). It is the first account of the generation of an overall neurotoxic environment, of worsening pathologies and of impeded regeneration processes, confirming and expanding previous postulations (5, 11, 18). In this study we focused explicitly on the roles of glial cells since our earlier investigations pointed to an inhibited OPC differentiation as well as to neurotoxic microglial polarization. Moreover, glial pathological reactions were also suggested by the outcome of clinical trials with a neutralizing anti-HERV-W ENV antibody (Temelimab) where reduced brain atrophy rates (suggesting a role in smoldering neurodegeneration) as well as preserved myelin integrities (supporting evidence for effects on oligodendroglial differentiation and myelin repair) were observed (19). Such an emphasis on de- and regeneration was additionally justified as these pathological processes still represent unmet clinical needs.

Astrocyte activation was unexpected and thus represents a novel observation. These cells appear to be sensitive to both, a direct impact of the ENV protein as well as to signals emanating from polarized microglial cells - pointing to cell/cell interactions as recently described in chronic MS (45). This finding certainly contributes to the understanding of the emerging pathological role of astrocytic cells in MS and related demyelinating diseases (46), promoting research into cell specific modularity approaches aiming at novel therapeutic opportunities. It will also be of interest to see whether and at what disease stages the here-described glial phenotypes can also be seen in MS tissue samples.

Our view on this viral protein's impact on myelin repair has also been refined. ENV appears not only to interfere with oligodendroglial maturation, but its presence also resulted in a lack of OPC recruitment mostly due to a reduction in proliferation rates. This was not seen in former ex vivo studies due to the use of post-mitotic primary cells. At present it is not known whether this relates to a direct effect on OPCs or whether lack of trophic input or the presence of an inhibitory milieu generated by microglia and/or astrocytes account for decreased cell numbers (47) – an issue that remains to be addressed in future studies.

Astrocyte and microglia/macrophage numbers were, however, increased in both lesion models also correlating with the larger lesions found in inflamed spinal cords of the EAE mice. Of note, in the transgenic background the degree of neurotoxic glial cells exceeded this ratio, arguing for a pronounced activation of neurotoxic phenotypes. Given that in mutant mice such astrocyte subtypes were also detected outside EAE lesions, a possible contribution to lesion growth can be suggested.

Finally, signs of increased axonal degeneration as seen by the development of APP-positive spheroids were detected in demyelinated, cuprizone treated transgenic mice. It is tempting to speculate that observed aggravated clinical symptoms in the EAE model also result (in part) from enhanced degeneration processes. This on the one hand confirms our previous description of an axon-degenerating microglial phenotype in response to ENV protein exposure (11), on the other hand it clearly demonstrates that even in a rather gentle and regenerative lesion set-up such as mediated via cuprizone feeding, mutant animals are more impaired – a remarkable consideration for this model system (48).

While we could prove that all CNS glial cells respond to this pathological protein expression in two different models mimicking MS features, additional observations in the EAE model provided evidence that also autoimmune processes might be altered, given that lesions were not more frequent but larger in size and were thus containing more Iba1-positive (myeloid) cells. It is unlikely that this can be explained via microglia phenotype consolidation only, also in light of the fact of a more pronounced macrophage contribution known to occur in EAE. It therefore remains to be shown whether previous reports on Th1-like type of Th cell differentiation or superantigen-like (SAg) activation of T cells (6, 7) can be confirmed in this in vivo model. Such a more detailed description of immune cells is out of scope of this study, and this also includes analyses of stem cells niches, endothelia as well as pericytes, all of which also being functionally implicated in autoimmune and neurodegenerative pathologies. Of note, a direct effect of HERV-W ENV exerted onto lymphoid cells was not supported by the clinical trials (19). But it must be kept in mind that this circumstance might relate to the particular MS patient cohort enrolled for these trials as for example addressing early autoimmunity events, such as for example conversion from clinically isolated syndrome (CIS) to relapsing-remitting MS, was not possible.

A notable limitation of our investigation relates to the applied transgenic mouse model. As we used a general and non-inducible transgene expression, possible role(s) in development can currently not be excluded. Yet, using multiple protein markers related to oligodendrogenesis, microglial- and astroglial phenotype generation we detected no differences between unchallenged (non-CPZ, non-EAE) transgenic mice and wildtype litter mates – at least at all ages analyzed here (Figs. 1, 2, 3, 4, 6). Also, no evidence of increased or predetermined axon degeneration was found providing further evidence that the model is valid and that HERV-W ENV obviously needs a pathological environment (toxin, infection, autoimmunity) to manifest its functionality as discussed previously (37, 38). Given the still rather unclear HERV-W activation process(es) involved in the pathology of MS, one might indeed suggest that this entity is part of a two-hit model the sequence of events still

remains to be determined (37). Moreover, more accurate overexpression models await the still non-existing information on the genomic nature of this pathological HERV-W element as it is currently not known to what degree fixed or unfixed copies and under which regulatory sequences account for activation and expression in the diverse diseases. Likewise, it will be difficult to measure any cognitive impairments or changes at sub-neuronal levels (synapses, plasticity) using the here-applied short-term experimental paradigms. Given the recent description of HERV-W ENV modulating synapse maturation in vitro (49) but also taking into account findings on the neutralizing antibody Temelimab conferring a rescue from brain volume loss (19), such important aspects need to be addressed in more chronic demyelination/degeneration scenarios.
Moreover, given the identification of EBV as being involved in the generation of MS on the long-term (15), a more recent description of antibodies directed against EBV and HERV-W related proteins in the CSF of MS patients (50) further supports exogenous to endogenous viral entities to be implicated in different disease processes. Such an activation scenario has previously already been suggested (13, 14).
In conclusion, we here present the long expected functional proof of HERV-W ENV's degenerative potential in vivo and demonstrate that mainly glial cells react and contribute to the generation of a neurotoxic parenchyma. It is tempting to speculate that our findings not only relate to pathological processes underlying MS but also to tissue changes in neurodevelopmental disorders or in long-Covid19 patients. Therefore, the further development of suitable neutralization strategies such as by preclinical and clinical examinations of the Temelimab antibody or of unrelated pharmacological approaches (17, 51) is highly warranted.

Materials and Methods

Ethics statements for animal experiments
All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The Institutional Review Board (IRB) of the ZETT (Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben) at the Heinrich-Heine-University Düsseldorf approved animal procedures related to tissue isolation under internal licenses O69/11, O90/15. The review board of the state government LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, North-Rhine Westphalia, Germany) approved all animal experimental procedures under licenses: Az.:84-02.04.2017.A137 and 81-02.04.2019.A063.

Animal models
Transgenic C57BL6/J;129P2/Ola-Hprttm(CAG-Env) (CAG-Env; 75% C57BL6/J + 25% 129P2/Ola) mice were used in this study. Animal genotypes were determined using genomic PCR for hypoxanthine-guanine phosphoribosyltransferase (Hprt) alleles as established previously (22). Briefly, genomic DNA was extracted from ear punches used for animal labelling according to the manufacturers protocol (PureLink genomic DNA-Minikit, Thermo Fisher Scientific, Waltham, MA, USA). Afterwards, alleles were amplified using Red HS Taq Master Mix (Biozym, Hessisch Oldendorf, Germany) and analyzed on a 2% agarose gel (LE Agarose, Biozym). For the detection of Hprt wildtype (wt) and transgenic (tg) alleles following primer pairs were used: wt_fwd: 5'- TGT CCT TAG AAA ACA CAT ATC CAG GGT TTA GG; wt_rev: 5'- CTG GCT TAA AGA CAA CAT CTG GGA GAA AAA and tg_fwd: 5'-ACG TCA GTA GTC ATA GGA ACT GCG GTC G; tg_rev: 5'-TAC AGG CGT GAA CCA CTG CTC CCT using temperature cycles: 94°C for 2 min, followed by 94°C for 30 s, 55°C (wt)/65°C(tg) for 30 s, 68°C for 60 s, repeated 35 times, then 68°C for 8 min resulting in a wt DNA fragment of 345 bp and a transgenic (tg) DNA fragment of 399 bp. Mice were bred by the ZETT and housed in a pathogen-free facility (SPF) with 12 h light/dark cycle and supplied with food/water ad libitum. Demyelination was induced in 8-week-old mice using a diet containing 0.2% w/w cuprizone [bis(cyclohexanone)oxaldihydrazone] (V-1534, Ssniff, Soest, Germany) similar as previously described (24-26). In order to achieve sufficient demyelination, animals had to be fed for 7 weeks with CPZ (Fig. 1E,F) and were changed afterwards to control diet without cuprizone (V-

1534, Ssniff) for 1, 2 and 3 weeks (1, 2, 3 wk rem). Control animals (unchallenged, no lesion formation) received chow without cuprizone (V-1534, Sniff). The diet was changed twice per week and animal bodyweights were monitored twice per week. All cuprizone experiments were performed with 6 animals (either sex) per group and time point according to the cohort size analysis (using G*Power 3.1.9.7; effect size: 2.6; α-level: 0.05; Power: 0.95). Experimental autoimmune encephalomyelitis (EAE) was induced as previously described (51). Briefly, 8-week-old mice (either sex) were immunized with 200 µg myelin oligodendrocyte glycoprotein fragment 35-55 (MOG35-55) (Biotrend, Cologne, Germany) followed by intraperitoneal injections of 200 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) at days 0 and 2. Afterwards, animals were monitored and the clinical EAE score (0-5) (52) was determined on a daily basis for the following 30 days. Cohort size analysis for EAE experiments (using G*Power 3.1.9.7; effect size: 1.6; α-level: 0.05; Power: 0.95) resulted in an optimal group size of 12 animals. Furthermore, only animals that developed clinical signs of paralysis (EAE score) were included in subsequent analyses.

Primary rat mixed glial cultures

Primary rat mixed glial cultures containing microglia and astrocytes were isolated as previously described (11). Briefly, dissociated P1 Wister rat cortices were cultured on T-75 cell culture flasks in Dulbecco′s Modified Eagle′s Medium (DMEM; Thermo Fisher Scientific) substituted with 10% fetal calf serum (FCS; Capricorn Scientific, Palo Alto, CA, USA) and 2 mM L-glutamine (Thermo Fisher Scientific), 50 U/ml penicillin/streptomycin (Thermo Fisher Scientific). After 10 days, flasks were shaken at 180 rpm/min at 37 °C and microglia-containing supernatants were collected after 2 h and 24h. Subsequently, astrocytes and microglia were purified using Magnetic-Activated-Cell-Sorting (MACS) according to the manufacturer's protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, the remaining astrocytes were dislodged by trypsin-ethylenediaminetetraacetic acid (EDTA)-treatment (Thermo Fisher Scientific), labelled using a combination of anti-ACSA1 biotinylated antibodies in combination with anti-biotin microbeads (Miltenyi Biotec) and applied to the isolation column. Microglial cells were detached by accutase treatment and labelled with anti-rat Cd11b/c microbeads (Miltenyi Biotec). The resulting cell suspensions were analyzed for cell viability and numbers using trypan blue staining. Average cell purities as assessed by Gfap/Iba1-positivity were consistently >98%. Astrocytes (30.000 cells/well, 2 wells/ condition) and microglia (100.000 cells/transwell, 2 wells/condition) were seeded on 24-well plates and respective transwells in DMEM medium containing 10% FCS, 2 mM L-glutamine and 50 U/ml penicillin/streptomycin (Thermo Fisher Scientific. After 24 h, cell cultures were stimulated with either 1 µg/ml recombinant HERV-W ENV protein (Protein'eXpert, Grenoble, France) or reconstitution buffer. To avoid side effects through the recombinant production of HERV-W ENV protein, Endotoxin levels were measured using the limulus amebocyte lysate (LAL)-test and found to be below the detection limit (<5EU=ml).

Tissue isolation for transcript and protein expression analysis

Briefly, animals were deeply anesthetized with isoflurane and transcardially perfused with 20 mL cold phosphate-buffered saline (PBS) to remove blood cells from the brain tissue. For the detection of HERV-W ENV protein using automated western blot techniques, whole brain were isolated and immediately frozen in liquid nitrogen. For cuprizone (CPZ) experiments, the brain was isolated and placed in a murine brain matrix (BSMAS001-1; Zivic instruments, Pittsburg, USA) and three 1 mm corpus callosum (corpus callosum) containing slices were isolated and placed in a drop DPBS. Immediately, corpus callosum was isolated using a binocular and scalpel and snap frozen in liquid nitrogen. All samples were stored at -80 °C until Protein and/or RNA isolation.

RNA extraction, cDNA synthesis and real time quantitative RT-PCR

For the RNA extraction from cell cultures, cells were lysed using 350 µl β-mercaptoethanol (Sigma-Aldrich) – RLT buffer (1:100, Qiagen, Hilden, Germany) and immediately snap frozen on dry ice. Afterwards, total RNA was isolated using the column-based RNeasy mini kit (Qiagen) according to the manufacturer's protocol. For the RNA extraction from snap frozen tissue, snap frozen tissue was homogenized by applying 1 ml TRIzol™ reagent (Thermo Fisher Scientific)/ 100 mg tissue

using Polytron PT 2100 homogenizer (Kinematica AG, Malters, Switzerland) . Afterwards, total RNA was isolated according to the manufacturer's protocol. RNA quality and concentration were quantified by a Nanodrop spectrophotometer (Thermo Fisher Scientific), and samples were stored at −80°C until analysis. For q-RT-PCR analysis, isolated RNA was first reverse transcribed using the high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative determination of gene expression levels was performed on a 7900HT sequence detection system (Thermo Fisher Scientific) using Power SybrGreen PCR master mix (Thermo Fisher Scientific) as previously published (11). Following primer sequences were generated via PrimerExpress 2.0 software (Thermo Fisher Scientific), as well as tested and determined: mGapdh forward: AGG TTG TCT CCT GCG ACT TCA, mGapdh reverse: CCA GGA AAT GAG CTT GAC AAA G, HERV-Wenv forward: TTT ACT CCT CTT TGG ACC CT, HERV-Wenv reverse: ATC TGG GGT TCC ATT TGA AG, mC1qa forward: GCC TGT GTG CTG ACC ATG AC, mC1qa reverse: GGGTGC TCG GCA GAC ATC T, mClec7a forward: CCT TGG AGG CCC ATT GC, mClec7a reverse: GCA ACC ACT ACT ACC ACA AAG CA, mCd74 forward: CCA ACG CGA CCT CAT CTC TAA, mCd74 reverse AGG GCG GTT GCC CAG TA, mC3 forward: CCG TGA ACA GGA GGA ACT TAA GG, mC3 reverse: ATG CTG CAG AAG GCT GGA TT, mLcn2 forward: CCC TGT ATG GAA GAA CCA AGG A, mLcn2 reverse: GCA AAG CGG GTG AAA CGT T, rGapdh forward: GAA CGG GAA GCT CAC TGG C, rGapdh reverse: GCA TGT CAG ATC CAC AAC GG, rS100a10 forward: GCC ATC CCA AAT GGA GCA T, rS100a10 reverse: CCC CTG CAA ACC TGT GAA AT, rSerping1 forward: GAC AGC CTG CCC TCT GAC A, rSerping1 reverse: GCA CTC AAG TAG ACG GCA TTG A, rC3 forward: GGT CTG CGG AAG TGT TGT GA, rC3 reverse: GGC GCT GGC AGC TGT ACT, rLcn2 forward: GGG CAG GTG GTT CGT TGT C, rLcn2 reverse: AGC GGC TTT GTC TTT CTT TCT G. Gapdh, which proved to be the most accurate and stable normalization gene among a number of others such as Hprt, Odc, Tbp, was used as reference gene. Relative gene expression levels were determined according to the ΔΔCt method (Thermo Fisher Scientific) and each sample was measured in duplicate.

Protein isolation and automated western blot analysis

For the detection of HERV-W ENV antigen, snap frozen mouse tissue was extracted according to MEM-PER manufactures instructions (Thermo Fisher Scientific). Mouse brains were homogenized with 3 cycles of 20 seconds of Precellys (CK14, Bertin instruments, Montigny-le-Bretonneux, France). After 10 min of centrifugation at 10,000 x g, supernatants were collected. HERV-W ENV antigen detection was analyzed on the Jess device using Simple Western technology an automated capillary-based size sorting and immunolabeling system (ProteinSimpleTM, Biotechne Miniapolis, MA, USA) as previously described (23). Anti-HERV-W ENV mAb GN_mAb_ENV01 (Geneuro, Geneva, Switzerland) was used at 20µg/mL to detect antigen. HERV-W ENV antigen was identified within the apparent molecular weight range (350-450 KDa) in this capillary matrix, using the Jess platform Compass TM software (ProteinSimpleTM, Biotechne Miniapolis, MA, USA).

Immunocytochemistry

For immunocytochemistry, astroglial-microglial cocultures were fixed for 10 min with 4% paraformaldehyde (PFA), D-PBS washed, blocked for 45 min according to the host of the secondary antibody (either 2% normal goat serum [NGS] or 10% normal donkey serum [NDS] respectively, both Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich) in D-PBS. Afterwards, cells were incubated at 4 °C overnight with primary antibody solution containing, 10% NDS and 0,1% Triton X-100 in D-PBS with rabbit anti-Iba1 (1/500, WAKO Pure Chemical Corporation, Osaka, Japan; RRID: AB_839504), goat anti-iNOS (1/250; Abcam, Cambridge, UK, RRID: AB_301857), chicken anti Gfap (1/1000; Abcam; RRID:AB_304558), rabbit anti hC3d (1/300; Agilent, Santa Clara, CA, USA; RRID:AB_578478) and goat anti Lcn2 (1/100; R and D Systems, Minneapolis, MN, USA, RRID:AB_355022). Following D-PBS washes species-appropriate Alexa fluorochrome-conjugated secondary antibody (1/200 in PBS, Thermo Fisher Scientific) and 4′, 6-diamidino-2-phenylindole (DAPI; 20 ng/ml, Roche, Basel, Switzerland) were incubated for 30 min at RT. Afterwards, cover slips were washed in PBS and embedded using Shandon™ Immu-Mount (Thermo Fisher Scientific).

Immunohistochemical procedures

EAE and CPZ-treated animals were deeply anesthetized with isoflurane and transcardially perfused with 20 mL cold PBS followed by 20 mL 4% paraformaldehyde (PFA, Sigma-Aldrich) Brains and/or spinal cord were isolated and post-fixed in the same fixative for 1 day at 4 °C, followed by 24 to 48 h cryoprotective dehydration in 30% sucrose at 4 °C. Afterwards, tissue was embedded in Tissue-Tek (Sakura Finetek Europe, Alphen aan den Rijn, Netherland), frozen and stored at -30 °C until preparation of 12 µm sections using a cryostat (Leica CM30510S, Leica, Wetzlar, Germany). For CPZ experiments, coronal sections of the caudal corpus callosum (Bregma: -0.70 - -2.06) were collected and for EAE experiments, transverse lumbar spinal cord sections were prepared. All sections were stored at -30 °C until immunohistochemical analysis avoiding any freeze-thaw cycles. To assess the relative myelination of the corpus callosum, Luxol fast blue (LFB, Sigma-Aldrich) staining was used. Slides containing 12 µm coronal section were incubated overnight in LFB solution (0.1% LFB, 4% glacial acetic acid in 96% ethanol) at 56 °C. Afterwards, redundant LFB staining was washed out using 0.05% lithium carbonate solution (in ddH2O), tissue was dehydrated and embedded in ROTI-Histokit II (Carl Roth, Karlsruhe, Germany). For immunohistochemistry, brain sections (CPZ) were thawed and left to dry for 15 min at RT. Afterwards, sections were rehydrated for 5 min in distilled water, post fixated for 5 min in 4% PFA and for another 5 min in -20°C acetone. Afterwards, sections were washed once in Tris-buffered saline (TBS, pH 7.6), once in TBS-T (TBS containing 0.02% Triton) for 5 min each and incubated for another 5 min in 0.3% H2O2 solution. Blocking was performed using 10% NGS (Sigma-Aldrich) and 5% biotin-free bovine serum albumin (BSA; Sigma-Aldrich; in TBS-T) for 30 min at RT, followed by the application of anti-amyloid precursor protein (APP; 1/200; Thermo Fisher Scientific, RRID:AB_2533902) in 10% NGS and 5% BSA in TBS over night at 4 °C. Afterwards, sections were washed twice in TBS (5 min each) and a biotinylated secondary antibody (goat anti-rabbit [1/200; Vector Laboratories, Burlingame, CA, USA]) was added for 30 min. Next, sections were washed twice in TBS and ABC reagent was incubated for another 30 min according to the manufacturer's protocol (Vectastain Elite ABC HRP kit; Vector Laboratories). Afterwards, sections were washed again twice for 5 min in TBS and peroxidase substrate was added for 5 min at RT (ImmPact DAB; Vector Laboratories). The reaction was stopped by two washing steps in ddH2O, followed by dehydration and embedding in ROTI-Histokitt II (Carl Roth). For Immunofluorescence staining, brain (CPZ) and/or spinal cord sections (EAE) were thawed, rehydrated for 5 min in distilled water, post fixated for 5 min in 4% PFA and for another 5 min in -20°C acetone. Before blocking, sections were washed once using Tris-buffered saline (TBS, pH 7.6) and once in TBS-T (TBS containing 0.02% Triton) for 5 min each. Blocking was performed with 10% serum according to the host of the secondary antibody (NGS or NDS respectively; Sigma-Aldrich) and 5% biotin-free BSA (Sigma-Aldrich; in TBS-T) for 30 min at RT, followed by application of the following antibodies (in 10% NGS/NDS, 5% BSA, TBS) and incubation overnight: goat anti-Pdgfrα (1/250; R and D Systems RRID:AB_2236897), rabbit anti-Ki67 (1/250; Abcam; RRID:AB_302459) rabbit anti-Sox10 (1/100, DCS Immunoline, Hamburg, Germany; RRID: AB_2313583), mouse anti-APC (CC1, 1/300, Sigma-Aldrich; RRID:AB_ 2057371), mouse anti-Bcas1 (1/200; Santa Cruz; Dallas, TX, USA; RRID:AB_10839529), rabbit anti-Iba1 (1/500, WAKO Pure Chemical Corporation; RRID: AB_839504), rat anti-Clec7a (Dectin1; 1/50; InvivoGen San Diego, CA, USA; RRID:AB_2753143), rat anti-Cd74 (1/200; Biolegend, San Diego, CA, USA; RRID:AB_2566502), chicken anti-Gfap (1/1000; Abcam; RRID:AB_304558), rabbit anti-C3d (1/300; Agilent; RRID:AB_578478), goat anti-Lcn2 (1/100; R and D Systems; RRID:AB_355022) and rat anti-MBP (1/300; Biorad, Hercules, CA, USA; RRID:AB_325004). Sections were washed two times for 5 min in TBS and incubated with the species-appropriate Alexa fluorochrome-conjugated secondary antibody (1/200 in TBS, Thermo Fisher Scientific) and 4′, 6-diamidino-2-phenylindole (DAPI; 20 ng/ml, Roche) for 30 min at RT. Afterwards, sections were washed once in TBS and once in TBS for 5 min each and embedded using Shandon™ Immu-Mount (Thermo Fisher Scientific).

Image acquisition and analysis
Images of astroglial cell cultures as well as of LFB and DAB stained tissue sections were captured on an Axioplan 2 microscope (Zeiss, Jena, Germany). All other Images were performed at a Zeiss CLSM microscope 510 (CLSM 510, Zeiss) always using the same exposure times, laser intensities and digital gains. The quantification of all microscopic images was performed using ImageJ software (National Institute of Health (NIH) Bethesda, MD, USA). For the analysis of microglial-astroglial cocultures 7 images per coverslip/insert and 2 coverslips/inserts per treatment were quantified, for the analysis brain/spinal cord tissue, for each marker setup 3 (CPZ) or 4 (EAE) sections were analyzed per condition and replicate. Scale bars were always adjusted to the respective microscope and all other setting (including thresholds) were identically applied to all images of a marker set. The in vitro analysis of iNOS-positive microglia as well as the number of C3/Lcn2-positive astrocytes were quantified manually, using the ImageJ tool "cell-counter". Similar is true for the quantification of oligodendroglial differentiation marker Pdgfrα, Sox10, APC, Bcas1 and Ki67, as well as for the APP-positive spheroids. To analyze the relative myelination of the corpus callosum, images of LFB staining were transformed to grey scale and the same threshold was applied to all images in order to creating a binary image. Afterwards the area of the corpus callosum as well as the LFB-positive area was determined and relative LFB-positive myelinated areas were calculated.
For the analysis of double and/or triple immune-positive cells, merged images were uploaded in ImageJ software, channels were split and a median filter (3) as well as background reduction (50, sliding parabolic, except for Gfap+ cells) was applied to all channels. Afterwards binary images were created, always using the same threshold for each channel of a marker setup, and the images were re-merged. Now a RGB color threshold was applied to detect and measure double and/or triple colocalizing areas, accordingly.

Statistical analysis
Data are presented as mean values ± standard error of the mean (SEM) in which n represents the number of independent replicates. Statistical analysis were conducted using Graph-Pad Prism 8.4.3 (GraphPad Software, San Diego, California USA). All data showed a normal distribution assessed by the Shapiro-Wilk test. Therefore, pairwise comparisons were analyzed using a two-tailed unpaired Student's t test, whereas multiple comparisons were assessed by 2-way analysis of variance (ANOVA) followed by Sidak's post hoc test. Furthermore, statistical significance of in vivo qPCR data was assessed using a two-tailed unpaired Student's t test of the calculated area under the curve. The experimental groups were considered significantly different at *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Data and materials availability
All data generated or analysed during this study are included in this published article.

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References

1. Reich DS, Lucchinetti CF, & Calabresi PA (2018) Multiple Sclerosis. *N Engl J Med* 378(2):169-180.
2. Lassmann H (2018) Multiple Sclerosis Pathology. *Cold Spring Harbor perspectives in medicine* 8(3).
3. Perron H*, et al.* (1989) Leptomeningeal cell line from multiple sclerosis with reverse transcriptase activity and viral particles. *Res Virol* 140(6):551-561.
4. Dolei A & Perron H (2009) The multiple sclerosis-associated retrovirus and its HERV-W endogenous family: a biological interface between virology, genetics, and immunology in human physiology and disease. *J Neurovirol* 15(1):4-13.
5. Perron H*, et al.* (2013) Human endogenous retrovirus protein activates innate immunity and promotes experimental allergic encephalomyelitis in mice. *PLoS One* 8(12):e80128.
6. Perron H*, et al.* (2001) Multiple sclerosis retrovirus particles and recombinant envelope trigger an abnormal immune response in vitro, by inducing polyclonal Vbeta16 T-lymphocyte activation. *Virology* 287(2):321-332.
7. Rolland A*, et al.* (2006) The envelope protein of a human endogenous retrovirus-W family activates innate immunity through CD14/TLR4 and promotes Th1-like responses. *J Immunol* 176(12):7636-7644.
8. Garson JA, Tuke PW, Giraud P, Paranhos-Baccala G, & Perron H (1998) Detection of virion-associated MSRV-RNA in serum of patients with multiple sclerosis. *Lancet* 351(9095):33.
9. Mameli G*, et al.* (2009) Novel reliable real-time PCR for differential detection of MSRVenv and syncytin-1 in RNA and DNA from patients with multiple sclerosis. *J Virol Methods* 161(1):98-106.
10. Perron H*, et al.* (2012) Human endogenous retrovirus type W envelope expression in blood and brain cells provides new insights into multiple sclerosis disease. *Mult Scler* 18(12):1721-1736.
11. Kremer D*, et al.* (2019) pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. *Proc Natl Acad Sci U S A* 116(30):15216-15225.
12. van Horssen J, van der Pol S, Nijland P, Amor S, & Perron H (2016) Human endogenous retrovirus W in brain lesions: Rationale for targeted therapy in multiple sclerosis. *Mult Scler Relat Disord* 8:11-18.
13. Mameli G*, et al.* (2013) Activation of MSRV-type endogenous retroviruses during infectious mononucleosis and Epstein-Barr virus latency: the missing link with multiple sclerosis? *PLoS One* 8(11):e78474.
14. Mameli G*, et al.* (2012) Expression and activation by Epstein Barr virus of human endogenous retroviruses-W in blood cells and astrocytes: inference for multiple sclerosis. *PLoS One* 7(9):e44991.
15. Bjornevik K*, et al.* (2022) Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science* 375(6578):296-301.
16. Duperray A*, et al.* (2015) Inflammatory response of endothelial cells to a human endogenous retrovirus associated with multiple sclerosis is mediated by TLR4. *Int Immunol* 27(11):545-553.
17. Göttle P*, et al.* (2019) Rescuing the negative impact of human endogenous retrovirus envelope protein on oligodendroglial differentiation and myelination. *Glia* 67(1):160-170.
18. Kremer D*, et al.* (2013) Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. *Ann Neurol* 74(5):721-732.
19. Hartung HP*, et al.* (2022) Efficacy and safety of temelimab in multiple sclerosis: Results of a randomized phase 2b and extension study. *Mult Scler* 28(3):429-440.
20. Kremer D, Perron H, & Küry P (2019) Reply to Ruprecht and Mayer: Unearthing genomic fossils in the pathogenesis of multiple sclerosis. *Proc Natl Acad Sci U S A* 116(40):19793-19794.

21. Ruprecht K & Mayer J (2019) On the origin of a pathogenic HERV-W envelope protein present in multiple sclerosis lesions. *Proc Natl Acad Sci U S A* 116(40):19791-19792.
22. Levet S*, et al.* (2017) An ancestral retroviral protein identified as a therapeutic target in type-1 diabetes. *JCI Insight* 2(17).
23. Charvet B*, et al.* (2021) Human Endogenous Retrovirus Type W Envelope from Multiple Sclerosis Demyelinating Lesions Shows Unique Solubility and Antigenic Characteristics. *Virologica Sinica* 36(5):1006-1026.
24. Göttle P*, et al.* (2023) Teriflunomide as a therapeutic means for myelin repair. *Journal of neuroinflammation* 20(1):7.
25. Manousi A*, et al.* (2021) Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. *EBioMedicine* 65:103276.
26. Silva Oliveira Junior M*, et al.* (2022) Myelin repair is fostered by the corticosteroid medrysone specifically acting on astroglial subpopulations. *EBioMedicine* 83:104204.
27. Fard MK*, et al.* (2017) BCAS1 expression defines a population of early myelinating oligodendrocytes in multiple sclerosis lesions. *Sci Transl Med* 9(419).
28. Keren-Shaul H*, et al.* (2017) A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* 169(7):1276-1290 e1217.
29. Krasemann S*, et al.* (2017) The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity* 47(3):566-581 e569.
30. Stratoulias V, Venero JL, Tremblay ME, & Joseph B (2019) Microglial subtypes: diversity within the microglial community. *EMBO J* 38(17):e101997.
31. Liddelow SA*, et al.* (2017) Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 541(7638):481-487.
32. Jin C*, et al.* (2021) A Unique Type of Highly-Activated Microglia Evoking Brain Inflammation via Mif/Cd74 Signaling Axis in Aged Mice. *Aging Dis* 12(8):2125-2139.
33. Zöller T, Attaai A, Potru PS, Russ T, & Spittau B (2018) Aged Mouse Cortical Microglia Display an Activation Profile Suggesting Immunotolerogenic Functions. *International journal of molecular sciences* 19(3).
34. Gorina R*, et al.* (2009) Astrocytes are very sensitive to develop innate immune responses to lipid-carried short interfering RNA. *Glia* 57(1):93-107.
35. Gharagozloo M*, et al.* (2021) Complement component 3 from astrocytes mediates retinal ganglion cell loss during neuroinflammation. *Acta Neuropathol* 142(5):899-915.
36. Bi F*, et al.* (2013) Reactive astrocytes secrete lcn2 to promote neuron death. *Proc Natl Acad Sci U S A* 110(10):4069-4074.
37. Gruchot J, Herrero F, Weber-Stadlbauer U, Meyer U, & Küry P (2023) Interplay between activation of endogenous retroviruses and inflammation as common pathogenic mechanism in neurological and psychiatric disorders. *Brain, behavior, and immunity* 107:242-252.
38. Küry P*, et al.* (2018) Human Endogenous Retroviruses in Neurological Diseases. *Trends in molecular medicine* 24(4):379-394.
39. Balestrieri E*, et al.* (2021) Evidence of the pathogenic HERV-W envelope expression in T lymphocytes in association with the respiratory outcome of COVID-19 patients. *EBioMedicine* 66:103341.
40. Charvet B*, et al.* (2023) SARS-CoV-2 awakens ancient retroviral genes and the expression of proinflammatory HERV-W envelope protein in COVID-19 patients. *iScience* 26(5):106604.
41. Gruchot J, Kremer D, & Kury P (2020) Human endogenous retroviruses: ammunition for myeloid cells in neurodegenerative diseases? *Neural regeneration research* 15(6):1043-1044.
42. Gruchot J, Kremer D, & Küry P (2019) Neural Cell Responses Upon Exposure to Human Endogenous Retroviruses. *Frontiers in genetics* 10:655.
43. Jordao MJC*, et al.* (2019) Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation. *Science* 363(6425).

44. Masuda T, *et al.* (2019) Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature* 566(7744):388-392.
45. Absinta M, *et al.* (2021) A lymphocyte-microglia-astrocyte axis in chronic active multiple sclerosis. *Nature* 597(7878):709-714.
46. Ponath G, Park C, & Pitt D (2018) The Role of Astrocytes in Multiple Sclerosis. *Frontiers in immunology* 9:217.
47. Galloway DA, Gowing E, Setayeshgar S, & Kothary R (2020) Inhibitory milieu at the multiple sclerosis lesion site and the challenges for remyelination. *Glia* 68(5):859-877.
48. Zhan J, *et al.* (2020) The Cuprizone Model: Dos and Do Nots. *Cells* 9(4).
49. Johansson EM, *et al.* (2020) Human endogenous retroviral protein triggers deficit in glutamate synapse maturation and behaviors associated with psychosis. *Science advances* 6(29):eabc0708.
50. Lanz TV, *et al.* (2022) Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. *Nature* 603(7900):321-327.
51. Göttle P, *et al.* (2021) TLR4 Associated Signaling Disrupters as a New Means to Overcome HERV-W Envelope-Mediated Myelination Deficits. *Frontiers in cellular neuroscience* 15:777542.
52. Dietrich M, *et al.* (2022) Increased Remyelination and Proregenerative Microglia Under Siponimod Therapy in Mechanistic Models. *Neurol Neuroimmunol Neuroinflamm* 9(3).
53. Dietrich M, *et al.* (2018) Early alpha-lipoic acid therapy protects from degeneration of the inner retinal layers and vision loss in an experimental autoimmune encephalomyelitis-optic neuritis model. *Journal of neuroinflammation* 15(1):71.

Figures and Tables

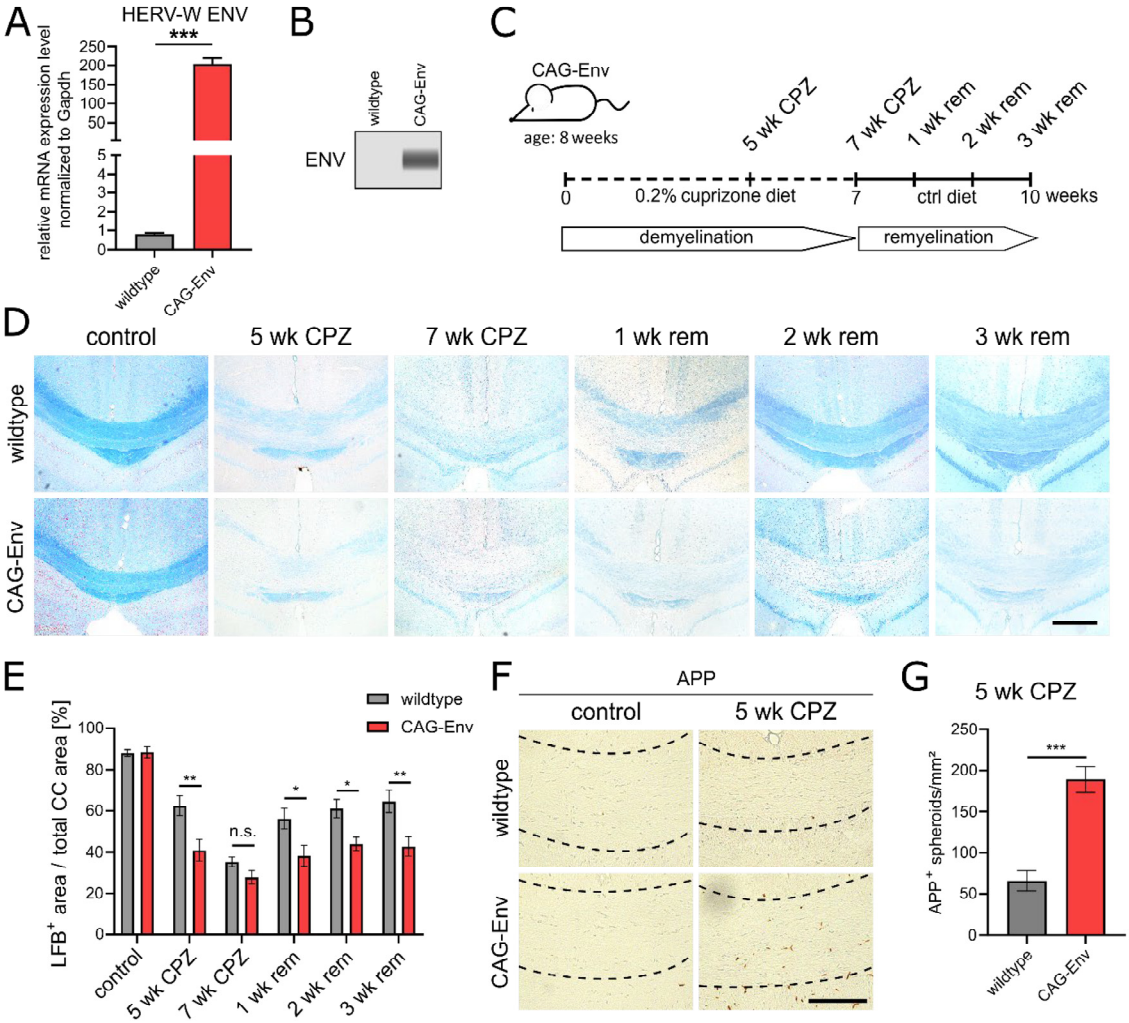

Figure 1. Transgenic HERV-W ENV expression fosters demyelination and alters remyelination upon cuprizone treatment. (A) Determination of the relative ENV transcript levels normalized to Gapdh in whole brain lysates of wildtype and CAG-Env transgenic mice. (B) Automated western blot analysis of whole brain lysates detecting HERV-W ENV protein in transgenic mice. (C) Schematic presentation of cuprizone (CPZ) de- and remyelination experiments. (D) Representative images of LFB-stained control- and CPZ challenged wildtype and transgenic tissue sections encompassing the caudal corpus callosum (corpus callosum) at time points 5 and 7 weeks of CPZ treatment and at 1, 2 and 3 weeks during remyelination. (E) Quantification of the percentage of LFB-positive, myelinated area of the corpus callosum. (F) Representative images of anti-APP stained wildtype and CAG-Env corpus callosum tissue sections at 5 weeks of demyelination. (G) Quantification of APP-positive spheroid densities in the corpus callosum of wildtype and transgenic animals. Data is presented as mean values (A-B: n = 3; E-H: n = 6) ± SEM. Significance of HERV-W ENV mRNA levels as well as APP-positive spheroids were assessed by Student's t-test and the significance of the relative LFB-positive areas was accessed by 2-way analysis of variance (ANOVA) followed by Sidak's post hoc. Data were considered as statistically significant (95% confidence interval) at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n.s. = not significant. CC = corpus callosum. Scale bar in E: 250 µm, scale bar in G: 100 µm.

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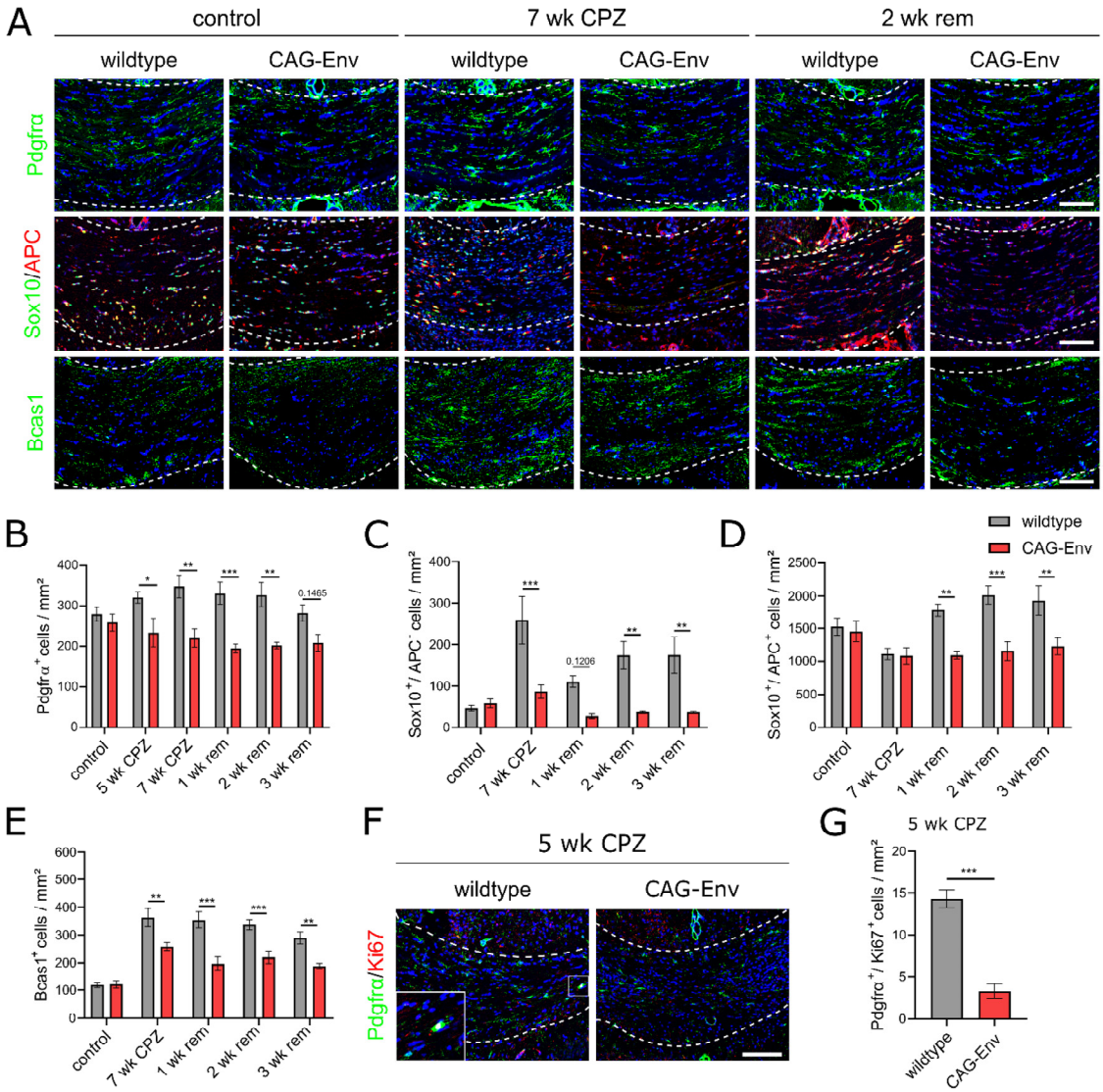

Figure 2. Transgenic HERV-W ENV expression affects oligodendroglial differentiation. (A) Representative immunohistochemical images of Pdgfrα-, Sox10/APC- and Bcas1- expression in unchallenged animals (wildtype and CAG-Env mice), after 7 weeks of CPZ treatment and after 2 weeks of CPZ withdrawal (2 wk rem). (B) Quantification of Pdgfrα-positive cell densities in the corpus callosum of control- vs CPZ treated animals. (C) Quantification of Sox10-positive, APC-negative cell densities in the corpus callosum of control- vs CPZ treated animals. (D) Quantification of Sox10/APC double-positive maturing oligodendroglial cell densities in the corpus callosum of control- vs CPZ treated mice. (E) Quantification of Bcas1-positive myelinating oligodendrocyte densities in the corpus callosum of control- vs CPZ treated animals. (F) Representative immunohistochemical pictures of Pdgfrα/Ki67-coexpressing cells in the corpus callosum of wildtype vs CAG-Env mice at 5 weeks of CPZ treatment. (G) Quantification of Ki67-positive proliferating OPCs in wildtype vs CAG-Env corpus callosum tissues after 5 weeks of CPZ diet. Data is presented as mean values (n = 6) ± SEM. Significance of Ki67-positive OPCs was analyzed by Student's unpaired t-test, whereas all other significances were accessed by 2-way analysis of variance

(ANOVA) followed by Sidak's post hoc test (95% confidence interval) at *p < 0.05, **p < 0.01, ***p < 0.001. Dashed lines indicate the area of corpus callosum. Scale bar: 100 µm.

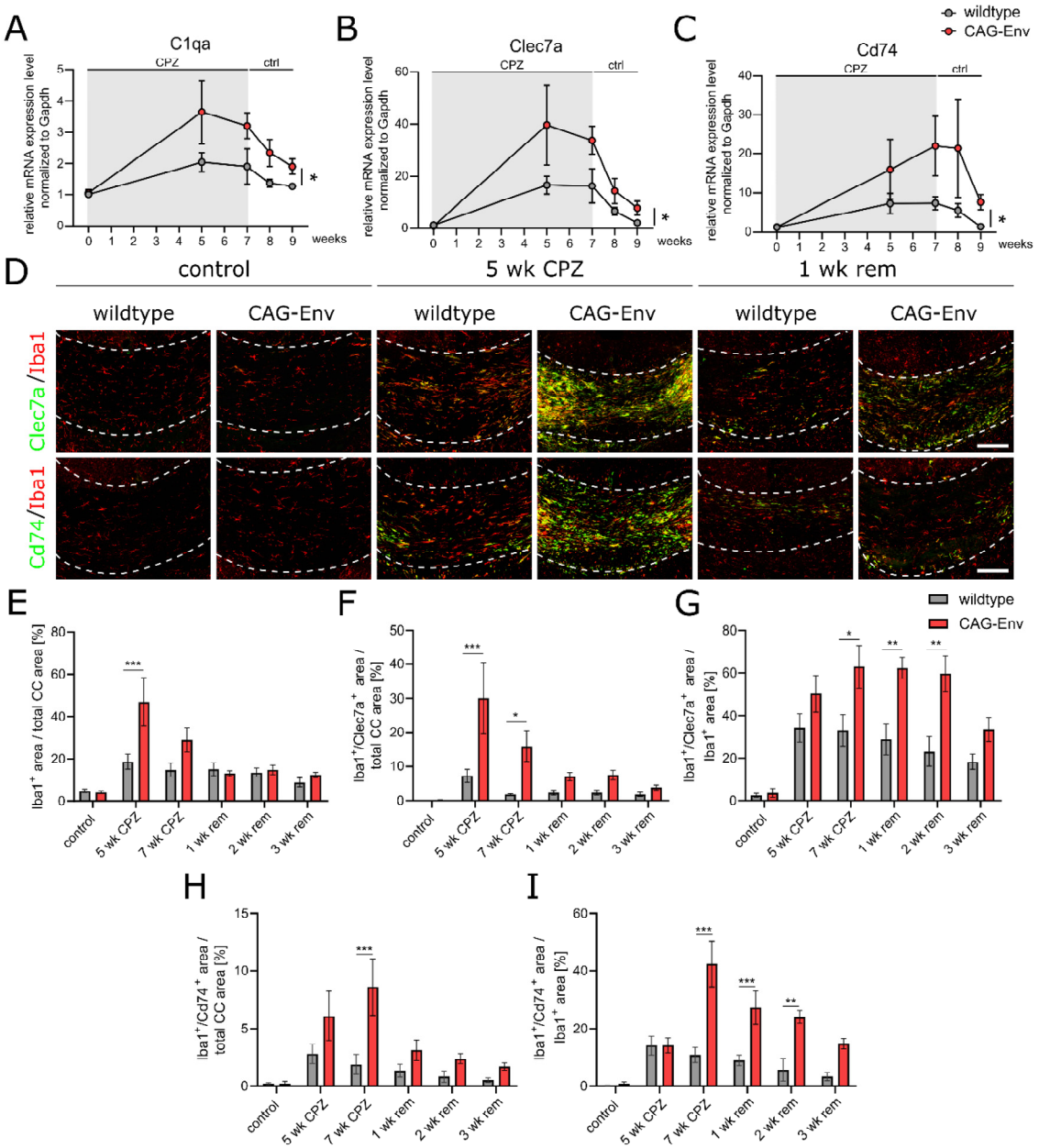

Figure 3. Transgenic HERV-W ENV expression activates microglial cells upon cuprizone treatment. (A-C) C1qa, Clec7A and Cd74 gene expression analysis in the corpus callosum before and during cuprizone treatment in wildtype vs transgenic CAG-Env mice. (D) Representative immunohistochemical pictures of Clec7a/Iba1- and Cd74/Iba1 coexpressing cells in wildtype and transgenic corpus callosum tissues (unchallenged- and CPZ treated animals). (E) Quantification of the Iba1-positive area over the total corpus callosum area in control- vs CPZ treated wildtype and CAG-Env mice. (F) Quantification of Clec7a/Iba1 double-positive areas over total corpus callosum areas in wildtype and transgenic CAG-Env mice (control- and under CPZ diet). (G) Quantification of the proportion of Clec7a-positive microglia in wildtype and transgenic mice (control- and under CPZ diet). (H) Quantification of Cd74-positive microglial- vs total corpus callosum areas in wildtype and CAG-Env mice (control- and under CPZ diet). (I) Quantification of the proportion of Cd74-

positive microglia in wildtype and transgenic mice (control- and under CPZ diet). Data is presented as mean values (n = 6) ± SEM. Significance of gene expression analysis was assessed by a Student's unpaired t-test of calculated AUCs whereas statistical significance of histological data was analyzed via 2-way analysis of variance (ANOVA) followed by Sidak's post hoc test. Data were considered as statistically significant (95% confidence interval) at *p < 0.05, **p < 0.01, ***p < 0.001. CC = corpus callosum. Dashed lines in D demarcate the corpus callosum. Scale bar in D: 100 µm.

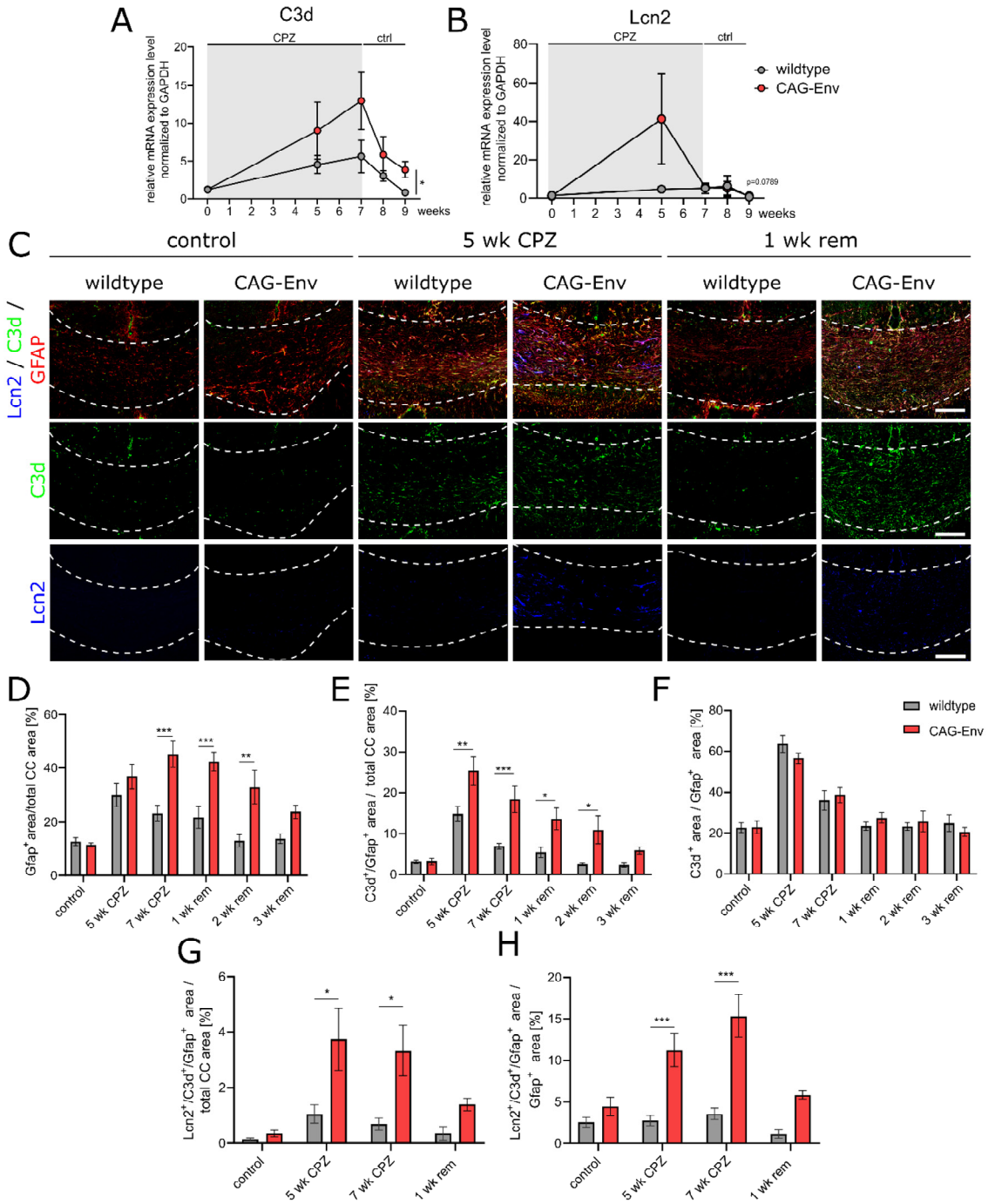

Figure 4. Transgenic expression of HERV-W ENV activates astrocytes. (A-B) C3d and Lcn2 expression gene expression analysis at various time points, before, during and after CPZ treatment in wildtype vs transgenic CAG-Env mice. (C) Representative immunohistochemical images of C3d-, Lcn2- and Gfap- (co)-expressing cells in wildtype and transgenic corpus callosum tissue sections (control- and CPZ treated). (D) Quantification of Gfap-positive areas within the corpus callosum of wildtype and CAG-Env mice (control- and CPZ treated). (E) Quantification of C3d/Gfap double-

positive areas within the corpus callosum of wildtype and CAG-Env mice (control- and CPZ treated). (F) Relative proportion of C3d/Gfap double-positive astrocytic areas in wildtype and CAG-Env (control- and CPZ treated). (G) Analysis of Lcn2/C3d/Gfap triple-positive astrocytic areas with the corpus callosum of wildtype and transgenic mice (control- and CPZ treated). (H) Relative proportion of Lcn2/C3d/Gfap triple-positive astrocytic areas within the corpus callosum of transgenic and control mice upon CPZ treatment. Data is presented as mean values (n = 6) ± SEM. Significance of gene expression analysis was assessed by Student's unpaired t-test of calculated AUCs whereas the statistical significance of histological data was analyzed via 2-way analysis of variance (ANOVA) followed by Sidak's post hoc test. Data were considered as statistically significant (95% confidence interval) at *p < 0.05, **p < 0.01, ***p < 0.001. CC = corpus callosum. Dashed lines in C demarcate the area of the corpus callosum. Scale bar in C: 100 µm.

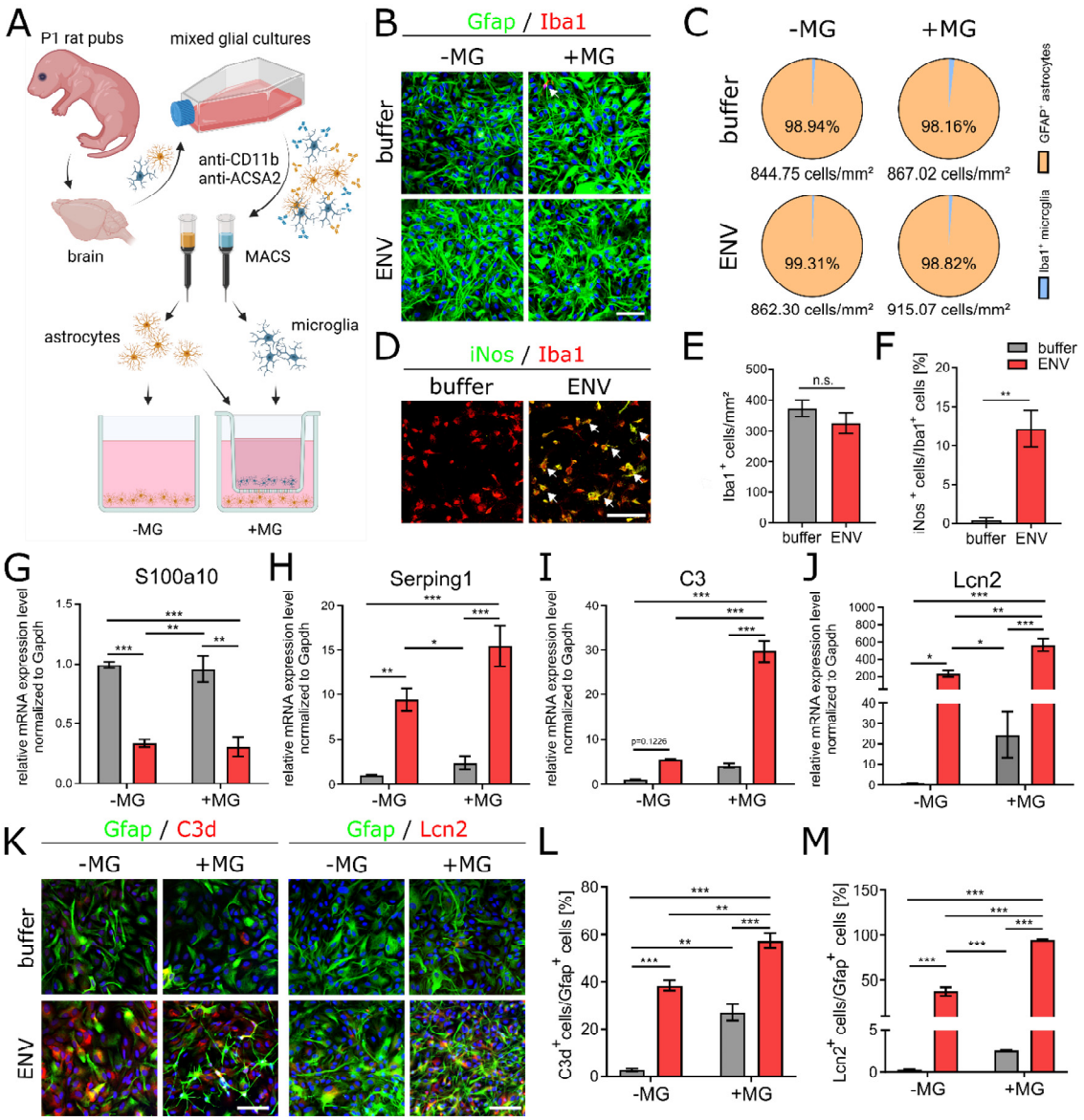

Figure 5. HERV-W ENV protein exposure leads to an activation of astroglial cells which is amplified by microglia. (A) Schematic presentation of experimental procedure to generate spatially separated primary cultures of microglia and astrocytes (created using BioRender.com). (B) Representative immunocytochemical images of Gfap-positive astrocytes in absence and presence of microglia, treated with buffer or recombinant HERV-W ENV protein for 24 h. (C) Quantification of astrocyte culture purities under all four conditions. (D) Representative immunocytochemical images of microglial cells grown on cell culture inserts expressing Iba1 and iNOS. Arrows point to iNOS-positive cells. (E,F) Quantification of Iba1-positive and iNos-positive microglia densities upon buffer and ENV protein stimulation after 24 h. (G-J) Astrocyte gene expression analysis in absence and presence of microglia and in response to buffer or recombinant HERV-W ENV protein treatment after 24 h. Relative expression levels of S100a10 (G), Serping1 (H), C3d (I) and Lcn2 (J) were assessed. (K) Representative immunocytochemical images of C3d and Lcn2 expressing (Gfap-positive astrocytes) under all four conditions and after 24 h. (L,M)

Quantification of C3d-positive and Lcn2-positive astrocytes under all four conditions and after 24 h. Data is presented as mean values (n = 3) ± SEM. Significance of microglia analyses (E-F) was assessed by Student's unpaired t-test whereas the significance of all other quantifications (G-J,L,M) was analyzed via 2-way analysis of variance (ANOVA) followed by Sidak's post hoc test. Data were considered as statistically significant (95% confidence interval) at *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar: 50 µm. The arrow in (B) points to a single Iba1-positive microglial cell. Arrows in (D) point to iNOS-positive microglial cells.

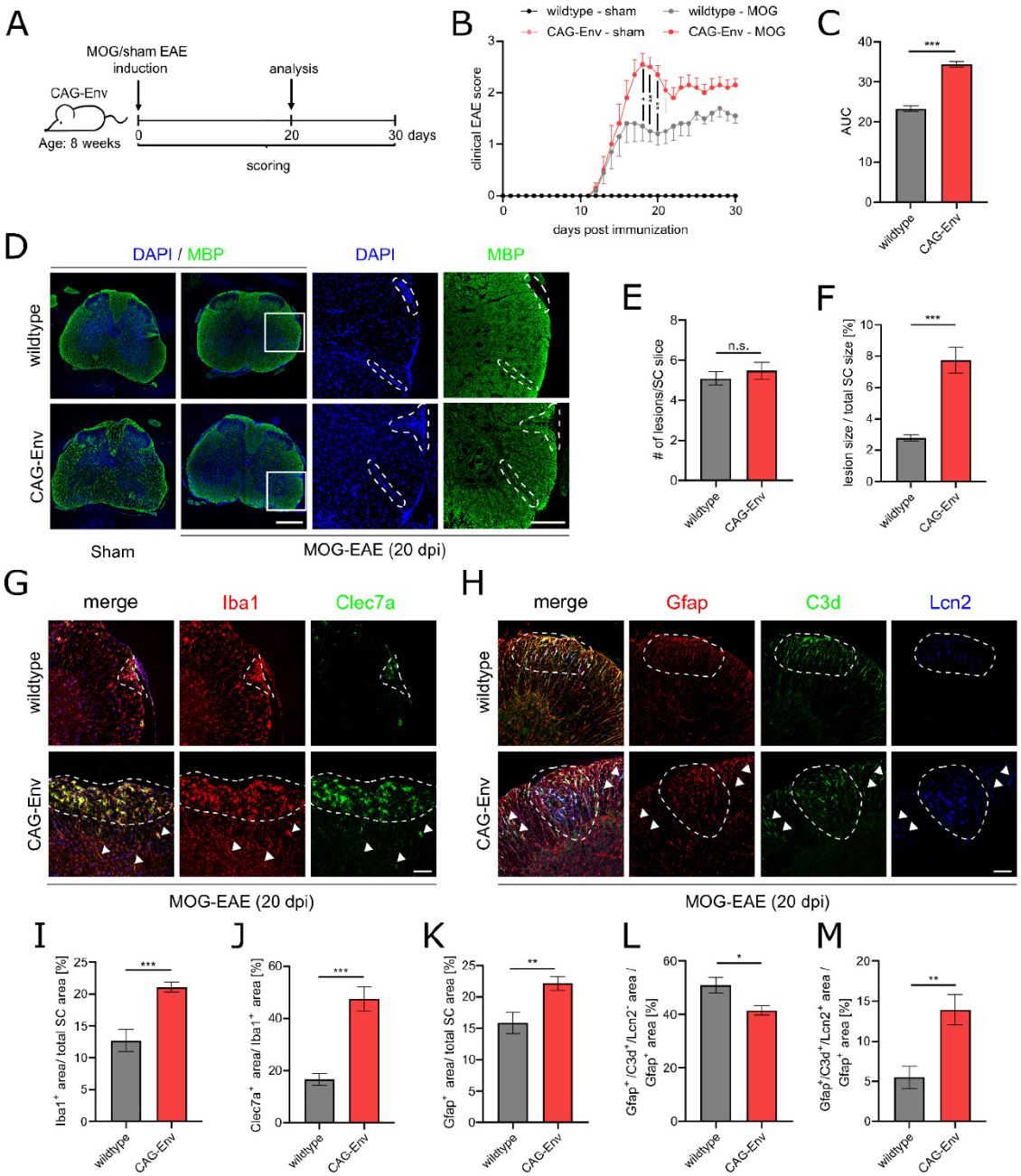

Figure 6. Increased microglial- and astroglial activation in transgenic mice upon induction of experimental autoimmune encephalomyelitis. (A) Schematic representation of EAE experiments. (B) Clinical score of sham- and MOG-induced wildtype and CAG-Env mice. (C) Determination of area under the curve (AUC) values of clinical scores in MOG-induced wildtype vs CAG-Env mice. (D) Representative images of MBP expression in lumbar spinal cords of sham vs MOG-induced wildtype and transgenic animals (20 dpi). (E-F) Quantification of lesion formation in MOG-induced animals displaying no differences in lesion numbers (E) but increased lesion sizes in CAG-Env mice (F) at 20 dpi. (G) Representative images of Iba1- and Clec7a expression patterns in lumbar spinal cords of MOG-induced wildtype vs CAG-Env mice (20 dpi). (H) Representative

images of Gfap-, C3d-, and Lcn2 expression patterns in lumbar spinal cords of MOG-induced wildtype vs transgenic animals (20 dpi). (I) Quantification of Iba1-positive areas (over total spinal cord areas) and (J) of Clec7a-positive areas (within Iba1-positive areas) in MOG-induced wildtype and CAG-Env mice at 20 dpi. (K) Quantification of Gfap-positive areas (over total spinal cord areas) in wildtype and CAG-Env mice at 20 dpi. (L) The proportion of C3d-positive but Lcn2-negative astrocytic areas relative to total Gfap-positive areas was reduced in transgenic CAG-Env mice. (M) On the other hand, the extent of Lcn2/C3d/Gfap-triple-positive areas (within Gfap-positive areas) was significantly increased in transgenic animals 20 days after MOG-EAE induction. Data is presented as mean values ± SEM. EAE course (B) was analyzed in n=10 mice and histological analysis (G-M) was performed in n=9 animals. Significance of the clinical EAE score (B) was assessed via 2-way analysis of variance (ANOVA) followed by Sidak's post hoc test and all other data were analyzed by Student's unpaired t-test. Data were considered as statistically significant (95% confidence interval) at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar in D (overview): 500 µm; scale bar in D (detailed): 200 µm; scale bars in G and H: 50 µm. Dashed lines in (C,G,H) delineate lesion boundaries and arrowheads point to either Clec7a-positive microglia (G) or Lcn2/C3d-positive astrocytes (H) outside lesions.

3. Discussion

Neurodegenerative diseases are the leading cause of disability and the second leading cause of death worldwide, making them a heavy burden for affected individuals and the health system. Therefore, in order to contribute to the improvement of putative treatment strategies for neurodegenerative diseases, the studies presented in this thesis address two major problems: I.) The CNS is one of the most important structures in the human body as it controls not only a behavior but also warrants movement, interaction with the environment and vegetative function. However, the human (diseased) brain is difficult to access and studies on humans and human samples come with well-founded ethic concerns. Animal models must therefore be used on a large scale, which of course can only be transferred to a limited extent – not only because laboratory animals mostly do not live as long as certain diseases need to progress to exert symptoms. The complex pathomechanisms of diverse neurodegenerative disorders are therefore far from being understood, leading to the fact that similar therapeutic approaches have been used to treat the same involved mechanisms for decades. Current therapeutic approaches still loose effectiveness in the long-term treatment, probably because the targeted mechanisms are rather universal. In order to improve future treatment strategies, the first aim of this thesis is to characterize mechanisms in more detail, which lead to the onset and progression of different neurodegenerative diseases. II.) The beginning of neurodegenerative diseases can hardly be recognized, since the first symptoms emerge after the neurodegeneration on a cellular level has already taken place. As a result, the reasons that led to the onset of many of the diseases have not been conclusively described, yet. In this context, however, an association between the expression of endogenous retroviruses and the development of neurological diseases has been often described. Therefore, the second aim that is addressed by the studies of this thesis is to develop a more detailed picture on the effects HERV-W ENV exerts on glial cells in order to understand whether this viral entity contributes to the onset of the diseases or whether it is a part of their pathomechanisms. In conclusion, the results of both aims will contribute to a better understanding of neurodegenerative diseases and thereby may help to improve current and future treatment strategies.

3.1 New insights into the microglial biology in the context of multiple sclerosis and other neurodegenerative diseases

Microglia are the primary source of immune cells in the CNS and therefore build the first line of defense when it comes to CNS infection and/or injury. In this context, reactive microglia setting the basis for the many different beneficial, but also harmful processes. They are involved in the tissue clearing and debris phagocytosis, the secretion of pro- and anti-

inflammatory cytokines to attract peripheral immune cells as well as to induce regeneration of the damaged tissue, but also to induce oxidative stress, chronic inflammation as well as the induction of BBB leakiness (Muzio et al., 2021). So far, it is highly debated whether microglia exhibit primarily beneficial or harmful functions in neurodegenerative disorders (Du et al., 2017). Understanding the complex biological role of microglia in neurodegenerative diseases will therefore also contribute to possible treatment strategies.

Up to now, the diagnosis of neurodegenerative diseases, such as MS, is based on established clinical criteria that are designed to assess cognitive impairment in patients (Gomez-Rio et al., 2016). However, the differential diagnosis between disorders can be difficult, especially in early phases or atypical variants. Furthermore, disease progression is also analyzed based on this system, leading to the problem that changes can only be detected when clinical disability has already advanced. To solve this issue, clinicians use an arsenal of diagnostic tests, including (functional) brain imaging techniques as well as invasive analysis of biomarker expression to gain an advantage in the fight against neurodegenerative diseases. A constant improvement of techniques is mandatory in order to start treatment as early as possible. A part of the research is focusing on assessing biomarkers that can be measured in CSF and/or blood of the patients, which, however, does have negative side effects as they are highly invasive (lumbar puncture) and not widely available, especially not outside of specialist centers (Hansson, 2021). Others focus on improving imaging techniques or defining the characterization of via brain imaging techniques accessible parameters for different stadia of neurodegenerative diseases (Barthel et al., 2022).

Over the last years, high-field structural magnetic resonance imaging (MRI) has become a powerful tool in deciphering and predicting specific brain pathology patterns (Calabrese et al., 2015; Steenwijk et al., 2016) and disease courses such as e.g. MS (Krämer et al., 2019). In particular, advances in brain imaging acquisition and post-processing have improved the field and made strong contributions to our understanding of the progression of neuroinflammation and neurodegeneration (Ciccarelli et al., 2014). The strength of MRI comes from its ability to provide quantifiable markers with high spatial accuracy that can trace disease trajectories both for research and for clinical studies (Cortese et al., 2019).

In the current study (Fleischer et al., 2021), our colleagues identified an early association between enlargement of the choroid plexus (ChP) and disease severity in two large cohorts of MS patients, including both treatment-naïve patients and patients under immunomodulatory treatment. The ChP is a highly vascularized tissue that extends on the floor of the lateral ventricles as well as the roof of the third and fourth ventricles (Talhada et al., 2020) and produces most of the CSF (Lun et al., 2015). The choroid plexus consists of modified ependymal cells surrounding a core of capillaries and loose connective tissue (Lun et al.,

2015). Furthermore, they established a method to measure the ChP enlargement in two mouse models of MS (cuprizone-mediated demyelination as well as experimental autoimmune encephalomyelitis). Both models displayed ChP enlargement that correlated with the numbers of myeloid as well as astroglial cells in the cortex. In order to contribute to the understanding of the mechanism of ChP enlargement, we were able to show elevated numbers of reactive Clec7a positive myeloid cell as well as CD3 positive T lymphocytes within the ChP parenchyma. This was of high interest, since cuprizone mode of action is mostly described in the whiter matter tracts of the brain and in some parts of the cortex (Zhan et al., 2020), similar to the EAE, where most of its effects can be seen in the lumbar spinal cord (Constantinescu et al., 2011) – both regions are far away from the ChP analyzed here. In addition, we were able to isolate ChP tissue from diseased mice (CPZ or EAE) for the first time in order to carry out a transcriptome analysis. The results revealed that the cuprizone model of demyelination as well as the EAE change the transcriptome of the ChP in a way that supports increased leukocyte adhesion, migration and activation. This indicates, that ChP enlargement in combination with the elevated presence of activated microglia and peripheral T lymphocytes is contributing to increased immune cell trafficking across the BBB. It is widely accepted that different compartments can regulate the peripheral immune cell infiltration across the epithelial cells of which the meninges, the perivascular regions as well as the ChP play the biggest role (Benarroch, 2016; Yakimov et al., 2019). Furthermore, the data obtain in the animal models go in line with the literature, as peripheral immune cell infiltration as well as BBB permeability are also well described in both models (Baxter, 2007; McMahon et al., 2002; Shelestak et al., 2020). It is thought, that astrocytes create a local inflammatory milieu that likely participates in destabilizing the BBB integrity mediated by down-regulation of tight junction proteins (Berghoff et al., 2017). In addition to infiltrating immune cells, a reduction in BBB integrity can also allow a variety of toxic molecules such as inflammatory cytokines and oxidative stress to enter the brain microenvironment from the periphery. These mediators can (directly and indirectly via neuronal damage) further activate microglial and astroglial cells, which in turn leads to the induction of neuroinflammatory damage and loss of BBB integrity (Hanisch & Kettenmann, 2007; Haruwaka et al., 2019), resulting in a positive feedback loop. However, in most cases, it is not possible to determine whether barrier compromise is causal in disease onset or a result of neurological disease progression. Nevertheless, it can often be seen that a barrier disorder contributes to the development of a pathology and aggravates it (Persidsky et al., 2006). In order to limit BBB permeability, recruitment of peripheral immune cells as well as microglia toxicity is seen as an important mechanism to regulate inflammatory lesion formation in early disease stages (Lassmann et al., 2012).

In the past, many treatments for MS aimed to suppress the immune system. However, it turned out that the suppression of the immune system has strong side effects and that most medications lose their effect after a short time. This leads to the fact that more and more medication is needed or the therapies have to be changed constantly (Vargas & Tyor, 2017). Therefore, current research is focusing on immune modulatory therapies in order to change the immune systems direction without blocking it completely (Vargas & Tyor, 2017).

In this context, siponimod (BAF312), an orally administered sphingosine-1-phophate receptor (S1PR) modulator that selectively binds to S1PR1 and S1PR5, is used to treat RRMS (Kappos et al., 2016a). It has proven in clinical trials that reduces relapse rate as well as inflammatory disease activity and is seen as one of the few drugs that can also be used in SPMS as it still slows down disease progression and brain atrophy (Kappos et al., 2018). Additionally, several studies have confirmed that siponimod reduces oligodendroglial death in organotypic slice cultures (O'Sullivan et al., 2016) and prevents synaptic loss in the MS animal model experimental autoimmune encephalomyelitis (EAE; Gentile et al., 2016). In general, sphingosine-1-phosphate (S1P) is a biologically active sphingolipid that regulates a wide range of physiological processes, e.g. lymphocyte circulation, cardiac function, or the maintenance of the BBB (Wang et al., 2020). Most S1P effects are mediated through one of the five G protein-coupled S1P receptor subtypes called S1PR1–5 (An et al., 1997). These receptors are expressed differently on cell types, such as lymphocytes, dendritic cells, cardiomyocytes, endothelial cells, smooth vascular muscle cells, or fibroblasts (Kipp, 2020), but also CNS cells such as astrocytes (Brana et al., 2014; Nishimura et al., 2010), microglia (Noda et al., 2013; Tham et al., 2003), and oligodendrocytes (Brana et al., 2014; Coelho et al., 2007).

Despite the fact that receptors are expressed by CNS cells, so far no one has investigated any effects of siponimod onto microglia, although a putative effect on CNS cells is highly debated (Kipp, 2020). In order to analyze the effects of siponimod onto reactive microglial cells, primary rat microglial cell were stimulated with LPS and siponimod (Gruchot et al., 2022). Our findings showed that siponimod protects microglia from LPS reactivity as it prevents microglial cells from adopting activated cytoskeletal architecture and morphologies, reduces iNOS protein expression, modulates microglial cytokine expression and decreases microglial immunological pathways and points therefore to specific effects of siponimod on microglial cells. These results go in line with previous experiments performed with another S1PR modulator, named fingolimod. Fingolimod, which acts via S1PR1, 3, and 5, was shown to modulate microglia to exert neuroprotective and remyelinating effects (Jackson et al., 2011; Noda et al., 2013), which could be further verified by PET-CT imaging of MS lesions (Sucksdorff et al., 2017). In contrast to fingolimod, siponimod exerts a more specific receptor

modulation as it only affects S1PR1 and S1PR5 (Kappos et al., 2016b). It is therefore tempting to hypothesize that S1PR mediated effects on microglia are primarily driven by S1PR1 and S1PR5, but not by S1PR3. To prove this hypothesis, possible experiments could include specific S1PR antagonists, such as W146, for the inhibition of S1PR1 signaling, CAY10444 for S1PR3 mediated effects and compound 15, a novel selective S1P5 antagonist (Ma et al., 2021). Although S1PR modulators are well tolerated by the patients, its receptors are still widely expressed by many cell types of the CNS as well as in the periphery, leading to potential side effects (Kipp, 2020). It is therefore important to continue focus on the effects of S1P modulators, such as siponimod, onto other cells such as e.g. astroglia as well as Schwann cells in the PNS.

Interestingly, we were able to characterize that siponimod stimulation specifically changes the pattern of microglial gene expression that cannot be assigned to a “classic” M1 or M2 characterization and appears to be more complex, as siponimod generates a completely new cytokine signature in LPS challenged pro-inflammatory microglia. The interplay of different cytokines is thought to be crucial for the onset and progression in MS, although it is not always clear yet, whether cytokines in general participate primarily in beneficial or harmful functions in neurodegenerative disorders (Becher et al., 2017). Therefore, having found an activated microglial subtype that cannot be classified as M1 or M2 and has an undescribed cytokine profile, this study is consistent with recent RNA sequencing and genome-wide association studies suggesting the existence of disease-specific microglial, often referred to as disease-associated microglia (DAMs; Deczkowska et al., 2018; Keren-Shaul et al., 2017; Pulido-Salgado et al., 2018; Tay et al., 2018). As in vivo single cell transcriptome techniques are developed and improved, future studies will reveal whether the putative DAM signatures described here are indeed disease specific and/or perhaps corroborate to mechanisms for a group of diseases.

In summary a causal correlation between ChP enlargement, BBB integrity and microglial activation is provided here for the first time. This knowledge might lead to the use of ChP measurement techniques via MRI in future diagnosis and prognosis of neurodegenerative diseases and, above all, MS. In addition, it could be shown that siponimod, a drug that is used to treat RR and SPMS, is not only affecting peripheral immune cell or oligodendrocytes (Dietrich et al., 2022; Gentile et al., 2016) but also microglia, by modulating their immune reaction. In the end, it is tempting to speculate that siponimod might also have beneficial effects onto the BBB integrity, as it modulates microglial cytokine expression as well as the production of nitrosative stress. Both factors are known to induce BBB permeability (Haruwaka et al., 2019). Although other immunomodulatory therapies are also able to effectively prevent or mitigate early disease progression in MS, siponimod is still of high interest, as it is one of

the few drugs that is approved for the treatment of active secondary progressive MS patients. As it is known from MS histopathology, microglia can be primed by invading peripheral immune cells during initial disease relapses, leading to a milieu of smoldering inflammation behind an intact BBB in SPMS (Giovannoni et al., 2022), which might be prevented by siponimod, if the here presented data are taken into account.

3.2 New insights onto the effects of HERV-W ENV onto glial cells

Human endogenous retroviruses are proposed to play important roles in health and disease. While some of them have been domesticated over time such as syncytin-1, others have kept their viral character and are thought to induce pathological effects (Küry et al., 2018; Xiang & Liang, 2021). Over time many different pathologies were found that show implications of HERV expression and the number is steadily increasing (Küry et al., 2018). This includes neurodegenerative diseases such as multiple sclerosis and amyotrophic lateral sclerosis, neurodevelopmental disorders such as attention deficit hyperactivity syndrome and autism, as well as neuropsychiatric diseases, such as schizophrenia bipolar disorder (Balestrieri et al., 2019; Balestrieri et al., 2014; Karlsson et al., 2001; Li et al., 2015; Perron et al., 1989; Tamouza et al., 2021; Yolken et al., 2000). As it is not completely clear how and via which CNS cells HERV-ENV exerts its neuropathological effect, the aim of this thesis was the investigation of HERV-W ENV's effects onto different cell types of the CNS as well as onto de- and remyelination and neurodegeneration.

3.2.1 Complex (re)awakening events leading to the expression of endogenous retroviruses

Although neurodegenerative diseases share certain commonalities in their pathomechanism, the exact mechanism leading to the activation and expression of HERVs is far from being understood. As part of this thesis, it was aimed to summarize the complex (re)awakening processes that lead to the expression of endogenous retroviruses.

Under physiological conditions, the majority of HERVs, particularly those with primarily pathological functions, are proposed to be in a dormant state and suppressed by molecular mechanisms of epigenetic silencing (Gruchot et al., 2023). The mechanism of epigenetic silencing include the localization of proviruses in the heterochromatin, blocking LTR access, CpG methylation and histone deacetylation. This, however, also implies that endogenous retroviral elements need to be epigenetically unlocked before they can be activated. To this end, several environmental factors, including infections, nutrition, and certain drugs were summarized (Gruchot et al., 2023) as putative key players in modulating epigenetic *status quo*. Altogether, the combination of these environmental factors together with the unique

interindividual setup of HERVs might lead to the complex prevalence in neurodegenerative, but also other neurological disorders.

Apart from unlocking the epigenetic barrier, a direct induction of HERV expression is also known. In this context, it was found that LTR sequences act as a key activator of HERV expression, as they can bind to inflammatory transcription factors leading to the expression of these viral entities (Manghera & Douville, 2013). Furthermore, infections were shown to induce HERV expression, such as with HHV6 and EBV (Küry et al., 2018). Although, it has to be stated clear, that numerous studies demonstrated that EBV has a direct mode of action on HERV activation (Mameli et al., 2013; Mameli et al., 2012), there is still a problem in the differentiation between viral or inflammation driven activation of HERVs. A direct induction of HERV expression by viral infections is often indistinguishable from an indirect activation through inflammatory, i.e. NF-κB signaling pathways, since both entities will activate similar signaling pathways. As a matter of fact, most of available studies were correlative in nature and therefore, fall short in answering the question whether infectious agents exert direct effects on HERV expression, or whether these effects are indirectly mediated by pro-inflammatory pathways and/or epigenetic unlocking processes discussed above. Indeed, because systemic inflammation and viral infections share similar signaling pathways, it is difficult to distinguish temporally between those two HERV effectors. As part of this thesis, it was therefore proposed that more longitudinal studies on HERV activation in neurodegenerative diseases have to be performed in order to better understand the complex sequence of events.

Similar to HERVs, the exact mechanism leading to the onset of neurodegenerative diseases is far from being understood. A recent study has set a milestone by conducting a longitudinal study within the US military. In this study, it was found that there is a 32-fold increased risk to develop MS after an infection with EBV (Bjornevik et al., 2022). In addition to the fact that EBV infections are known to induce an arsenal of epigenetic modulators as well as systemic inflammation, it is also proposed to be a regulatory mechanism of HERV activation (Küry et al., 2018). Interestingly, EBV also represent a risk factor for the development of certain HERV-associated diseases, including MS (Römer, 2021). This notion is supported by the fact, that the exposure of B cells to EBV was found to cause a genome-wide activation of LTR sequences in these cells and the EBV-mediated activation of LTRs was further associated with local DNA hypomethylation (Leung et al., 2018). As it was multiple times shown, EBV infection itself is able to change host epigenetics on the long-term, thereby counteracting the immune reaction and might further unlock endogenous retroviral elements (Buschle & Hammerschmidt, 2020). In the end, additional longitudinal studies are again warranted to decipher the temporal sequences of molecular events acting on inserted viral elements and

leading to their release – prior or concomitant to disease onset and development. Since none of the available studies have ascertained the epigenetic *status quo*, it remains unclear whether prior epigenetic de-repression is a necessary step to prime cells for HERV responses, thereby enabling HERV activation in response to infection and/or inflammation. It was therefore proposed, that future studies should not only focus on the detection of HERV expression, but rather also include information on the current epigenetic state. Although such approaches are very labor intensive, lengthy and expensive, it goes in line with the current literature as recent articles also demand the inclusion of epigenetic and longitudinal analyses in neurodegenerative disorders (Kashani et al., 2021; Koulousakis et al., 2023; Leng et al., 2019; Martinez-Iglesias et al., 2021).

Finally, it can be concluded that this work made a detailed summary on the complex activation mechanism of HERVs. Moreover, clear future suggestions were made that correlate with the scientific view in other fields of neurodegenerative diseases.

3.2.2 Effects of HERV-W ENV onto oligodendroglial lineage cells

Mature oligodendrocytes are mandatory for the electrical isolation of CNS neurons thereby warranting the saltatory signal transduction (Zalc et al., 2008). In multiple sclerosis, oligodendrocytes are attacked by immune cells and die focally, leading to demyelinating lesions. In general, oligodendroglial precursor cells would be able to replace the attacked oligodendrocytes and remyelinate demyelinated axons, however this process was shown to be very limited and inefficient at least in MS ((Kremer et al., 2011; Kuhlmann et al., 2008) as well as reviewed by (Gruchot et al., 2019b)). It was therefore of high interest, to understand whether HERV-W ENV exerts an effect onto oligodendroglial linage cells. In 2013, Kremer and colleagues were able to show in MS patients that HERV-W ENV is present in close proximity to TLR4-expressing oligodendroglial progenitor cells. Furthermore it was found, that the ENV protein itself could inhibit oligodendroglial differentiation *in vitro* in a TLR4 dependent manner (Kremer et al., 2013). Follow up studies showed, that this effect can be prevented when OPCs were co-incubated with an ENV neutralizing antibody named Temelimab (Kremer et al., 2015) and that HERV-W ENV is not only blocking the differentiation of OPCs, but also the myelination (Göttle et al., 2019). In order to analyze HERV-W ENV dependent effects onto oligodendroglial lineage cells in a more complex cellular structure, the de- and remyelination processes in an ENV expressing mouse model (CAG-Env) were analyzed upon exposure to the demyelinating agent cuprizone (CPZ; Gruchot et al., submitted). Upon CPZ feeding, CAG-Env mice showed an induction in demyelination processes and upon withdrawal of the CPZ diet transgenic animals were impaired in remyelination processes. While the increased demyelination processes are most likely due to increased inflammation and immune cell

activation as already indicated by Kremer and colleagues in 2019 (Kremer et al., 2019), another effect of HERV-W ENV protein onto oligodendroglial cells could be revealed, as the decreased remyelination capacity of CAG-Env mice turned out to be not only based on impaired OPC differentiation but also on decreased OPC recruitment. An effect that was most likely mediated by decreased OPC proliferation. This refined our view of the viral protein's impact on myelin repair, as ENV appears to induce multiple effects onto oligodendroglial lineage cells. One the one hand HERV-W ENV protein interferes with oligodendroglial maturation as demonstrated before (Göttle et al., 2019; Kremer et al., 2019; Kremer et al., 2013), but on the other hand its presence also caused a lack of OPC recruitment. This was not seen in previous *ex vivo* studies due to the use of post-mitotic primary cells. As a matter of fact, however, it remains to be shown whether the lack in OPC recruitment is only based on impaired OPC proliferation or whether OPC migration and infiltration of the corpus callosum is impaired as well. It is well described that the lack of trophic input and the inhibitory milieu generated by microglia and astrocytes can also lead to a lack of OPC recruitment (Galloway et al., 2020). Indeed, in the coming chapters it will be shown that astrocytes and microglia are also activated by HERV-W ENV expression. Therefore, it is even very likely that the effects described here have multiple casualties consisting of direct and indirect effects on oligodendroglial lineage cells.

Interestingly, these findings go in line with recent transcriptome and histological analysis from MS patients (Jäkel et al., 2019), as Jäkel and colleagues were able to show that the number of OPCs is decreased in rim of MS lesions compared to normal appearing white matter (NAWM). Furthermore, they suggest that a proportion of mature oligodendrocytes might be able to contribute to remyelination in MS (Jäkel et al., 2019). It would be therefore of high interest to perform fate mapping studies of CPZ challenged CAG-Env mice in order to identify additional reasons for the deficit in remyelination capacity. The usage Cre recombinase (Cre) inducible OPC specific reporter mice (such as PDGFRα-Cre^{ER} x *Rosa26-eYFP* or NG2-Cre^{ER} x *Rosa26-tdTomato* mice) would enable it to fate trace oligodendroglial lineage cells and therefore to analyze whether the differentiation is blocked at a certain stages. Furthermore, it could be investigated whether oligodendroglial lineage cells get lost or whether there is an impaired infiltration of OPCs during the course of cuprizone-mediated demyelination as well as in remyelination phases.

Moreover, not only OPCs contribute to the total remyelination capacity of the brain, but also brain resident neural stem cells (NSCs), primarily of the subventricular zone, have the ability to contribute to remyelination (Moyon et al., 2023). Although recent findings suggest, that OPCs are the primary source for maturing oligodendrocytes in the cuprizone model of demyelination (Moyon et al., 2023), a blockage of OPC differentiation led to a significant

increase in the recruitment and differentiation of NSC-derived cells into the demyelinated corpus callosum. Therefore, in order to refine our current results of the HERV-W dependent remyelination deficit, it would be of high interest to include the analysis of NSC derived remyelination in future studies.

Taken together, this thesis improves our view on the effects of HERV-W ENV onto oligodendroglial lineage cells as it identified an OPC recruitment deficit upon HERV-W ENV expression. This effect together with the described oligodendroglial differentiation deficit upon HERV-W ENV stimulation resulted in a decreased remyelination capacity. These findings might therefore contribute to the discovery of novel drug targets thereby further supporting treatment strategies and improving patient health.

3.2.3 Effects of HERV-W ENV onto microglia

Microglia are described as an important cell type, as their expression and secretion of pro-inflammatory cytokines as well as their phagocytosis ability contribute to the onset and progression of neurodegenerative diseases (Lassmann et al., 2012). Furthermore, microglia express either the HERV-W ENV receptor TLR4 as well as HERV-W ENV itself in the rim of MS lesions (Kremer et al., 2013; Rolland et al., 2006; van Horssen et al., 2016). It was therefore considered of high interest to analyze the effects HERV-W ENV has onto microglial cells. In the initial study (Kremer et al., 2019) we were able to proof once again that HERV-W ENV is expressed in microglia. Moreover, it was also found that these ENV positive myeloid cells were in a very close proximity to myelinated axons (Kremer et al., 2019), leading to the hypothesis that these cells contribute to neurodegeneration. The following *in vitro* studies then revealed that microglia increasingly express pro-inflammatory cytokines as well as nitrosative stress markers and suppress anti-inflammatory cytokines as well as phagocytosis upon ENV protein exposure. Similar to our findings, Rolland and colleagues showed, that HERV-W ENV leads to an increased expression and secretion of pro-inflammatory cytokines in PBMC cultures (Rolland et al., 2006). However, the role of cytokines in neurodegenerative diseases is highly debated (Kany et al., 2019). Upon a damage or infection event, cytokines are necessary to guide the immune reaction and thereby leads to the clearance of damaged tissues or cells, as well as to regeneration. On the other side, chronic long-term inflammation is associated with a number of different devastating diseases such as neurodegenerative diseases (Murakami & Hirano, 2012). In this case, cytokines not only lead to the clearance of injured or infected tissues and/or cells but also damages healthy tissue (Murakami & Hirano, 2012). In contrast to cytokines, nitrosative stress has been described more clearly. While it is seen to be important at low levels in physiological processes, higher levels, such as detected in neurodegenerative disorders, lead to neuronal dysfunction and death (Butterfield, 2006). In

order to proof this hypothesis, myelinated co-cultures with microglia were stimulated with recombinant HERV-W ENV. Similar to the MS histology, it was found, that HERV-W ENV leads to an increased association of microglia with myelinated axons. Furthermore, ELISA secretion assays were able to show increased levels of MBP, NF-H and SYP in the media of co-cultures with microglia and HERV-W ENV - an effect that was only seen when microglia and HERV-W ENV were present in the culture, indicating that neurodegeneration and demyelination upon HERV-W exposure is dependent on microglia. However, since the co-culture used in these experiments also contained astrocytes, but an effect was only seen when microglia and HERV-W ENV were present, it is tempting to speculate that astrocytic cells do not contribute the same amount to the occurring neurodegeneration and demyelination as microglia. However, the next chapter will look at this thoughts from a different perspective and might therefore come to a more complex result.

At this point, it is worth mentioning that microglia are well described of being able to induce neuronal death. Important molecules that are expressed by microglia and are associated with the induction of neurodegeneration are TNFα (Kitaoka et al., 2006) as well as NO (Butterfield, 2006) – two effectors that were also detected in the co-culture experiments. Although TNFα can have beneficial roles, the literature clearly indicates that this cytokine is mediating neurodegeneration upon chronic long-term exposure (Jayaraman et al., 2021). This process is most likely mediated by TNF receptor-1-mediated necroptosis pathway, via phosphorylation of RIPK3 and MLKL (Jayaraman et al., 2021). In contrast, however, the inhibition of TNFα resulted in increased disease activity and lesion load progression (The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1999), indicating that certain amount of cytokines is necessary. Nitrosative stress mediated by nitric oxide, on the other hand, is also well described to induce neuronal degeneration (Smith et al., 2001). Furthermore, multiple studies have demonstrated a significant involvement of NO in neurodegenerative diseases such as AD (Duda et al., 2000), PD (Good et al., 1998) but also MS (Smith & Lassmann, 2002). However, the exact mechanism on how NO leads to neurodegeneration remains to be shown.

As a next step, the polarization of microglial cells was analyzed in a HERV-W ENV expressing transgenic mouse line (CAG-Env), challenged with different MS models. Upon cuprizone-mediated demyelination as well as experimental autoimmune encephalomyelitis, transgenic mice displayed an elevated area of microglia/myeloid cells in the respective regions of interest. In general, infiltration of microglial cells into the corpus callosum (upon CPZ treatment) or within EAE lesions of the spinal cord, are well described in both models (Montilla et al., 2023; Zhan et al., 2020). However, our observation that HERV-W ENV amplifies these effects indicates the occurrence of increased inflammation. These finding also go in line with our *in*

vitro analysis, as we have seen in the mono- as well as in co-culture model, that microglia exhibit enlarged morphologies and increased cell numbers upon HERV-W ENV stimulation. Of note, a limitation of our EAE studies still lies in the lack of markers to distinguish between microglia and macrophages. Therefore, while in CPZ experiments Iba1 positive cells are primarily characterized by microglia, Iba1 positive cells of EAE experiments cannot be differentiated between microglia and macrophages and are therefore referred as myeloid cells.

Our results, furthermore, revealed an increased myeloid expression of Clec7a, which is a transmembrane protein containing an immunoreceptor tyrosine-based activation (ITAM)-like motif on its intracellular tail and was shown to be induced in microglia in many different neurodegenerative disorders such as MS, ALS, AD and PD (Keren-Shaul et al., 2017; Krasemann et al., 2017; Paolicelli et al., 2022; Stratoulias et al., 2019). Furthermore, it is thought that Clec7a signaling can be activated by amyloid beta, myelin debris and apoptotic cells leading to the induction of a neurodegenerative microglial phenotypes via spleen tyrosine kinase (SYK)-dependent pathways (Schafer & Stillman, 2022). However, a different group showed that Clec7a deficient mice have decreased axonal regeneration in a model of LPS induced optic neuritis (Baldwin et al., 2015), indicating that the exact molecular mechanism leading to neurodegeneration remains to be identified. Moreover, our experiments were not able to identify, whether Clec7a expression is a direct consequence of HERV-W ENV exposure leading to neuronal death or a secondary effect that is induced by the increased neurodegeneration. A recently published study showed, that Clec7a expression in monocytes can be induced by the TLR4 agonist LPS (Wang et al., 2022). In this context, it is tempting to speculate, that HERV-W ENV might also be able to induce Clec7a expression, although future experiments should focus on a proof of principle.

Additionally, we found increased expression of complement component 1q A chain (C1qa) as well as CD74 upon cuprizone mediated demyelination. C1q is a polyprotein complex composed of 18 polypeptide chains: six A-chains, six B-chains, and six C-chains. It is considered as a central part of the complement system and the adaptive immune system. In the CNS, it is primarily expressed by microglial cells (Fonseca et al., 2017) and besides other cytokines leads to the activation of reactive neurotoxic astrocytes (Liddelow et al., 2017). CD74 on the other hand, is involved in the antigen presentation and is a marker for highly activated microglia in neurodegenerative diseases (Jin et al., 2021). In addition, similar findings were shown by a different group that was able to identify increased CD74 expression in microglia upon stimulation with LPS – an effect that could be rescued by TGFβ co-stimulation (Jahn et al., 2022).

In summary, this work provides first principle evidence that HERV-W ENV stimulates microglia-dependent functions such as decreased phagocytes, expression of pro-

inflammatory cytokines, and down-regulation of anti-inflammatory cytokines. These effects not only lead to a negative milieu leading to OPC differentiation (Galloway et al., 2020) but are also associated with increased neurodegeneration and disease progression. It is very likely that the observed effects of HERV-W ENV onto OPCs are rather mixed effects of direct and indirect inhibition of OPC differentiation. Furthermore, analyzing novel microglial polarization marker, it could be shown, that HERV-W ENV challenged microglia express neurodegenerative associated proteins such as Clec7a and CD74 in two distinct models of MS. This finding contributes significantly to the characterization of HERV-W ENV triggered microglia in the context of neurodegenerative diseases.

3.2.4 Effects of HERV-W ENV onto astroglia

Besides microglial cells, astrocytes are also described as important immune-competent cells of the CNS, contributing to the onset and progression of neurodegenerative diseases (Absinta et al., 2021). Although astroglia express TLRs (Gorina et al., 2009), the knowledge about the effects of HERVs onto astrocytic cells is very limited. Observations on the related syncytin-1 displayed increased astrocytic release of redox reactants, which are known to have cytotoxic effects onto oligodendrocytes (Antony et al., 2007). Furthermore, syncytin-1 overexpression in human astroglia was reported to activate the inflammatory marker CRP via TLR3 signaling, notably a receptor that is known to bind double-stranded RNA but is prone to nucleic acid artifacts in transfection experiments (Wang et al., 2018). However, it is highly debated whether the physiological expressed syncytin-1 and the pathogenic form of HERV-W ENV exert similar effects. At least at biochemical level, these two proteins display important differences that might also interfere with receptor activation (Charvet et al., 2021), indicating that there is a clear need for more and in depth analysis of the effects of HERV-W ENV onto astrocytes.

To this end, astroglial polarization upon CPZ treatment as well as after induction of the EAE was analyzed. The results indicated an elevation in cleaved C3 (C3d) and Lcn2 positive reactive astrocytes upon cuprizone mediated demyelination as well as experimental autoimmune encephalomyelitis in transgenic vs wildtype brains (Gruchot et al., submitted). This marker combination in astrocytes is highly associated with neurodegeneration as well as impaired remyelination (Al Nimer et al., 2016; Bi et al., 2013; Liddelow et al., 2017). As we have already identified e.g. C1qa expression in microglia that could lead to an activation of astrocytes, a closer look was taken in order to identify whether these effects are specific for an interaction between astroglia and HERV-W ENV or just a correlation of microglial activation. To this end, an astroglial-microglia co-culture model was established in which it was found, that these cells appear to be sensitive to both, a direct impact of the ENV protein as well as to signals emanating from polarized microglial cells (Gruchot et al., submitted). Of note, since

the cell types were separated by cell culture inserts, a direct cell-cell contact was not necessary for this type of polarization. Similar interactions were recently described by Absinta and colleagues who described a strong interaction between microglia, astrocytes and peripheral immune cells (Absinta et al., 2021) and therefore contributes to the understanding of the emerging pathological role of astrocytes in MS and related disorders (Ponath et al., 2018). Moreover, our data provided here go in line with the literature, as C3 expression and cleavage to C3d was shown for many neurodegenerative diseases such as AD, PD, ALS and MS (Ingram et al., 2014; Loeffler et al., 2006; Mantovani et al., 2014; Rogers et al., 1992; Watkins et al., 2016). It is therefore seen as an important candidate for the identification and treatment of neurodegenerative activated astroglia (Schartz & Tenner, 2020). While C1qa expression and secretion, by e.g. microglia, are described to have a direct influence onto the expression of C3 and cleavage to C3d (Schartz & Tenner, 2020), Liddelow and colleagues have shown that C1qa signaling alone is not sufficient to induce Lcn2 expression in astrocytes (Liddelow et al., 2017). In contrast, they were able to show that Lcn2 expressing astrocytes can be induced by LPS stimulation – a known agonist of TLR4 signaling (Bohannon et al., 2013). Since TLR4 is also considered to be an important receptor for HERV-W ENV-mediated effects (Rolland et al., 2006), our data fit very well into the scientific literature. Furthermore, it can be hypothesized that Lcn2-positive astrocytes are most likely not induced by microglial C1qa secretion but by the HERV-W ENV protein itself. Similar to the complement activation, secreted Lcn2 is detected in the serum and/or CSF of patients suffering from neurodegenerative disorder such as AD, ALS and MS, where it is often described as an important biomarker and signaling pathway (Al Nimer et al., 2016; Berard et al., 2012; Naude et al., 2012; Petrozziello et al., 2020). However, it must also be considered that the boundaries of astroglial classification are fluid (Escartin et al., 2021) and although our proposed data here are more indicative of a neurotoxic astroglial phenotype, it could also be true that neuroprotective markers point in a different direction. In this context, recent studies have shown astroglial subpopulations that are thought to contribute to myelin repair (Silva et al., 2023) and thereby suggested to have a closer look into cell-specific modularity approaches aiming at novel therapeutic opportunities. As suggested by Escartin and a consortium of astroglial researchers, future studies should therefore focus on a multilevel analysis of the astroglial polarization including bulk and/or single cell transcriptome analysis (Escartin et al., 2021). Since astroglia contribute to the integrity of the BBB (Sofroniew & Vinters, 2010), it is tempting to speculate that HERV-W ENV will also have an influence on the infiltration of peripheral immune cells via astroglial polarization. Of note, Duperray and colleagues have already shown that HERV-W ENV has pro-inflammatory effects onto endothelial cells (Duperray et al., 2015). This effect was mediated by TLR4 signaling and lead to an increased

expression of intercellular adhesion molecule (ICAM)-1, a major mediator of lymphocyte adhesion to endothelial cells.

In conclusion, this thesis describes for the first time a direct effect of HERV-W ENV on astrocytes. Exposure of astrocytes to HERV-W ENV protein resulted in a reactive neurotoxic phenotype that was independent from microglial activation but can be potentiated by microglia. Since this was the first description of an effect of HERV-W onto astrocytic cells, future studies should focus on an in depth characterization, e.g. using transcriptome analysis.

3.2.5 HERV-W ENV contributes to neurodegeneration

Since the mammalian CNS lacks any regeneration, dying neurons are very likely not been replaced, leading to the progression of the diseases as well as to permanent worsening of the clinical disabilities. To this end, a wide variety of different methods was used to detect HERV-dependent neurodegeneration.

Initially, the MS histology provided first insights into HERV-W ENV mediated neurodegeneration, as ENV positive microglia could be shown in a close proximity to myelinated axons that displayed a swollen morphology (Kremer et al., 2019) – a phenotype often described for axonal transections (Coleman & Perry, 2002). This axonal swelling in the brain was increased compared to other neurodegenerative diseases such as ALS and control patients without neurological disorders, which suggests a fundamental involvement of ENV-positive microglia in MS pathology. Additionally, a microglia and HERV-W ENV dependent increase in neurofilament levels in the media of a myelinated co-culture was observed (Kremer et al., 2019). HERV-W ENV expressing mice (CAG-Env) also showed an increased number of amyloid precursor protein (APP) positive spheroids upon cuprizone mediated demyelination (Gruchot et al., submitted) – an indicator of axonal transection *in vivo* (Coleman & Perry, 2002). Interestingly, a rather gentle and regenerative lesion set-up, as mediated via cuprizone feeding, led to significantly increased neurodegeneration in transgenic mice, which can be seen as a remarkable consideration for this model system (Zhan et al., 2020). Similar is true for the clinical EAE score, which was significantly increased in CAG-Env mice. The clinical EAE score is highly associated with spinal cord inflammation and neurodegeneration (Liu et al., 2008). Therefore, it is tempting to speculate that the observed worsening in clinical symptoms in the EAE model also result (in part) from enhanced neurodegenerative processes.

Our results provided here, along with published knowledge, open up various possibilities of direct and indirect mechanisms leading to neurodegeneration. In this context, it was previously shown, that HERV-K, a retroviral entity associated with ALS, can be expressed by neurons and upon expression leads to the death of neurons (Li et al., 2015). This process is described to be mediated by TDP-43 signaling (Douville & Nath, 2017). While Li and colleagues were

able to show direct effects of an endogenous viral entity, our model, showed no spontaneous induction of neurodegeneration in control transgenic animals (Gruchot et al., submitted).

However, taken into account that impaired de- and remyelination processes were observed (Gruchot et al., submitted), it is well described that impaired myelination leads to neurodegeneration (Ettle et al., 2016). While most of this evidence is based on studies on MS, also other neurodegenerative diseases are characterized by myelin dysfunction or demyelination, such as AD (Bartzokis, 2004), indicating that proper myelin function is essential for the neuronal integrity and hence long-term neuronal survival.

Although this effect was not described in our ex vivo studies from 2019, we were also able to provide strong evidence that also astroglial cells contribute to the occurring neurodegeneration as our recent results clearly demonstrate an increased expression of the neurotoxic marker Lcn2 (Bi et al., 2013). The exact mechanism on how Lcn2 induces neurodegeneration is, however, not fully understood, yet. One possible mechanism could be related to Egr1, as a recent publication showed Lcn2 dependent upregulation of Egr1 in oligodendrocytes (Li et al., 2022). In neurons, Egr1 is known as a stress signal associated with neuropsychiatric diseases (Brito et al., 2022; Tallafuss et al., 2022). Of note, it was also recently shown by Johannson and colleagues that HERV-W ENV stimulated hippocampal slice cultures exhibit a deficit in glutamate synapse maturation and when it is transfected postnatal in mice it leads to altered behaviors associated with psychosis. This effect, however, was shown to be mediated by glial cells, similar to our observations (Johansson et al., 2020). Although they did not analyze Lcn2 expression, they identified increased levels of pro-inflammatory cytokines to be responsible for the deficiency in glutamate synapse maturation (Johansson et al., 2020).

A third possibility that is described in this thesis points towards microglial cells that induce neurodegeneration. In general, it is well described that microglia can induce neuronal death, and this thesis identified different pathways such as mediated by cytokines, oxidative stress and/or via Clec7a signaling. Of note, Perron and colleagues could show a different mechanism, which is dependent on peripheral immune cells. In 2013, they were able to show that HERV-W ENV can be used as adjuvant for the induction of EAE that exceed the clinical scores in classically induced mice (Perron et al., 2013). This effect could be rescued by the treatment with an ENV blocking antibody named Temelimab (Perron et al., 2013). However, the authors hypothesized that these effects were mediated by an activation of T lymphocytes, as they were able to show, that cultured T cells of MOG/ENV immunized mice responded with a significant secretion of IFNγ, compared to cells of immunized control animals (Perron et al., 2013). Similarly, previous reports have shown a Th1-like type of Th cell differentiation or superantigen-like (SAg) activation of T cells (Perron et al., 2012; Rolland et al., 2006). Although it cannot be excluded that peripheral immune cells contribute to the here described

neurotoxic effects, our data primarily point towards microglia, astrocytes as well as increased demyelination as mediators for neurodegeneration.

In conclusion, the data shown in this thesis indicate that multiple pathways could mediate the effects of HERV-W ENV on neurodegeneration. Although a direct effect of HERV-W ENV onto neurons as well as effects of peripheral immune cells cannot be excluded, this thesis provides unequivocal evidence that microglial and astrocytic cells play a significant role in this context.

3.3 The importance of these results and future perspectives

Both, the experimental work studying the influence of microglia onto the BBB integrity in MS (Fleischer et al., 2021) as well as analyzing the effects of siponimod onto reactive microglia, (Gruchot et al., 2022) together with the review that summarizes the current mechanisms leading remyelination failure (Gruchot et al., 2019b), contribute to a more detailed knowledge on the biology of neurodegenerative diseases. Within the framework of the two experimental studies, it was possible to have a direct influence on the elucidation of the reasons for remyelination errors summarized in the review.

Regardless of extensive research efforts on neurodegenerative diseases, the causative mechanisms are still obscure. With the here provided study (Fleischer et al., 2021) that analyzed the correlation of BBB enlargement and BBB integrity in MS an unmet need for novel molecular, cellular, and imaging biomarkers predicting loss of BBB integrity is addressed, thereby aiming in characterizing the disease onset and severity in patients with neurodegenerative diseases. With the better understanding of how microglia and peripheral immune cells contribute to BBB leakiness during the course of MS, this thesis may also be able to improve the prognosis and medication of MS patients. However, BBB integrity is not only important for MS pathology, as described above, increased peripheral immune cell infiltration is reported for all neurodegenerative diseases (Doty et al., 2015; Rezai-Zadeh et al., 2009). Furthermore, an improved knowledge in the dynamics of BBB integrity might lead to a better understanding in how therapeutic molecules will be transported across the BBB. Of note, the protection of BBB integrity is not only important for the symptomatic improvement of neurodegenerative diseases, but also plays crucial roles in aging processes and the protection against infectious diseases (Knox et al., 2022), indicating the wide impact of this study. However, the success of this study is also reflected by its citations within the scientific field. As it could be shown, that ChP enlargement also plays significant roles in depression (Althubaity et al., 2022), and was shown to be associated with cognitive decline and Apolipoprotein E (ApoE) expression (Mantovani et al., 2014). Furthermore, choroid plexus volumetric are meanwhile proposed to be accepted as a predictor of MS treatment response (Murck et al., 2023).

Since siponimod has been shown to penetrate the intact BBB as well (Gentile et al., 2016), a significant contribution to the understanding on how reactive microglia can be targeted to prevent the smoldering inflammation known to also reside behind a closed BBB could be made. At this point, it should be emphasized that our RNAseq analysis went in line the proposed recommendations for the analysis of microglial cells (Paolicelli et al., 2022). The categorization of microglia with outdated terms like “dormant versus activated” or “M1 versus M2” led to widespread problems, so in this publication (Gruchot et al., 2022) we have taken a different approach that reflects the current technical advantages and thereby provides a larger scale of information. As the gene ontology analysis revealed that there is an increase in the reaction to unfolded proteins, it would be of interest to see to what extend siponimod has an influence onto the endoplasmic reticulum and whether siponimod has any effects onto the immune metabolism. All together, these would be important information to fully reveal the beneficial but also harmful effects of siponimod treatment and might improve future siponimod medication.

Furthermore, based on the first two reviews (Gruchot et al., 2019a, 2020), together with the novel description of the HERV-W ENV-dependent activation of microglial and astroglial cells and how this leads to demyelination as well as neurodegeneration and impaired remyelination (Gruchot et al., submitted; Kremer et al., 2019), an important contribution to the field of HERVs but also neurodegenerative field could be made. Since recent single cell RNAseq experiments have shed light onto the high complexity of microglial and astroglial immune polarization (Escartin et al., 2021; Paolicelli et al., 2022), it would be an important next step to analyze the transcriptome of microglia and astrocytes derived from CAG-Env mice upon cuprizone-mediated demyelination and/or EAE. These data would be a great benefit for the scientific community, as it could reveal the complex HERV-W ENV specific genetic signature that can be compared with already existing data on HERV-W independent activated microglia and astrocytes. This comparison would shed light on activation mechanisms that are either shared or CAG-Env specific. In addition, the bioinformatics community is evolving very rapidly, and once these data are released, other laboratories might be able to perform even deeper *in silico* analyses, which could potentially reveal new biomarkers for detecting early forms of e.g. MS and advance the field of human endogenous retroviral diseases. In addition, there is still very limited information about the effects of HERV-W onto stem cells niches, endothelial cells as well as pericytes, all of which are also functionally associated in autoimmune and neurodegenerative diseases (Hou & Hong, 2008; Lendahl et al., 2019; Yuan et al., 2023). Combined with the here provided ChP analysis and its implication in MS, it would be of interest to analyze ChP enlargement as well as BBB integrity in CAG-Env mice upon different MS models. In this context, it is proposed, that endothelial cells exert an inflammatory response

upon exposure to HERV-W ENV, characterized by an increased expression of ICAM-1 as well as cytokines, which in conclusion led to increased transmigration of activated immune cells (Duperray et al., 2015).

Although we made great progress in the characterization of HERV-W's effects onto glial cells, the EAE model provided evidence that also autoimmune processes might be altered. Given that the lesion formation in the EAE model was not found to be more frequent but larger in size and was thus containing more myeloid cells, it is very likely that also peripheral immune cells, that are known to infiltrate the CNS during the course of the EAE, play a significant role. It would therefore be of interest to analyze and compare the populations of peripheral immune cells inside vs outside of the CNS. As described above, HERV-W ENV can influence the T helper cell differentiation as well as the activation of T cells via the formation of SAgs (Perron et al., 2012; Rolland et al., 2006). However, a direct effect of HERV-W ENV onto lymphoid cells was not supported by the clinical trials (Hartung et al., 2022).

While most reports (including our) point towards TLR4 mediated effects of HERV-W ENV others propose different signaling pathways such as mediated by major facilitator superfamily domain-containing protein (MFSD)-2 (Esnault et al., 2008), neutral amino acid transporter A (ASCT1; Antony et al., 2007), neutral amino acid transporter B(0) (ASCT2; Blond et al., 2000) and monocarboxylat-Transporter (MCT)-1 (Blanco-Melo et al., 2017). The question therefore remains which of these candidate receptors is important for the here described glial effects. Future experiments should therefor focus on different knock down/knock out experiments using e.g. TLR4-KO mice and/or apply specific blocking reagents in order to proof a specific signaling pathway. Interestingly, a recent study has shown that natalizumab treatment of MS patients modulates the expression of HERV-W as well as its response to it (Arru et al., 2015). In this context, other immunomodulatory therapies such as S1PR-modulators might also have beneficial effects onto the expression of HERV-W ENV. Of note and as indicated by this thesis the effects described for HERV-W ENV and LPS might be mediated by the same receptor signaling (TLR4). It is therefore tempting to speculate, that siponimod might also exert beneficial effects onto ENV triggered microglia. This underlines the fact that HERV-W ENV expression should be included in future serological analysis of treated MS patients.

In the end, the epigenetic *status quo* of endogenous viral sequences as well as the activation mechanisms leading to the expression of endogenous retroviruses was summarized and thereby contributed to shape future suggestions in order to solve the current open questions in the field of endogenous retroviruses (Gruchot et al., 2023). In this context, we also shed light onto mouse endogenous retroviruses, which have a great potential for future analysis.

While most of the here described experiments were based on MS specific models, it should again be pointed out that there are many homologous pathways known between the different neurodegenerative diseases (characterized in the Introduction). Furthermore, recent transcriptome analyses are also aiming in the identification of common and different signatures in microglia, astroglia but also peripheral immune cells (Absinta et al., 2021; Escartin et al., 2021; Paolicelli et al., 2022). Since HERVs can be found in many neurodegenerative diseases such as MS, ALS but also AD (Römer, 2021), it remains to be shown, whether these viral entities might characterize a common mechanism leading to chronic inflammation, autoimmunity as well as neurodegeneration.

Of note, in late December 2019, an outbreak of an unknown disease later identified to be caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) quickly developed into a worldwide pandemic and caused high morbidity and mortality (He et al., 2020). Besides the disease's severity, numerous long-lasting post-infectious symptoms were observed among patients who suffered from SARS-CoV-2 infections (Lopez-Leon et al., 2021). Apart from the direct effects mediated by the SARS-CoV-2 infection, a contribution of endogenous retroviruses in particular HERV-W ENV onto the severity as well as onto post-infectious symptoms was recently described (Balestrieri et al., 2021; Charvet et al., 2023). A similar type of HERV activation was already described before, since induction of HERV-W expression was shown to be mediated by HHV-6 and CD46 receptor interaction (Charvet et al., 2018). Given the involvement of HERV-W in the pathogenesis of multiple sclerosis, as well as its association with the pathology of certain inflammatory neuropsychiatric diseases (Johansson et al., 2020; Tamouza et al., 2021), a correlation of the observed HERV-W ENV expression with neurological and cognitive symptoms is inevitable. Indeed, neurological symptoms and cognitive impairments are seen as a hallmark post-acute COVID-19 syndrome (Boldrini et al., 2021; Förster et al., 2020). It therefore remains to be shown, whether the here described effects of HERV-W onto the different glial cell populations might also play a role in post-acute COVID-19 syndrome as well as whether currently established therapeutic strategies such as for example treatment with the ENV neutralizing antibody Temelimab, might be applicable for post COVID-19 patients in the future. This, once more, underlines the importance of the here presented studies as there is first functional evidence that this viral entity contributes to impairments in neurological and cognitive symptoms of COVID-19 patients.

3.4 Conclusion

In conclusion, this thesis describes novel mechanisms on how microglia contribute to the onset and progression of neurodegenerative diseases and how immunomodulatory treatment strategies such as siponimod administration can influence microglial activation states. In addition, a better understanding about the molecular basis leading to remyelination failure in MS, as well as about the complex mechanisms of HERV activation in health and disease was provided here. By using latest glial polarization markers in combination with the novel HERV-W ENV expressing mouse model, new insights of HERV's mode of action could be described. In this context, it could be shown that HERV-W ENV protein exerts effects onto all four glial cell types, leading to severe impairments in de- and remyelination events as well as neurodegeneration. The fact that the expression of endogenous viral entities is associated with a constant rising numbers of highly inflammatory disorders underlines the importance of this research and it remains to be shown, whether future discoveries in the context of HERVs might lead to the development of more effective treatment strategies.

References

Aaronson, S. A., Todaro, G. J., & Scolnick, E. M. (1971). Induction of murine C-type viruses from clonal lines of virus-free BALB-3T3 cells. *Science, 174*(4005), 157-159. doi: 10.1126/science.174.4005.157

Absinta, M., Maric, D., Gharagozloo, M., Garton, T., Smith, M. D., Jin, J., . . . Reich, D. S. (2021). A lymphocyte-microglia-astrocyte axis in chronic active multiple sclerosis. *Nature, 597*(7878), 709-714. doi: 10.1038/s41586-021-03892-7

Al Nimer, F., Elliott, C., Bergman, J., Khademi, M., Dring, A. M., Aeinehband, S., . . . Piehl, F. (2016). Lipocalin-2 is increased in progressive multiple sclerosis and inhibits remyelination. *Neurol Neuroimmunol Neuroinflamm, 3*(1), e191. doi: 10.1212/NXI.0000000000000191

Alfahad, T., & Nath, A. (2013). Retroviruses and amyotrophic lateral sclerosis. *Antiviral Res, 99*(2), 180-187. doi: 10.1016/j.antiviral.2013.05.006

Alonso, A. C., Li, B., Grundke-Iqbal, I., & Iqbal, K. (2008). Mechanism of tau-induced neurodegeneration in Alzheimer disease and related tauopathies. *Curr Alzheimer Res, 5*(4), 375-384. doi: 10.2174/156720508785132307

Althubaity, N., Schubert, J., Martins, D., Yousaf, T., Nettis, M. A., Mondelli, V., . . . Veronese, M. (2022). Choroid plexus enlargement is associated with neuroinflammation and reduction of blood brain barrier permeability in depression. *Neuroimage Clin, 33*, 102926. doi: 10.1016/j.nicl.2021.102926

Alvarez-Lafuente, R., De las Heras, V., Bartolome, M., Picazo, J. J., & Arroyo, R. (2004). Relapsing-remitting multiple sclerosis and human herpesvirus 6 active infection. *Arch Neurol, 61*(10), 1523-1527. doi: 10.1001/archneur.61.10.1523

Amor, S., Puentes, F., Baker, D., & van der Valk, P. (2010). Inflammation in neurodegenerative diseases. *Immunology, 129*(2), 154-169. doi: 10.1111/j.1365-2567.2009.03225.x

An, S., Bleu, T., Huang, W., Hallmark, O. G., Coughlin, S. R., & Goetzl, E. J. (1997). Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. *FEBS Lett, 417*(3), 279-282. doi: 10.1016/s0014-5793(97)01301-x

Antony, J. M., Ellestad, K. K., Hammond, R., Imaizumi, K., Mallet, F., Warren, K. G., & Power, C. (2007). The human endogenous retrovirus envelope glycoprotein, syncytin-1, regulates neuroinflammation and its receptor expression in multiple sclerosis: a role for endoplasmic reticulum chaperones in astrocytes. *J Immunol, 179*(2), 1210-1224. doi: 10.4049/jimmunol.179.2.1210

Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., . . . Oda, T. (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun, 351*(3), 602-611. doi: 10.1016/j.bbrc.2006.10.093

Arru, G., Caggiu, E., Leoni, S., Mameli, G., Pugliatti, M., Sechi, G. P., & Sechi, L. A. (2015). Natalizumab modulates the humoral response against HERV-Wenv73-88 in a follow-up study of Multiple Sclerosis patients. *J Neurol Sci, 357*(1-2), 106-108. doi: 10.1016/j.jns.2015.07.007

Azzouz, M., Poindron, P., Guettier, S., Leclerc, N., Andres, C., Warter, J. M., & Borg, J. (2000). Prevention of mutant SOD1 motoneuron degeneration by copper chelators in vitro. *J Neurobiol, 42*(1), 49-55. doi: 10.1002/(sici)1097-4695(200001)42:1<49::aid-neu5>3.0.co;2-7

Baldwin, K. T., Carbajal, K. S., Segal, B. M., & Giger, R. J. (2015). Neuroinflammation triggered by beta-glucan/dectin-1 signaling enables CNS axon regeneration. *Proc Natl Acad Sci U S A, 112*(8), 2581-2586. doi: 10.1073/pnas.1423221112

Balestrieri, E., Cipriani, C., Matteucci, C., Benvenuto, A., Coniglio, A., Argaw-Denboba, A., . . . Sinibaldi-Vallebona, P. (2019). Children With Autism Spectrum Disorder and Their Mothers Share Abnormal Expression of Selected Endogenous Retroviruses Families and Cytokines. *Front Immunol, 10*, 2244. doi: 10.3389/fimmu.2019.02244

Balestrieri, E., Minutolo, A., Petrone, V., Fanelli, M., Iannetta, M., Malagnino, V., . . . Matteucci, C. (2021). Evidence of the pathogenic HERV-W envelope expression in T lymphocytes in association with the respiratory outcome of COVID-19 patients. *EBioMedicine, 66*, 103341. doi: 10.1016/j.ebiom.2021.103341

Balestrieri, E., Pitzianti, M., Matteucci, C., D'Agati, E., Sorrentino, R., Baratta, A., . . . Pasini, A. (2014). Human endogenous retroviruses and ADHD. *World J Biol Psychiatry, 15*(6), 499-504. doi: 10.3109/15622975.2013.862345

Barnham, K. J., Masters, C. L., & Bush, A. I. (2004). Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov, 3*(3), 205-214. doi: 10.1038/nrd1330

Barthel, H., Villemagne, V. L., & Drzezga, A. (2022). Future Directions in Molecular Imaging of Neurodegenerative Disorders. *J Nucl Med, 63*(Suppl 1), 68S-74S. doi: 10.2967/jnumed.121.263202

Bartzokis, G. (2004). Age-related myelin breakdown: a developmental model of cognitive decline and Alzheimer's disease. *Neurobiol Aging, 25*(1), 5-18; author reply 49-62. doi: 10.1016/j.neurobiolaging.2003.03.001

Baxter, A. G. (2007). The origin and application of experimental autoimmune encephalomyelitis. *Nat Rev Immunol, 7*(11), 904-912. doi: 10.1038/nri2190

Becher, B., Spath, S., & Goverman, J. (2017). Cytokine networks in neuroinflammation. *Nat Rev Immunol, 17*(1), 49-59. doi: 10.1038/nri.2016.123

Benarroch, E. E. (2016). Choroid plexus--CSF system: Recent developments and clinical correlations. *Neurology, 86*(3), 286-296. doi: 10.1212/WNL.0000000000002298

Bentvelzen, P., Daams, J. H., Hageman, P., & Calafat, J. (1970). Genetic transmission of viruses that incite mammary tumor in mice. *Proc Natl Acad Sci U S A, 67*(1), 377-384. doi: 10.1073/pnas.67.1.377

Berard, J. L., Zarruk, J. G., Arbour, N., Prat, A., Yong, V. W., Jacques, F. H., . . . David, S. (2012). Lipocalin 2 is a novel immune mediator of experimental autoimmune encephalomyelitis pathogenesis and is modulated in multiple sclerosis. *Glia, 60*(7), 1145-1159. doi: 10.1002/glia.22342

Berghoff, S. A., Duking, T., Spieth, L., Winchenbach, J., Stumpf, S. K., Gerndt, N., . . . Saher, G. (2017). Blood-brain barrier hyperpermeability precedes demyelination in the cuprizone model. *Acta Neuropathol Commun, 5*(1), 94. doi: 10.1186/s40478-017-0497-6

Bi, F., Huang, C., Tong, J., Qiu, G., Huang, B., Wu, Q., . . . Zhou, H. (2013). Reactive astrocytes secrete lcn2 to promote neuron death. *Proc Natl Acad Sci U S A, 110*(10), 4069-4074. doi: 10.1073/pnas.1218497110

Binder, L. I., Frankfurter, A., & Rebhun, L. I. (1985). The distribution of tau in the mammalian central nervous system. *J Cell Biol, 101*(4), 1371-1378. doi: 10.1083/jcb.101.4.1371

Bjornevik, K., Cortese, M., Healy, B. C., Kuhle, J., Mina, M. J., Leng, Y., . . . Ascherio, A. (2022). Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science, 375*(6578), 296-301. doi: 10.1126/science.abj8222

Blackshaw, S. (2022). Why Has the Ability to Regenerate Following CNS Injury Been Repeatedly Lost Over the Course of Evolution? *Front Neurosci, 16*, 831062. doi: 10.3389/fnins.2022.831062

Blanco-Melo, D., Gifford, R. J., & Bieniasz, P. D. (2017). Co-option of an endogenous retrovirus envelope for host defense in hominid ancestors. *Elife, 6*. doi: 10.7554/eLife.22519

Block, M. L., & Hong, J. S. (2005). Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog Neurobiol, 76*(2), 77-98. doi: 10.1016/j.pneurobio.2005.06.004

Block, M. L., Zecca, L., & Hong, J. S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci, 8*(1), 57-69. doi: 10.1038/nrn2038

Blond, D., Raoul, H., Le Grand, R., & Dormont, D. (2000). Nitric oxide synthesis enhances human immunodeficiency virus replication in primary human macrophages. *J Virol, 74*(19), 8904-8912. doi: 10.1128/jvi.74.19.8904-8912.2000

Bohannon, J. K., Hernandez, A., Enkhbaatar, P., Adams, W. L., & Sherwood, E. R. (2013). The immunobiology of toll-like receptor 4 agonists: from endotoxin tolerance to immunoadjuvants. *Shock, 40*(6), 451-462. doi: 10.1097/SHK.0000000000000042

Boldrini, M., Canoll, P. D., & Klein, R. S. (2021). How COVID-19 Affects the Brain. *JAMA Psychiatry, 78*(6), 682-683. doi: 10.1001/jamapsychiatry.2021.0500

Bowen, L. N., Tyagi, R., Li, W., Alfahad, T., Smith, B., Wright, M., . . . Nath, A. (2016). HIV-associated motor neuron disease: HERV-K activation and response to antiretroviral therapy. *Neurology, 87*(17), 1756-1762. doi: 10.1212/WNL.0000000000003258

Boyle, P. A., Wilson, R. S., Yu, L., Barr, A. M., Honer, W. G., Schneider, J. A., & Bennett, D. A. (2013). Much of late life cognitive decline is not due to common neurodegenerative pathologies. *Ann Neurol, 74*(3), 478-489. doi: 10.1002/ana.23964

Brana, C., Frossard, M. J., Pescini Gobert, R., Martinier, N., Boschert, U., & Seabrook, T. J. (2014). Immunohistochemical detection of sphingosine-1-phosphate receptor 1 and 5 in human multiple sclerosis lesions. *Neuropathol Appl Neurobiol, 40*(5), 564-578. doi: 10.1111/nan.12048

Brito, V., Montalban, E., Sancho-Balsells, A., Pupak, A., Flotta, F., Masana, M., . . . Giralt, A. (2022). Hippocampal Egr1-Dependent Neuronal Ensembles Negatively Regulate Motor Learning. *J Neurosci, 42*(27), 5346-5360. doi: 10.1523/JNEUROSCI.2258-21.2022

Brown, R. C., Lockwood, A. H., & Sonawane, B. R. (2005). Neurodegenerative diseases: an overview of environmental risk factors. *Environ Health Perspect, 113*(9), 1250-1256. doi: 10.1289/ehp.7567

Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A., & Hof, P. R. (2000). Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev, 33*(1), 95-130. doi: 10.1016/s0165-0173(00)00019-9

Bunge, R. P. (1968). Glial cells and the central myelin sheath. *Physiol Rev, 48*(1), 197-251. doi: 10.1152/physrev.1968.48.1.197

Buschle, A., & Hammerschmidt, W. (2020). Epigenetic lifestyle of Epstein-Barr virus. *Semin Immunopathol, 42*(2), 131-142. doi: 10.1007/s00281-020-00792-2

Butterfield, D. A. (2006). Oxidative stress in neurodegenerative disorders. *Antioxid Redox Signal, 8*(11-12), 1971-1973. doi: 10.1089/ars.2006.8.1971

Calabrese, M., Reynolds, R., Magliozzi, R., Castellaro, M., Morra, A., Scalfari, A., . . . Monaco, S. (2015). Regional Distribution and Evolution of Gray Matter Damage in Different

Populations of Multiple Sclerosis Patients. *PLoS One, 10*(8), e0135428. doi: 10.1371/journal.pone.0135428

Campbell, A. (2004). Inflammation, neurodegenerative diseases, and environmental exposures. *Ann N Y Acad Sci, 1035*, 117-132. doi: 10.1196/annals.1332.008

Carson, M. J., Doose, J. M., Melchior, B., Schmid, C. D., & Ploix, C. C. (2006). CNS immune privilege: hiding in plain sight. *Immunol Rev, 213*, 48-65. doi: 10.1111/j.1600-065X.2006.00441.x

Charvet, B., Brunel, J., Pierquin, J., Iampietro, M., Decimo, D., Queruel, N., . . . Horvat, B. (2023). SARS-CoV-2 awakens ancient retroviral genes and the expression of proinflammatory HERV-W envelope protein in COVID-19 patients. *iScience, 26*(5), 106604. doi: 10.1016/j.isci.2023.106604

Charvet, B., Pierquin, J., Brunel, J., Gorter, R., Quetard, C., Horvat, B., . . . Perron, H. (2021). Human Endogenous Retrovirus Type W Envelope from Multiple Sclerosis Demyelinating Lesions Shows Unique Solubility and Antigenic Characteristics. *Virol Sin, 36*(5), 1006-1026. doi: 10.1007/s12250-021-00372-0

Charvet, B., Reynaud, J. M., Gourru-Lesimple, G., Perron, H., Marche, P. N., & Horvat, B. (2018). Induction of Proinflammatory Multiple Sclerosis-Associated Retrovirus Envelope Protein by Human Herpesvirus-6A and CD46 Receptor Engagement. *Front Immunol, 9*, 2803. doi: 10.3389/fimmu.2018.02803

Chio, A., Mazzini, L., D'Alfonso, S., Corrado, L., Canosa, A., Moglia, C., . . . Al-Chalabi, A. (2018). The multistep hypothesis of ALS revisited: The role of genetic mutations. *Neurology, 91*(7), e635-e642. doi: 10.1212/WNL.0000000000005996

Ciccarelli, O., Barkhof, F., Bodini, B., De Stefano, N., Golay, X., Nicolay, K., . . . Miller, D. H. (2014). Pathogenesis of multiple sclerosis: insights from molecular and metabolic imaging. *Lancet Neurol, 13*(8), 807-822. doi: 10.1016/S1474-4422(14)70101-2

Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med, 48*(6), 749-762. doi: 10.1016/j.freeradbiomed.2009.12.022

Coelho, R. P., Payne, S. G., Bittman, R., Spiegel, S., & Sato-Bigbee, C. (2007). The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. *J Pharmacol Exp Ther, 323*(2), 626-635. doi: 10.1124/jpet.107.123927

Cohen, C. C. H., Popovic, M. A., Klooster, J., Weil, M. T., Mobius, W., Nave, K. A., & Kole, M. H. P. (2020). Saltatory Conduction along Myelinated Axons Involves a Periaxonal Nanocircuit. *Cell, 180*(2), 311-322 e315. doi: 10.1016/j.cell.2019.11.039

Coleman, M. P., & Perry, V. H. (2002). Axon pathology in neurological disease: a neglected therapeutic target. *Trends Neurosci, 25*(10), 532-537. doi: 10.1016/s0166-2236(02)02255-5

Colton, C., & Wilcock, D. M. (2010). Assessing activation states in microglia. *CNS Neurol Disord Drug Targets, 9*(2), 174-191. doi: 10.2174/187152710791012053

Constantinescu, C. S., Farooqi, N., O'Brien, K., & Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol, 164*(4), 1079-1106. doi: 10.1111/j.1476-5381.2011.01302.x

Cook, C., Carlomagno, Y., Gendron, T. F., Dunmore, J., Scheffel, K., Stetler, C., . . . Petrucelli, L. (2014). Acetylation of the KXGS motifs in tau is a critical determinant in modulation of tau aggregation and clearance. *Hum Mol Genet, 23*(1), 104-116. doi: 10.1093/hmg/ddt402

Cortese, R., Collorone, S., Ciccarelli, O., & Toosy, A. T. (2019). Advances in brain imaging in multiple sclerosis. *Ther Adv Neurol Disord, 12*, 1756286419859722. doi: 10.1177/1756286419859722

Deaton, A. M., & Bird, A. (2011). CpG islands and the regulation of transcription. *Genes Dev, 25*(10), 1010-1022. doi: 10.1101/gad.2037511

Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., & Amit, I. (2018). Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell, 173*(5), 1073-1081. doi: 10.1016/j.cell.2018.05.003

Deuschl, G., Beghi, E., Fazekas, F., Varga, T., Christoforidi, K. A., Sipido, E., . . . Feigin, V. L. (2020). The burden of neurological diseases in Europe: an analysis for the Global Burden of Disease Study 2017. *Lancet Public Health, 5*(10), e551-e567. doi: 10.1016/S2468-2667(20)30190-0

Dickson, D. W., Braak, H., Duda, J. E., Duyckaerts, C., Gasser, T., Halliday, G. M., . . . Litvan, I. (2009). Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria. *Lancet Neurol, 8*(12), 1150-1157. doi: 10.1016/S1474-4422(09)70238-8

Dietrich, M., Hecker, C., Martin, E., Langui, D., Gliem, M., Stankoff, B., . . . Albrecht, P. (2022). Increased Remyelination and Proregenerative Microglia Under Siponimod Therapy in Mechanistic Models. *Neurol Neuroimmunol Neuroinflamm, 9*(3). doi: 10.1212/NXI.0000000000001161

Dimou, L., & Gallo, V. (2015). NG2-glia and their functions in the central nervous system. *Glia, 63*(8), 1429-1451. doi: 10.1002/glia.22859

Dolei, A. (2018). The aliens inside us: HERV-W endogenous retroviruses and multiple sclerosis. *Mult Scler, 24*(1), 42-47. doi: 10.1177/1352458517737370

Doty, K. R., Guillot-Sestier, M. V., & Town, T. (2015). The role of the immune system in neurodegenerative disorders: Adaptive or maladaptive? *Brain Res, 1617*, 155-173. doi: 10.1016/j.brainres.2014.09.008

Douville, R., Liu, J., Rothstein, J., & Nath, A. (2011). Identification of active loci of a human endogenous retrovirus in neurons of patients with amyotrophic lateral sclerosis. *Ann Neurol, 69*(1), 141-151. doi: 10.1002/ana.22149

Douville, R. N., & Nath, A. (2017). Human Endogenous Retrovirus-K and TDP-43 Expression Bridges ALS and HIV Neuropathology. *Front Microbiol, 8*, 1986. doi: 10.3389/fmicb.2017.01986

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev, 82*(1), 47-95. doi: 10.1152/physrev.00018.2001

Du, L., Zhang, Y., Chen, Y., Zhu, J., Yang, Y., & Zhang, H. L. (2017). Role of Microglia in Neurological Disorders and Their Potentials as a Therapeutic Target. *Mol Neurobiol, 54*(10), 7567-7584. doi: 10.1007/s12035-016-0245-0

Duda, J. E., Giasson, B. I., Chen, Q., Gur, T. L., Hurtig, H. I., Stern, M. B., . . . Trojanowski, J. Q. (2000). Widespread nitration of pathological inclusions in neurodegenerative synucleinopathies. *Am J Pathol, 157*(5), 1439-1445. doi: 10.1016/S0002-9440(10)64781-5

Dugger, B. N., & Dickson, D. W. (2017). Pathology of Neurodegenerative Diseases. *Cold Spring Harb Perspect Biol, 9*(7). doi: 10.1101/cshperspect.a028035

Duperray, A., Barbe, D., Raguenez, G., Weksler, B. B., Romero, I. A., Couraud, P. O., . . . Marche, P. N. (2015). Inflammatory response of endothelial cells to a human endogenous retrovirus associated with multiple sclerosis is mediated by TLR4. *Int Immunol, 27*(11), 545-553. doi: 10.1093/intimm/dxv025

Dutta, R., & Trapp, B. D. (2014). Relapsing and progressive forms of multiple sclerosis: insights from pathology. *Curr Opin Neurol, 27*(3), 271-278. doi: 10.1097/WCO.0000000000000094

Escartin, C., Galea, E., Lakatos, A., O'Callaghan, J. P., Petzold, G. C., Serrano-Pozo, A., . . . Verkhratsky, A. (2021). Reactive astrocyte nomenclature, definitions, and future directions. *Nat Neurosci, 24*(3), 312-325. doi: 10.1038/s41593-020-00783-4

Esnault, C., Priet, S., Ribet, D., Vernochet, C., Bruls, T., Lavialle, C., . . . Heidmann, T. (2008). A placenta-specific receptor for the fusogenic, endogenous retrovirus-derived, human syncytin-2. *Proc Natl Acad Sci U S A, 105*(45), 17532-17537. doi: 10.1073/pnas.0807413105

Ettle, B., Schlachetzki, J. C. M., & Winkler, J. (2016). Oligodendroglia and Myelin in Neurodegenerative Diseases: More Than Just Bystanders? *Mol Neurobiol, 53*(5), 3046-3062. doi: 10.1007/s12035-015-9205-3

Falcao, A. M., van Bruggen, D., Marques, S., Meijer, M., Jakel, S., Agirre, E., . . . Castelo-Branco, G. (2018). Disease-specific oligodendrocyte lineage cells arise in multiple sclerosis. *Nat Med, 24*(12), 1837-1844. doi: 10.1038/s41591-018-0236-y

Farina, C., Aloisi, F., & Meinl, E. (2007). Astrocytes are active players in cerebral innate immunity. *Trends Immunol, 28*(3), 138-145. doi: 10.1016/j.it.2007.01.005

Faucard, R., Madeira, A., Gehin, N., Authier, F. J., Panaite, P. A., Lesage, C., . . . Creange, A. (2016). Human Endogenous Retrovirus and Neuroinflammation in Chronic Inflammatory Demyelinating Polyradiculoneuropathy. *EBioMedicine, 6*, 190-198. doi: 10.1016/j.ebiom.2016.03.001

Faulkner, G. J., & Carninci, P. (2009). Altruistic functions for selfish DNA. *Cell Cycle, 8*(18), 2895-2900. doi: 10.4161/cc.8.18.9536

Fawcett, J. W. (2020). The Struggle to Make CNS Axons Regenerate: Why Has It Been so Difficult? *Neurochem Res, 45*(1), 144-158. doi: 10.1007/s11064-019-02844-y

Feigin, V. L., Vos, T., Nichols, E., Owolabi, M. O., Carroll, W. M., Dichgans, M., . . . Murray, C. (2020). The global burden of neurological disorders: translating evidence into policy. *Lancet Neurol, 19*(3), 255-265. doi: 10.1016/S1474-4422(19)30411-9

Fereshtehnejad, S. M., Vosoughi, K., Heydarpour, P., Sepanlou, S. G., Farzadfar, F., Tehrani-Banihashemi, A., . . . Global Burden of Disease Study Eastern Mediterranean Region Collaborators - Neurological Diseases, S. (2019). Burden of neurodegenerative diseases in the Eastern Mediterranean Region, 1990-2016: findings from the Global Burden of Disease Study 2016. *Eur J Neurol, 26*(10), 1252-1265. doi: 10.1111/ene.13972

Fleischer, V., Gonzalez-Escamilla, G., Ciolac, D., Albrecht, P., Küry, P., Gruchot, J., . . . Groppa, S. (2021). Translational value of choroid plexus imaging for tracking neuroinflammation in mice and humans. *Proc Natl Acad Sci U S A, 118*(36). doi: 10.1073/pnas.2025000118

Fonseca, M. I., Chu, S. H., Hernandez, M. X., Fang, M. J., Modarresi, L., Selvan, P., . . . Tenner, A. J. (2017). Cell-specific deletion of C1qa identifies microglia as the dominant source of C1q in mouse brain. *J Neuroinflammation, 14*(1), 48. doi: 10.1186/s12974-017-0814-9

Förster, M., Weyers, V., Küry, P., Barnett, M., Hartung, H. P., & Kremer, D. (2020). Neurological manifestations of severe acute respiratory syndrome coronavirus 2-a controversy 'gone viral'. *Brain Commun, 2*(2), fcaa149. doi: 10.1093/braincomms/fcaa149

Frank, O., Giehl, M., Zheng, C., Hehlmann, R., Leib-Mosch, C., & Seifarth, W. (2005). Human endogenous retrovirus expression profiles in samples from brains of patients with schizophrenia and bipolar disorders. *J Virol, 79*(17), 10890-10901. doi: 10.1128/JVI.79.17.10890-10901.2005

Galloway, D. A., Gowing, E., Setayeshgar, S., & Kothary, R. (2020). Inhibitory milieu at the multiple sclerosis lesion site and the challenges for remyelination. *Glia, 68*(5), 859-877. doi: 10.1002/glia.23711

Garcia-Montojo, M., Doucet-O'Hare, T., Henderson, L., & Nath, A. (2018). Human endogenous retrovirus-K (HML-2): a comprehensive review. *Crit Rev Microbiol, 44*(6), 715-738. doi: 10.1080/1040841X.2018.1501345

Garson, J. A., Tuke, P. W., Giraud, P., Paranhos-Baccala, G., & Perron, H. (1998). Detection of virion-associated MSRV-RNA in serum of patients with multiple sclerosis. *Lancet, 351*(9095), 33. doi: 10.1016/s0140-6736(98)24001-3

Garson, J. A., Usher, L., Al-Chalabi, A., Huggett, J., Day, E. F., & McCormick, A. L. (2019). Quantitative analysis of human endogenous retrovirus-K transcripts in postmortem premotor cortex fails to confirm elevated expression of HERV-K RNA in amyotrophic lateral sclerosis. *Acta Neuropathol Commun, 7*(1), 45. doi: 10.1186/s40478-019-0698-2

Gentile, A., Musella, A., Bullitta, S., Fresegna, D., De Vito, F., Fantozzi, R., . . . Centonze, D. (2016). Siponimod (BAF312) prevents synaptic neurodegeneration in experimental multiple sclerosis. *J Neuroinflammation, 13*(1), 207. doi: 10.1186/s12974-016-0686-4

Gifford, R. J., Blomberg, J., Coffin, J. M., Fan, H., Heidmann, T., Mayer, J., . . . Johnson, W. E. (2018). Nomenclature for endogenous retrovirus (ERV) loci. *Retrovirology, 15*(1), 59. doi: 10.1186/s12977-018-0442-1

Giovannoni, G., Popescu, V., Wuerfel, J., Hellwig, K., Iacobaeus, E., Jensen, M. B., . . . Scalfari, A. (2022). Smouldering multiple sclerosis: the 'real MS'. *Ther Adv Neurol Disord, 15*, 17562864211066751. doi: 10.1177/17562864211066751

Göke, J., & Ng, H. H. (2016). CTRL+INSERT: retrotransposons and their contribution to regulation and innovation of the transcriptome. *EMBO Rep, 17*(8), 1131-1144. doi: 10.15252/embr.201642743

Gomez-Rio, M., Caballero, M. M., Gorriz Saez, J. M., & Minguez-Castellanos, A. (2016). Diagnosis of Neurodegenerative Diseases: The Clinical Approach. *Curr Alzheimer Res, 13*(5), 469-474. doi: 10.2174/1567205013666151116141603

Good, P. F., Hsu, A., Werner, P., Perl, D. P., & Olanow, C. W. (1998). Protein nitration in Parkinson's disease. *J Neuropathol Exp Neurol, 57*(4), 338-342. doi: 10.1097/00005072-199804000-00006

Gorina, R., Santalucia, T., Petegnief, V., Ejarque-Ortiz, A., Saura, J., & Planas, A. M. (2009). Astrocytes are very sensitive to develop innate immune responses to lipid-carried short interfering RNA. *Glia, 57*(1), 93-107. doi: 10.1002/glia.20738

Göttle, P., Förster, M., Gruchot, J., Kremer, D., Hartung, H. P., Perron, H., & Küry, P. (2019). Rescuing the negative impact of human endogenous retrovirus envelope protein on oligodendroglial differentiation and myelination. *Glia, 67*(1), 160-170. doi: 10.1002/glia.23535

Grabski, D. F., Hu, Y., Sharma, M., & Rasmussen, S. K. (2019). Close to the Bedside: A Systematic Review of Endogenous Retroviruses and Their Impact in Oncology. *J Surg Res, 240*, 145-155. doi: 10.1016/j.jss.2019.02.009

Grandi, N., & Tramontano, E. (2017). Type W Human Endogenous Retrovirus (HERV-W) Integrations and Their Mobilization by L1 Machinery: Contribution to the Human

Transcriptome and Impact on the Host Physiopathology. *Viruses, 9*(7). doi: 10.3390/v9070162

Grandi, N., & Tramontano, E. (2018). Human Endogenous Retroviruses Are Ancient Acquired Elements Still Shaping Innate Immune Responses. *Front Immunol, 9*, 2039. doi: 10.3389/fimmu.2018.02039

Gros-Louis, F., Gaspar, C., & Rouleau, G. A. (2006). Genetics of familial and sporadic amyotrophic lateral sclerosis. *Biochim Biophys Acta, 1762*(11-12), 956-972. doi: 10.1016/j.bbadis.2006.01.004

Gruchot, J., Herrero, F., Weber-Stadlbauer, U., Meyer, U., & Küry, P. (2023). Interplay between activation of endogenous retroviruses and inflammation as common pathogenic mechanism in neurological and psychiatric disorders. *Brain Behav Immun, 107*, 242-252. doi: 10.1016/j.bbi.2022.10.007

Gruchot, J., Kremer, D., & Küry, P. (2019a). Neural Cell Responses Upon Exposure to Human Endogenous Retroviruses. *Front Genet, 10*, 655. doi: 10.3389/fgene.2019.00655

Gruchot, J., Kremer, D., & Küry, P. (2020). Human endogenous retroviruses: ammunition for myeloid cells in neurodegenerative diseases? *Neural Regen Res, 15*(6), 1043-1044. doi: 10.4103/1673-5374.270311

Gruchot, J., Lein, F., Lewen, I., Reiche, L., Weyers, V., Petzsch, P., . . . Kremer, D. (2022). Siponimod Modulates the Reaction of Microglial Cells to Pro-Inflammatory Stimulation. *Int J Mol Sci, 23*(21). doi: 10.3390/ijms232113278

Gruchot, J., Lewen, I., Dietrich, M., Reiche, L., Sindi, M., Hecker, C., . . . Küry, P. (submitted). Transgenic expression of the HERV-W envelope protein leads to polarized glial cell populations and a neurodegenerative environment. *Proc Natl Acad Sci U S A*.

Gruchot, J., Weyers, V., Göttle, P., Förster, M., Hartung, H. P., Küry, P., & Kremer, D. (2019b). The Molecular Basis for Remyelination Failure in Multiple Sclerosis. *Cells, 8*(8). doi: 10.3390/cells8080825

Hanisch, U. K., & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci, 10*(11), 1387-1394. doi: 10.1038/nn1997

Hansson, O. (2021). Biomarkers for neurodegenerative diseases. *Nat Med, 27*(6), 954-963. doi: 10.1038/s41591-021-01382-x

Hardiman, O., Al-Chalabi, A., Chio, A., Corr, E. M., Logroscino, G., Robberecht, W., . . . van den Berg, L. H. (2017). Amyotrophic lateral sclerosis. *Nat Rev Dis Primers, 3*, 17071. doi: 10.1038/nrdp.2017.71

Hartung, H. P., Derfuss, T., Cree, B. A., Sormani, M. P., Selmaj, K., Stutters, J., . . . Barkhof, F. (2022). Efficacy and safety of temelimab in multiple sclerosis: Results of a randomized phase 2b and extension study. *Mult Scler, 28*(3), 429-440. doi: 10.1177/13524585211024997

Haruwaka, K., Ikegami, A., Tachibana, Y., Ohno, N., Konishi, H., Hashimoto, A., . . . Wake, H. (2019). Dual microglia effects on blood brain barrier permeability induced by systemic inflammation. *Nat Commun, 10*(1), 5816. doi: 10.1038/s41467-019-13812-z

Hauser, S. L., & Oksenberg, J. R. (2006). The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. *Neuron, 52*(1), 61-76. doi: 10.1016/j.neuron.2006.09.011

He, R., Lu, Z., Zhang, L., Fan, T., Xiong, R., Shen, X., . . . Geng, Q. (2020). The clinical course and its correlated immune status in COVID-19 pneumonia. *J Clin Virol, 127*, 104361. doi: 10.1016/j.jcv.2020.104361

Henderson, J. M., Carpenter, K., Cartwright, H., & Halliday, G. M. (2000). Loss of thalamic intralaminar nuclei in progressive supranuclear palsy and Parkinson's disease: clinical and therapeutic implications. *Brain, 123 (Pt 7)*, 1410-1421. doi: 10.1093/brain/123.7.1410

Hill, R. A., Li, A. M., & Grutzendler, J. (2018). Lifelong cortical myelin plasticity and age-related degeneration in the live mammalian brain. *Nat Neurosci, 21*(5), 683-695. doi: 10.1038/s41593-018-0120-6

Hirsch, E., Graybiel, A. M., & Agid, Y. A. (1988). Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature, 334*(6180), 345-348. doi: 10.1038/334345a0

Hottinger, A. F., Fine, E. G., Gurney, M. E., Zurn, A. D., & Aebischer, P. (1997). The copper chelator d-penicillamine delays onset of disease and extends survival in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Eur J Neurosci, 9*(7), 1548-1551. doi: 10.1111/j.1460-9568.1997.tb01511.x

Hou, L., & Hong, T. (2008). Stem cells and neurodegenerative diseases. *Sci China C Life Sci, 51*(4), 287-294. doi: 10.1007/s11427-008-0049-1

Hua-Van, A., Le Rouzic, A., Boutin, T. S., Filee, J., & Capy, P. (2011). The struggle for life of the genome's selfish architects. *Biol Direct, 6*, 19. doi: 10.1186/1745-6150-6-19

Ingram, G., Loveless, S., Howell, O. W., Hakobyan, S., Dancey, B., Harris, C. L., . . . Morgan, B. P. (2014). Complement activation in multiple sclerosis plaques: an immunohistochemical analysis. *Acta Neuropathol Commun, 2*, 53. doi: 10.1186/2051-5960-2-53

Jackson, S. J., Giovannoni, G., & Baker, D. (2011). Fingolimod modulates microglial activation to augment markers of remyelination. *J Neuroinflammation, 8*, 76. doi: 10.1186/1742-2094-8-76

Jahn, J., Bollensdorf, A., Kalischer, C., Piecha, R., Weiss-Muller, J., Potru, P. S., . . . Spittau, B. (2022). Microglial CD74 Expression Is Regulated by TGFbeta Signaling. *Int J Mol Sci, 23*(18). doi: 10.3390/ijms231810247

Jäkel, S., Agirre, E., Mendanha Falcao, A., van Bruggen, D., Lee, K. W., Knuesel, I., . . . Castelo-Branco, G. (2019). Altered human oligodendrocyte heterogeneity in multiple sclerosis. *Nature, 566*(7745), 543-547. doi: 10.1038/s41586-019-0903-2

Jayaraman, A., Htike, T. T., James, R., Picon, C., & Reynolds, R. (2021). TNF-mediated neuroinflammation is linked to neuronal necroptosis in Alzheimer's disease hippocampus. *Acta Neuropathol Commun, 9*(1), 159. doi: 10.1186/s40478-021-01264-w

Jin, C., Shao, Y., Zhang, X., Xiang, J., Zhang, R., Sun, Z., . . . Shi, L. (2021). A Unique Type of Highly-Activated Microglia Evoking Brain Inflammation via Mif/Cd74 Signaling Axis in Aged Mice. *Aging Dis, 12*(8), 2125-2139. doi: 10.14336/AD.2021.0520

Johansson, E. M., Bouchet, D., Tamouza, R., Ellul, P., Morr, A. S., Avignone, E., . . . Groc, L. (2020). Human endogenous retroviral protein triggers deficit in glutamate synapse maturation and behaviors associated with psychosis. *Sci Adv, 6*(29), eabc0708. doi: 10.1126/sciadv.abc0708

Kabashi, E., Valdmanis, P. N., Dion, P., Spiegelman, D., McConkey, B. J., Vande Velde, C., . . . Rouleau, G. A. (2008). TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat Genet, 40*(5), 572-574. doi: 10.1038/ng.132

Kany, S., Vollrath, J. T., & Relja, B. (2019). Cytokines in Inflammatory Disease. *Int J Mol Sci, 20*(23). doi: 10.3390/ijms20236008

Kappos, L., Arnold, D. L., Bar-Or, A., Camm, J., Derfuss, T., Kieseier, B. C., . . . Harada, T. (2016a). Safety and efficacy of amiselimod in relapsing multiple sclerosis (MOMENTUM): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol, 15*(11), 1148-1159. doi: 10.1016/S1474-4422(16)30192-2

Kappos, L., Bar-Or, A., Cree, B. A. C., Fox, R. J., Giovannoni, G., Gold, R., . . . Investigators, E. C. (2018). Siponimod versus placebo in secondary progressive multiple sclerosis (EXPAND): a double-blind, randomised, phase 3 study. *Lancet, 391*(10127), 1263-1273. doi: 10.1016/S0140-6736(18)30475-6

Kappos, L., Li, D. K., Stuve, O., Hartung, H. P., Freedman, M. S., Hemmer, B., . . . Selmaj, K. (2016b). Safety and Efficacy of Siponimod (BAF312) in Patients With Relapsing-Remitting Multiple Sclerosis: Dose-Blinded, Randomized Extension of the Phase 2 BOLD Study. *JAMA Neurol, 73*(9), 1089-1098. doi: 10.1001/jamaneurol.2016.1451

Karch, C. M., & Goate, A. M. (2015). Alzheimer's disease risk genes and mechanisms of disease pathogenesis. *Biol Psychiatry, 77*(1), 43-51. doi: 10.1016/j.biopsych.2014.05.006

Karlsson, H., Bachmann, S., Schroder, J., McArthur, J., Torrey, E. F., & Yolken, R. H. (2001). Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia. *Proc Natl Acad Sci U S A, 98*(8), 4634-4639. doi: 10.1073/pnas.061021998

Karran, E., Mercken, M., & De Strooper, B. (2011). The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov, 10*(9), 698-712. doi: 10.1038/nrd3505

Kashani, A. H., Asanad, S., Chan, J. W., Singer, M. B., Zhang, J., Sharifi, M., . . . Ringman, J. M. (2021). Past, present and future role of retinal imaging in neurodegenerative disease. *Prog Retin Eye Res, 83*, 100938. doi: 10.1016/j.preteyeres.2020.100938

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., . . . Amit, I. (2017). A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell, 169*(7), 1276-1290 e1217. doi: 10.1016/j.cell.2017.05.018

Kim, C., Ho, D. H., Suk, J. E., You, S., Michael, S., Kang, J., . . . Lee, S. J. (2013). Neuron-released oligomeric alpha-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. *Nat Commun, 4*, 1562. doi: 10.1038/ncomms2534

Kipp, M. (2020). Does Siponimod Exert Direct Effects in the Central Nervous System? *Cells, 9*(8). doi: 10.3390/cells9081771

Kirby, L., Jin, J., Cardona, J. G., Smith, M. D., Martin, K. A., Wang, J., . . . Calabresi, P. A. (2019). Oligodendrocyte precursor cells present antigen and are cytotoxic targets in inflammatory demyelination. *Nat Commun, 10*(1), 3887. doi: 10.1038/s41467-019-11638-3

Kitaoka, Y., Kitaoka, Y., Kwong, J. M., Ross-Cisneros, F. N., Wang, J., Tsai, R. K., . . . Lam, T. T. (2006). TNF-alpha-induced optic nerve degeneration and nuclear factor-kappaB p65. *Invest Ophthalmol Vis Sci, 47*(4), 1448-1457. doi: 10.1167/iovs.05-0299

Knox, E. G., Aburto, M. R., Clarke, G., Cryan, J. F., & O'Driscoll, C. M. (2022). The blood-brain barrier in aging and neurodegeneration. *Mol Psychiatry, 27*(6), 2659-2673. doi: 10.1038/s41380-022-01511-z

Koch-Henriksen, N., Thygesen, L. C., Stenager, E., Laursen, B., & Magyari, M. (2018). Incidence of MS has increased markedly over six decades in Denmark particularly with late onset and in women. *Neurology, 90*(22), e1954-e1963. doi: 10.1212/WNL.0000000000005612

Kordower, J. H., Olanow, C. W., Dodiya, H. B., Chu, Y., Beach, T. G., Adler, C. H., . . . Bartus, R. T. (2013). Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain, 136*(Pt 8), 2419-2431. doi: 10.1093/brain/awt192

Kotter, M. R., Stadelmann, C., & Hartung, H. P. (2011). Enhancing remyelination in disease--can we wrap it up? *Brain, 134*(Pt 7), 1882-1900. doi: 10.1093/brain/awr014

Koulousakis, P., Tiane, A., Hellings, N., Prickaerts, J., van den Hove, D., & Vanmierlo, T. (2023). A perspective on causality assessment in epigenetic research on neurodegenerative disorders. *Neural Regen Res, 18*(2), 331-332. doi: 10.4103/1673-5374.343898

Krämer, J., Bruck, W., Zipp, F., Cerina, M., Groppa, S., & Meuth, S. G. (2019). Imaging in mice and men: Pathophysiological insights into multiple sclerosis from conventional and advanced MRI techniques. *Prog Neurobiol, 182*, 101663. doi: 10.1016/j.pneurobio.2019.101663

Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., El Fatimy, R., . . . Butovsky, O. (2017). The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity, 47*(3), 566-581 e569. doi: 10.1016/j.immuni.2017.08.008

Kremer, D., Aktas, O., Hartung, H. P., & Küry, P. (2011). The complex world of oligodendroglial differentiation inhibitors. *Ann Neurol, 69*(4), 602-618. doi: 10.1002/ana.22415

Kremer, D., Förster, M., Schichel, T., Göttle, P., Hartung, H. P., Perron, H., & Küry, P. (2015). The neutralizing antibody GNbAC1 abrogates HERV-W envelope protein-mediated oligodendroglial maturation blockade. *Mult Scler, 21*(9), 1200-1203. doi: 10.1177/1352458514560926

Kremer, D., Gruchot, J., Weyers, V., Oldemeier, L., Göttle, P., Healy, L., . . . Küry, P. (2019). pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. *Proc Natl Acad Sci U S A, 116*(30), 15216-15225. doi: 10.1073/pnas.1901283116

Kremer, D., Schichel, T., Förster, M., Tzekova, N., Bernard, C., van der Valk, P., . . . Küry, P. (2013). Human endogenous retrovirus type W envelope protein inhibits oligodendroglial precursor cell differentiation. *Ann Neurol, 74*(5), 721-732. doi: 10.1002/ana.23970

Kuhlmann, T., Miron, V., Cui, Q., Wegner, C., Antel, J., & Bruck, W. (2008). Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain, 131*(Pt 7), 1749-1758. doi: 10.1093/brain/awn096

Kurtzke, J. F. (2000). Multiple sclerosis in time and space--geographic clues to cause. *J Neurovirol, 6 Suppl 2*, S134-140.

Küry, P., Nath, A., Creange, A., Dolei, A., Marche, P., Gold, J., . . . Perron, H. (2018). Human Endogenous Retroviruses in Neurological Diseases. *Trends Mol Med, 24*(4), 379-394. doi: 10.1016/j.molmed.2018.02.007

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., . . . International Human Genome Sequencing, C. (2001). Initial sequencing and analysis of the human genome. *Nature, 409*(6822), 860-921. doi: 10.1038/35057062

Lassmann, H., van Horssen, J., & Mahad, D. (2012). Progressive multiple sclerosis: pathology and pathogenesis. *Nat Rev Neurol, 8*(11), 647-656. doi: 10.1038/nrneurol.2012.168

Lavie, L., Kitova, M., Maldener, E., Meese, E., & Mayer, J. (2005). CpG methylation directly regulates transcriptional activity of the human endogenous retrovirus family HERV-K(HML-2). *J Virol, 79*(2), 876-883. doi: 10.1128/JVI.79.2.876-883.2005

Lee, A., & Gilbert, R. M. (2016). Epidemiology of Parkinson Disease. *Neurol Clin, 34*(4), 955-965. doi: 10.1016/j.ncl.2016.06.012

Lendahl, U., Nilsson, P., & Betsholtz, C. (2019). Emerging links between cerebrovascular and neurodegenerative diseases-a special role for pericytes. *EMBO Rep, 20*(11), e48070. doi: 10.15252/embr.201948070

Leng, Y., Musiek, E. S., Hu, K., Cappuccio, F. P., & Yaffe, K. (2019). Association between circadian rhythms and neurodegenerative diseases. *Lancet Neurol, 18*(3), 307-318. doi: 10.1016/S1474-4422(18)30461-7

Leung, A., Trac, C., Kato, H., Costello, K. R., Chen, Z., Natarajan, R., & Schones, D. E. (2018). LTRs activated by Epstein-Barr virus-induced transformation of B cells alter the transcriptome. *Genome Res, 28*(12), 1791-1798. doi: 10.1101/gr.233585.117

Leung, D. C., & Lorincz, M. C. (2012). Silencing of endogenous retroviruses: when and why do histone marks predominate? *Trends Biochem Sci, 37*(4), 127-133. doi: 10.1016/j.tibs.2011.11.006

Lewis, E. B. (1950). The phenomenon of position effect. *Adv Genet, 3*, 73-115. doi: 10.1016/s0065-2660(08)60083-8

Li, J., O, W., Li, W., Jiang, Z. G., & Ghanbari, H. A. (2013). Oxidative stress and neurodegenerative disorders. *Int J Mol Sci, 14*(12), 24438-24475. doi: 10.3390/ijms141224438

Li, Q., Ru, X., Yang, Y., Zhao, H., Qu, J., Chen, W., . . . Feng, H. (2022). Lipocalin-2-Mediated Insufficient Oligodendrocyte Progenitor Cell Remyelination for White Matter Injury After Subarachnoid Hemorrhage via SCL22A17 Receptor/Early Growth Response Protein 1 Signaling. *Neurosci Bull, 38*(12), 1457-1475. doi: 10.1007/s12264-022-00906-w

Li, W., Lee, M. H., Henderson, L., Tyagi, R., Bachani, M., Steiner, J., . . . Nath, A. (2015). Human endogenous retrovirus-K contributes to motor neuron disease. *Sci Transl Med, 7*(307), 307ra153. doi: 10.1126/scitranslmed.aac8201

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., . . . Barres, B. A. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature, 541*(7638), 481-487. doi: 10.1038/nature21029

Liu, J., & Wang, F. (2017). Role of Neuroinflammation in Amyotrophic Lateral Sclerosis: Cellular Mechanisms and Therapeutic Implications. *Front Immunol, 8*, 1005. doi: 10.3389/fimmu.2017.01005

Liu, Z., Li, Y., Zhang, J., Elias, S., & Chopp, M. (2008). Evaluation of corticospinal axon loss by fluorescent dye tracing in mice with experimental autoimmune encephalomyelitis. *J Neurosci Methods, 167*(2), 191-197. doi: 10.1016/j.jneumeth.2007.08.013

Loeffler, D. A., Camp, D. M., & Conant, S. B. (2006). Complement activation in the Parkinson's disease substantia nigra: an immunocytochemical study. *J Neuroinflammation, 3*, 29. doi: 10.1186/1742-2094-3-29

LoGerfo, A., Chico, L., Borgia, L., Petrozzi, L., Rocchi, A., D'Amelio, A., . . . Siciliano, G. (2014). Lack of association between nuclear factor erythroid-derived 2-like 2 promoter gene polymorphisms and oxidative stress biomarkers in amyotrophic lateral sclerosis patients. *Oxid Med Cell Longev, 2014*, 432626. doi: 10.1155/2014/432626

Lopez-Leon, S., Wegman-Ostrosky, T., Perelman, C., Sepulveda, R., Rebolledo, P. A., Cuapio, A., & Villapol, S. (2021). More than 50 long-term effects of COVID-19: a systematic review and meta-analysis. *Sci Rep, 11*(1), 16144. doi: 10.1038/s41598-021-95565-8

Lun, M. P., Johnson, M. B., Broadbelt, K. G., Watanabe, M., Kang, Y. J., Chau, K. F., . . . Lehtinen, M. K. (2015). Spatially heterogeneous choroid plexus transcriptomes encode

positional identity and contribute to regional CSF production. *J Neurosci, 35*(12), 4903-4916. doi: 10.1523/JNEUROSCI.3081-14.2015

Ma, B., Guckian, K. M., Liu, X. G., Yang, C., Li, B., Scannevin, R., . . . Walzer, T. (2021). Novel Potent Selective Orally Active S1P5 Receptor Antagonists. *ACS Med Chem Lett, 12*(3), 351-355. doi: 10.1021/acsmedchemlett.0c00631

MacDonald, V., & Halliday, G. M. (2002). Selective loss of pyramidal neurons in the pre-supplementary motor cortex in Parkinson's disease. *Mov Disord, 17*(6), 1166-1173. doi: 10.1002/mds.10258

MacGowan, D. J., Scelsa, S. N., Imperato, T. E., Liu, K. N., Baron, P., & Polsky, B. (2007). A controlled study of reverse transcriptase in serum and CSF of HIV-negative patients with ALS. *Neurology, 68*(22), 1944-1946. doi: 10.1212/01.wnl.0000263188.77797.99

Makin, S. (2018). The amyloid hypothesis on trial. *Nature, 559*(7715), S4-S7. doi: 10.1038/d41586-018-05719-4

Mameli, G., Astone, V., Arru, G., Marconi, S., Lovato, L., Serra, C., . . . Dolei, A. (2007). Brains and peripheral blood mononuclear cells of multiple sclerosis (MS) patients hyperexpress MS-associated retrovirus/HERV-W endogenous retrovirus, but not Human herpesvirus 6. *J Gen Virol, 88*(Pt 1), 264-274. doi: 10.1099/vir.0.81890-0

Mameli, G., Madeddu, G., Mei, A., Uleri, E., Poddighe, L., Delogu, L. G., . . . Dolei, A. (2013). Activation of MSRV-type endogenous retroviruses during infectious mononucleosis and Epstein-Barr virus latency: the missing link with multiple sclerosis? *PLoS One, 8*(11), e78474. doi: 10.1371/journal.pone.0078474

Mameli, G., Poddighe, L., Astone, V., Delogu, G., Arru, G., Sotgiu, S., . . . Dolei, A. (2009). Novel reliable real-time PCR for differential detection of MSRVenv and syncytin-1 in RNA and DNA from patients with multiple sclerosis. *J Virol Methods, 161*(1), 98-106. doi: 10.1016/j.jviromet.2009.05.024

Mameli, G., Poddighe, L., Mei, A., Uleri, E., Sotgiu, S., Serra, C., . . . Dolei, A. (2012). Expression and activation by Epstein Barr virus of human endogenous retroviruses-W in blood cells and astrocytes: inference for multiple sclerosis. *PLoS One, 7*(9), e44991. doi: 10.1371/journal.pone.0044991

Mandelkow, E. M., & Mandelkow, E. (2012). Biochemistry and cell biology of tau protein in neurofibrillary degeneration. *Cold Spring Harb Perspect Med, 2*(7), a006247. doi: 10.1101/cshperspect.a006247

Manghera, M., & Douville, R. N. (2013). Endogenous retrovirus-K promoter: a landing strip for inflammatory transcription factors? *Retrovirology, 10*, 16. doi: 10.1186/1742-4690-10-16

Mantovani, S., Gordon, R., Macmaw, J. K., Pfluger, C. M., Henderson, R. D., Noakes, P. G., . . . Woodruff, T. M. (2014). Elevation of the terminal complement activation products C5a and C5b-9 in ALS patient blood. *J Neuroimmunol, 276*(1-2), 213-218. doi: 10.1016/j.jneuroim.2014.09.005

Marsden, C. D. (1983). Neuromelanin and Parkinson's disease. *J Neural Transm Suppl, 19*, 121-141.

Martin, L., Page, G., & Terro, F. (2011). Tau phosphorylation and neuronal apoptosis induced by the blockade of PP2A preferentially involve GSK3beta. *Neurochem Int, 59*(2), 235-250. doi: 10.1016/j.neuint.2011.05.010

Martin, M. A., Bryan, T., Rasheed, S., & Khan, A. S. (1981). Identification and cloning of endogenous retroviral sequences present in human DNA. *Proc Natl Acad Sci U S A, 78*(8), 4892-4896. doi: 10.1073/pnas.78.8.4892

Martinez-Iglesias, O., Naidoo, V., Cacabelos, N., & Cacabelos, R. (2021). Epigenetic Biomarkers as Diagnostic Tools for Neurodegenerative Disorders. *Int J Mol Sci, 23*(1). doi: 10.3390/ijms23010013

Mayeux, R., & Stern, Y. (2012). Epidemiology of Alzheimer disease. *Cold Spring Harb Perspect Med, 2*(8). doi: 10.1101/cshperspect.a006239

Mc, C. B. (1950). The origin and behavior of mutable loci in maize. *Proc Natl Acad Sci U S A, 36*(6), 344-355. doi: 10.1073/pnas.36.6.344

McCann, H., Cartwright, H., & Halliday, G. M. (2016). Neuropathology of alpha-synuclein propagation and braak hypothesis. *Mov Disord, 31*(2), 152-160. doi: 10.1002/mds.26421

McCormick, A. L., Brown, R. H., Jr., Cudkowicz, M. E., Al-Chalabi, A., & Garson, J. A. (2008). Quantification of reverse transcriptase in ALS and elimination of a novel retroviral candidate. *Neurology, 70*(4), 278-283. doi: 10.1212/01.wnl.0000297552.13219.b4

McMahon, E. J., Suzuki, K., & Matsushima, G. K. (2002). Peripheral macrophage recruitment in cuprizone-induced CNS demyelination despite an intact blood-brain barrier. *J Neuroimmunol, 130*(1-2), 32-45. doi: 10.1016/s0165-5728(02)00205-9

Metodiewa, D., & Koska, C. (2000). Reactive oxygen species and reactive nitrogen species: relevance to cyto(neuro)toxic events and neurologic disorders. An overview. *Neurotox Res, 1*(3), 197-233. doi: 10.1007/BF03033290

Mey, G. M., Mahajan, K. R., & DeSilva, T. M. (2023). Neurodegeneration in multiple sclerosis. *WIREs Mech Dis, 15*(1), e1583. doi: 10.1002/wsbm.1583

Meyer, T. J., Rosenkrantz, J. L., Carbone, L., & Chavez, S. L. (2017). Endogenous Retroviruses: With Us and against Us. *Front Chem, 5*, 23. doi: 10.3389/fchem.2017.00023

Mizuno, Y., Amari, M., Takatama, M., Aizawa, H., Mihara, B., & Okamoto, K. (2006). Transferrin localizes in Bunina bodies in amyotrophic lateral sclerosis. *Acta Neuropathol, 112*(5), 597-603. doi: 10.1007/s00401-006-0122-4

Montilla, A., Zabala, A., Er-Lukowiak, M., Rissiek, B., Magnus, T., Rodriguez-Iglesias, N., . . . Domercq, M. (2023). Microglia and meningeal macrophages depletion delays the onset of experimental autoimmune encephalomyelitis. *Cell Death Dis, 14*(1), 16. doi: 10.1038/s41419-023-05551-3

Morishima, M., & Ihara, Y. (1994). Posttranslational modifications of tau in paired helical filaments. *Dementia, 5*(5), 282-288. doi: 10.1159/000106736

Moyon, S., Holloman, M., & Salzer, J. L. (2023). Neural stem cells and oligodendrocyte progenitor cells compete for remyelination in the corpus callosum. *Front Cell Neurosci, 17*, 1114781. doi: 10.3389/fncel.2023.1114781

Murakami, M., & Hirano, T. (2012). The molecular mechanisms of chronic inflammation development. *Front Immunol, 3*, 323. doi: 10.3389/fimmu.2012.00323

Murck, H., Fava, M., Cusin, C., Chin-Fatt, C., & Trivedi, M. (2023). Brain Ventricle and Choroid Plexus Morphology as Predictor of Treatment Response: Findings from the EMBARC Study. *Res Sq*. doi: 10.21203/rs.3.rs-2618151/v1

Muzio, L., Viotti, A., & Martino, G. (2021). Microglia in Neuroinflammation and Neurodegeneration: From Understanding to Therapy. *Front Neurosci, 15*, 742065. doi: 10.3389/fnins.2021.742065

Naude, P. J., Nyakas, C., Eiden, L. E., Ait-Ali, D., van der Heide, R., Engelborghs, S., . . . Eisel, U. L. (2012). Lipocalin 2: novel component of proinflammatory signaling in Alzheimer's disease. *FASEB J, 26*(7), 2811-2823. doi: 10.1096/fj.11-202457

Nave, K. A. (2010). Myelination and the trophic support of long axons. *Nat Rev Neurosci, 11*(4), 275-283. doi: 10.1038/nrn2797

Negi, N., & Das, B. K. (2018). CNS: Not an immunoprivilaged site anymore but a virtual secondary lymphoid organ. *Int Rev Immunol, 37*(1), 57-68. doi: 10.1080/08830185.2017.1357719

Nellaker, C., Keane, T. M., Yalcin, B., Wong, K., Agam, A., Belgard, T. G., . . . Ponting, C. P. (2012). The genomic landscape shaped by selection on transposable elements across 18 mouse strains. *Genome Biol, 13*(6), R45. doi: 10.1186/gb-2012-13-6-r45

Nelson, P. N., Carnegie, P. R., Martin, J., Davari Ejtehadi, H., Hooley, P., Roden, D., . . . Murray, P. G. (2003). Demystified. Human endogenous retroviruses. *Mol Pathol, 56*(1), 11-18. doi: 10.1136/mp.56.1.11

Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., . . . Lee, V. M. (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science, 314*(5796), 130-133. doi: 10.1126/science.1134108

Niedzielska, E., Smaga, I., Gawlik, M., Moniczewski, A., Stankowicz, P., Pera, J., & Filip, M. (2016). Oxidative Stress in Neurodegenerative Diseases. *Mol Neurobiol, 53*(6), 4094-4125. doi: 10.1007/s12035-015-9337-5

Nishimura, H., Akiyama, T., Irei, I., Hamazaki, S., & Sadahira, Y. (2010). Cellular localization of sphingosine-1-phosphate receptor 1 expression in the human central nervous system. *J Histochem Cytochem, 58*(9), 847-856. doi: 10.1369/jhc.2010.956409

Noda, H., Takeuchi, H., Mizuno, T., & Suzumura, A. (2013). Fingolimod phosphate promotes the neuroprotective effects of microglia. *J Neuroimmunol, 256*(1-2), 13-18. doi: 10.1016/j.jneuroim.2012.12.005

O'Sullivan, C., Schubart, A., Mir, A. K., & Dev, K. K. (2016). The dual S1PR1/S1PR5 drug BAF312 (Siponimod) attenuates demyelination in organotypic slice cultures. *J Neuroinflammation, 13*, 31. doi: 10.1186/s12974-016-0494-x

Ohl, K., Tenbrock, K., & Kipp, M. (2016). Oxidative stress in multiple sclerosis: Central and peripheral mode of action. *Exp Neurol, 277*, 58-67. doi: 10.1016/j.expneurol.2015.11.010

Ohtani, H., Liu, M., Zhou, W., Liang, G., & Jones, P. A. (2018). Switching roles for DNA and histone methylation depend on evolutionary ages of human endogenous retroviruses. *Genome Res, 28*(8), 1147-1157. doi: 10.1101/gr.234229.118

Okamoto, K., Mizuno, Y., & Fujita, Y. (2008). Bunina bodies in amyotrophic lateral sclerosis. *Neuropathology, 28*(2), 109-115. doi: 10.1111/j.1440-1789.2007.00873.x

Owens, T., Bechmann, I., & Engelhardt, B. (2008). Perivascular spaces and the two steps to neuroinflammation. *J Neuropathol Exp Neurol, 67*(12), 1113-1121. doi: 10.1097/NEN.0b013e31818f9ca8

Paolicelli, R. C., Sierra, A., Stevens, B., Tremblay, M. E., Aguzzi, A., Ajami, B., . . . Wyss-Coray, T. (2022). Microglia states and nomenclature: A field at its crossroads. *Neuron, 110*(21), 3458-3483. doi: 10.1016/j.neuron.2022.10.020

Pekna, M., & Pekny, M. (2021). The Complement System: A Powerful Modulator and Effector of Astrocyte Function in the Healthy and Diseased Central Nervous System. *Cells, 10*(7). doi: 10.3390/cells10071812

Perron, H., Dougier-Reynaud, H. L., Lomparski, C., Popa, I., Firouzi, R., Bertrand, J. B., . . . Marche, P. N. (2013). Human endogenous retrovirus protein activates innate immunity and promotes experimental allergic encephalomyelitis in mice. *PLoS One, 8*(12), e80128. doi: 10.1371/journal.pone.0080128

Perron, H., Geny, C., Laurent, A., Mouriquand, C., Pellat, J., Perret, J., & Seigneurin, J. M. (1989). Leptomeningeal cell line from multiple sclerosis with reverse transcriptase activity and viral particles. *Res Virol, 140*(6), 551-561. doi: 10.1016/s0923-2516(89)80141-4

Perron, H., Germi, R., Bernard, C., Garcia-Montojo, M., Deluen, C., Farinelli, L., . . . Hartung, H. P. (2012). Human endogenous retrovirus type W envelope expression in blood and brain cells provides new insights into multiple sclerosis disease. *Mult Scler, 18*(12), 1721-1736. doi: 10.1177/1352458512441381

Perron, H., Jouvin-Marche, E., Michel, M., Ounanian-Paraz, A., Camelo, S., Dumon, A., . . . Lafon, M. (2001). Multiple sclerosis retrovirus particles and recombinant envelope trigger an abnormal immune response in vitro, by inducing polyclonal Vbeta16 T-lymphocyte activation. *Virology, 287*(2), 321-332. doi: 10.1006/viro.2001.1045

Petrozziello, T., Mills, A. N., Farhan, S. M. K., Mueller, K. A., Granucci, E. J., Glajch, K. E., . . . Sadri-Vakili, G. (2020). Lipocalin-2 is increased in amyotrophic lateral sclerosis. *Muscle Nerve, 62*(2), 272-283. doi: 10.1002/mus.26911

Piller, C. (2022). Blots on a field? *Science, 377*(6604), 358-363. doi: 10.1126/science.add9993

Ponath, G., Park, C., & Pitt, D. (2018). The Role of Astrocytes in Multiple Sclerosis. *Front Immunol, 9*, 217. doi: 10.3389/fimmu.2018.00217

Popa-Wagner, A., Mitran, S., Sivanesan, S., Chang, E., & Buga, A. M. (2013). ROS and brain diseases: the good, the bad, and the ugly. *Oxid Med Cell Longev, 2013*, 963520. doi: 10.1155/2013/963520

Pugliatti, M., Sotgiu, S., & Rosati, G. (2002). The worldwide prevalence of multiple sclerosis. *Clin Neurol Neurosurg, 104*(3), 182-191. doi: 10.1016/s0303-8467(02)00036-7

Pulido-Salgado, M., Vidal-Taboada, J. M., Barriga, G. G., Sola, C., & Saura, J. (2018). RNA-Seq transcriptomic profiling of primary murine microglia treated with LPS or LPS + IFNgamma. *Sci Rep, 8*(1), 16096. doi: 10.1038/s41598-018-34412-9

Ransohoff, R. M. (2016). A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci, 19*(8), 987-991. doi: 10.1038/nn.4338

Rezai-Zadeh, K., Gate, D., & Town, T. (2009). CNS infiltration of peripheral immune cells: D-Day for neurodegenerative disease? *J Neuroimmune Pharmacol, 4*(4), 462-475. doi: 10.1007/s11481-009-9166-2

Rogers, J., Cooper, N. R., Webster, S., Schultz, J., McGeer, P. L., Styren, S. D., . . . et al. (1992). Complement activation by beta-amyloid in Alzheimer disease. *Proc Natl Acad Sci U S A, 89*(21), 10016-10020. doi: 10.1073/pnas.89.21.10016

Rolland, A., Jouvin-Marche, E., Viret, C., Faure, M., Perron, H., & Marche, P. N. (2006). The envelope protein of a human endogenous retrovirus-W family activates innate immunity through CD14/TLR4 and promotes Th1-like responses. *J Immunol, 176*(12), 7636-7644. doi: 10.4049/jimmunol.176.12.7636

Römer, C. (2021). Viruses and Endogenous Retroviruses as Roots for Neuroinflammation and Neurodegenerative Diseases. *Front Neurosci, 15*, 648629. doi: 10.3389/fnins.2021.648629

Rosati, G. (2001). The prevalence of multiple sclerosis in the world: an update. *Neurol Sci, 22*(2), 117-139. doi: 10.1007/s100720170011

Schafer, D. P., & Stillman, J. M. (2022). Microglia are SYK of Abeta and cell debris. *Cell, 185*(22), 4043-4045. doi: 10.1016/j.cell.2022.09.043

Schartz, N. D., & Tenner, A. J. (2020). The good, the bad, and the opportunities of the complement system in neurodegenerative disease. *J Neuroinflammation, 17*(1), 354. doi: 10.1186/s12974-020-02024-8

Shelestak, J., Singhal, N., Frankle, L., Tomor, R., Sternbach, S., McDonough, J., . . . Clements, R. (2020). Increased blood-brain barrier hyperpermeability coincides with mast cell activation early under cuprizone administration. *PLoS One, 15*(6), e0234001. doi: 10.1371/journal.pone.0234001

Silva, A. L., Oliveira, J. L., do Nascimento, R. P., Santos, L. O., de Araujo, F. M., Dos Santos, B. L., . . . Costa, S. L. (2023). Monocrotaline induces acutely cerebrovascular lesions, astrogliosis and neuronal degeneration associated with behavior changes in rats: A model of vascular damage in perspective. *Neurotoxicology, 94*, 59-70. doi: 10.1016/j.neuro.2022.10.017

Sinha, K., Das, J., Pal, P. B., & Sil, P. C. (2013). Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Arch Toxicol, 87*(7), 1157-1180. doi: 10.1007/s00204-013-1034-4

Small, S. A., & Duff, K. (2008). Linking Abeta and tau in late-onset Alzheimer's disease: a dual pathway hypothesis. *Neuron, 60*(4), 534-542. doi: 10.1016/j.neuron.2008.11.007

Smith, K. J., & Lassmann, H. (2002). The role of nitric oxide in multiple sclerosis. *Lancet Neurol, 1*(4), 232-241. doi: 10.1016/s1474-4422(02)00102-3

Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol, 119*(1), 7-35. doi: 10.1007/s00401-009-0619-8

Spencer, T. E., Mura, M., Gray, C. A., Griebel, P. J., & Palmarini, M. (2003). Receptor usage and fetal expression of ovine endogenous betaretroviruses: implications for coevolution of endogenous and exogenous retroviruses. *J Virol, 77*(1), 749-753. doi: 10.1128/jvi.77.1.749-753.2003

Sreedharan, J., Blair, I. P., Tripathi, V. B., Hu, X., Vance, C., Rogelj, B., . . . Shaw, C. E. (2008). TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science, 319*(5870), 1668-1672. doi: 10.1126/science.1154584

Steele, A. J., Al-Chalabi, A., Ferrante, K., Cudkowicz, M. E., Brown, R. H., Jr., & Garson, J. A. (2005). Detection of serum reverse transcriptase activity in patients with ALS and unaffected blood relatives. *Neurology, 64*(3), 454-458. doi: 10.1212/01.WNL.0000150899.76130.71

Steenwijk, M. D., Geurts, J. J., Daams, M., Tijms, B. M., Wink, A. M., Balk, L. J., . . . Pouwels, P. J. (2016). Cortical atrophy patterns in multiple sclerosis are non-random and clinically relevant. *Brain, 139*(Pt 1), 115-126. doi: 10.1093/brain/awv337

Stocking, C., & Kozak, C. A. (2008). Murine endogenous retroviruses. *Cell Mol Life Sci, 65*(21), 3383-3398. doi: 10.1007/s00018-008-8497-0

Stratoulias, V., Venero, J. L., Tremblay, M. E., & Joseph, B. (2019). Microglial subtypes: diversity within the microglial community. *EMBO J, 38*(17), e101997. doi: 10.15252/embj.2019101997

Streit, W. J., Conde, J. R., Fendrick, S. E., Flanary, B. E., & Mariani, C. L. (2005). Role of microglia in the central nervous system's immune response. *Neurol Res, 27*(7), 685-691. doi: 10.1179/016164105X49463a

Sucksdorff, M., Rissanen, E., Tuisku, J., Nuutinen, S., Paavilainen, T., Rokka, J., . . . Airas, L. (2017). Evaluation of the Effect of Fingolimod Treatment on Microglial Activation Using Serial PET Imaging in Multiple Sclerosis. *J Nucl Med, 58*(10), 1646-1651. doi: 10.2967/jnumed.116.183020

Sulzer, D., Alcalay, R. N., Garretti, F., Cote, L., Kanter, E., Agin-Liebes, J., . . . Sette, A. (2017). T cells from patients with Parkinson's disease recognize alpha-synuclein peptides. *Nature, 546*(7660), 656-661. doi: 10.1038/nature22815

Szpakowski, S., Sun, X., Lage, J. M., Dyer, A., Rubinstein, J., Kowalski, D., . . . Lizardi, P. M. (2009). Loss of epigenetic silencing in tumors preferentially affects primate-specific retroelements. *Gene, 448*(2), 151-167. doi: 10.1016/j.gene.2009.08.006

Talhada, D., Costa-Brito, A. R., Duarte, A. C., Costa, A. R., Quintela, T., Tomas, J., . . . Santos, C. R. A. (2020). The choroid plexus: Simple structure, complex functions. *J Neurosci Res, 98*(5), 751-753. doi: 10.1002/jnr.24571

Tallafuss, A., Stednitz, S. J., Voeun, M., Levichev, A., Larsch, J., Eisen, J., & Washbourne, P. (2022). Egr1 Is Necessary for Forebrain Dopaminergic Signaling during Social Behavior. *eNeuro, 9*(2). doi: 10.1523/ENEURO.0035-22.2022

Tamouza, R., Meyer, U., Foiselle, M., Richard, J. R., Wu, C. L., Boukouaci, W., . . . Leboyer, M. (2021). Identification of inflammatory subgroups of schizophrenia and bipolar disorder patients with HERV-W ENV antigenemia by unsupervised cluster analysis. *Transl Psychiatry, 11*(1), 377. doi: 10.1038/s41398-021-01499-0

Tan, C. F., Eguchi, H., Tagawa, A., Onodera, O., Iwasaki, T., Tsujino, A., . . . Takahashi, H. (2007). TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. *Acta Neuropathol, 113*(5), 535-542. doi: 10.1007/s00401-007-0206-9

Tansey, M. G., McCoy, M. K., & Frank-Cannon, T. C. (2007). Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. *Exp Neurol, 208*(1), 1-25. doi: 10.1016/j.expneurol.2007.07.004

Tay, T. L., Sagar, Dautzenberg, J., Grun, D., & Prinz, M. (2018). Unique microglia recovery population revealed by single-cell RNAseq following neurodegeneration. *Acta Neuropathol Commun, 6*(1), 87. doi: 10.1186/s40478-018-0584-3

Teleanu, D. M., Niculescu, A. G., Lungu, II, Radu, C. I., Vladacenco, O., Roza, E., . . . Teleanu, R. I. (2022). An Overview of Oxidative Stress, Neuroinflammation, and Neurodegenerative Diseases. *Int J Mol Sci, 23*(11). doi: 10.3390/ijms23115938

Tham, C. S., Lin, F. F., Rao, T. S., Yu, N., & Webb, M. (2003). Microglial activation state and lysophospholipid acid receptor expression. *Int J Dev Neurosci, 21*(8), 431-443. doi: 10.1016/j.ijdevneu.2003.09.003

The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. (1999). TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. *Neurology, 53*(3), 457-465.

Thomas, J., Perron, H., & Feschotte, C. (2018). Variation in proviral content among human genomes mediated by LTR recombination. *Mob DNA, 9*, 36. doi: 10.1186/s13100-018-0142-3

Thompson, P. J., Macfarlan, T. S., & Lorincz, M. C. (2016). Long Terminal Repeats: From Parasitic Elements to Building Blocks of the Transcriptional Regulatory Repertoire. *Mol Cell, 62*(5), 766-776. doi: 10.1016/j.molcel.2016.03.029

Tong, J., Wong, H., Guttman, M., Ang, L. C., Forno, L. S., Shimadzu, M., . . . Furukawa, Y. (2010). Brain alpha-synuclein accumulation in multiple system atrophy, Parkinson's disease and progressive supranuclear palsy: a comparative investigation. *Brain, 133*(Pt 1), 172-188. doi: 10.1093/brain/awp282

Trapp, B. D., & Nave, K. A. (2008). Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci, 31*, 247-269. doi: 10.1146/annurev.neuro.30.051606.094313

van Horssen, J., van der Pol, S., Nijland, P., Amor, S., & Perron, H. (2016). Human endogenous retrovirus W in brain lesions: Rationale for targeted therapy in multiple sclerosis. *Mult Scler Relat Disord, 8*, 11-18. doi: 10.1016/j.msard.2016.04.006

Vargas, D. L., & Tyor, W. R. (2017). Update on disease-modifying therapies for multiple sclerosis. *J Investig Med, 65*(5), 883-891. doi: 10.1136/jim-2016-000339

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., . . . Zhu, X. (2001). The sequence of the human genome. *Science, 291*(5507), 1304-1351. doi: 10.1126/science.1058040

Villarreal, L. P. (2011). Viral ancestors of antiviral systems. *Viruses, 3*(10), 1933-1958. doi: 10.3390/v3101933

Wagner, H. J., Munger, K. L., & Ascherio, A. (2004). Plasma viral load of Epstein-Barr virus and risk of multiple sclerosis. *Eur J Neurol, 11*(12), 833-834. doi: 10.1111/j.1468-1331.2004.00871.x

Wakamatsu, K., Fujikawa, K., Zucca, F. A., Zecca, L., & Ito, S. (2003). The structure of neuromelanin as studied by chemical degradative methods. *J Neurochem, 86*(4), 1015-1023. doi: 10.1046/j.1471-4159.2003.01917.x

Wang, X., Liu, Z., Wang, P., Li, S., Zeng, J., Tu, X., . . . Zhu, F. (2018). Syncytin-1, an endogenous retroviral protein, triggers the activation of CRP via TLR3 signal cascade in glial cells. *Brain Behav Immun, 67*, 324-334. doi: 10.1016/j.bbi.2017.09.009

Wang, Y., Li, X., Xu, X., Yu, J., Chen, X., Cao, X., . . . Ding, X. (2022). Clec7a expression in inflammatory macrophages orchestrates progression of acute kidney injury. *Front Immunol, 13*, 1008727. doi: 10.3389/fimmu.2022.1008727

Wang, Z., Zheng, Y., Wang, F., Zhong, J., Zhao, T., Xie, Q., . . . Zhu, J. (2020). Mfsd2a and Spns2 are essential for sphingosine-1-phosphate transport in the formation and maintenance of the blood-brain barrier. *Sci Adv, 6*(22), eaay8627. doi: 10.1126/sciadv.aay8627

Watkins, L. M., Neal, J. W., Loveless, S., Michailidou, I., Ramaglia, V., Rees, M. I., . . . Howell, O. W. (2016). Complement is activated in progressive multiple sclerosis cortical grey matter lesions. *J Neuroinflammation, 13*(1), 161. doi: 10.1186/s12974-016-0611-x

Weiss, R. A. (1969a). The Host Range of bryan Strain Rous Sarcoma Virus Synthesized in the Absence of Helper Virus. *Journal of General Virology, 5*(4), 511-528. doi: https://doi.org/10.1099/0022-1317-5-4-511

Weiss, R. A. (1969b). Interference and Neutralization Studies with Bryan Strain Rous Sarcoma Virus Synthesized in the Absence of Helper Virus. *Journal of General Virology, 5*(4), 529-539. doi: https://doi.org/10.1099/0022-1317-5-4-529

Weiss, R. A. (2006). The discovery of endogenous retroviruses. *Retrovirology, 3*, 67. doi: 10.1186/1742-4690-3-67

Wijesekera, L. C., & Leigh, P. N. (2009). Amyotrophic lateral sclerosis. *Orphanet J Rare Dis, 4*, 3. doi: 10.1186/1750-1172-4-3

Wishart, C. L., Spiteri, A. G., Locatelli, G., & King, N. J. C. (2023). Integrating transcriptomic datasets across neurological disease identifies unique myeloid subpopulations driving disease-specific signatures. *Glia, 71*(4), 904-925. doi: 10.1002/glia.24314

Xiang, Y., & Liang, H. (2021). The Regulation and Functions of Endogenous Retrovirus in Embryo Development and Stem Cell Differentiation. *Stem Cells Int, 2021*, 6660936. doi: 10.1155/2021/6660936

Yakimov, V., Schweiger, F., Zhan, J., Behrangi, N., Horn, A., Schmitz, C., . . . Kipp, M. (2019). Continuous cuprizone intoxication allows active experimental autoimmune encephalomyelitis induction in C57BL/6 mice. *Histochem Cell Biol, 152*(2), 119-131. doi: 10.1007/s00418-019-01786-4

Yang, Q., Wang, G., & Zhang, F. (2020). Role of Peripheral Immune Cells-Mediated Inflammation on the Process of Neurodegenerative Diseases. *Front Immunol, 11*, 582825. doi: 10.3389/fimmu.2020.582825

Yokoseki, A., Shiga, A., Tan, C. F., Tagawa, A., Kaneko, H., Koyama, A., . . . Onodera, O. (2008). TDP-43 mutation in familial amyotrophic lateral sclerosis. *Ann Neurol, 63*(4), 538-542. doi: 10.1002/ana.21392

Yolken, R. H., Karlsson, H., Yee, F., Johnston-Wilson, N. L., & Torrey, E. F. (2000). Endogenous retroviruses and schizophrenia. *Brain Res Brain Res Rev, 31*(2-3), 193-199. doi: 10.1016/s0165-0173(99)00037-5

Yuan, Y., Sun, J., Dong, Q., & Cui, M. (2023). Blood-brain barrier endothelial cells in neurodegenerative diseases: Signals from the "barrier". *Front Neurosci, 17*, 1047778. doi: 10.3389/fnins.2023.1047778

Zalc, B., Goujet, D., & Colman, D. (2008). The origin of the myelination program in vertebrates. *Curr Biol, 18*(12), R511-512. doi: 10.1016/j.cub.2008.04.010

Zecca, L., Zucca, F. A., Costi, P., Tampellini, D., Gatti, A., Gerlach, M., . . . Sulzer, D. (2003). The neuromelanin of human substantia nigra: structure, synthesis and molecular behaviour. *J Neural Transm Suppl*(65), 145-155. doi: 10.1007/978-3-7091-0643-3_8

Zeis, T., Enz, L., & Schaeren-Wiemers, N. (2016). The immunomodulatory oligodendrocyte. *Brain Res, 1641*(Pt A), 139-148. doi: 10.1016/j.brainres.2015.09.021

Zhan, J., Mann, T., Joost, S., Behrangi, N., Frank, M., & Kipp, M. (2020). The Cuprizone Model: Dos and Do Nots. *Cells, 9*(4). doi: 10.3390/cells9040843

Zhou, B., Zuo, Y. X., & Jiang, R. T. (2019). Astrocyte morphology: Diversity, plasticity, and role in neurological diseases. *CNS Neurosci Ther, 25*(6), 665-673. doi: 10.1111/cns.13123

Zhou, J., Broe, M., Huang, Y., Anderson, J. P., Gai, W. P., Milward, E. A., . . . Halliday, G. M. (2011). Changes in the solubility and phosphorylation of alpha-synuclein over the course of Parkinson's disease. *Acta Neuropathol, 121*(6), 695-704. doi: 10.1007/s00401-011-0815-1

Appendix - Publication List

Gruchot, J., Lewen, I., Dietrich, M., Reiche, L., Sindi, M., Hecker, C., Herrero, F., Charvet, B., Weber-Stadlbauer, U., Hartung, H. P., Albrecht, P., Perron, H. Meyer, U. & Küry, P. (submitted). Transgenic expression of the HERV-W envelope protein leads to polarized glial cell populations and a neurodegenerative environment. Proceedings of the National Academy of Sciences.

Göttle, P., Groh, J., Reiche, L., **Gruchot, J.**, Rychlik, N., Werner, L., Samper Agrelo, I., Akkermann, R., Zink, A., Prigione, A., Hartung, H. P., Martini, R. & Küry, P. (2023). Teriflunomide as a therapeutic means for myelin repair. Journal of Neuroinflammation, 20(1), 1-16.

Gruchot, J., Herrero, F., Weber-Stadlbauer, U., Meyer, U., & Küry, P. (2022). Interplay between activation of endogenous retroviruses and inflammation as common pathogenic mechanism in neurological and psychiatric disorders. Brain, Behavior, and Immunity, 107, 242-252.

Herrero, F., Mueller, F. S., **Gruchot, J.**, Küry, P., Weber-Stadlbauer, U., & Meyer, U. (2023). Susceptibility and resilience to maternal immune activation are associated with differential expression of endogenous retroviral elements. Brain, Behavior, and Immunity, 107, 201-214.

Schroeter, C. B., Rolfes, L., Gothan, K. S., **Gruchot, J.**, Herrmann, A. M., Bock, S., Fazio, L., Henes, A., Narayanan, V., Pfeuffer, S., Nelke, C., Räuber, S., Huntemann, N., Duarte-Silva, E., Dobelmann, V., Hundehege, P., Wiendl, H., Raba, K., Küry, P., Kremer, D., Ruck, T., Müntefering, T., Budde, T., Cerina, M. & Meuth, S. G. (2022). Cladribine treatment improves cortical network functionality in a mouse model of autoimmune encephalomyelitis. Journal of Neuroinflammation, 19(1), 1-19.

Gruchot, J., Lein, F., Lewen, I., Reiche, L., Weyers, V., Petzsch, P., Göttle, P., Köhrer, K., Hartung, H. P., Küry, P., & Kremer, D. (2022). Siponimod Modulates the Reaction of Microglial Cells to Pro-Inflammatory Stimulation. International Journal of Molecular Sciences, 23(21), 13278.

Junior, M. S. O., Schira-Heinen, J., Reiche, L., Han, S., de Amorim, V. C. M., Lewen, I., **Gruchot, J.**, Göttle, P., Akkermann, R., Azim, K. & Küry, P. (2022). Myelin repair is fostered by the corticosteroid medrysone specifically acting on astroglial subpopulations. EBioMedicine, 83, 104204.

Dietrich, M., Hecker, C., Martin, E., Langui, D., Gliem, M., Stankoff, B., Lubetzki, C, **Gruchot, J.**, Göttle, P., Issberger, A., Nasiri, M., Ramseier, P., Beerli, C., Tisserand, S., Beckmann, N., Shimshek, D., Petzsch, P., Akbar, D., Levkau, B., Stark, H., Köhrer, K., Hartung, H. P., Küry, P., Meuth, S. G., Bigaud, M., Zalc, B. & Albrecht, P. (2022). Increased Remyelination and Proregenerative Microglia Under Siponimod Therapy in Mechanistic Models. Neurology-Neuroimmunology Neuroinflammation, 9(3).

Pfeuffer, S., Müntefering, T., Rolfes, L., Straeten, F. A., Eichler, S., **Gruchot, J.**, Dobelmann, V., Prozorovski, T., Görg, B., Vucur, M., Berndt, C., Küry, P., Ruck, T., Bittner, S., Bettenworth, D., Budde, T., Lüdde, T. & Meuth, S. G. (2022). Deficiency of the Two-Pore Potassium Channel KCNK9 Impairs Intestinal Epithelial Cell Survival and Aggravates Dextran Sodium Sulfate-Induced Colitis. Cellular and Molecular Gastroenterology and Hepatology, 14(6), 1199-1211.

Fleischer, V., Gonzalez-Escamilla, G., Ciolac, D., Albrecht, P., Küry, P., **Gruchot, J.**, Dietrich, M., Hecker, C., Müntefering, T., Bock, S., Ohsaghi, M., Radetz, A., Cerine, M., Krämer, J., Wachsmuth, L., Faber, C., Lassmann, H., Ruck, T., Meuth, S. G. Muthuraman, M. & Groppa, S. (2021). Translational value of choroid plexus imaging for tracking neuroinflammation in mice and humans. Proceedings of the National Academy of Sciences, 118(36), e2025000118.

Manousi, A., Göttle, P., Reiche, L., Cui, Q. L., Healy, L. M., Akkermann, R., **Gruchot, J.**. Schira-Heinen, J., Antel, J. P., Hartung, H. P. & Küry, P. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276.

Kremer, D., Weyers, V., **Gruchot, J.**, Göttle, P., Hartung, H. P., Perron, H., & Küry, P. (2020). Meeting report: “Human endogenous retroviruses: HERVs or transposable elements in autoimmune, chronic inflammatory and degenerative diseases or cancer”, Lyon, France, november 5th and 6th 2019–an MS scientist's digest. Multiple Sclerosis and Related Disorders, 42, 102068.

Gruchot, J., Kremer, D., & Küry, P. (2020). Human endogenous retroviruses: ammunition for myeloid cells in neurodegenerative diseases?. Neural Regeneration Research, 15(6), 1043.

Gruchot, J., Weyers, V., Göttle, P., Förster, M., Hartung, H. P., Küry, P., & Kremer, D. (2019). The molecular basis for remyelination failure in multiple sclerosis. Cells, 8(8), 825.

Kremer, D., **Gruchot, J**., Weyers, V., Oldemeier, L., Göttle, P., Healy, L., Ho Jang, J., Xu, Y. K. T., Volsko, C., Dutta, R., Trapp B., Perron, H., Hartung H. P. & Küry, P. (2019). pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. Proceedings of the National Academy of Sciences, 116(30), 15216-15225.

Gruchot, J., Kremer, D., & Küry, P. (2019). Neural cell responses upon exposure to human endogenous retroviruses. Frontiers in genetics, 10, 655.

Göttle, P., Förster, M., **Gruchot, J.**, Kremer, D., Hartung, H. P., Perron, H., & Küry, P. (2019). Rescuing the negative impact of human endogenous retrovirus envelope protein on oligodendroglial differentiation and myelination. Glia, 67(1), 160-170.

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Eidesstattliche Erklärung

Ich, Joel Gruchot, versichere an Eides Statt, dass die vorliegende Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Textstellen, die wörtlich oder abgewandelt aus anderen Arbeiten stammen, habe ich mit einer Quellenangabe versehen. Diese Arbeit wurde weder vollständig noch in Teilen einem anderen Prüfungsamt zur Erlangung eines akademischen Grades vorgelegt.

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31.05.2023, Joel Gruchot