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Proinflammatory effects of the pHERV-W envelope protein on primary neonatal microglia of the Wistar rat related to multiple sclerosis

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Zusammenfassung

Multiple Sklerose (MS) ist die häufigste chronisch-entzündliche und demyelinisierende Autoimmunerkrankung des menschlichen zentralen Nervensystems (ZNS) unbekannter Ätiologie. Sie ist gekennzeichnet durch einen Entzündungsprozess, gefolgt von Demyelinisierung und Verlust von Axonen, was zu irreversibler Neurodegeneration führt. Verschiedene Viren, darunter das humane endogene Retrovirus der Familie W (HERV-W) und im speziellen sein Hüllprotein, die im Gehirn und Blut von MS-Patienten nachgewiesen werden können, werden mit dem Ausbruch und/oder dem Fortschreiten von MS in Verbindung gebracht. Bisherige Studien haben gezeigt, dass das Hüllprotein (pHERV-W Env) proinflammatorische Wirkungen auf Immunzellen und auf im ZNS-ansässige Gliazellen, wie oligodendrogliale Vorläuferzellen (OPCs) und Mikroglia, ausübt. Vermittelt durch den *Toll-like*-Rezeptor 4 (TLR4), induziert das pHERV-W Env-Protein die Produktion von Stickstoffmonoxid (NO), was zu nitrosativem Stress führt, der mit axonalen Schäden verbunden ist. Es wurde vermutet, dass in erster Linie Mikroglia die negativen Auswirkungen des pHERV-W Env-Proteins vermitteln und damit effektiv zur MS-Pathogenese beitragen könnten. Die in dieser Dissertation vorgestellten Ergebnisse zeigen, dass die Exposition primärer neonataler Rattenmikrogliazellen zu rekombinantem pHERV-W Env-Protein zur Induktion eines proinflammatorischen Phänotyps führt und einen Rückgang der neuro-unterstützenden Eigenschaften der Mikroglia bewirkt, was sich beispielsweise in einer extremen Verringerung der mikroglialen Freisetzung des insulinähnlichen Wachstumsfaktors 1 (IGF-1) zeigt, ein Molekül, das bekanntermaßen OPC-Differenzierungsprozesse fördert. Außerdem wurde beobachtet, dass die Transkription von Genen, die an der Regulierung der mikroglialen Myelinaufnahme beteiligt sind, wie z.B. MerTK, und für ordnungsgemäße Reparaturprozesse zur Erleichterung der Remyelinisierung unerlässlich sind, drastisch herunterreguliert wurden - was letztlich zu einer stark beeinträchtigten Myelin-Phagozytose führte. Bei den hier vorgestellten Versuchen, die durch das pHERV-W Env-Protein vermittelten proinflammatorischen Effekte auf Mikroglia *in vitro* zu neutralisieren, wurde Interleukin 4 (IL-4) als vielversprechender Kandidat identifiziert. Bemerkenswerterweise konnte der Anti-Env-Protein-Antikörper Temelimab den durch die Stimulation mit dem pHERV-W Env-Protein ausgelösten mikroglialen nitrosativen Stress nicht vermindern. Dies könnte die überraschende Beobachtung erklären, dass aktuelle klinische Studien zu Temelimab keine Auswirkungen auf den neuroinflammatorischen Zustand bei MS-Patienten zeigen. In dieser Hinsicht unterstreicht diese Dissertation eine biomedizinische Rationale für das pHERV-W Env-Protein als vielversprechendes Ziel für künftige therapeutische Ansätze, da es offenbar einen bedeutenden mikroglia-abhängigen Beitrag zu Schlüsselproblemen bei MS vermittelt: entzündliche und demyelinisierende Aspekte.

Abstract

Multiple sclerosis (MS) is the most frequent chronic inflammatory and demyelinating autoimmune disease of the human central nervous system (CNS) of unknown aetiology and is characterized by inflammation followed by demyelination and loss of axons, resulting in irreversible neurodegeneration. Different viruses, amongst them the human endogenous retrovirus of the family W (HERV-W) and particularly its envelope protein which both can be detected in the brain and blood of MS patients are associated with the onset and/or progression of MS. Previous research revealed that the envelope protein (pHERV-W Env) exerts proinflammatory effects on immune cells and on CNS resident glial cells, such as oligodendroglial precursor cells (OPCs) and microglia. Mediated by the toll-like receptor 4 (TLR4), exposure to the pHERV-W Env protein induces the production of nitric oxide (NO) consequently leading to nitrosative stress, which is associated with axonal damage. It was assumed that primarily microglia might impart the negative effects of the pHERV-W Env protein, thereby effectively contributing to MS pathogenesis. Results presented within this dissertation show that the exposure of primary neonatal rodent microglia to the recombinant pHERV-W Env protein leads to the induction of a proinflammatory phenotype and reveals a decline in the neuro-supportive properties of microglia, as indicated, for example, by an extreme reduction in microglial release of insulin-like growth factor 1 (IGF-1), a molecule known to promote OPC differentiation processes. In addition, transcript levels of genes involved in microglial myelin uptake, such as MerTK, essential for proper repair processes facilitating remyelination were observed to be drastically downregulated - ultimately resulting in a severely impaired myelin phagocytosis. The here presented attempts to neutralize pHERV-W Env protein mediated microglial proinflammatory effects *in vitro* identified interleukin 4 (IL-4) as a promising candidate. Remarkably, the anti-Env protein antibody temelimab failed to diminish microglial nitrosative stress induced by pHERV-W Env protein stimulation. This may explain the surprising observation of current clinical trials of temelimab demonstrating no effects on the neuroinflammation state in MS patients. In this respect, this dissertation highlights a biomedical rationale for the pHERV-W Env protein as a promising target for future therapeutic approaches as it appears to mediate a substantial microglial-dependent contribution to key issues in MS: inflammatory as well as demyelinating aspects.

Abbreviations

°C *Degrees celsius*
µg *Mikrogram*
µl *Mikrolitre*
µM *Mikromolar*
AE *Adverse effect*
ALS *Amyotrophic lataral sclerosis*
ANOVA *Analysis of variance*
APC *Antigen-presenting cell*
APRIL *A proliferation inducing ligand*
AQP4 *Aquaporin 4*
Arg-1 *Arginase-1*
Axl *Axl receptor tyrosine kinase*
BBB *Blood-brain-barrier*
BSA *Bovine serum albumin*
CC3 *Cleaved caspase 3*
CCL5 *CC-chemokine ligand 5*
CD 206 *Cluster of differentiation 206*
CD14 *Cluster of differentiation 14*
CD20 *Cluster of differentiation 20*
CD52 *Cluster of differentiation 52*
cDNA *Complementary deoxyribonucleic acid*
CHO *Chinese hamster ovary cells*
cm *Centimetre*
CNS *Central nervous system*
COX2 *Cyclooxygenase 2*
CPZ *Cuprizone*
CSF *Cerebrospinal fluid*
CSF-1 *Colony stimulating factor - 1*
CXCL1 *C-X-C motif chemokine ligand 1*
CXCL10 *C-X-C motif chemokine ligand 10*
d *Day*
DAMP *Damage-associated molecular pattern*
DAPI *4'6-diamidino-2phenylindole*
DMD *Disease modifying drug*
DMT *Disease modifying therapy*
DNA *Deoxyribonucleic acid*
E *Embryonic day*
EAE *Experimental autoimmune encephalomyelitis*
EBV *Epstein-Barr virus*
ELISA *Enzyme-linked immunosorbent assay*
env *Envelope gene*
EU *Endotoxin units*
FCS *Fetal calf serum*
FGF-2 *Fibroblast growth factor - 2*
gag *Group-specific antigen*
h *Hour*
HERV *Human endogenous retrovirus*
HHV-6 *Human herpes simplex virus 6*
HLA *Human leukocyte antigen*
HLA-DR *Human leukocyte antigen-DR isotype*
HSV-1 *Herpes simplex virus 1*
i.v. *intravenous*
IFNγ *Interferon γ*
IGF-1 *Insulin-like growth factor 1*
IL *Interleukin*
iNOS *Inducible nitric oxide synthase*
LM7 *Leptomeningeal cell line 7*
LPS *Lipopolysaccharide*
LTR *Long terminal repeats*
M1 *Proinflammatory microglial cell*
M2 *Neuroprotective microglial cell*
MACS *Magnetic cell sorting*
MAPK *Mitogen-activated protein kinase*
MBP *Myelin basic protein*
MCP-1 *Monocyte chemoattractant protein-1*
MerTK *Mer receptor tyrosine kinase*
mg *Milligram*
MHC *Major histocompatibility complex*
min *Minute*
ml *Millilitre*
mM *Millimolar*
MOG *Myelin/oligodendrocyte glycoprotein*
MRI *Magnetic resonance imaging*
mRNA *Messenger ribonucleic acid*
MS *Multiple sclerosis*
MTR *Magnetization transfer ratio*
mu-GNbAC1 *murine GNbAC1*
NEDA *No evidence of disease activity*
NFL *Neurofilament*
NF-κB *Nuclear factor 'kappa-light-chain-enhancer' of activated B cells*
ng *Nanogram*
NG2 *Nerve/glial antigen 2*
NLR *NOD-like receptor*
nm *Nanometre*
NO *Nitric oxide*
NOD *Nucleotide-binding oligomerization domain*
ODC *Ornithine decarboxylase*
OL *Oligodendrocyte*
OND *Other neurological diseases*
OPC *Oligodendrocyte precursor cell*
ORF *Open reading frame*
p.o *per os*
PAMP *Pathogen-associated molecular pattern*
PBMC *Peripheral blood mononuclear cell*
PBS *Phosphate-buffered saline*
PFA *Paraformaldehyde*
pHERV-W Env protein *pathogenic human endogenous retrovirus type W Envelope protein*
pHERV-W Env-SU *Subunit of the pHERV-W Env protein*

pHERV-W Env-T *Full length of the pHERV-W Env protein*
PLP *Proteolipid protein*
pmol *Picomol*
PMXB *Polymyxin B*
PNS *Peripheral nervous system*
pol *Polymerase gene*
PPMS *Primary-progressive multiple sclerosis*
pro *Protease gene*
PRR *Pattern recognition receptor*
PTX3 *Pentraxin 3*
qRT-PCR *Quantitative real-time polymerase chain reaction*
RA *Rheumatoid arthritis*
RNA *Ribonucleic acid*
ROS *Reactive oxygen species*
rpm *Revolutions per minute*
RPMS *Relapsing-progressive multiple sclerosis*
RRMS *Relapsing-remitting multiple sclerosis*
RT *Room temperature*
s.c. *subcutaneous*
SA *Serum albumin*
SARS-CoV-2 *Severe adute respiratory syndrome coronavirus type 2*
SEM *Standard error of the mean*
SLE *Systemic lupus erythematosus*
SNP *Single nucleotide polymorphism*
SPMS *Secondary-progressive multiple sclerosis*
SR *Scavenger receptor*
SU *Subunit*
SYP *Synaptophysin*
T1D *Type 1 diabetes*
TE *Transposable element*
TGFβ *Transforming growth factor β*
TLR4 *Toll-like receptor 4*
TNF *Tumour necrosis factor*
TNF-α *Tumour necrosis factor- α*
TREM *Triggering receptor expressed on myeloid cells*
TREM2 *Triggering receptor expressed on myeloid cells 2*
tRNA *Transfer ribonucleic acid*
Tyro3 *Tyrosine kinase receptor 3*
VZV *Varicella-zoster virus*
w/v *Weight per volume*
YS *Yolc sac*
ZETT *Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben*
ΔΔCt *Comparative cycle threshold method*

Table of content

1 Introduction

1.1 The central nervous system and its different cell types

The highly complex human nervous system is subdivided into the central nervous system (CNS), comprising the brain and the spinal cord, and the peripheral nervous system (PNS). Nerves of the PNS transmit signals to and from the CNS. The brain is responsible for the integration and processing of stimuli which affect the human organism, coordinates motor function and synchronizes subsidiary organ systems. Functionally, multiple subsystems such as the sensory, the motor, the visual, the auditory, the gustatory and the limbic system exist, which receive, process and evaluate information affecting the organism and subsequently generate appropriate reactions.

Macroscopically, the CNS can be subdivided into two main tissues: the grey- and the white matter. Microscopically, the different cell types of the CNS are generally assigned to two main classes – neurons and glial cells (Kettenmann and Verkhratsky, 2008). Neurons can be considered as the main functional units of the CNS and form multiple networks. As electrically excitable cells, they transmit signals via electric impulses, the so-called action potentials. Thereto, a piecewise saltatory impulse conduction between the nodes of Ranvier, which are unsheathed axonal segments (Ranvier, 1871), and myelinated internodes takes place (Tasaki, 1939). Simplified, all other cell types of the CNS which are not able to transmit or generate action potentials upon stimulation are categorized into one big class and termed glial cells (Kuffler et al., 1966). To date, it is known that these cells play essential roles in CNS homeostasis, development, plasticity and disease (Barres, 2008, Allen and Barres, 2009, Zuchero and Barres, 2015, Jakel and Dimou, 2017, Allen and Lyons, 2018). Glial cells are further subclassified into macroglia and microglia. Macroglia comprises of astrocytes, oligodendrocytes (OLs) and their progenitor cells, radial glial cells and ependymal cells. These cell types originate, as neurons, from progenitor cells of the neuroepithelium (Rowitch and Kriegstein, 2010). Microglia, which are part of the CNS' innate immunity derive from a mesodermal haematopoietic origin and enter the CNS in early stages of development (Ginhoux and Garel, 2018).

Astrocytes are the most abundant glial cell type of the CNS and are a heterogenous cell group, based on their variety of functions (Tabata, 2015, Miller, 2018). They are involved in the regulation of ion homeostasis, energy metabolism, synaptic transmission, neuronal synaptogenesis, neuronal excitability and homeostasis of neurotransmitters, and thereby effectively modulate CNS microenvironment (Markiewicz and Lukomska, 2006, Kimelberg, 2010, Kimelberg and Nedergaard, 2010). Besides, astrocytes are known to be highly secretory cells and strongly communicate with all cell types within the CNS (Verkhratsky et al., 2016). Moreover, astrocytes form a continuous layer, termed glia limitans, which is part of the blood-brain-barrier (BBB) (Abbott et al., 2006). As a diffusion barrier, the BBB controls the influx of molecules from blood circulation into the CNS due to their size and polarity, thereby constituting a physical and immunological barrier (Ballabh et al., 2004). Thus, astrocytes play crucial roles in supporting and maintaining the BBB integrity (Anderson and Nedergaard, 2003). Additionally, astrocytes regulate the water homeostasis via the membranous water channel protein Aquaporin 4 (AQP4) (Nagelhus and Ottersen, 2013, Satoh et al., 2007, Mader and Brimberg, 2019) and the vascular tone (Takano et al., 2006, MacVicar and Newman, 2015). Besides microglia, astrocytes are relevant mediators in the inflamed CNS as they can turn into reactive astrocytes upon e.g., CNS injury (Giovannoni and Quintana, 2020, Yu et al., 2021). Reactive astrocytes can adopt either a neurotoxic phenotype contributing to neuroinflammation or a neuroprotective phenotype promoting CNS recovery (Liddelow and Barres, 2017). Intriguingly, the appearance of the neurotoxic astrocytic phenotype was shown to be promoted by activated microglia (Liddelow et al., 2017).

Mature OLs are the myelinating glial cells of the CNS, producing a lipid-rich membrane, termed myelin, which enwraps axons in a concentric way (Rome et al., 1986). Myelin sheaths electrically insulate axons, enable saltatory nerve conduction and ensure axonal integrity (Stadelmann et al., 2019). Moreover, OLs maintain long-term axonal integrity by providing metabolic and trophic support (Fünfschilling et al., 2012). In addition, OLs secrete a variety of molecules which positively influence neuronal survival (Byravan et al., 1994, Wilkins et al., 2001, Wilkins et al., 2003, Philips and Rothstein, 2017).

Myelinating OLs are generated from oligodendrocyte precursor cells (OPCs) in a tightly controlled process consisting of migration, proliferation and differentiation (Bradl and Lassmann, 2010, Kuhn et al., 2019). This differentiation process occurs predominantly in the developing CNS but is also present to a lower degree in the mature CNS as resident OPCs, termed nerve/glial antigen 2 (NG2) glial cells, represent a source of migratory and proliferative precursor cells, which can replace extinct or damaged OLs in the adult brain (Berry et al., 2002, Nishiyama et al., 2016). Anatomically, multiple origins within the developing CNS were identified of generating OPCs, such as the ventral ventricular zone, the anterior- and dorsal spinal cord and the embryonic ventral neural tube (Raff et al., 1983, Pringle and Richardson, 1993, Timsit et al., 1995). From these origins, OPCs migrate and populate the CNS, especially the white matter, and either directly differentiate into mature myelinating OLs or remain and comprise NG2 glia. Additionally, OPCs exert immunomodulatory properties as they express several cytokine receptors and respond to inflammatory stimuli (Baerwald and Popko, 1998, Falcao et al., 2018). Notably, due to their lifelong ability to differentiate into myelinating OLs, OPCs are pivotal players, especially in demyelinating diseases of the CNS, as they can initiate remyelination processes by reestablishment of the myelin sheath in demyelinated areas (Franklin and Ffrench-Constant, 2008, Miron, 2017).

1.2 Microglia – the innate immunity of the CNS

1.2.1 Origin

Microglia are the resident macrophages of the CNS and were first described as a small subpopulation of phagocytic and migratory cells (Rio-Hortega, 1932, Rio-Hortega, 1939). They constitute approximately 10-15% of CNS glial cells (Lawson et al., 1990). These cells feature a hematopoietic origin and derive from the yolk sac (YS), invading the CNS in early stages of development before BBB formation (Alliot et al., 1999, Ginhoux and Prinz, 2015). Primitive macrophage-like cells occur at embryonic day 8.5/9.0 (E8.5/E9.0) in mice brains (Alliot et al., 1991). YS derived macrophages are highly proliferative cells with their numbers increasing dramatically from E9.0/E9.5 until 2 weeks after birth (Alliot et al., 1999). In humans, microglia were found to populate the embryonic brain from 4.5 weeks of gestation

(Monier et al., 2007). Once, the BBB is generated, renewal and differentiation of microglia exclusively takes place within the CNS. Notably, a hallmark of CNS-resident microglia is their capacity for self-renewal throughout the entire life of an organism, both in health and disease (Ajami et al., 2007).

1.2.2 Microglial properties in the CNS

Under physiological conditions, microglia adopt a resting ramified phenotype and are primarily responsible for maintaining tissue homeostasis. Therefore, they constantly move with their dynamic processes through the tissue and monitor the CNS parenchyma for potential pathogens, molecules or cell debris that could be harmful to the CNS (Nimmerjahn et al., 2005, Hanisch and Kettenmann, 2007). Permanent intensive and reciprocal communication with their neighbouring cells is essential for keeping microglia in a quiescent state (Cardona et al., 2006, Hoarau et al., 2011, Eggen et al., 2013). Remarkably, in the healthy CNS, quiescent microglia exhibit quite sparse antigen-presenting properties (Shrikant and Benveniste, 1996, Mack et al., 2003). In the developing CNS, phagocytosis, as one of the key functions of microglia, is necessary to eliminate overproduced neurons and synapses, which further permits the building of new neuronal networks. Additionally, microglia are responsible for the removal of apoptotic cells or cell debris in the developing as well as in the adult CNS (Schafer and Stevens, 2015, Galloway et al., 2019). Several molecules and receptors regulate phagocytic processes, such as the TAM family receptor tyrosine kinases (Tyro3, Axl and MerTK) and Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), which play crucial roles, particularly in the regulation of microglia's phagocytosis of myelin (Takahashi et al., 2005, Lemke, 2013, Fourgeaud et al., 2016, Healy et al., 2016, Healy et al., 2017). The latter is of major importance in the context of demyelinating diseases (see introduction, section 1.3.4). Intriguingly, microglia can initiate oligodendrogenesis via secretion of diverse molecules, such as insulin-like growth factor 1 (IGF-1) which is well known to induce the differentiation process of OPCs into mature and myelinating OLs (Ye et al., 2002, Hagemeyer et al., 2017).

1.2.3 Microglia upon exposure to CNS threatening agents

As immunocompetent cells of the CNS, microglia immediately react to stimuli which compromise tissue homeostasis (Aloisi, 2001, Davalos et al., 2005). The turnover

from a quiescent into an activated state is basically characterized by changes from a ramified to ameboid morphology, and by changes of the gene expression pattern resulting in the release of several molecules (Suzumura et al., 1991, Colton, 2009, Lynch, 2009, Orecchioni et al., 2019). Depending on their environment, activated microglia can adopt different phenotypes, simply subclassified into M1 and M2, contributing to inflammation (M1) and neuroprotection (M2) (Mills et al., 2000, Orihuela et al., 2016). Belonging to the innate immunity, microglia express different pattern recognition receptors (PRRs) on the cell surface, among them receptors of the toll-like family, predominantly toll-like receptor 4 (TLR4), triggering receptors expressed on myeloid cells (TREMs), nucleotide-binding oligomerization domains (NODs), NOD-like receptors (NLRs) and several scavenger receptors (SRs) (Ransohoff and Brown, 2012). Each PRR displays a specific affinity for pathogen-associated molecular patterns (PAMPs), such as certain viral, bacterial or fungal particles, or damage-associated molecular patterns (DAMPs), such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or proteins (Takeuchi and Akira, 2010). The TLR4 signalling pathway is activated by bacterial lipopolysaccharide (LPS), promoting a proinflammatory microglial phenotype *in vitro* and *in vivo* (Hoshino et al., 1999, Nakamura et al., 1999, Lund et al., 2006). When LPS binds to microglial TLR4 and its co-receptor, cluster of differentiation 14 (CD14), a signalling cascade is induced which leads to the activation of the nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathway (Lu et al., 2008, Liu et al., 2017). The subsequent signalling cascade results in microglial production of certain inflammatory molecules such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-12, inducible nitric oxide synthase (iNOS) for nitric oxide (NO) production, reactive oxygen species (ROS), cyclooxygenase-2 (COX2), chemokines (C-X-C motif chemokine ligand 1 (CXCL1), C-X-C motif chemokine ligand 10 (CXCL10), CC-chemokine ligand 5 (CCL5), monocyte chemoattractant protein-1 (MCP-1)) and others, allocating microglia to the M1 phenotype (Hanisch and Kettenmann, 2007, Saijo et al., 2013, Lively and Schlichter, 2018). Moreover, acute-phase proteins, such as pentraxin 3 (PTX3), are released, thereby modulating microglia's phagocytic capacities (Jeon et al., 2010). Notably, reactive microglia express human leukocyte antigen – DR isotype (HLA-DR) and major histocompatibility complex (MHC) class I and II receptors and turn into antigen-

presenting cells (APCs), thus mediating and triggering the adaptive immunity (Hayes et al., 1987, McGeer et al., 1988, Ulvestad et al., 1994, Shaked et al., 2004, Schetters et al., 2017). Next to the classical activation of microglia, there exists an alternative activation which results in a restorative and neuro-supportive microglial phenotype (M2) (Stein et al., 1992, Franco and Fernandez-Suarez, 2015). This phenotype is predominantly induced in response to IL-4, IL-10 and IL-13, resulting in an upregulation of cluster of differentiation 206 (CD 206), arginase-1 (Arg-1), IGF-1, transforming growth factor β (TGFβ) and other molecules associated with neuroprotection, tissue repair and downregulation of inflammatory cells (Gadani et al., 2012, Cherry et al., 2014). To date, the stringent M1 or M2 activation paradigm is too simplistic and needs to be revised, as there are several hints that activated microglia consist of quite heterogenous groups of cells which respond differently to the same stimuli and can switch between the reported different activation states (Ransohoff, 2016, Hammond et al., 2019, Stratoulias et al., 2019).

1.3 Multiple Sclerosis

1.3.1 Clinical presentation and subtypes

Multiple sclerosis (MS) is the most frequent chronic inflammatory and demyelinating autoimmune disease of the CNS, characterized by inflammation followed by demyelination and subsequently neurodegeneration. Multiple focal lesions are found in CNS tissues of MS patients which are the results of demyelinating processes and axonal damage. MS presents itself as a multi-faceted heterogenous disease affecting visual, sensory, motor and cerebellar functions as well as the vegetative nerve system and cognition. Neurological symptoms differ from patient to patient depending on the locations of the lesions within the CNS (Filippi et al., 2018). Based on its clinical sequence of events, MS is further subclassified into diverse forms. The most abundant subtype (80%) that occurs in patients who are first diagnosed with MS is the relapsing-remitting form (RRMS). Patients suffering from RRMS experience exacerbations of acute neurological worsening from which they recover partially or completely. Between these relapses no disease progression is observed. Secondary-progressive MS (SPMS) derives from RRMS and is characterized by progressive neurological deterioration occurring with or without

superimposed exacerbations. About 80% of patients initially diagnosed with RRMS transform to SPMS. A minority of patients (10 to 20%) first diagnosed with MS are referred to a severe form: the primary-progressive form (PPMS). These patients suffer from constantly progressive neurological symptoms directly from the onset of the disease without the presence of complete recovery. At best, these patients show intermittent plateau phases. Additionally, another severe presentation of MS, relapsing-progressive MS (RPMS), is described, in which patients suffer from permanent progressive neurological symptoms from disease onset, as do patients of PPMS, but additionally experience superimposed relapses (Bitsch and Bruck, 2002, Lublin et al., 2014, Klineova and Lublin, 2018).

1.3.2 Epidemiology and aetiology

MS is mainly responsible for the occurrence of non-traumatic disabilities in young adults. To date, the worldwide prevalence of MS has increased up to 2.8 million people. Females are more affected than males, with a ratio from 2:1 to even 4:1 in some regions of the world (Coetzee and Thompson, 2020, Walton et al., 2020). The average disease onset is reported at the age of 30 years, but disease onset can also occur after the age of 50 years, termed late-onset MS (Martinelli et al., 2004, Howard et al., 2016, Gbaguidi et al., 2022).

To date, the aetiology of MS is still unknown. Several environmental and genetic risk factors are linked to promoting the disease onset, but none of these risk factors have been clearly identified to induce MS. The prevalence of MS seems to be dependent on the earth latitude increasing towards the poles. This suggests that specific environmental risk factors, such as low ultraviolet radiation and diminished vitamin D serum levels, contribute to MS development (Kurtzke, 1975, van der Mei et al., 2003, Munger et al., 2004, Simpson et al., 2011). Interestingly, migration studies revealed that immigrants from low-risk areas who migrate to high-risk areas retain the initial risk of developing MS of their place of birth, if migration occurred after the age of 15 years. The same is observed vice versa. However, children of these immigrants adopt the prevalence of MS risk levels of their birthplace, corroborating a correlation of individuals' exposure to environmental factors and the origin of MS (Dean et al., 1976, Visscher et al., 1977, Elian et al., 1990). Moreover, influenceable lifestyle factors, such as smoking, are reported to strongly increase the risk of MS (Alfredsson and Olsson, 2019). Surprisingly, some ethnic groups,

such as Hispanics and Asians (Wallin et al., 2012, Langer-Gould et al., 2013), Aboriginals (Miller et al., 1990), and Lacandonians (Flores et al., 2012) seem to be more resistant to contracting MS, additionally indicating race and genetic factors being involved in the risk of developing the disease (Amezcua and McCauley, 2020). Moreover, a genetic component of contracting MS is corroborated by family- (O'Gorman et al., 2013, Harirchian et al., 2018) and twin studies (Willer et al., 2003) and recent meta-analyses revealed a worldwide prevalence of familiar MS of about 12.6% (Balcerac and Louapre, 2022). Furthermore, some individual genetic risk factors have been identified so far, which predispose an individual to MS. The strongest correlations are reported for variations in the human leukocyte antigen (HLA) system and especially the MHC class II alleles HLA-DRB1*15:01, HLA-DRB1*13:03, HLA-DRB1*03:01, HLA-DRB1*08:01 and HLA-DQB1*03:02 which were identified to have the strongest association with MS (Sawcer et al., 2014, Canto and Oksenberg, 2018). In addition, several infectious diseases are attributed to the pathogenesis of MS. The strongest correlations are reported for infectious mononucleosis evoked by the Epstein-Barr virus (EBV) (Warner and Carp, 1981, Ascherio et al., 2001). In addition, varicella-zoster virus (VZV) (Brettschneider et al., 2009, Sotelo and Corona, 2011), human herpes simplex virus 6 (HHV-6) (Challoner et al., 1995, Soldan et al., 1997), herpes simplex virus 1 (HSV-1) (Duarte et al., 2022) and human endogenous retroviruses (HERVs) (Perron et al., 2005) are associated with higher risks of subsequently developing MS. Notably, severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) is assumed to be able to induce MS (Sarwar et al., 2021) and to promote relapses by significantly reducing protective effects of disease modifying therapies (DMTs) (Finsterer, 2022).

1.3.3 Histopathology

Macroscopically, the histopathological correlates of MS are multiple focal heterogenous cortical grey and white matter lesions, the so-called plaques, which can be found on magnetic resonance imaging (MRI) scans of MS patients CNS. These plaques are focal areas of myelin loss which are the results of autoimmune mediated inflammatory demyelination processes (Rindfleisch, 1863, Pirko et al., 2007, Popescu and Lucchinetti, 2012). Basically, these lesions predominantly occur in specific regions of the CNS, such as the optic nerves, the juxtacortical and

periventricular white matter, the cerebellum, the brainstem and the spinal cord (Filippi et al., 2019).

Microscopically, a heterogenous constitution of MS plaques is detected depending on the disease phase (acute or chronic) displaying variable dimensions of inflammation, axonal degradation, gliosis and even processes of remyelination (Lucchinetti et al., 2000). Commonly, MS lesions are found around small veins and venules showing perivascular and parenchymal signs of inflammatory infiltrates resulting from BBB disruption (Adams et al., 1989). Consequently, activated microglia or macrophages, but particularly immune cells from the periphery such as CD8-positive cytotoxic T cells, CD4-positive T helper cells, B cells and plasma cells, invade the CNS, with B cells predominantly found in the perivascular spaces (Baecher-Allan et al., 2018). More importantly, CNS invading T cells trigger activation and recruitment of peripheral macrophages and CNS resident microglia, thereby fuelling inflammatory processes. Together, these autoreactive immune cells induce inflammation mediated loss of OLs and thereby promote demyelination consequently resulting in irreversible neurodegeneration and axonal destruction (Dutta and Trapp, 2007). Due to their histological appearance and the variably evoked immune responses, active plaques are further allocated to one out of four patterns. Pattern I is the result of microglia/macrophage and T cell mediated cytotoxic inflammation with equal loss of myelin and OLs. In the inactive plaque center OLs reappear and initiate remyelination to a certain extent. The MS predominant pattern II histologically appears like pattern I, but elevated levels of immunoglobulins and activated complement are found in the cerebrospinal fluid (CSF), indicating primarily antibody- and complement mediated inflammatory events. On the contrary, pattern III is the result of direct inflammatory mediated impairment of OLs through the production of cytotoxic molecules, such as NO or ROS, without signs of remyelination. Pattern IV is characterized by a metabolic failure of OLs rendering them more susceptible to toxic inflammatory molecules (Lucchinetti et al., 2000). These different patterns evoke the yet unresolved question as to whether MS is predominantly an autoimmune mediated disease or an oligodendrogliopathy or a combination of both (Nakahara et al., 2010, Nakahara et al., 2012). All above-described patterns finally cause the same outcome – axonal demyelination and subsequently irreversible axonal degradation, with the latter

being reported to occur in up to 80% of MS lesions (Lassmann, 2003). Next to acute active plaques, chronic active and chronic inactive plaques are also found in CNS tissue of MS patients. Chronic active plaques are characterized by a hypocellular demyelinated centre which is surrounded by myelin phagocyting macrophages or microglia expanding the lesion. Chronic inactive plaques are sharply restricted completely demyelinated lesions displaying no inflammatory activity with predominant occurrence of astrocytes forming the glial scar (Popescu and Lucchinetti, 2012, Popescu et al., 2013).

To date, several histopathological analyses exist and try to explain various cellular mechanisms which might be responsible for contributing to chronic neurodegeneration and axonal loss in MS, but none of them was identified of being causative (Bjartmar et al., 2003, Brück, 2005, Su et al., 2009, Criste et al., 2014). Notably, remyelinated plaques are also identified in MS tissue, especially in patients displaying disease patterns I or II, whereas they are not found in MS patients expressing disease patterns III or IV. These so-called “shadow plaques” exhibit thinly myelinated axons, resulting from newly produced myelin sheaths, following the recruitment and differentiation of resident NG2-glia (Bramow et al., 2010, Popescu et al., 2013). However, in MS the complete remyelination of lesions fails, which is a central hallmark of the disease (Franklin, 2002, Gruchot et al., 2019).

1.3.4 Microglia and their role in MS – a double-edged sword

Microglia are always present in acute or chronic active lesions of MS patients (Lassmann, 2012). Their roles in mediating, exacerbating, or terminating inflammatory and neurodegenerative processes in MS are complex and not completely understood. Microglia are predominantly found in close contact to demyelinated axons, thus indicating that they adopt a rather proinflammatory phenotype exerting myelin destructive properties and thereby might contribute to the pathogenesis of MS (Benveniste, 1997, Gandhi et al., 2010, Guerrero and Sicotte, 2020). Within the course of MS, microglia display a certain dynamic plasticity and switch between their proinflammatory and neuro-supportive phenotypic profiles. They change their morphologies, gene expression patterns and secreted molecules and were further identified during the phases of remyelination to efficiently phagocyte myelin debris and recruit OPCs to lesion sites (Olah et al., 2012, Voß et al., 2012, Miron et al., 2013, Peferoen et al., 2015). As disrupted myelin

sheaths need to be primarily removed before efficient remyelination can start, myelin phagocyting microglia constitute important and active players in limiting the progress of MS (Lampron et al., 2015). In order to create and promote the required remyelinating milieu in MS, microglia foster an intense crosstalk between the other CNS resident glial cells (Domingues et al., 2016). However, a microglial phenotype which promotes demyelination and axonal degradation seems to predominate during the whole disease course of MS. Proinflammatory activated microglia were identified to be the major sources of nitrosative- and oxidative stress, by producing NO radicals and ROS (Liu et al., 2001). In MS lesions, huge amounts of such microglial/macrophage produced nitrosative- and oxidative molecules are present (Bagasra et al., 1995, Smith and Lassmann, 2002), subsequently promoting a neurotoxic atmosphere (Calabrese et al., 2007). This proinflammatory microglial phenotype is assumed to be a major source of exacerbation of the disease course and seems to hamper remyelination processes (Haider et al., 2011). Additionally, microglia maintain a strong crosstalk between immune cells and as APCs, they trigger and regulate the activation of T cells in MS (Schetters et al., 2017). With microglial expression of MHC I and MHC II proteins on their cell surfaces, they recruit T cells, to the CNS which subsequently become activated (Ransohoff and Perry, 2009). In response, these T cells proliferate and secrete a diversity of cytokines which further promote microglial activation. Depending on the predominant subtype of T cells (Th1- or Th2) microglia are triggered to produce proinflammatory molecules or molecules which support a neuro-supportive atmosphere in MS. This intense and reciprocal crosstalk between these immune cells seems to significantly affect and drive the course of MS (Sriram and Rodriguez, 1997, Ebner et al., 2013, Legroux and Arbour, 2015). Yet to date, it is still unclear who exactly is the initiator of inflammatory processes in MS. If it is microglia or T cells or both to same parts and if there exists a kind of super-agent which induces the inflammatory onset of MS is still not decoded and requires further research.

1.3.5 Current approved treatment options

To date, since the origin and pathogenesis of MS is still obscure, no curative therapy is available. Basically, today's strategies for treating MS focus on three different aspects: 1) quick control and termination of relapses, 2) DMTs with use of approved disease modifying drugs (DMDs), and 3) abatement of MS coexisting symptoms

(Hemmer et al., 2021). Relapsing episodes, which usually come along with acute clinical deterioration of neurological symptoms, are first-line treated with high-dose intravenous steroids. The aim of this state-of-the-art therapy is to quickly induce regression of neurological symptoms due to the well-known immunosuppressive and strong anti-inflammatory effect of glucocorticoids. However, a long-term effect of positively influencing the disease course of MS is not reported for glucocorticoids (Milligan et al., 1987, Sloka and Stefanelli, 2005). Long-term control of MS activity is achieved using DMDs. Superior aims of this approach are a) significant reduction of relapse rates, b) significant reduction of disease activity with achieving the status of “no evidence of disease activity (NEDA)”, c) delay of disease progress, and d) maintenance of patient’s quality of life. For choosing an effective MS long-term medication, patient’s subtype of MS and its level of disease activity must be clearly defined. Moreover, further comorbidities, status of family planning, contraindications or preferences of drug application must be considered to choose the most promising DMT for the individual (Hemmer et al., 2021).

As of now, a variety of DMDs featuring immunomodulatory or immunosuppressive properties is available. DMDs of category I (table 1) were identified to reduce relapse rates by up to 50% and are approved for patients suffering from RRMS in its mild or moderate form, or in the case of β-interferons, are additionally approved for the active form of SPMS. Category II DMDs (table 2) reduce relapse rates by up to 60% and these drugs are basically administered to patients who suffer from highly active forms of RRMS or SPMS. Category III DMDs (table 3) are approved for the treatment of extreme active forms of RRMS and SPMS with ocrelizumab being the only DMD approved for the treatment of PPMS so far (Hemmer et al., 2021).

DMD	mechanism of action	references
Recombinant β-interferons • administered s.c.	not completely identified • Decreasing proliferation rates of T cells • Inducing apoptosis of autoreactive T cells • Shifting T cell populations to anti-inflammatory T2 cells • Reduction of transmigration of inflammatory cells across the BBB	• Yong et al. (1998) • Bermel and Rudick (2007)
Glatiramer acetate (GA)	not completely identified	• Weber et al. (2007)

• administered s.c.	• Shifting T cell populations to anti-inflammatory T2 cells • Increasing the presence of regulatory T cells • Modulation of Class II APCs	• Lalive et al. (2011)
Dimethyl fumarate (DMF) • administered p.o.	not completely identified • Conversion of cytokine production towards a T2 cell profile • Antioxidative properties • Triggering endogenous production of anti-oxidative molecules	• Bomprezzi (2015) • Linker and Haghikia (2016)
Teriflunomide • administered p.o.	• Inhibition of proliferation of lymphocytes • Disruption of interaction between T cells and APCs • Promoting OPCs differentiation properties	• Bar-Or et al. (2014) • Göttle et al. (2018)

Table 1: DMDs of category I

DMD	mechanism of action	references
Cladribine • administered p.o.	• Disruption of DNA synthesis, especially of lymphocytes	• Carson et al. (1983) • Brousil et al. (2006)
Sphingosine-1-phosphate receptor modulators Fingolimod, Siponimod, Ozanimod, Ponesimod • administered p.o.	• Diminishing migration of lymphocytes out of lymph nodes • Exerting immunomodulatory properties on CNS resident glial cells	• Brinkmann et al. (2010) • Bordet et al. (2020)

Table 2: DMDs of category II

DMD	mechanism of action	references
Natalizumab • administered i.v. • administered s.c.	• Reduction of migration of lymphocytes into the CNS	• Yednock et al. (1992) • Ransohoff (2007) • Lopez et al. (2021) • Trojano et al. (2021)

Ocrelizumab • administered i.v.	• Depletion of CD20 positive lymphocytes via inducing antibody dependent cellular cytotoxicity	• Frau et al. (2018) • Mulero et al. (2018) • Mancinelli et al. (2021)
Alemtuzumab • administered i.v.	• CD52 antibody, induction of cytolysis of B and T cells	• Hu et al. (2009) • Ruck et al. (2015)
Ofatumumab • administered s.c.	• Complement mediated CD20 B cell lysis	• Sorensen et al. (2014) • Kang and Blair (2022)
Mitoxantrone • administered i.v.	• Inhibition of proliferation of B-, T cells and macrophages • Reduction of proinflammatory cytokines	• Neuhaus et al. (2004) • Martinelli et al. (2009)

Table 3: DMDs of category III

1.4. Human endogenous retroviruses and their relation to MS

HERVs are retroviral DNA insertions resulting from certain exogenous retroviral infections and have integrated themselves into the primate germline millions of years ago (Belshaw et al., 2004). Since then, their genetic material is being inherited in a Mendelian manner and became constantly implanted in the human genome (Nelson et al., 2003). About 8% of the human genome consists of such retroviral material (Lander et al., 2001), which is commonly inactivated or non-functional. HERVs are Class I transposable elements (TEs) (Rebollo et al., 2012), and structurally resemble their endogenous retroviral origin, containing open reading frames (ORFs) in the following genes: the group-specific antigen gene (gag), the protease gene (pro), the polymerase gene (pol) and the envelope gene (env). These genes are enclosed by regions of long terminal repeats (LTRs), comprising silencer and enhancer sequences which are crucial for modulation of retroviral gene expression (Villesen et al., 2004). To date, 40 different HERV families have been identified in the human genome, with each family resulting from a certain exogenous viral infection of the ancestral germline (Gifford and Tristem, 2003). Basically, these families are classified by the specific form of transfer ribonucleic acid (tRNA), which is used for initiating reverse transcription (Nelson et al., 2003). Hereby, elements of the HERV-K family were shown to be most intact and biologically active (Marchi et al., 2014). Intense research related different HERV families to be somehow involved

in the pathogenesis of several autoimmune diseases, such as MS (Perron et al., 1989), amyotrophic lateral sclerosis (ALS) (Douville et al., 2011), systemic lupus erythematosus (SLE) (Wu et al., 2015), rheumatoid arthritis (RA) (Mameli et al., 2017) and type 1 diabetes (T1D) (Levet et al., 2019). To date, the exact mechanisms and degree of HERVs' influence or modulation of disease course are still a matter of debate, as it is unknown if HERVs initiate disease onset or trigger disease progression or do both.

Various HERV families have been associated to the pathogenesis of MS, such as HERV-H, HERV-K and HERV-W, with HERV-W displaying the strongest association so far (Brudek et al., 2009). Transactivation by habitual exogenous viral infections, which are already linked to the pathogenesis of MS, such as EBV, HHV-6, VZV and HSV-1, is assumed of causing HERVs reactivation (Nellaker et al., 2006, Perron et al., 2009, Bello-Morales et al., 2021). Here, the strongest correlations leading to HERVs‘ reactivation in MS are reported for EBV (Mameli et al., 2012, Mameli et al., 2013, Latifi et al., 2022). The first hint for HERVs being involved in the pathogenesis of MS was observed in an isolated leptomeningeal cell line (LM7) obtained from the CSF of a patient suffering from MS, as these cells showed properties of retroviral activity (Perron et al., 1989, Perron et al., 1991). These observations were further corroborated by identifying viral RNA in supernatants of peripheral blood mononuclear cells (PBMCs) and the presence of retrovirus-like particles in monocytes, originating from the blood of MS patients (Perron et al., 1991, Mameli et al., 2007, Dolei and Perron, 2009). The new detected retroviral particles were identified as genetically deriving from HERVs and according to their tRNA, a new HERV family class was introduced and termed HERV-W. Additionally, several studies confirmed the presence of HERV-W virions in blood samples of MS patients, with a sample positivity of almost 53%-100% (Perron et al., 1997, Garson et al., 1998, Serra et al., 2001, Dolei et al., 2002). Moreover, it was demonstrated that increased HERV-W DNA copy numbers in blood samples of MS patients correlate with disease severity and progression. These patients suffered from multiple relapses without accomplishing complete recovery and displayed severe disease patterns, subsequently leading to a poorer prognosis (Sotgiu et al., 2002, Sotgiu et al., 2006b, Rolland et al., 2005, Garcia-Montojo et al., 2013). Notably, blood samples of female patients with MS displayed higher levels of HERV-W loads

compared to males, due to the presence of a single nucleotide polymorphism (SNP) in a 3-kb region of chromosome Xq22.3, which contains a HERV-W Env locus (Garcia-Montojo et al., 2013). Previously, a multicentric study revealed higher levels of HERV-W particles in patients with inflammatory other neurological diseases (OND) compared to those suffering from ONDs without inflammatory components, indicating that HERV-W is especially involved in inflammatory processes (Arru et al., 2007). Intriguingly, HERV-W RNA and their encoded retroviral proteins Gag and Env were detected in brain tissues of MS patients (Perron et al., 2005, Mameli et al., 2007). It was further demonstrated that these retroviral proteins are, to a certain degree, physiologically expressed in human brain tissue, but especially the envelope protein was subsequently linked to mediate inflammatory processes. On cellular level, a physiological expression of HERV-W Env antigens were identified in leucocytes, microglia and endothelial cells of human brain tissues. However, in active MS plaques an elevated positivity for the HERV-W Env protein was detected, especially present in microglia and endothelial cells (Perron et al., 2005). These findings supported the already existing hypothesis that the HERV-W Env protein might exert harmful retroviral properties, thereby promoting the disease course of MS (Perron et al., 1992). Intriguingly, huge amounts of the HERV-W Env protein were detected in active MS lesions, correlating with the level of inflammation and demyelination (Mameli et al., 2007). Due to its obvious harmful contribution to the disease course of MS, the name of HERV-W Env protein was adapted to pathogenic HERV-W Env protein (pHERV-W Env protein). Furthermore, Rolland and colleagues deciphered that the pHERV-W Env protein binds with its surface unit (SU) to the PRR TLR4 and its co-receptor CD14, predominantly expressed on immune cells of the innate immunity, consequently inducing the activation of the NF-κB signalling pathway, which results in the release of a repertoire of proinflammatory molecules (Rolland et al., 2006). Moreover, it was demonstrated *in vitro* that pHERV-W Env protein mediated TLR4 activation resulted in the production of molecules associated with nitrosative stress and thereby efficiently inhibited differentiation and proliferation of OPCs (Kremer et al., 2013). This *in vitro* study provided further evidence regarding a possible pHERV-W Env protein mediated contribution to demyelinating processes in MS. To investigate effects *in vivo*, the experimental autoimmune encephalomyelitis (EAE) animal model is the most common used model to mimic the pathologic key features of MS. Here,

autoimmunity against myelin is induced by using the Freund's adjuvant and myelin peptides such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin/oligodendrocyte glycoprotein (MOG) 35-55 (Constantinescu et al., 2011). In this way, Perron and colleagues were able to induce TLR4 mediated EAE in mice via administration of MOG 35-55 and the pHERV-W Env protein instead of the Freund's adjuvant and provided a suitable *in vivo* model for MS using a stimulation agent which seems to trigger neuroinflammation and neurodegeneration in MS patients (Perron et al., 2013).

1.5 Temelimab and clinical trials

Temelimab, formerly termed GNbAC1, is a full-length humanized recombinant monoclonal antibody of the IgG4/kappa subclass, which was designed to specifically target the subunit (SU) of the pHERV-W Env protein (pHERV-W Env-SU) as well as its full length, pHERV-W Env-T, without destroying TLR4 expressing cells (Curtin et al., 2015). The first antibody against the pHERV-W Env protein was designed and tested in mice (mu-GNbAC1) and was shown to efficiently neutralize the observed induction of proinflammatory cytokines upon exposure. Based on these observations, a humanized form of temelimab was designed temporarily using chimeric forms, ch-GNbAC1-IgG1 and ch-GNbAC1-IgG4, which were tested regarding therapeutic efficacy in pHERV-W Env protein induced EAE in mice. The most promising results regarding survival of the animals and reduction of clinical symptoms were obtained for the IgG1 and IgG4 subtypes. A dose dependent mediated complement-dependent cytotoxicity was observed for the IgG1 but not for the IgG4 version, which consecutively resulted in the choice of the IgG4 version for designing the human variant of temelimab (Curtin et al., 2015). After administering temelimab in healthy mice without observing adverse effects (AEs) at doses of 30mg/kg, a first clinical phase I study (ClinicalTrials.gov: NCT01699555) was launched in 33 healthy male participants, who received a single intravenous injection of temelimab at different doses (highest 6mg/kg) or a placebo, which showed no AEs and a favourable pharmacokinetic profile of the antibody (Curtin et al., 2012). Next, a randomized double-blind placebo-controlled dose escalation clinical phase IIa study in 10 MS patients was launched, where participants firstly received intravenous infusions of temelimab at doses of 2 or 6 mg/kg or a placebo,

and secondly were again randomized to receive 2 or 6 mg/kg temelimab monthly for 11 months. This phase was followed by a 6-month open-label phase (ClinicalTrials.gov: NCT01639300). Interestingly, upon treatment with temelimab, transcripts of HERV-W decreased and 9 patients showed a stable lesion load on brain MRI scans (Derfuss et al., 2015). Consecutively, a clinical phase IIb study was launched including 270 RRMS patients (CHANGE-MS study (ClinicalTrials.gov: NCT02782858)). Participants intravenously received 6, 12 or 18mg/kg temelimab or a placebo in a 4-week-interval for 24 weeks and afterwards were re-randomized and intravenously received 6,12 or 18mg/kg temelimab for another 48 weeks. Results showed that temelimab was not able to significantly influence or diminish acute neuroinflammation at the highest administered dose, but seems to mediate decreasing neurodegenerative effects. Brain MRI scans revealed a reduction of brain atrophy, a decreasing magnetization transfer ratio (MTR), which is used to quantify the integrity of the myelinated white matter (Schmierer et al., 2004), and showed fewer new T1 hypointense lesions. These results were further corroborated by a follow-up study (ANGEL-MS (ClinicalTrials.gov: NCT03239860)) (Hartung et al., 2022). In a recent performed clinical study (ProTEct-MS (ClinicalTrials.gov: NCT04480307)), temelimab was administered to 41 relapsing MS patients with severe disease activity, in higher doses (up to 54mg/kg) and in combination with rituximab, which is known to diminish inflammation by eliminating CD20 expressing immune cells and is already used *off-label* for MS patients (Hauser et al., 2008, Brancati et al., 2021). The administered combination of both agents was well tolerated and a decrease of neurodegenerative features was again observed on brain MRI scans, which offers quite a promising approach to efficiently treating MS patients with severe disease courses in the future (GeNeuro press release, 2022/03/21). Interestingly, in all performed clinical trials it is not reported whether study participants were positively tested for the presence of the pHERV-W Env protein.

1.6. Aim of this study

Previous studies demonstrated that the pHERV-W Env protein is a strong agonist of the TLR4 receptor, resulting in the induction of the NF-κB signalling pathway (Rolland et al., 2006). Prior performed *in vitro* studies identified OPCs, based on

their TLR4 receptor expression, as target cells for the pHERV-W Env protein. Remarkably, the pHERV-W Env protein was shown to initiate nitrosative stress in OPCs, subsequently disrupting their differentiation capacities and was thereby identified as efficiently impairing remyelination processes, which are crucial for limiting axonal degeneration in MS (Kremer et al., 2013, Förster, 2020). Histological studies demonstrated the presence of the pHERV-W Env protein in CNS tissues of MS patients, predominantly occurring in myeloid cells/microglia (Perron et al., 2005) and in microglia neighbouring degenerated myelin sheaths and damaged axons (Kremer et al., 2019b). Besides, it is known that degenerated myelin sheaths, which are not efficiently removed, inhibit processes of remyelination (Kotter et al., 2006). These findings lead to the hypothesis that microglia, which express the TLR4 receptor, might represent yet another target for the pHERV-W Env protein within the CNS. Previous *in vitro* studies performed by other members of the research group indicated a microglial transformation towards a rather proinflammatory phenotype upon exposure to the pHERV-W Env protein, with an increase of microglia's production of proinflammatory cytokines in response (Sogorski, 2022). It is hypothesized, that microglia might play important roles in directly mediating effects of the pHERV-W Env protein and thereby might efficiently contribute to the pathogenesis of MS. The aim of this project was to investigate to what extent the pHERV-W Env protein influences microglia's behaviour and modulates central aspects of their cell functions. Next to the already postulated increase of microglia's repertoire of proinflammatory cytokines upon pHERV-W Env protein exposure, the question was addressed whether microglia's release of molecules promoting a neuro-supportive atmosphere is also affected upon contact. In addition, a central aspect of this project was to investigate whether the pHERV-W Env protein modulates microglia's phagocytosis rates specifically regarding the uptake of myelin – a key function, crucial for supporting remyelination processes. Finally, this project addressed the question whether abrogation of especially pHERV-W Env protein mediated proinflammatory effects on microglia is achievable. In this regard, another aim of this project was to determine if the observed microglial proinflammatory effects, mediated by the pHERV-W Env protein, can be neutralized by temelimab. For this purpose, primary microglia obtained from neonatal Wistar rats were used and several *in vitro* methods were performed, as described in detail in the following section.

2 Materials and Methods

2.1 Materials

2.1.1 Organisms

Species	Line	Supplier
Rattus norvegicus	Wistar	Janvier Labs, Saint-Berthevin, France

2.1.2 Laboratory equipment and software

Equipment	Supplier
7900HT Fast Real-Time PCR System	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Autoclave GLA30	Fritz Gössner GmbH, Hamburg, Germany
Axioplan 2 Fluorescence microscope	Carl Zeiss, Oberkochen, Germany
Axiovision 4.2 software	Carl Zeiss, Oberkochen, Germany
BBD 6220 CO_2 incubator	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Centrifuge	Heraeus Holding GmbH, Hanau, Germany
Endnote X7.7.1	Thomas Reuters, New York City, USA
Eppendorf Centrifuge 5804	Eppendorf, Wesseling-Berzdorf, Germany
Excella E24 Incubator shaker	New Brunswick Scientific, Nürtingen, Germany
Graphpad PRISM software 8.0.2	GraphPad Prism, San Diego, USA
Herasafe HSP 12 sterile bench	Heraeus, Hanau, Germany
ImageJ software	National Institute of Health, Rockville, USA
MACS MultiStand	Miltenyi Biotec, Bergisch Gladbach, Germany
MiniMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Minishaker MS2; vortexer	IKAR Works, Inc. Wilmington, USA
MS Office 2016	Microsoft Corporation, Redmond, USA
NanoDrop ND 1000	PeqLab, Erlangen, Germany
Primer Express 3.0.1	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Sequence Detection System (Version 2.3)	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Tecan i-control (Version 1.7.1.12)	Tecan, Männedorf, Switzerland
Tecan Microplate reader Infinite 200pro	Tecan, Männedorf, Switzerland

Thoma counting chamber (Depth: 0.100mm; Area: 0.0025 mm^2)	Optik Labor, Görlitz, Germany
Veriti thermocycler	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Water bath	GFL, Burgwedel, Germany

2.1.3 Chemicals/reagents, enzymes and media

Substance	**Supplier**
2-propanol	Merck, Darmstadt, Germany
4',6-Diamidino-2-phenylindole	Roche Diagnostic GmbH, Mannheim, Germany
Accutase	PAA Laboratories, Pasching, Austria
Albumin BSA-V	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
β-mercaptoethanol	Sigma-Aldrich Chemie GmbH, München, Germany
Purified bovine myelin	Lab intern production
Bovine serum albumin (BSA) Fraktion V 7.5%	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Rat-CD11b magnetic microbeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Citifluor mounting medium	Citifluor, London, UK
Dulbecco's modified eagle medium (DMEM); low glucose	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Dulbecco's modified eagle medium (DMEM); high glucose	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Dulbecco's modified eagle medium (DMEM); low glucose, -phenol red	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
DNase I	Cell Systems, Lakewood, USA
Dulbecco´s phosphate buffered saline (PBS)	Sigma-Aldrich, St. Louis, USA
Ethanol ≥ 96%	Merck, Darmstadt, Germany
Fetal calf serum (FCS)	Lonza, Basel, Switzerland
Isoflurane	Piramal-Healthcare, Mumbai, India
L-cystein	Sigma-Aldrich-Aldrich, St. Louis, USA
LiChrosolv® water	Merck, Darmstadt, Germany
Leibovitz's medium L-15	Sigma-Aldrich-Aldrich, St. Louis, USA
L-glutamine	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Normal goat serum	Sigma-Aldrich, St. Louis, USA
Normal donkey serum	Sigma-Aldrich, St. Louis, USA
Minimum Essential Medium	ThermoFisher, Scientific, Life Technologies, Darmstadt, Germany
Papain	Worthington / Cell Systems, Lakewood, USA
Paraformaldehyde	Merck, Darmstadt, Germany

Penicillin / Streptomycin	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
pHrodo™Red, succinimidyl ester	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
RNase-free water	Qiagen, Hilden, Germany
Triton X-100	Sigma-Aldrich-Aldrich, St. Louis, USA
Trypan blue 0.4%	Sigma-Aldrich-Aldrich, St. Louis, USA
Trypsin inhibitor	Sigma-Aldrich-Aldrich, St. Louis, USA

2.1.4 Consumables

Material	**Supplier**
Cell culture plates: 6 well, 24 well, 96 well, 100 mm Ø petri dish	Greiner Bio-One GmbH, Frickenhausen, Germany
Culture flasks, 75 cm^2	Sarstedt AG und Co. KG, Nümbrecht, Germany
0.2 µm filtropur S, filter	Sarstedt AG & Co. KG, Nümbrecht, Germany
MicroAmp®Fast 96-well Reaction Plate (0.1mL)	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Micro-reaction tubes 0.5 ml 1.5 ml 2.0 ml	Sarstedt AG und Co. KG, Nümbrecht, Germany
MACS® Columns, type MS	Miltenyi Biotec, Bergisch Gladbach, Germany
Pipette tips / Stripettes 10 µl 10/20 µl 100 µl 200 µl 1000 µl 5 ml 10 ml 25 ml	• Eppendorf GmbH, Wesseling-Berdorf, Germany • Gilson, Limburg-Offheim, Germany • Greiner Bio-One GmbH, Frickenhausen, Germany
Pasteur pipettes	Brand GmbH & CO., Wertheim, Germany
Tubes 5 ml 15 ml 50 ml	Sarstedt AG und Co. KG, Nümbrecht, Germany
X-well cell culture chamber, 2-well, on PCA slide, removable frame	Sarstedt AG und Co. KG, Nümbrecht, Germany
X-well cell culture chamber, 8-well, on PCA slide, removable frame	Sarstedt AG und Co. KG, Nümbrecht, Germany

2.1.5 Kits

Kit	Supplier
RNeasy Mini Kit	Qiagen, Hilden, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
High-Capacity cDNA Reverse Transcription Kit	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
SYBR Green Master Mix	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Nitric Oxide Assay Kit, Colorimetric	Calbiochem®, EMD Biosciences, Inc., Darmstadt, Germany
Quantikine® ELISA Mouse/Rat IGF-1	R&D Systems,Inc., Minneapolis, USA

2.1.6 Antibodies

Antibody	Dilution	Supplier
Anti-APRIL polyclonal rabbit antibody (ab 189263)	1:500	Abcam, Cambridge, UK
Anti-Iba1 polyclonal goat antibody (ab 5076)	1:500	Abcam, Cambridge, UK
Anti-iNOS polyclonal rabbit antibody (ab 15323)	1:250	Abcam, Cambridge, UK
Anti-Ki67 monoclonal rabbit antibody (ab16667)	1:250	Abcam, Cambridge, UK
Anti-cleaved caspase-3 polyclonal rabbit antibody (#9661)	1:400	Cell Signaling Technology, Massachusetts, USA
Goat anti rabbit Alexa Flour 488	1:500	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Goat anti rabbit Alexa Flour 594	1:500	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Donkey anti rabbit Alexa Flour 488	1:500	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany
Donkey anti goat Alexa Flour 594	1:500	ThermoFisher Scientific, Life Technologies, Darmstadt, Germany

2.1.7 Primer for qRT-PCR analysis

Name	Sequence foward	Sequence reverse
r-ODC	GGT TCC AGA GGC CAA ACA TC	GTT GCC ACA TTG ACC GTG AC
r-TNFα	AGC CCT GGT ATG AGC CCA TGT A	CCG GAC TCC GTG ATG TCT AAG T

r-iNOS	CTC AGC ACA GAG GGC TCA AAG	TGC ACC CAA ACA CCA AGG T
r-IL 6	GTT GTG CAA TGG CAA TTC TGA	TCT GAC AGT GCA TCA TCG CTG
r-IL1β	GAA ACA GCA ATG GTC GGG AC	AAG ACA CGG GTT CCA TGG TG
r-TREM2	CCA AGG AGC CAA TCA GGA AA	GGC CAG GAG GAG AAG AAT GG
r-MerTK	TCT GAC AGA GAC CGC AGT CTT C	TGG ACA CCG TCA GTC CTT TG
r-IGF-1	AGA CGG GCA TTG TGG ATG A	ACA TCT CCA GCC TCC TCA GAT C
r-FGF 2	TGG TAT GTG GCA CTG AAA CGA	CCA GGC CCC GTT TTG G
r-CSF 1	CGA GGT GTC GGA GCA CTG TA	TCA ACT GCT GCA AAA TCT GTA GGT

2.2 Composition of cell culture media and other reagents

2.2.1 Cell culture media and reagents

Media	**Contents and concentrations**
Microglia cell culture medium	• Dulbecco's modified eagle medium (DMEM); low glucose • FCS (10%) • L-Glutamine (2 mM) • Penicillin/Streptomycin (50 U/ml)
Microglia cell culture medium for NO Assay	• Dulbecco's modified eagle medium (DMEM); low glucose, -phenol red • FCS (10%) • L-Glutamine (2 mM) • Penicillin/Streptomycin (50 U/ml)
Mixed rat brain culture medium	• Dulbecco's modified eagle medium (DMEM); low glucose • FCS (10%) • L-Glutamine (2 mM) • Penicillin/Streptomycin (50 U/ml)
Trypsin inhibitor medium	• Leibovitz's medium L-15 • Trypsin inhibitor (0,1mg/ml) • Albumin BSA-V (5 mg/ml) • DNase I (0,4 µg/ml)
Digestion medium	• 2% DNase I (40 µg/ml) • 20% L-cystein (0,24 mg/ml) • 10% papain (30 U/ml)

2.2.2 Immunocytochemistry

Reagent	Contents and concentration
Paraformaldehyde	• Dulbecco´s phosphate buffered saline (PBS) • PFA (4%)
Blocking solution I	• Dulbecco´s phosphate buffered saline (PBS) • Normal goat serum (NGS, 2%) • Triton X-100 (0,5%)
Blocking solution II	• Dulbecco´s phosphate buffered saline (PBS) • Normal donkey serum (NDS, 10%) • Triton X-100 (0,5%)
Antibody solution I	• Dulbecco´s phosphate buffered saline (PBS) • Normal goat serum (NGS, 2%) • Triton X-100 (0,1%)
Antibody solution II	• Dulbecco´s phosphate buffered saline (PBS) • Normal donkey serum (NDS, 10%) • Triton X-100 (0,2%)

2.3 Reagents for stimulation of microglial cells

Reagent	supplier
Recombinant HERV-W Env-T protein	GeNeuro, Plan-les-Ouates, Switzerland
HERV-W Env-T buffer	GeNeuro, Plan-les-Ouates, Switzerland
Anti-pHERV-W Env antibody GNbAC1	GeNeuro, Plan-les-Ouates, Switzerland
GNbAC1 buffer	GeNeuro, Plan-les-Ouates, Switzerland
Polymyxin B (PMXB)	InvivoGen, California, USA
Soluble IL-4	R&D Systems, Minneapolis, USA
MACS buffer	• Dulbecco´s phosphate buffered saline (PBS), Sigma-Aldrich, St. Louis, USA • Bovine serum albumin (BSA) Fraktion V (0,5%), ThermoFisher Scientific, Life Technologies, Darmstadt, Germany

2.3.1 Composition of the pHERV-W Env protein and control buffer

pHERV-W Env is a recombinant protein consisting of 548 amino acids (61,44 kDa) produced in BL21 Escherichia coli under defined sterile conditions by PX'Therapeutics, Grenoble, France. For all experiments the batch of May 2016 was used (stock concentration 2,54 mg/ml; provided by GeNeuro SA). Endotoxin levels were found to be under the detection limit of <5EU/ml (Kremer et al., 2013). Upon reception, parts of the recombinant pHERV-W Env stock solution were diluted with buffer, comprising of Tris-HCl 20 mM pH 7.5, NaCl 150 mM, SDS 1.5% and DTT 10 mM, to a concentration of 1000 ng/µl and both, aliquots and stock solution were stored at -80°C until use. Treatments with exclusively buffer served as controls.

2.3.2 Composition of the antibody GNbAC1 (temelimab) and control buffer

The recombinant monoclonal antibody GNbAC1 was produced in epithelial cell lines from Chinese hamster ovary cells (CHO) under defined sterile conditions at Polymun, Klosterneuburg, Austria. The antibody and its dilution buffer were provided by GeNeuro SA, Plan-les-Ouates, Switzerland. The delivered stock solution had a concentration of 50 mg/ml diluted in a buffer comprising of 20 mM histidine, 5% (w/v) sucrose and 0.01% (w/v) polysorbate 20 at pH 6.0. The buffer alone was used as a control. Upon reception GNbAC1 and its buffer were stored at 4°C until use.

2.4 Methods

2.4.1 Cell culture methods

2.4.1.1 Preparation of primary rat microglia

Primary rat microglia were obtained from a mixed brain cell culture according to a protocol initially described by McCarthy and de Vellis in 1980 (McCarthy and de Vellis, 1980). For this purpose, cortices of new-born Wistar rats (P0 and P1) were used. Animals were bred under defined conditions and were conducted under the file number O69/11 in the Animal Research Institute of the Heinrich-Heine-University Düsseldorf, Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben (ZETT). All animal procedures were accomplished in

consensus with the experimental guidelines approved by the regional authorities and ZETT. Preparation and purification of the cortices were performed by laboratory technicians. After isoflurane anaesthesia and decapitation, skin and skull were removed and brains were collected in MEM-HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) medium. Afterwards, brain hemispheres were separated followed by removal of meninges. The remaining cortices were cut into small pieces which were collected in centrifuge tubes containing 50 ml MEM-HEPES medium and spun down at 2.000 rpm for 1 min. Supernatants were aspirated and 1 ml of digestion medium was added followed by an incubation at 37°C, in 5% CO_2 and 98% humidity for 45 min to achieve a proper digestion and dissociation of the tissue. Subsequently, 1 ml of trypsin inhibitor medium was added to stop digestion followed by an incubation at RT for another 5 min. Next, the cell suspension was carefully mixed using glass pasteur pipettes. Afterwards, 10 ml of DMEM high glucose medium containing 10% FCS were added and cell suspension was again centrifuged at 1.200 rpm for 5 min. After discarding the supernatant, cells were resuspended in 15 ml mixed rat brain culture medium and seeded in T75 cell culture flasks finally containing cells derived from cortices of two new-born Wistar rats. Cells were incubated at 37°C, 5% CO_2 and 98% humidity for 10 d and medium was changed twice a week. Afterwards, flasks were placed on a shaker (Excella E24 incubator) at 180 rpm and 37°C for 2 h. Next, supernatants mainly including detached microglia were collected, transferred to a centrifuge tube, and spun down at 1.200 rpm for 5 min. Pooled cells were resuspended in 1 ml mixed rat brain culture medium and added to 9 ml mixed rat brain culture medium on a 10 cm bacterial dish. Detached microglial cells were pooled on the same bacterial dish to diminish loss of cells and were incubated at 37°C, 5% CO_2 and 98% humidity overnight. Remaining flasks predominantly including adherent OPCs and astrocytes were refilled with 10 ml mixed rat brain culture medium and replaced on the shaker at 180 rpm and 37°C for another 22 h. At the next day, supernatants containing large amounts of OPCs and to a lower degree astrocytes and microglia were transferred to 10 cm bacterial dishes. Within an incubation time of 20 min at 37°C, 5% CO_2 and 98% humidity, microglia attached to dish surfaces whereas OPCs and astrocytes remained in the supernatants. After removing the supernatant, attached microglia were incubated with 10 ml mixed rat brain culture at 37°C, 5% CO_2 and 98% humidity for another 2 h. In sum, microglia were obtained from these two

independent shakes (2 h and 22 h) and were pooled to receive maximum amount of cells for the following sorting procedure.

2.4.1.2 Sorting of microglia via magnetic cell sorting

All following steps were performed under sterile conditions. For microglia sorting the magnetic cell sorting system (MACS system) from Miltenyi Biotech was used. It consists of MACS buffer, CD11b/c conjugated magnetic microbeads and a strong magnetic field, the MACS separator. Since the sorting procedure is time-consuming, all media and buffers were kept on wet ice to protect cells from apoptosis. Initially, medium was removed from microglial cell cultures and cells were washed once with PBS. Afterwards, 3 ml accutase were added to each dish following an incubation at 37°C, 5% CO_2 and 98% humidity for 5 min to detach microglial cells from dish surfaces. The enzymatic reaction was stopped using 4 ml of microglial cell culture medium per dish and cell suspension was immediately collected and transferred to 50 ml tubes. Next, cell suspension was centrifuged at 1.200 rpm, 4°C for 5 min. Supernatants were discarded and cells were carefully resuspended in 80 µl MACS buffer. Afterwards, 20 µl CD11b/c conjugated magnetic microbeads were added and cells were incubated at 2-8°C for another 15 min. Subsequently, 2 ml MACS buffer were added, and cell suspension was spun down at 1.200 rpm, 4°C for 5 min. Supernatants were discarded and cells were resuspended in 500 µl MACS buffer. Accordingly, the MACS separator was prepared by placing MS-columns into the magnetic field and received 500 µl MACS buffer. Hereafter, cells were added to the columns. After three washing steps with 500 µl MACS buffer each, columns were removed from the magnetic field and placed into centrifugation tubes. By adding 1 ml MACS buffer the magnetic labelled microglial cells were immediately pressed through the column, spun down at 1.200 rpm, 4°C for 5 min and resuspended in 500 µl MACS buffer. To achieve a high purity of microglial cell cultures the magnetic separation was repeated once. Microglia of all columns were pooled in one centrifugation tube. A last centrifugation step was performed at 1.200 rpm, 4°C for 5 min and supernatant was discarded. The cell pellet was resuspended in 1 ml microglia cell culture medium (prewarmed at 37°C). To count vital microglia, 10 µl of the cell suspension were stained with 10 µl trypan blue and microglia were counted using a Thoma counting chamber. Finally, microglia were seeded and used

for different experiments after an incubation at 37°C, 5% CO_2 and 98% humidity for 1d. A purity of 98% of microglial cell cultures was obtained as assessed by Iba1 staining and specified as a percentage related to the total cell number (as visualized via 4',6-diamidino-2phenylindole (DAPI) staining, data not shown).

2.4.1.3 Treatment of microglia

Cells were stimulated according to already established protocols of the research group (Kremer et al., 2019b). To this end, soluble recombinant pHERV-W Env protein (or control buffer in parallel) was diluted in microglial cell culture medium to a concentration of 1000 ng/ml. Subsequently, media was changed, and stimulated cells were placed back into the incubator at 37°C, 5% CO_2 and 98% humidity for 1-3 d.

For pHERV-W Env protein neutralizing experiments the humanized monoclonal antibody GNbAC1 (temelimab) was used. Based on previous neutralizing experiments with OPCs (Kremer et al., 2015), the stimulation protocol was adapted to microglia. In a first experimental setup, soluble pHERV-W Env protein was incubated with GNbAC1 in autoclaved baker glasses, which provide suitable binding conditions. Four autoclaved baker glasses were prepared and 100 µl sterile filtrated FCS were added to each glass. Next, a combination of the following agents was added: a) control $buffer_{pHERV\text{-}W\ Env\ protein}$ and control $buffer_{GNbAC1}$, b) control $buffer_{pHERV\text{-}W\ Env\ protein}$ and GNbAC1, c) control $buffer_{GNbAC1}$ and pHERV-W Env protein and d) pHERV-W Env protein and GNbAC1. In accordance with previously performed experiments in OPCs, a ratio of GNbAC1 and pHERV-W Env protein of 30:1 was used here as this ratio seems to be sufficient for an appropriate neutralization reaction (Kremer et al., 2015). The combination of both buffers (condition a) was used as negative control. First, 6 µl GNbAC1 antibody and control buffer were added to the FCS up to a concentration of 600 µg/ml. Then, 2µl pHERV-W Env protein and control buffer were added to the suspensions up to a final concentration of 20 µg/ml and then mixed gently. Hereafter baker glasses were covered with aluminium foil following an incubation at RT for 1 h. In the meantime, four reaction tubes were prepared with 1 ml microglial cell culture medium. Prepared antibody and protein combinations were mixed again and 55.5 µl of each condition

were added to the corresponding reaction tube up to a concentration of 1000 ng/ml soluble pHERV-W Env protein and 30 µg/ml GNbAC1. Finally, culture media of microglia was changed and cells were stimulated with the corresponding mixes. Stimulated cells were replaced into the incubator at 37°C, 5% CO_2 and 98% humidity for 1d. In addition, stimulation procedures were modified in parallel to the initial experiments described above.

1. A higher ratio of GNbAC1 to pHERV-W Env protein (150:1) was used to increase neutralization rates.
2. Possible LPS residues of the used pHERV-W Env protein were blocked by using polymyxin B (PMXB) – an endotoxin-binding polypeptide. PMXB was added to each baker glass in a concentration of 10 µg/ml. Again, a ratio of GNbAC1 to pHERV-W Env protein of 150:1 was used. Except for the addition of PMXB all stimulation steps remained the same.
3. As the used FCS might interact with the pHERV-W Env protein possibly inhibiting a successful binding to GNbAC1, microglia were incubated with lower FCS concentrations (0.5%). All other steps of the neutralization and stimulation procedure were the same as described above. Again, a ratio of GNbAC1 to pHERV-W Env protein of 150:1 was used.

condition		setup 1	setup 2	setup 3	setup 4
a	control $buffer_{pHERV\text{-}W\ Env\ protein}$ and control $buffer_{GNbAC1}$	ratio GNbAC1 to pHERV-W Env protein 30:1	ratio GNbAC1 to pHERV-W Env protein 150:1	blocking possible LPS residues using PMXB ratio GNbAC1 to pHERV-W Env protein 150:1	lower FCS (0,5%) instead of FCS (10%) ratio GNbAC1 to pHERV-W Env protein 150:1
b	control $buffer_{pHERV\text{-}W\ Env\ protein}$ and GNbAC1				
c	control $buffer_{GNbAC1}$ and pHERV-W Env protein				
d	pHERV-W Env protein and GNbAC1				

Table 4: Overview of different experimental setups of pHERV-W Env protein neutralizing experiments using the monoclonal antibody GNbAC1.

In parallel to pHERV-W Env protein neutralizing experiments, stimulation experiments were carried out using recombinant soluble IL-4. According to the manufacturer's protocol, lyophilized IL-4 was diluted to a concentration of 50µg/ml using sterile PBS and 0,5% BSA. Hereafter, parts of the stock solution were further diluted using sterile PBS and 0,5% BSA to a concentration of 20 ng/ml and both, aliquots and the stock solution were stored at -80 C° until use. The combination of sterile PBS and 0,5% BSA (dilution buffer) was used as a control. For IL-4 stimulation procedures a concentration of 40 ng/ml was used. Cells were stimulated as followed: a) control buffer$_{\text{pHERV-W Env protein}}$ and control buffer$_{\text{IL-4}}$, b) control buffer$_{\text{pHERV-W Env protein}}$ and IL-4, c) control buffer$_{\text{IL4}}$ and pHERV-W Env protein, d) pHERV-W Env protein and IL-4. The combination of both buffers (condition a) was used as negative control. After stimulation microglia were placed back into the incubator at 37°C, 5% CO_2 and 98% humidity for 1 d.

2.4.2 Immunocytochemistry

For immunocytochemistry, removable PCA lab tek chamber slides comprising of 8 separated chambers were used. Therefore 50.000 microglia were seeded per chamber (0.8 cm^2) and stimulated with 300 µl of the above-described solutions. For single stimulation experiments, four chambers and for neutralizing experiments two chambers were used per condition, respectively. After an incubation of 1 d or 3 d medium was discarded, cells were washed once with PBS and fixed with 4% paraformaldehyde (PFA) at RT for 10 min. Hereafter, PFA solution was removed and cells were washed three times with PBS. Subsequently, fixed cells were permeabilized and blocked with blocking solution I at RT for 40 min. For co-staining with anti-iNOS or anti-APRIL and anti-Iba1 antibodies blocking solution II was used. Next, blocking solution was carefully aspirated and primary antibodies diluted in antibody solution I were added to the cells. Antibody solution II was used for staining with anti-iNOS or anti-APRIL and anti-Iba1. Primary antibody solutions were then added to the cells and incubated at 4°C over night. Then, antibody solutions were discarded and cells were carefully washed three times with PBS. Cells were next incubated with fluorophore conjugated secondary antibodies (1:500) diluted in PBS, additionally supplemented with 4',6-diamidin-2phenylindol (DAPI; 1:100) to counterstain cell nuclei at RT for 2 h protected from light. Afterwards, secondary

antibody solution was discarded, and cells were washed again with PBS for three times. Finally, walls of cell culture chambers were removed and the remaining microscope slide was embedded using Citiflour mounting medium. Staining was visualized using an Axioplan 2 fluorescence microscope (Zeiss) and Axiovision 4.2 software (Zeiss). Marker expression analyses were performed by counting 9 representative and randomly chosen fields per chamber. The total cell number per field was determined by counting DAPI positive cell nuclei. For quantification, numbers of positive cells were counted related to the total cell number and expressed as percentage.

2.4.3 Myelin phagocytosis assay

For myelin phagocytosis assays, 100.000 microglia were seeded into one well of a two-well lab tek chamber slide (4.4 cm^2) and subsequently stimulated with pHERV-W Env protein for 1 d. The experimental design was adapted from a protocol published by Healy and colleagues in 2016 (Healy et al., 2016). Briefly, 1 mg of bovine myelin was solved in 1 ml PBS at pH 8. Next, 10 µl pHRodo were added and incubated on a shaker at RT for 1 h protected from light. After centrifugation at 1.500 rpm for 10 min the pHRodo labelled bovine myelin pellet was resuspended in 1 ml fresh PBS at pH 8 and diluted to a concentration of 20 µg/ml. A volume of 20 µl myelin/pHRodo/PBS suspension was then added to pHERV-W Env protein pre-stimulated microglia and incubated for another 3 h. Subsequently, cells were gently washed three times with pre-warmed PBS and fixed with 4% PFA at RT for 10 min. After another washing step with PBS, nuclei were counterstained with DAPI (1:100). Pictures of 9 randomly chosen fields per chamber were taken at 10x magnification using the Axioplan 2 fluorescence microscope (Zeiss) and Axiovision 4.2 (Zeiss) software. Quantitative analyses were carried out by counting the total number of DAPI positive cell nuclei (blue). The counted number of pHRodo labelled myelin positive cells (red) was related to the number of DAPI positive nuclei and was expressed as percentage.

2.4.4 Extraction of ribonucleic acid

The extraction of RNA from cultured microglial cells was accomplished using the RNeasy Mini Kit from Qiagen according to the manufacturer's protocol. For this purpose, a 6 well cell culture plate was used with 500.000 microglia per well (9.6 cm^2) in duplicates for each condition and timepoint. Stimulated microglial cells were removed from the incubator and media was discarded. For lysis of microglial cell cultures 350 µl RLT lysis buffer containing 0,1 M β-mercaptoethanol (1:100) was used per condition and cells were mechanically removed from the wells via scratching with a pipette tip. To receive a homogenous cell extract, samples were transferred to QIAshredder and immediately centrifuged at 14.000 rpm, 4°C for 2 min. Next, eluates were diluted with 350 µl of 70% ethanol and transferred to RNeasy columns. Subsequently, columns were again centrifuged at 10.000 rpm, 4°C for 2 min. Eluates were discarded and a washing step with 350 µl of RW1 buffer was conducted. After another centrifugation step at 10.000 rpm, 4°C for 2 min, genomic DNA was removed using 80 µl of RNAse free DNAse buffer consisting of 10 µl DNAse and 70 µl RDD buffer. After 15 min of incubation at room temperature, 350 µl RW1 buffer were added and columns were centrifuged again at 10.000 rpm, 4°C for 2 min. To get rid of residual ethanol two washing steps were performed using 500 µl RPE buffer. Between these washing steps another centrifugation step was conducted. Eluates were discarded each time. Afterwards, 21 µl of RNAse free water were applied to each column, incubated for 3 minutes at RT and centrifuged at 10.000 rpm, 4°C for 2 min. Finally, the obtained levels of RNA were measured for each sample using the NanoDrop ND 1000. The RNA containing eluates were either stored at -80°C or directly reverse-transcribed into complementary deoxyribonucleic acid (cDNA).

2.4.5 Synthesis of cDNA

cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit according to the manufacturer's protocol (ThermoFisher). For every sample, a total of 250 ng of microglial RNA were applied. Therefore, 10.2 µl of cDNA master mix (table 2) were added to 19.8 µl RNA solution up to a total volume of 30 µl. All samples were placed into a thermocycler and cDNA synthesis was performed

following a defined heating program. Synthesis starts with a first step at 25°C for 10 min, followed by a second step at 37°C for 120 min and a terminal step at 85°C for 5 s. Finally, 170 µl of LiChrosolv® water were added to each sample and used for quantitative real-time polymerase chain reaction (qRT-PCR), respectively.

component	volume [µl]
10x RT buffer	3
10x RT random primers	3
RNase inhibitor, 20 U/ml	1,5
reverse transcriptase, 50 U/µl	1,5
dNTPs, 100 mM	1,2
total volume	**10,2**

Table 5: cDNA master mix: components and their used volumes for one sample

2.4.6 Quantitative real-time PCR

qRT-PCR was carried out using Power SYBRGreen universal master mix (ThermoFisher), on the ABI 7900 Fast-Real-Time sequence detection system. All samples were prepared according to the manufacturer's protocol listed below. Each oligonucleotide primer was used at a concentration of 15 pmol. Briefly, for each sample 16 µl of Power SYBRGreen universal master mix supplemented with oligonucleotide primers (table 3) were added to 4 µl diluted cDNA resulting in a total volume of 20 µl. Reagents were kept on ice. All cDNA samples were measured in duplicates. For measurements a MicroAmp®Fast 96-well reaction plate and a defined amplification profile was used. After activating the polymerase in a first step at 50°C for 2 min, denaturation was initiated to remove dimers in a second step at 95°C for 10 min. Subsequently, 40 cycles were performed consisting of 1 min at 60°C and 15 s at 95°C. Initially, sequence specific oligonucleotides anneal to single stranded DNA and are extended into double stranded DNA using the polymerase. In a next step, the produced double stranded DNA is denatured, and a next identical cycle is initiated. The number of generated double stranded amplicons is measured via intercalation of SYBRGreen dye. SYBRGreen dye ties to every single produced double stranded DNA amplicon resulting in an increase of the emitted intensity of fluorescence which is proportional to the produced number of double stranded amplicons. Ornithine decarboxylase (ODC) was used as the endogenous reference

gene and relative gene expression levels were calculated using the comparative cycle threshold method (ΔΔCt) (ThermoFisher Scientific, Applied Biosystems, Darmstadt, Germany). Results were normalized to control buffer treated cells at 1 d.

component	volume [µl]
oligonucleotide forward primer	1,2
oligonucleotide reverse primer	1,2
LiChrosolv® water	3,6
SYBRGreen master mix	10
total volume	**16**

Table 6: SYBRGreen universal master mix components and their used volumes for one sample

2.4.7 Nitric oxide colorimetric assay

For nitric oxide colorimetric assay, 50.000 microglia were seeded per well of a 96 well plate (0.34 cm^2) and stimulated with soluble recombinant pHERV-W Env protein and incubated for 1 d. For later detection culture medium without phenol red was used (50 µl per well). To be able to detect NO levels in cell culture supernatants using the Nitric Oxide Assay Kit (Calbiochem®), it was considered to use maximal cell numbers per well versus minimal amounts of media. Microglia were stimulated in triplicates per condition, supernatants were pooled and afterwards measured in duplicates. All steps of the assay were performed according to the manufacturer's protocol. As NO is rapidly converted to nitrate and nitrite in aqueous solutions, measurement of both molecules is necessary. To get reliable results of the overall produced NO, in a first step nitrate must be reduced to nitrite accomplished by the enzyme nitrate reductase. In a second step quantification of nitrite is visualized using the Griess Reagent which consists of an aniline derivative and a coupling agent. Thus, nitrite containing samples transform to a red to pink colour according to nitrite concentrations. Standard solutions were prepared according to the manufacturer's protocol to have a detection range from 0 µM to 100 µM NO. Next, 85 µl standard or sample were applied to each well of a 96-well plate. After adding 10 µl of nitrate reductase to each well, 10 µl of 2 mM NADH were added and the plate was placed on a shaker at room temperature for 20 min. Hereafter, 50 µl of Color Reagent #1 were applied and after a brief shake 50 µl of Color Reagent #2

were added to each well. Again, the plate was placed on a shaker at RT for 5 min protected from light. Then, absorbances were measured via Infinite M200 Pro (Tecan) microplate reader at 540 nm and quantification of NO was analysed via its software Tecan i-control (version 1.7.1.12). Next, data were transferred to Microsoft Excel (MS office 2016) and a linear standard absorption-concentration curve (0 µM to 100 µM) was calculated. Finally, concentration of NO in µM of each sample was determined by interpolation from the received standard curve.

2.4.8 Determination of IGF-1 concentrations by enzyme-linked immunosorbent assay

Determination of IGF-1 concentrations in cell culture supernatants were carried out using the Mouse/Rat IGF-1 Quantikine® ELISA Kit (R&D Systems) according to the manufacturer's protocol. The kit uses the quantitative sandwich enzyme immunoassay technique. Primarily, a monoclonal antibody specific for mouse/rat IGF-1 is pre-coated onto a microplate and IGF-1 which is present in cell culture supernatants ties to the immobilized antibody. Next, an enzyme linked polyclonal antibody specific for mouse/rat IGF-1 is applied and binds to the immobilized antibody-IGF-1 complex. After adding a substrate solution, provided by the manufacturer, the enzyme reaction shows a blue product or when stop solution is added a yellow product whose colour intensity proportionally represents IGF-1 amounts. A 24-well plate was used, and 300.000 microglia were seeded per well (1.9 cm^2). Again, cells were stimulated with soluble recombinant pHERV-W Env protein or in combination with IL-4 as described above and incubated for 1 d. To enable determination of microglia secreted IGF-1 levels in cell culture supernatants, maximal cell numbers versus minimal volumes of stimulation media were used. Thus, media volume was limited to 300 µl per well. After microglial cells were stimulated in duplicates, cell culture supernatants were pooled per condition and samples were stored at -80°C until use. All samples were measured in triplicates. Briefly, standard solutions were prepared to have a detection range of IGF-1 from at least 0 pg/ml to 2.000 pg/ml. According to the manufacturer's protocol, 50 µl of Calibrator Diluent RD5-38 were applied to each well. Next, 50 µl of samples or standards were added and incubated on a microplate shaker at RT for 2 h. After a washing step, 100 µl of mouse/rat IGF-1 conjugate was applied to each well and

again an incubation was accomplished on a microplate shaker at room temperature for 2 h. Subsequently, another washing step was done followed by application of 100 µl of substrate solution which was incubated at RT for 30 min protected from light. Afterwards, 100 µl stop solution were added and optical density of each well was determined within 30 min via Infinite M200 Pro (Tecan) microplate reader at 450nm. For precise determination of IGF-1 levels a second measurement was accomplished at 570 nm and readings were subtracted. Quantification of IGF-1 was analysed via the software Tecan i-control (version 1.7.1.12). Data were transferred to Microsoft Excel (MS office 2016) and a concentration-density standard curve (0 pg/ml to 2.000 pg/ml) was calculated. The concentration of IGF-1 in pg/ml of each sample was calculated with reference to the standard curve.

2.4.9 Statistics

Data are presented as mean values ± standard error of the mean (SEM). Graphs and statistical analysis were performed using Excel and the GraphPad Prism 8.0.2 software (GraphPad Prism, San Diego, CA, RRID:SCR_002798). All datasets passed the Shapiro-Wilk normality for Gaussian distribution. Two-sided Students t-test was applied for comparing two groups and one-way or two-way analysis of variance (ANOVA) with Tukey post-test for multiple comparisons was applied comparing three or more groups. Statistical significance thresholds were set as follows: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ and ns = not significant. “n” represents the number of independent experiments.

3 Results

3.1 pHERV-W Env protein induces proinflammatory gene expression and nitrosative stress in microglia

Previous experiments indicated that cultured primary rat microglia transform towards a proinflammatory phenotype upon pHERV-W Env protein exposure. To reproduce and analyse the effect of pHERV-W Env protein in more detail, microglia cultures were stimulated with recombinant pHERV-W Env protein (1000 ng/ml) for 1 d and 3 d and relative gene expression levels of proinflammatory genes were analysed by qRT-PCR. The obtained results showed a strong induction of the proinflammatory genes TNFα, iNOS, IL-6, and IL-1β after 1 d of pHERV-W Env protein exposure compared to buffer controls which decreased after 3 d (Fig. 1 A-D). These results confirmed the initial experiments performed by other members of the research group (Sogorski, 2022). Based on the qRT-PCR results, although not statistically significant for iNOS, it was of interest to investigate whether microglia secrete molecules which are involved in nitrosative stress and known to be harmful to OPCs and neuronal axons (Kremer et al., 2013, Kremer et al., 2019b) upon exposure to the protein. To this end, microglia were stimulated with pHERV-W Env protein for 1 d and afterwards the NO concentration was measured in cell culture supernatants. The NO concentration was significantly higher after pHERV-W Env protein stimulation compared to control stimulation (Fig. 1 E). Additionally, immunofluorescent staining for iNOS was performed to further confirm the presence of the NO catalysing enzyme (Fig. 2). Therefore, microglia were exposed to pHERV-W Env protein for 1 d and 3 d. To clearly identify iNOS positive cells as microglia, cells were counterstained for Iba1. For quantification, only cells positive for Iba1 were accepted. Quantitative analysis revealed a significant increase of iNOS positive microglia exposed to pHERV-W Env protein, whereas no iNOS positive microglia were detectable upon exposure to the buffer control (Fig. 2 A). The obtained results confirmed the already measured relative gene expression levels of iNOS (Fig. 1 B).

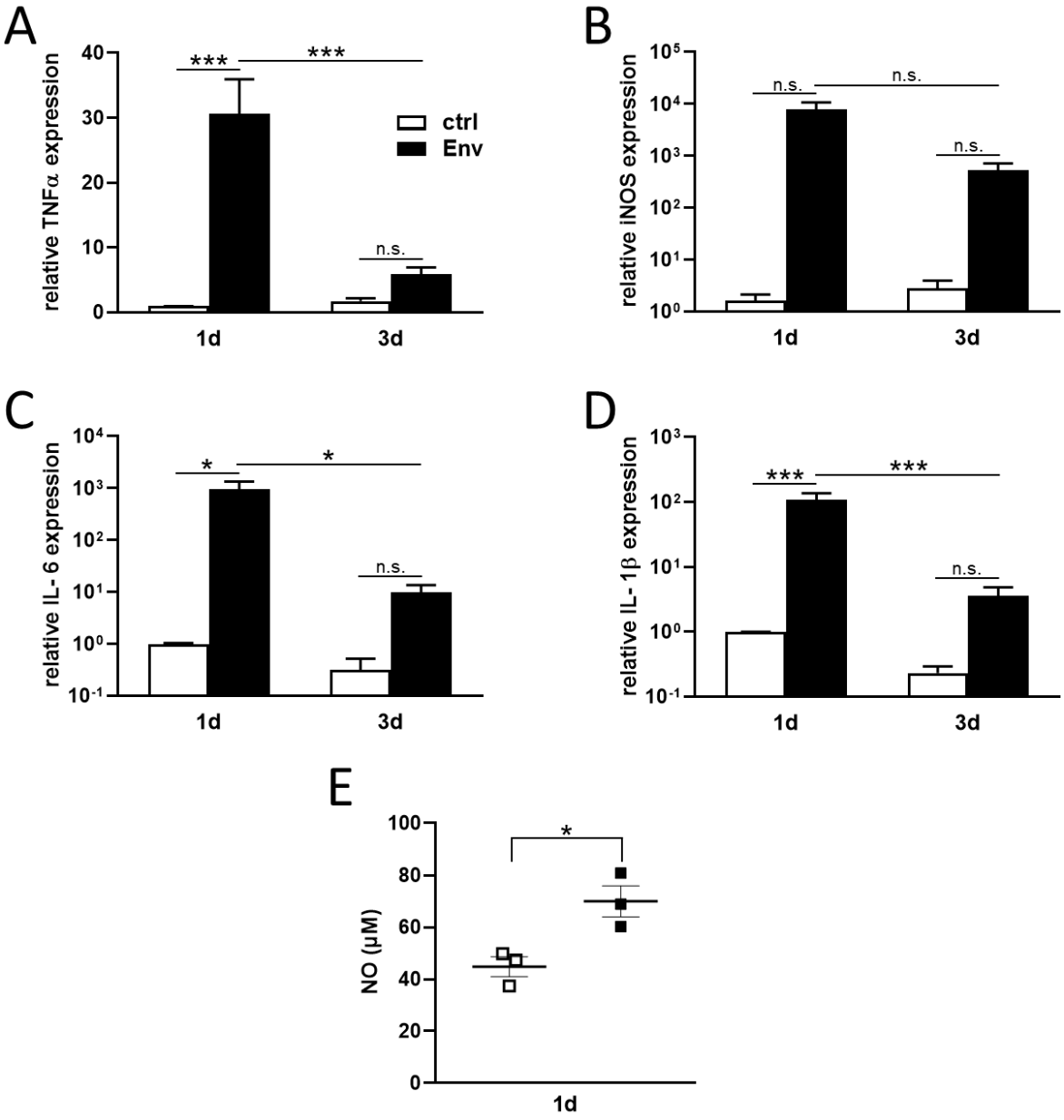

Fig. 1: Proinflammatory gene expression profiles and spectrometric analysis of microglial NO secretion upon pHERV-W Env protein exposure.

(A-D) qRT-PCR analysis of relative (A) TNFα, (B) iNOS, (C) IL-6, and (D) IL-1β transcript levels upon microglial exposure to buffer control and pHERV-W Env protein (1000 ng/ml) for 1 d and 3 d. Results showed an increased expression of all genes at day 1 which decreased over time but was still present at day 3. Results were normalised to buffer controls at day 1. (E) Spectrometric analysis of NO production in cell culture supernatants of buffer control and pHERV-W Env protein stimulated microglial cells after 1 d showed a significant increase of NO production upon pHERV-W Env protein exposure. Data are mean values ± SEM from n=6 independent experiments (A-D) and n=3 independent experiments (E). (A-D) Two-way ANOVA with Tukey post-test: *p≤0.05, ***p≤0.001, n.s. not significant; (E) Student's T-test: *p≤0.01; (A-E) modified according to Kremer et al., 2019 "pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis" https://doi.org/10.1073/pnas.1901283116 with permission from National Academy of Sciences, USA; PNAS is not responsible for the accuracy of this translation.

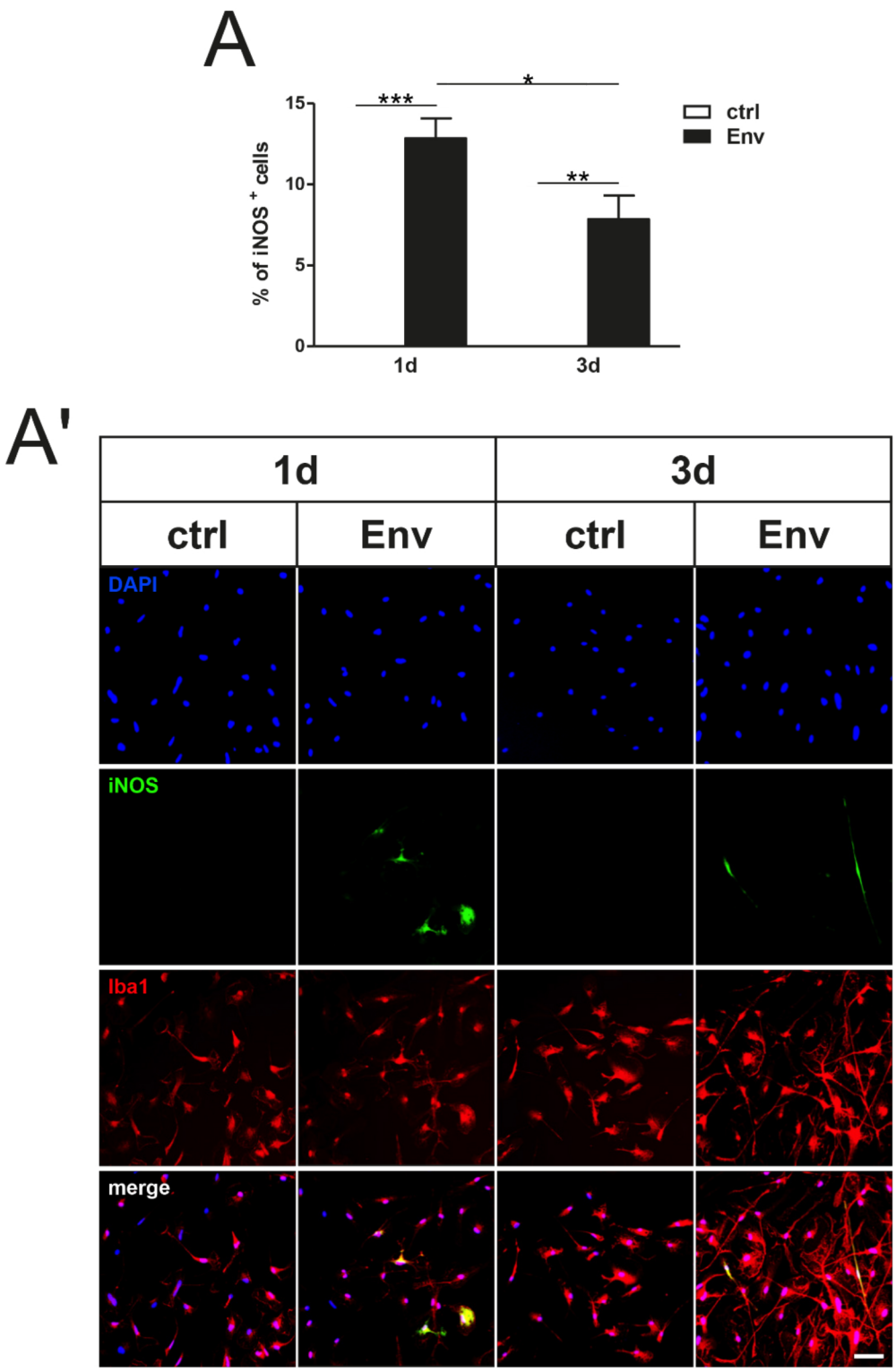

Fig. 2: Visualisation of pHERV-W Env protein mediated microglial nitrosative stress.

Quantitative analysis of iNOS positive microglia (A) and representative pictures (A') of anti-Iba1 (red) and anti-iNOS (green) double-stained microglia upon buffer control and pHERV-W Env protein (1000 ng/ml) exposure for 1d and 3 d. Here, a strong and significant increase of iNOS positive microglia upon pHERV-W Env protein exposure was detectable at day 1 which decreased over time and was still present at day 3. No iNOS positive microglia were detected upon exposure to buffer control. Nuclei are counterstained with DAPI (blue). Data are mean values ± SEM from n=4 independent experiments. Two-way ANOVA with Tukey post-test: *p≤0,05, **p≤0.01, ***p≤0.001; 20X magnification; scale bar: 100 µm

3.2 pHERV-W Env protein reduces microglial expression of neuroregenerative genes and secretion of IGF-1

Since microglia can adopt a proinflammatory as well as a restorative phenotype, it was of interest to investigate the impact of pHERV-W Env protein on the expression of neuroregeneration associated genes and OPC differentiation promoting genes (Zeger et al., 2007, Peferoen et al., 2014, Domingues et al., 2016, Miron, 2017). Therefore, analyses focused on genes regulating microglia-oligodendrocyte crosstalk and pro-oligodendroglial factors. Relative gene expression levels were analysed by qRT-PCR after incubation of microglia with pHERV-W Env protein or buffer control for 1 d and 3 d. Results indicated a strong and significant decrease of transcript levels of IGF-1, fibroblast growth factor-2 (FGF-2), and colony stimulating factor-1 (CSF-1) after 1 d which increased over time but was still present in case of IGF-1 and CSF-1 on d 3 (Fig. 3 A-C). In addition, to confirm obtained qRT-PCR data, IGF-1 ELISA of cell culture supernatants was carried out after 1 d of pHERV-W Env protein stimulation. Here, a significant reduction of microglial secreted IGF-1 was measured upon pHERV-W Env protein exposure compared to the buffer control (Fig. 3 D).

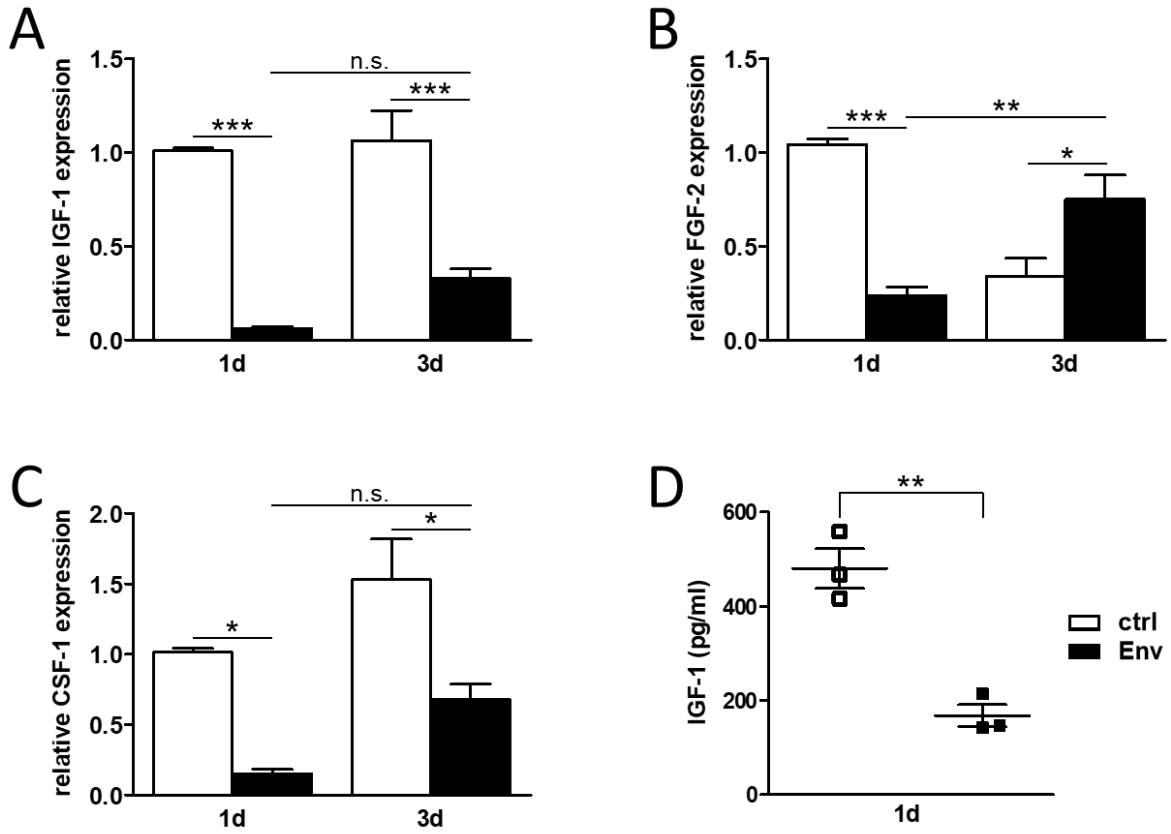

Fig. 3: Expression of genes related to neuroregeneration and microglial IGF-1 release upon stimulation with pHERV-W Env protein.

(A-C) qRT-PCR analysis of buffer control and pHERV-W Env protein (1000 ng/ml) stimulated microglia after 1 d and 3 d showed significantly decreasing (A) IGF-1, (B) FGF-2, and (C) CSF-1 relative transcript levels at day 1 which were in case of (A) IGF-1 and (C) CSF-1 still present on day 3. (D) ELISA based analysis of IGF-1 secretion of buffer control and pHERV-W Env protein stimulated microglia after 1 d. A significant reduction of microglial produced IGF-1 upon pHERV-W Env protein exposure was measured compared to buffer controls. Data are mean values ± SEM from n=6 independent experiments (A-C) and n=3 independent experiments (D). (A-C) Two-way ANOVA with Tukey post-test: *p≤0.05, **p≤0.01, ***p≤0.001, n.s. not significant; (D) Student's t-test: **p≤0.01; modified according to Kremer et al., 2019 "pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis" https://doi.org/10.1073/pnas.1901283116 with permission from National Academy of Sciences, USA; PNAS is not responsible for the accuracy of this translation.

3.3 pHERV-W Env protein decreases the expression of the pro-neuroregenerative marker APRIL in microglia

A proliferation inducing ligand (APRIL) is a member of the tumour necrosis (TNF) superfamily (Hahne et al., 1998) and is expressed by several cells (Dillon et al., 2006). Previous studies showed an increased APRIL expression in peripheral blood monocytes and T cells of MS patients. Additionally, reactive astrocytes were identified as a main source of APRIL expressing cells within the CNS and it was demonstrated that in MS patients these cells display an upregulation of APRIL (Thangarajh et al., 2005, Thangarajh et al., 2007). Next to reactive astrocytes, a recent study identified infiltrating macrophages as well as APRIL expressing cells in EAE lesions and in lesions of MS patients. The hereby secreted APRIL was shown to specifically target astrocytes, driving them to produce IL-10 as an anti-inflammatory response (Baert et al., 2019). Since APRIL was identified as an anti-inflammatory modulator in MS lesions and macrophages are one APRIL source, it was of interest to investigate whether pHERV-W Env protein exerts any modulating effect on the APRIL expression in microglia. Therefore, microglia exposed to pHERV-W Env protein were stained for APRIL. To clearly identify microglia, cells were again counterstained for Iba1 and only Iba1 positive cells were quantified. Results showed a highly significant reduction of APRIL positive microglia after 1 d of pHERV-W Env protein exposure which was still present and decreasing after 3 d (Fig. 4 A, A’).

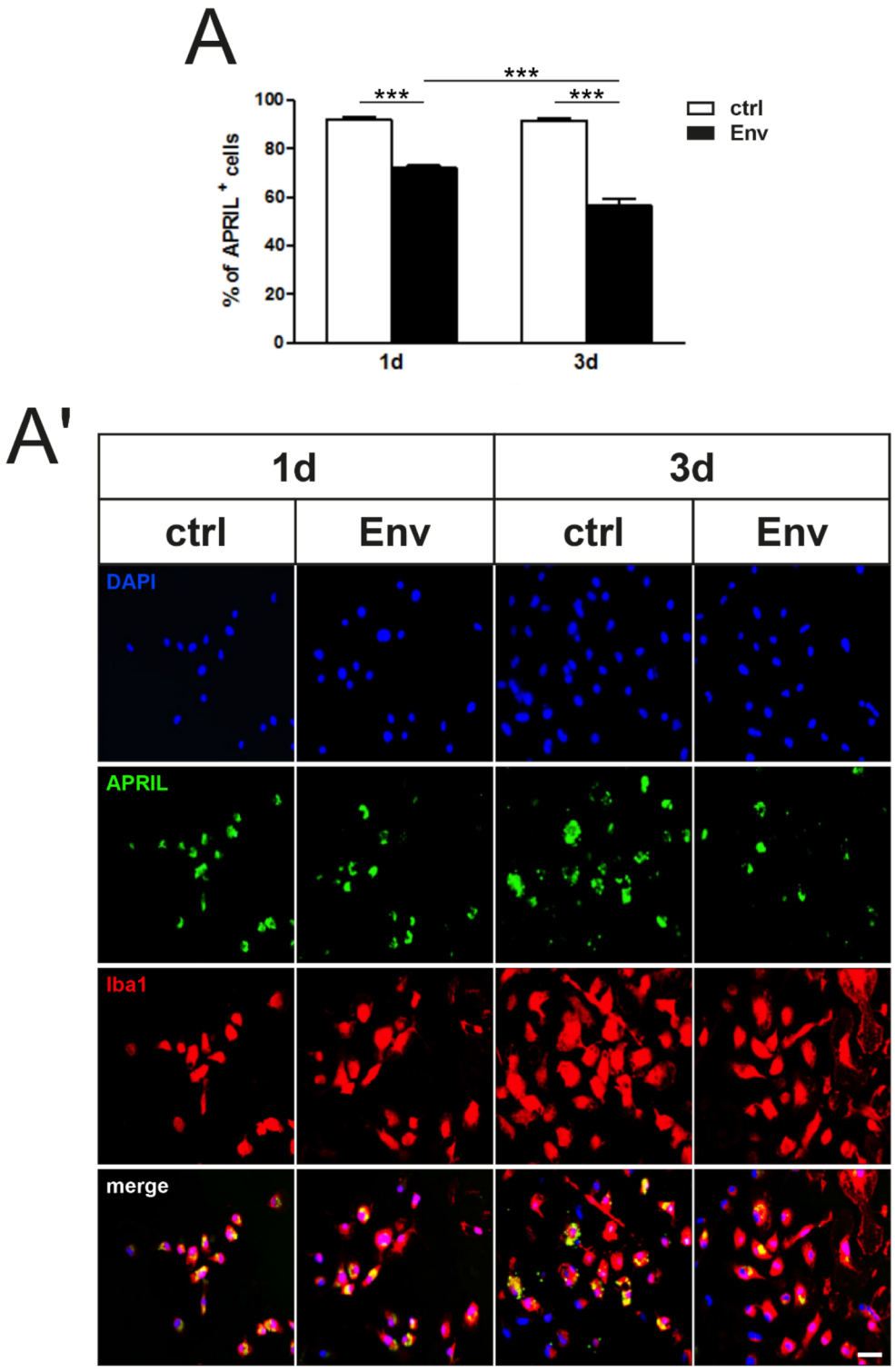

Fig. 4: Visualisation of pHERV-W Env protein modulated microglial expression of pro-neuroregenerative marker APRIL.

(A) Quantitative analysis of APRIL positive microglia and (A') representative images of anti-APRIL (green) and anti-Iba1 (red) co-stained microglia after buffer control and pHERV-W Env protein (1000 ng/ml) incubation for 1d and 3 d. Nuclei are counterstained with DAPI (blue). APRIL positive microglia significantly decreased upon pHERV-W Env protein exposure after 1 d and this decrease was still present on d 3. Data are mean values ± SEM and from n=5 independent experiments. Two-way ANOVA with Tukey post-test: ***p≤0.001; 40X magnification, scale bar: 50 µm

3.4 pHERV-W Env protein promotes microglial proliferation

In a next step, the question was addressed whether pHERV-W Env protein exerts an impact on proliferation capacity and the apoptosis rate of microglia. Therefore, microglia were exposed to pHERV-W Env protein (1000 ng/ml) and control buffer for 3 d. Afterwards, microglia were stained for Ki-67, a marker to visualise mitotic cells, and cleaved caspase 3 (CC3), an early marker protein for apoptosis. Quantitative analyses of anti-Ki-67 staining showed a significant increase of proliferating microglia upon pHERV-W Env protein stimulation in comparison to buffer controls (Fig. 5 A, A'). In addition, no difference in the apoptosis rate was detected, and in both groups apoptotic cells were rarely identified (Fig. 5 B, B').

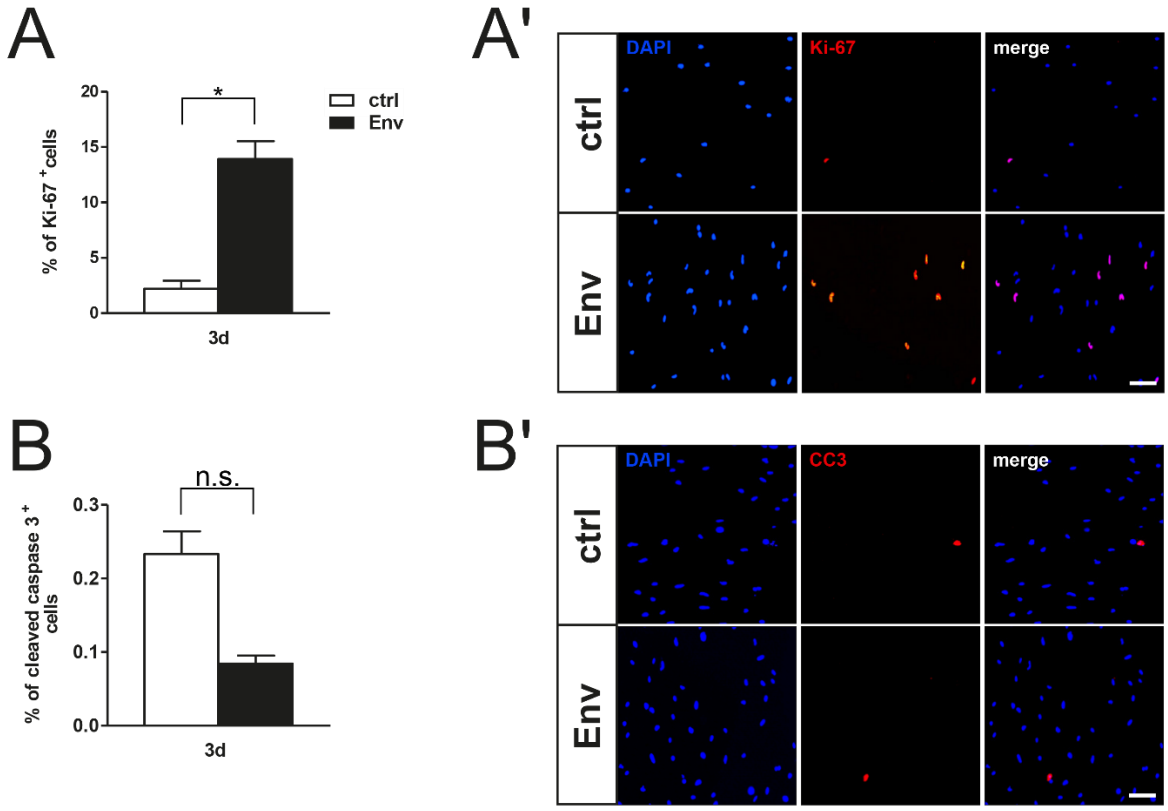

Fig. 5: Influence of pHERV-W Env protein on microglial proliferation and apoptosis rates

(A) Quantitative analysis of microglial proliferation showed a significant increase of Ki-67 positive microglia upon exposure to pHERV-W Env protein for 3 d, whereas microglial apoptosis rates analysed via cleaved caspase 3 (CC3) staining (B) were unaffected. Representative pictures of (A') anti-Ki-67 and (B') anti-CC3 staining of buffer control and pHERV-W Env protein (1000 ng/ml) stimulated microglia after 3 d. Nuclei are counterstained with DAPI. Data are shown as mean values ± SEM from n=3 independent experiments. Student's T-test: *p≤0.05, n.s. not significant; 20x magnification; scale bar: 100 µm. Ki-67 (A) modified according to Kremer et al., 2019 "pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis" https://doi.org/10.1073/pnas.1901283116 with permission from National Academy of Sciences, USA; PNAS is not responsible for the accuracy of this translation.

3.5 pHERV-W Env protein impairs the microglia phagocytosis rate

A key function of microglia is to phagocyte cell debris, dead cells and pathogens which might threaten CNS homeostasis. Since previous experiments pointed out a microglial transformation towards a rather proinflammatory phenotype, microglia phagocytosis capacity was investigated upon pHERV-W Env exposure. Therefore, microglial cells were treated with pHERV-W Env protein for 1 d and relative gene expression of TREM2 and MerTK known to regulate myelin debris clearance (Takahashi et al., 2007, Healy et al., 2016, Healy et al., 2017, Cignarella et al., 2020) was analysed. A significant decrease of both genes upon treatment with pHERV-W Env protein was observed compared to control treated microglial cells (Fig. 6 A, B). Moreover, validation experiments were conducted via performing an *in vitro* myelination assay adapted from Healy and colleagues (Healy et al., 2016). Bovine myelin, which was labelled with rhodamine was applied to microglia after 1 d of pHERV-W Env exposure and quantitative analysis of incorporated myelin was performed. Results showed a highly significant reduction of incorporated myelin by microglia exposed to pHERV-W Env protein in comparison to buffer controls (Fig. 6 C, C').

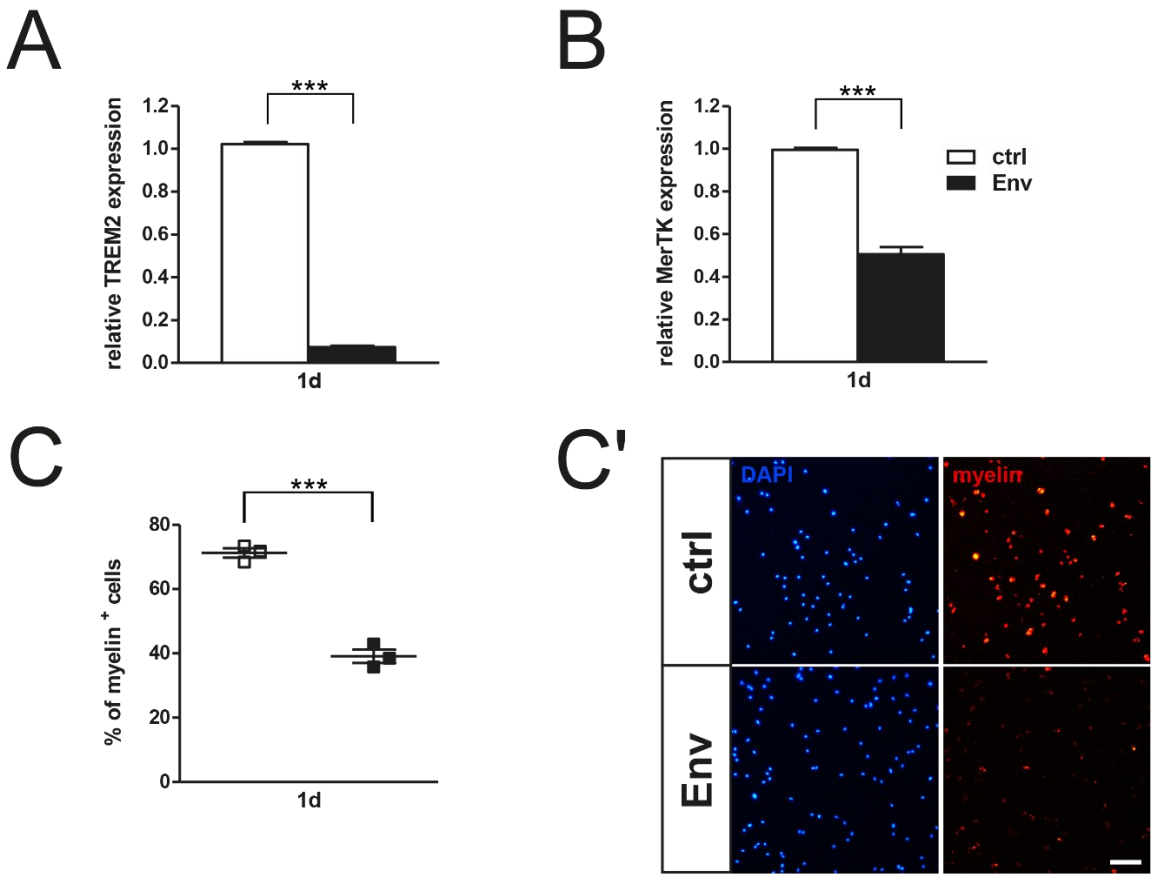

Fig. 6: pHERV-W Env protein mediated modulation of microglial myelin debris clearance.

qRT-PCR analysis of (A) TREM2 and (B) MerTK relative transcript levels revealed a significant decrease of pHERV-W Env protein exposure at 1 d. (C) Quantitative analysis of bovine myelin incorporation showed a significantly decreased myelin uptake upon pHERV-W Env protein exposure on 1 d. (C') Representative pictures of myelin uptake (red). Nuclei are counterstained with DAPI (blue). 10X magnification, scale bar: 200 µm. Data are mean values ± SEM from n=6 independent experiments (A, B) and n=3 independent experiments (C, C'). Student's T-test: ***p≤0.001; modified according to Kremer et al., 2019 "pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis" https://doi.org/10.1073/pnas.1901283116 with permission from National Academy of Sciences, USA; PNAS is not responsible for the accuracy of this translation.

3.6 IL-4 abrogates pHERV-W Env protein mediated proinflammatory effects and boosts microglial release of IGF-1

IL-4 is known to be neurosupportive, anti-inflammatory and promotes microglia towards a restorative phenotype (Park et al., 2005, Ponomarev et al., 2007, Gadani et al., 2012, Orihuela et al., 2016). Hence, the observed proinflammatory effect of pHERV-W Env protein might be reduced by IL-4. Therefore, microglia were stimulated simultaneously with pHERV-W Env protein and commercially available soluble IL-4 and their corresponding buffer controls for 1 d. The proinflammatory genes iNOS and TNFα were significantly increased upon pHERV-W Env protein exposure (see also Fig. 1 A, B) whereas after combined stimulation with pHERV-W Env protein and IL-4 the proinflammatory effect was reduced, although not being statistically significant for TNFα (Fig. 7 A, B). In case of iNOS, the effect of pHERV-W Env protein incubation was nearly abolished by IL-4 co-stimulation (Fig 7 A). Concerning the measurements of relative gene expression levels of IGF-1, the previously observed down-regulation was again detectable upon single pHERV-W Env protein exposure (see also Fig. 3 A). A pHERV-W Env protein reducing effect was observed upon simultaneous exposure to pHERV-W Env protein and IL-4 revealed by slight but statistically not significant increase of relative IGF-1 transcript levels (Fig. 7 C). ELISA measurements confirmed the gene expression analysis results. Upon exposure to IL-4, a significant increase of microglial IGF-1 release was observed which was almost three-fold higher compared to buffer controls. Compared to IL-4 exposure, microglial IGF-1 secretion was significantly decreased upon pHERV-W Env protein stimulation and was even lower than baseline secretion. A significantly increased IGF-1 secretion upon co-stimulation with pHERV-W Env protein and IL-4 was detected compared to pHERV-W Env protein exposure alone. Here, microglial IGF-1 secretion was higher than baseline levels, showing a complete abrogation of the previous observed impaired IGF-1 secretion upon microglial exposure to pHERV-W Env protein (Fig. 7 D).

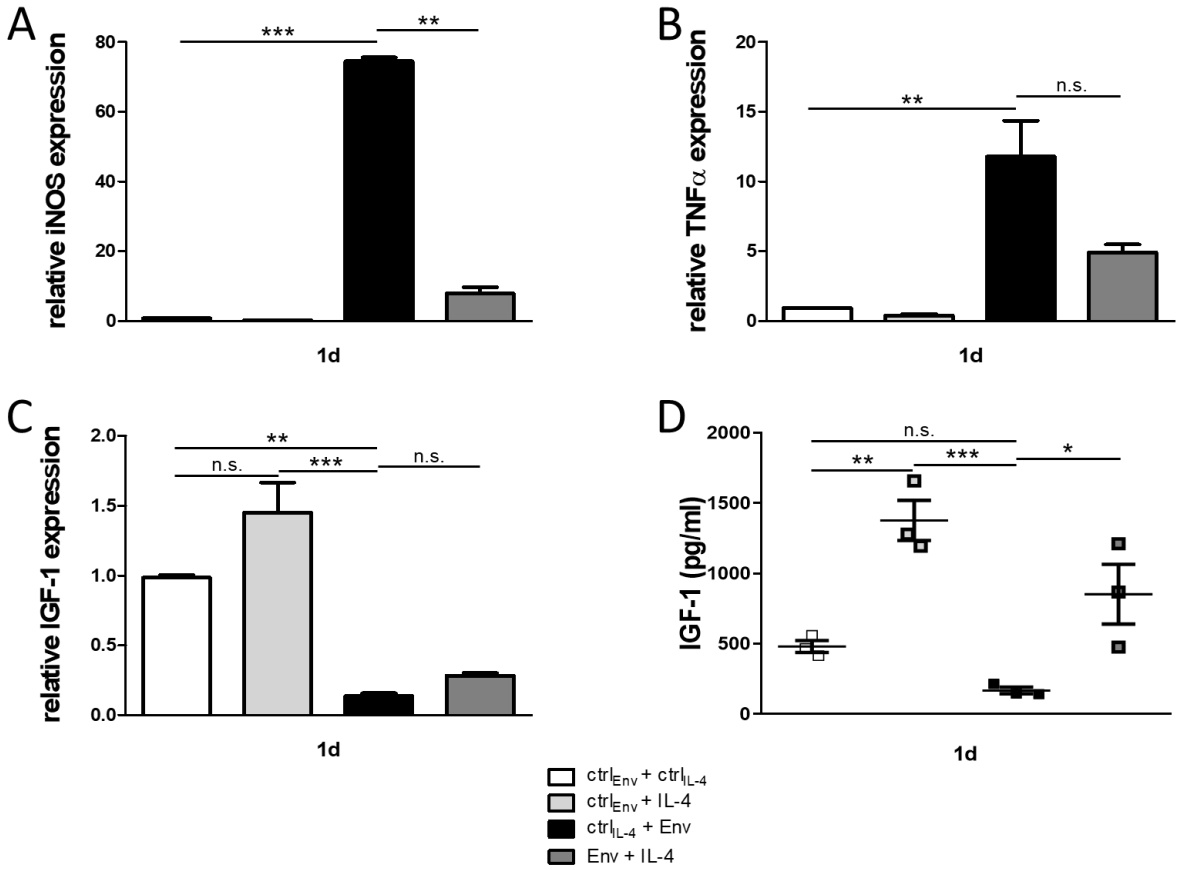

Fig. 7: IL-4 mediated reduction of pHERV-W Env protein induced proinflammatory effects on microglia.

qRT-PCR analysis of relative (A) iNOS, (B) TNFα and (C) IGF-1 transcript levels of buffer control and co-stimulated microglia with pHERV-W Env protein (1000 ng/ml) and IL-4 (40 ng/ml) after 1 d. Relative (A) iNOS and (B) TNFα gene expression were reduced upon exposure to both proteins compared to pHERV-W Env protein incubation. In case of TNFα, a slightly reduced expression was observed. After IL-4 stimulation (C) IGF-1 expression was slightly increased, which significantly decreased upon single pHERV-W Env protein exposure and slightly increased upon co-stimulation with pHERV-W Env protein and IL-4. (D) ELISA based analysis revealed an increase of IGF-1 secretion upon IL-4 stimulation. IGF-1 secretion decreased almost below baseline levels after single stimulation with pHERV-W Env protein after 1 d. Upon co-stimulation with pHERV-W Env protein and IL-4 a significant increase of microglial IGF-1 release was measured compared to pHERV-W Env protein exposure. Data are mean values ± SEM from n=3 independent experiments. One-way ANOVA with Tukey post-test: *p≤0.05, **p≤0.01, ***p≤0.001, n.s. not significant.

3.7 GNbAC1 does not antagonize pHERV-W Env protein induced microglial nitrosative stress

It was previously demonstrated that neutralization of pHERV-W Env protein by using the monoclonal antibody GNbAC1 leads to a reduction of nitrosative stress in OPCs (Kremer et al., 2015). Therefore, in a first approach, according to the initial experiments performed with OPCs, microglia were co-stimulated with GNbAC1 and pHERV-W Env protein. A ratio of 30:1 of the monoclonal antibody GNbAC1 and pHERV-W Env protein was used. Relative gene expression levels were measured for iNOS. As revealed by previous experiments an increased iNOS expression was detected upon pHERV-W Env protein exposure in comparison to buffer controls. However, iNOS expression was not reduced after co-stimulation with pHERV-W Env protein and GNbAC1 at 1 d (data not shown). Thus, it was suggested that a higher ratio of GNbAC1 and pHERV-W Env protein is needed for microglia compared to OPCs. Therefore, the ratio was raised to 150:1 of the monoclonal antibody GNbAC1 and pHERV-W Env protein. Immunofluorescent staining for iNOS was carried out after 1 d of stimulation. To clearly identify microglia, cells were co-stained against Iba1. As expected, pHERV-W Env protein stimulation led to increased numbers of iNOS positive microglia when combined with GNbAC1 control buffer. In addition, again a significant increase of iNOS positive microglia upon single pHERV-W Env protein exposure was detected. However, the number of iNOS positive cells was slightly but not significantly reduced after co-stimulation with pHERV-W Env protein and GNbAC1 (Fig. 8 A, A').

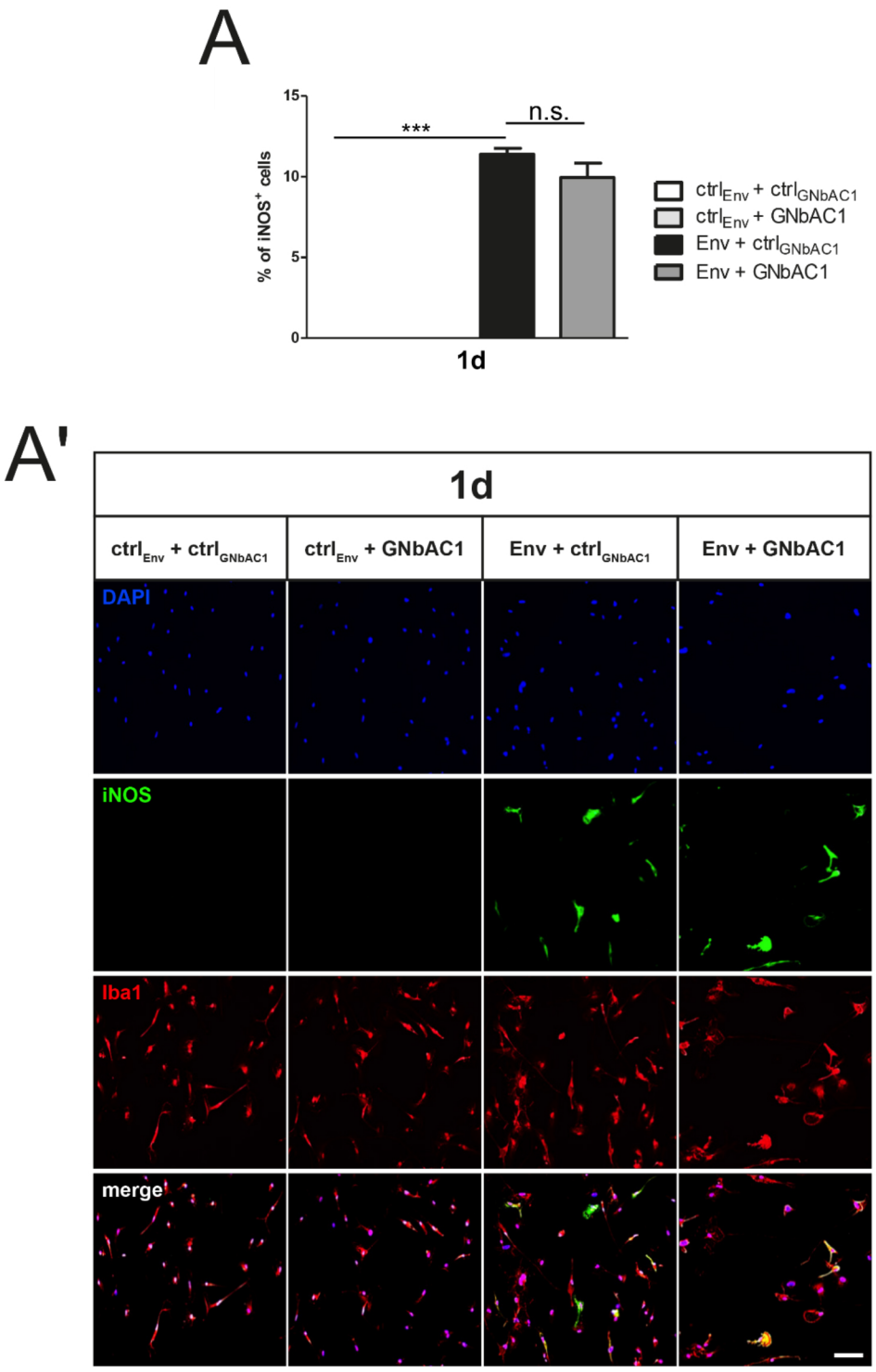

Fig. 8: Visualisation of microglial nitrosative stress upon co-stimulation with pHERV-W Env protein and GNbAC1.

(A-A') Quantitative analysis and representative pictures of anti-iNOS (green) and anti-Iba1 (red) double-stained microglial cells after 1 d of co-stimulation with pHERV-W Env protein (1000 ng/ml) and monoclonal antibody GNbAC1 (50 mg/ml) compared to buffer controls. Nuclei are counterstained with DAPI (blue). Quantification revealed a significant increase of iNOS positive microglia when exposed to the pHERV-W Env protein. Upon co-stimulation with the pHERV-W Env protein and GNbAC1 a slight but not significant decrease of iNOS positive microglia was detected. Data are mean values ± SEM from n=3 independent experiments. One-way ANOVA with Tukey post-test: ***p≤0.001, n.s. not significant; 20X magnification, scale bar: 100 µm

Since pHERV-W Env protein is produced in E. coli bacteria an LPS contamination was assumed, which might further activate microglia subsequently inducing a proinflammatory phenotype with microglial release of NO. Therefore, polymyxin B was added during the preincubation step to neutralize LPS residues. Immunofluorescent staining revealed a significant increase of iNOS positive microglia upon exposure to pHERV-W Env protein. However, this increase was less high compared to the experiments performed in absence of polymyxin B. In addition, a non-significant increase of iNOS positive microglia was detectable upon co-stimulation with GNbAC1 and pHERV-W Env protein (Fig. 9 A, A').

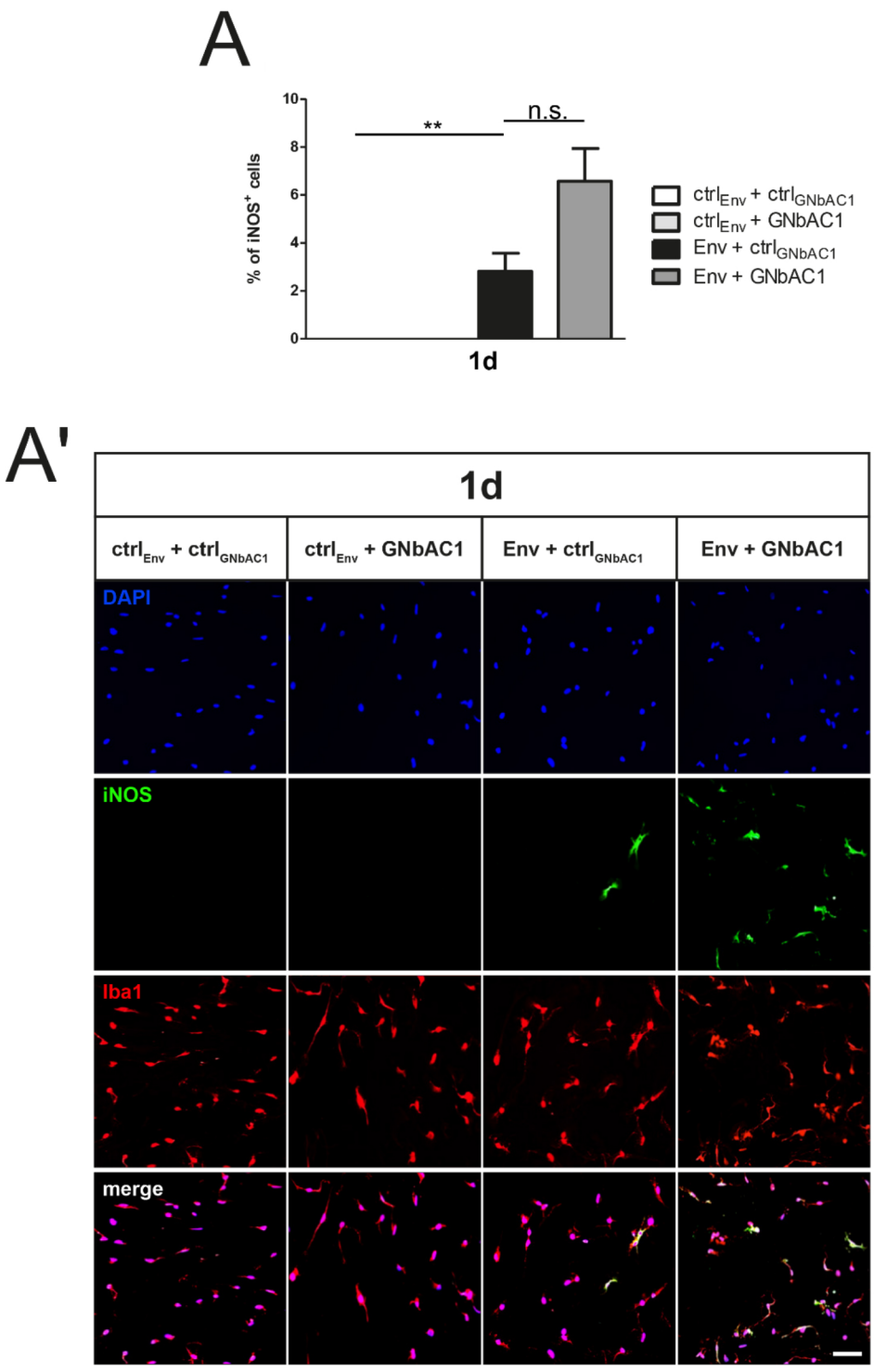

Fig. 9: Preincubation with polymyxin B does not reduce pHERV-W Env mediated microglial nitrosative stress upon co-stimulation with GNbAC1.

(A-A') Quantitative analysis and representative pictures of anti-Iba1 (red) and anti-iNOS (green) co-stained microglial cells after 1 day of co-stimulation with pHERV-W Env protein (1000 ng/ml) and monoclonal antibody GNbAC1 (50 mg/mL) compared to buffer controls. Nuclei are counterstained with DAPI (blue). Again, a significant increase of iNOS positive microglia was detected upon exposure to the pHERV-W Env protein. Here, a slight increase of iNOS positive microglia upon exposure to both agents was detectable. Data are mean values ± SEM from n=3 independent experiments. One-way ANOVA with Tukey post-test: **p≤0.01, n.s. not significant; 20X magnification, scale bar: 100 µm

Since no GNbAC1 mediated positive effect on nitrosative stress in microglia exposed to pHERV-W Env protein was observed, a third experimental setup was designed. As FCS might interact with the pHERV-W Env protein and thereby possibly inhibits a successful binding to the antibody, a reduced FCS concentration was used for microglia cultures (0.5 % FCS instead of 10 % FCS). Immunofluorescent staining showed a significant increase of iNOS positive microglia after incubation with pHERV-W Env protein. In comparison to the above used ratio of pHERV-W Env protein and GNbAC1 of 150:1 (see Fig. 8), the amount of iNOS positive microglia was two-fold higher after cultivation with 0.5 % FCS. Moreover, a slight increase of iNOS positive microglia was detected upon co-stimulation with pHERV-W Env protein and GNbAC1. However, a GNbAC1 neutralizing effect on microgilial nitrosative stress upon pHERV-W Env protein exposure was not detected (Fig. 10; A, A').

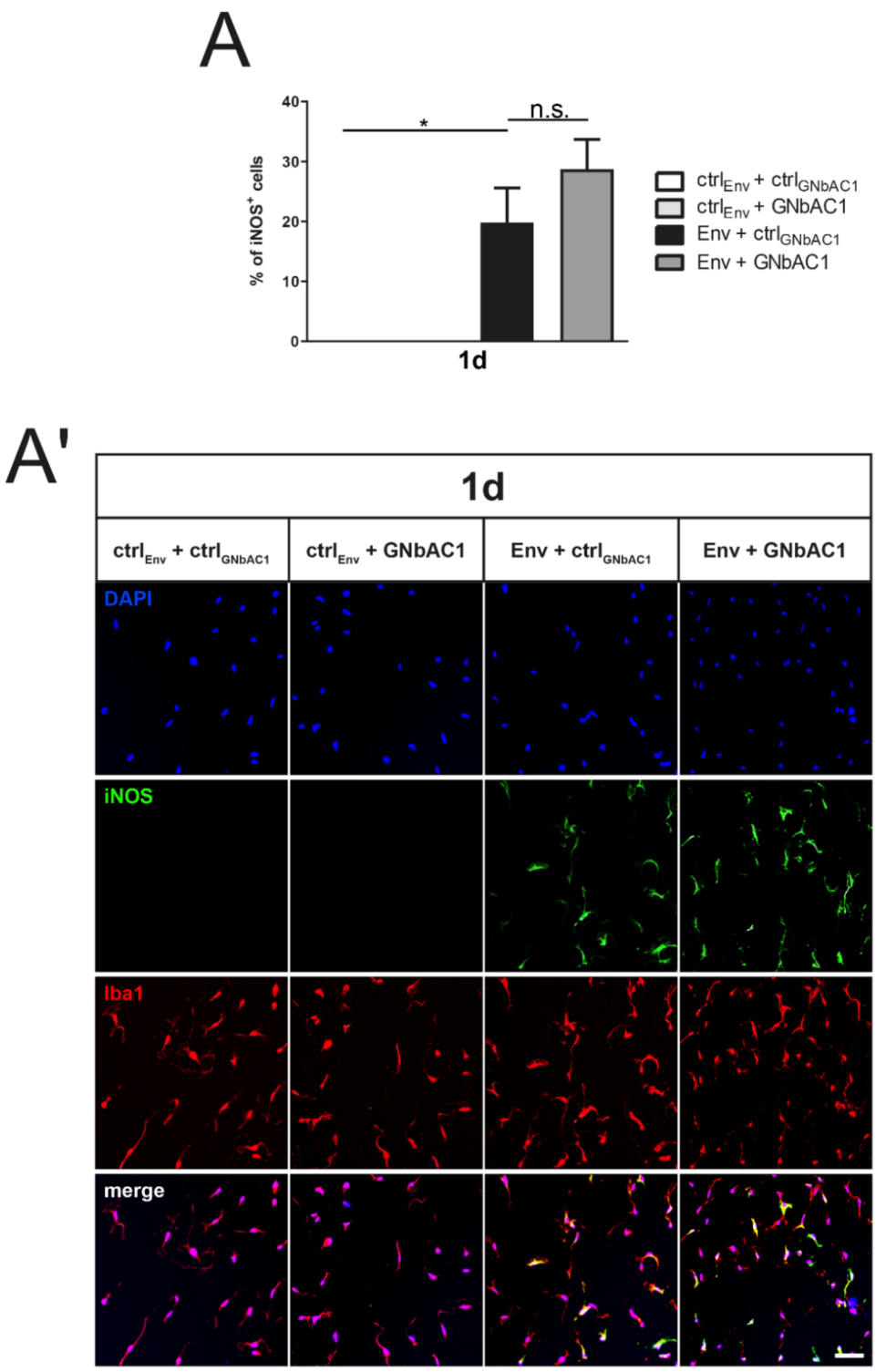

Fig. 10: Reduced FCS concentration does not diminish microglial nitrosative stress upon exposure to pHERV-W Env protein and GNbAC1.

(A-A') Quantitative analysis and representative pictures of anti-iNOS (green) and anti-Iba1 (red) co-stained microglia after 1 d of co-stimulation with pHERV-W Env protein (1000 ng/ml) and GNbAC1 (50 mg/ml) compared to buffer controls upon 0.5% FCS concentration instead of 10%. Nuclei are counterstained with DAPI (blue). Quantification showed a significant increase of iNOS positive microglia when exposed to pHERV-W Env protein. Upon co-stimulation with pHERV-W Env protein and GNbAC1 a slight increase of iNOS positive microglia was detected. Data are mean values ± SEM from n=3 independent experiments. One-way ANOVA with Tukey post-test: *p≤0.05, n.s. not significant; 20X magnification, scale bar: 100 µm

4 Discussion

MS is the most common chronic inflammatory and demyelinating autoimmune disease of the CNS of unknown aetiology, which is mainly responsible for non-traumatic disabilities of especially young people (Brownlee et al., 2017). Basically, the main hallmarks of MS are characterized by inflammation, which is followed by demyelination and loss of axons resulting in irreversible neurodegeneration. Neurodegeneration was most likely investigated for neurons and OLs, but innate immune responses via microglia seem to significantly influence the disease course. Microglia are always present in MS lesions (Lassmann, 2012) and besides their reported proinflammatory and myelin destructive phenotype (Benveniste, 1997) they are assumed to play central roles in promoting a remyelinating environment in the CNS, beyond secreting a wide repertoire of pro-oligodendroglial factors (Voß et al., 2012, Lloyd and Miron, 2019). The fact that microglia can switch between their phenotypic profiles exposes them to important players and targets for inhibiting the progress of MS (Lampron et al., 2015). To date, their complex roles in promoting an inflammatory or a neuro-supportive atmosphere and, moreover, the needed microenvironment for shifting microglia towards an anti-neurodegenerative phenotype in MS are not completely understood and still challenging (Luo et al., 2017, Guerrero and Sicotte, 2020, Yong, 2022). Regarding the pathogenesis of MS, several infectious diseases such as EBV, VZV, HHV-6, HSV-1 and HERVs have been associated to promote MS (Ascherio and Munger, 2007, Sedighi et al., 2023), but none of them have been identified of being causative. In this case, the pHERV-W Env protein seems to obtain a major role, since a blood sample positivity of almost 53 % - 100 % is reported for MS patients (Dolei et al., 2002, Sotgiu et al., 2002). Notably, the presence of the pHERV-W Env protein in MS patients is associated with severe disease courses and with extremely active forms (Sotgiu et al., 2002, Sotgiu et al., 2006a, Perron et al., 2012, Garcia-Montojo et al., 2013). In the CNS tissue of MS patients, the pHERV-W Env protein is predominantly attributed to microglia (Perron et al., 2005) and was found to be present in microglia neighbouring degenerated axons (Kremer et al., 2019b). To date, the exact role of the pHERV-W Env protein in the pathogenesis of MS is not deciphered and needs to be further confirmed (Morris et al., 2019, Charvet et al., 2021). In this respect this project aimed at investigating effects the pHERV-W Env protein directly exerts on microglia and at

investigating to what extent microglial cell functions are modulated upon exposure. Pre-existing studies performed by other members of the research group demonstrated that induced effects upon pHERV-W Env protein stimulation are closely related to its interaction with the TLR4 receptor (Kremer et al., 2013, Förster, 2020). Interestingly, both rodent and human microglia express the TLR4 and its co-receptor CD14, required for pHERV-W Env protein binding, on the cell surface and in the cytoplasm (Bsibsi et al., 2002, Jack et al., 2005, Kremer et al., 2019b, Sogorski, 2022). In this respect, this work demonstrates that rodent microglia are an additional important target cell type of the retroviral pHERV-W Env protein and upon its exposure, induce drastic modulations of their cell functions and behaviour.

4.1 pHERV-W Env protein induces a microglial proinflammatory phenotype and disrupts their neuro-supportive properties

4.1.1 pHERV-W Env protein forces microglia to produce NO via iNOS induction

Microglia are a major source of iNOS within the CNS and iNOS upregulation is linked to the activated proinflammatory microglial phenotype (Possel et al., 2000). Besides, increased iNOS-based microglial NO production is being reported in inflammatory, viral and traumatic events in the CNS and can obtain excessive amounts, as it has been described for many neurodegenerative disorders such as MS (Smith and Lassmann, 2002, Tewari et al., 2021). The so produced NO is extremely neurotoxic, as it triggers the formation of harmful reactive oxygen species and nitrosative stress (Calabrese et al., 2007). Increased NO levels are also found in MS lesions and elevated NO products, such as nitrate and nitrite, are detected in MS patients CSF, blood, and urine (Smith and Lassmann, 2002, Lukác et al., 2013, Förster et al., 2021). In the pathogenesis of MS, NO is known for triggering disruption of the BBB (Thiel and Audus, 2001), the loss of OLs by promoting mitochondrial disfunction (Mitrovic et al., 1994, Lan et al., 2018), or is known for directly impairing myelin sheaths (Hill et al., 2004) and axons (Smith and Lassmann, 2002). Hence, as a first step this project emphasizes the already assumed immediate proinflammatory microglial response upon exposure to the pHERV-W Env protein (Kremer et al., 2019b, Sogorski, 2022). Env induced effects on rodent microglia were elucidated

via measuring the mRNA levels of genes related to inflammation and stress response (Luo et al., 2017) via qRT-PCR. Thus, microglia respond to pHERV-W Env protein exposure by inducing several proinflammatory genes such as TNFα, iNOS, IL-6, and IL-1β (Fig.1). Intriguingly, microglial iNOS transcript levels showed the tendency of a huge induction upon pHERV-W Env protein exposure (Fig. 1, B). One point which might be causative for the observed strong variations of microglial iNOS gene expression levels could be, that different primary cell batches variably respond to the same stimulation pulse, consequently leading to different periods of time of the induced proinflammatory peak with answers which vary in their strength. Since it is known that obtained results are related closer to physiological processes *in vivo* and hence closer to the human species (Horvath et al., 2008), the use of primary microglial cells for this project was more suitable. Based on formerly performed *in vitro* studies with OPCs, which revealed an increase of iNOS expression upon contact to the pHERV-W Env protein (Kremer et al., 2013, Förster, 2020), the NO catalysing enzyme iNOS was chosen for further downstream analyses. To further confirm the proinflammatory and harmful microglial profile upon exposure to the pHERV-W Env protein, the amount of microglial produced NO was measured in cell culture supernatants and the presence of the NO catalysing enzyme iNOS was analysed via immunofluorescent staining, consequently identifying microglia as a source of noxious NO secretion – in direct response (Fig. 1, E; Fig. 2). Interestingly, control buffer exposed microglia showed no iNOS protein expression at all, whereas pHERV-W Env protein stimulation induced a clear and strong induction of iNOS (Fig. 2) which is compatible with the performed qRT-PCR and NO measurements. This may be attributed to technical limitations, as CT-values of control buffer treated microglia were found to be close to the detection limit. In this regard, it appears feasible that the amount of transcribed and translated microglial iNOS protein upon control buffer stimulated cells is far too low and thereby latent for the used iNOS antibody in staining procedures. However, measurements of NO levels in microglial cell culture supernatants demonstrate a certain baseline secretion of NO in control buffer stimulated cells (Fig.1, E). These obtained results might be explained with the fact that low NO levels are physiologically needed in the CNS for regulating e.g., sleep-wake cycles (Cespuglio et al., 2012). As immunocompetent cells, microglia react immediately towards CNS threatening agents and turn upon contact from their quiescent state into an activated one, by

inducing non-directed inflammatory responses, which are necessary for limiting the potential damage of the CNS parenchyma and are usually downregulated when CNS homeostasis is rebuilt (Orihuela et al., 2016, Kabba et al., 2018). In this way, the pHERV-W Env protein seems to constitute such a CNS threatening agent by inducing microglial release of a wide repertoire of inflammatory molecules in response. Due to its probably prolonged presence in MS patients, the pHERV-W Env protein might be causative for not completely terminating the microglial induced proinflammatory response. The here presented results reinforce a direct harmful contribution of the pHERV-W Env protein to the pathogenesis of MS by colossal release of iNOS induced microglial cytotoxic NO, which might boost both the inflammatory and the neurodegenerative aspects of the disease.

4.1.2 pHERV-W Env protein disrupts microglial release of pro-oligodendroglial differentiation factors

In accordance with the increased transcript levels of inflammatory-associated genes and excessive iNOS protein expression along with increased NO release of microglia upon pHERV-W Env protein exposure, this work additionally demonstrates that this protein forces microglia to downregulate their neuro-supportive properties. As CNS resident immune cells, microglia are known to maintain an intense crosstalk between other resident glial cells such as OLs and neurons for keeping up CNS homeostasis (Domingues et al., 2016). In this context, this study reveals a significant decrease of especially microglial released OPC promoting differentiation factors upon exposure to the pHERV-W Env protein (Fig. 3). Microglial secreted IGF-1 fulfils diverse paracrine and autocrine neuroprotective features, such as shielding OLs from early apoptosis and OPCs from cell death (Barres et al., 1992, Lin et al., 2005) and induces differentiation of OPCs, thereby boosting myelination processes (Voß et al., 2012, Miron, 2017). Remarkably, results of the here presented project demonstrate an intense downregulation of microglial IGF-1 mRNA levels and subsequent highly diminished microglial IGF-1 protein release upon exposure to the pHERV-W Env protein (Fig. 3 A, D), indicating a pHERV-W Env protein induced microglial mediated and -dependent arrest of OLs and their OPCs.

The same trends were obtained for CSF-1 and FGF-2 gene expression levels of pHERV-W Env protein stimulated microglia at least for the first 24h of stimulation

(Fig. 3 B, C). Unexpectedly, FGF-2 transcript levels showed a significant increase upon pHERV-W Env protein exposure on day 3 (Fig. 3 B). As a growth factor, FGF-2 boosts oligodendrogenesis by inducing progenitor cell proliferation (Armada-Moreira et al., 2015, Miron, 2017) and was identified to exert neuroprotective properties by promoting oligodendroglial differentiation processes via FGF-2 gene knockout studies in an EAE mouse model, which is among others a commonly used animal model to study MS (Rottlaender et al., 2011). Furthermore, an *in vitro* study performed by Clemente and colleagues attributed the attraction of OPCs to lesion sites to an increased microglial/macrophage FGF-2 secretion, with subsequent replacement of degenerated myelin sheaths (Clemente et al., 2011). On the contrary, other studies revealed an elevated FGF-2 concentration especially in demyelinated lesions of MS patients' tissue, thus negatively correlating with myelination (Thümmler et al., 2019) and showed elevated FGF-2 levels correlating with the loss of OLs and myelin *in vivo* (Butt and Dinsdale, 2005), indicating that FGF-2 signalling might exert pleiotropic features in MS. Although not corroborated on protein level in the here presented project, the obtained qRT-PCR results for microglial FGF-2 expression (Fig. 3 B) upon exposure to the pHERV-W Env protein could correspond with the reported pleiotropic effects of FGF-2 signalling in MS. The initial decrease of microglial FGF-2 transcript levels after a 1 d stimulation might accentuate a microglial pHERV-W Env protein induced inhibition of oligodendroglial differentiation processes (Armstrong et al., 2002), especially when compared to obtained IGF-1 and CSF-1 measurements and is considered in accordance with the observed strong elevated microglial secreted NO levels upon exposure. However, the measured increase of FGF-2 transcript levels on day 3 can be interpreted differently. Specifically, the obtained microglial FGF-2 increase may be based on the decline of pHERV-W Env protein levels in microglial cell culture media, as microglia received just one stimulation pulse. Furthermore, it might reflect a late promotion of oligodendrogenesis, since it is known that among others, members of the FGF family can stimulate OPC migration and promote an oligodendroglial differentiation (Murtie et al., 2005, Clemente et al., 2011). Considering that an elevated FGF-2 concentration in progressive MS tissues correlates with the level of demyelination, this scenario could also be reflected here (Armstrong et al., 2002, Armstrong et al., 2006). To gain a more precise insight into the correlations of FGF-2 signalling and the stimulation of microglial cells with the pHERV-W Env protein,

long-term studies and further FGF-2 protein secretion analyses would be needed. Altogether, this work here clearly highlights a pHERV-W Env protein mediated microglial-dependent inhibition of the release of oligodendroglial promoting differentiation factors and concurrent induction of the release of proinflammatory molecules, thus providing a potential biomedical rationale for ongoing demyelination and inefficient remyelination processes in MS.

4.1.3 The negative regulation of neuroprotective marker APRIL

Since in MS lesions not only oligodendroglial lineage cells play a crucial role for remyelination, it was also of interest to further investigate aspects of microglial crosstalk to astroglial populations. Apart from an influence on the crosstalk to oligodendroglia, this study further demonstrates a negative pHERV-W Env protein mediated microglial influence on microglial-astrocyte crosstalk by significantly decreasing microglial APRIL expression (Fig. 4). In MS lesions, infiltrating macrophages were identified as a source of APRIL and were shown to specifically promote astrocyte-derived IL-10 secretion as an anti-inflammatory response *in vitro* (Baert et al., 2019). Examining whether astrocytic IL-10 release is effectively diminished upon contact to microglia exposed to the pHERV-W Env protein was out of scope for this study and must be further clarified, as for example via microglia-astrocytes co-culture experiments. In general, the results of the present study here once more highlight a pHERV-W Env protein mediated microglial transformation towards a proinflammatory phenotype, possibly fuelling inflammatory and degenerative processes in MS by efficiently modulating resident glial cells' crosstalk.

4.2 pHERV-W Env protein induces microglial proliferation

An increased mitotic activity of classically activated microglia is well established (Fukushima et al., 2015, Cunha et al., 2016). In addition to the above described pHERV-W Env protein mediated proinflammatory microglial transformation, this study further demonstrates that the pHERV-W Env protein boosts microglial proliferation (Fig. 5 A, A'), whereas microglial apoptosis rates are not affected (Fig. 5 B, B'). To date, a successful elimination of the pHERV-W Env protein in positively tested MS patients has not been reported. In this way, results of the here presented study might support the idea of an enduring microglial presence of the pHERV-W

Env protein by a permanent increased microglial proliferation rate. Consequently, the so prolonged presence of the pHERV-W Env protein in MS patients might perpetuate progressing inflammatory and degenerative aspects of the disease, since a termination of the predominant proinflammatory microglial phenotype might be impeded. At this point, further research is needed, since mechanisms which maintain a prolonged presence of the retroviral pHERV-W Env protein are still unclear. In that way it would also be interesting to further investigate whether a target cell mediated induction of microglial endogenous synthesis of the protein exists, which could be causative for a prolonged or permanent presence.

4.3 Microglia lose their key function upon pHERV-W Env protein exposure and feature downregulated myelin debris clearance capacities

Chronic demyelination is a central hallmark in the pathogenesis of MS (Bruck and Stadelmann, 2003, Simkins et al., 2021). Although the adult brain, with its NG2 glia, contains a source of CNS resident glial cells which exhibit a lifelong potential to differentiate into mature OLs, and are subsequently capable of remyelinating axons and replacing degenerated myelin sheaths to a certain extent, a successful remyelination remains limited in MS (Chang et al., 2000, Zhang et al., 2022). Up to today, the problem of remyelination failure due to immune reactions and the influence of inhibitory factors in MS lesions is not overcome and is still a matter of intense research (Franklin, 2002, Kuhlmann et al., 2008, Hagemeier et al., 2012, Göttle et al., 2019, Gruchot et al., 2019). Moreover, no drugs have yet been developed which could successfully boost remyelination processes in MS (Kremer et al., 2019a). As phagocyting cells, microglia are responsible for performing an efficient myelin debris clearance, which is of major importance in the context of MS (Sen et al., 2022). It was demonstrated in several studies, that degenerated myelin which is not completely removed particularly impedes OPCs in their differentiation potential (Robinson and Miller, 1999, Kotter et al., 2006, Lampron et al., 2015), thus consequently promoting disease progression. Microglia express huge amounts of TREM2 which was identified to especially regulate and boost their myelin debris clearance capacities *in vitro* and in cuprizone (CPZ)-treated mice, which is another potent animal model for MS. These mice received a TREM2 agonistic antibody via intraperitoneal injection 4 days before the start of the CPZ-diet and once a week for

the duration of the experiment and were shown to display a faster OPC recruitment and differentiation, eventually resulting in an accelerated remyelination (Cignarella et al., 2020). Besides, MerTK, which is also largely expressed on microglia, has been linked to the regulation of myelin phagocytosis in human myeloid cells (Healy et al., 2016, Healy et al., 2017). The response of CPZ-treated MerTK-knockout mice demonstrated, that MerTK plays essential roles in regulating microglial myelin debris clearance as well and subsequently promotes remyelination (Shen et al., 2021). Additionally, Shen and colleagues (2021) showed that myelin debris stimulates microglial release of interferon gamma (IFNγ) *in vitro*, which contributes to a disrupted differentiation capacity of OPCs and to a diminished microglial phagocytosis capacity (Shen et al., 2021). Hence, the investigation of the response of these crucial players upon pHERV-W Env protein stimulation within this doctoral thesis was of huge interest. And indeed, the pHERV-W Env protein induced an extensive downregulation of both essential regulators of microglial myelin debris clearance capacities (Fig. 6). qRT-PCR measurements revealed a decrease of almost 90% and 50% of the relative TREM2 and MerTK transcript levels in microglia, respectively (Fig. 6 A, B). These results are further corroborated by assessing the myelin uptake of microglia via quantifying the internalised pH-rhodo labelled bovine myelin, which is significantly impaired upon exposure to the pHERV-W Env protein (Fig. 6 C, C'). Regarding the pathogenesis of MS, this *in vitro* study identifies the pHERV-W Env protein to fiercely disrupt microglia's myelin debris clearance capacities, crucial for initiating remyelination processes. This again provides a biomedical rationale for progressive demyelination during disease progression. In summary, the picture of a pHERV-W Env protein induced and mediated microglial transformation towards a proinflammatory and disease promoting phenotype gets more concise, which emphasizes microglia and of course the pHERV-W Env protein itself as challenging targets for future pharmacological approaches.

4.4 Can pHERV-W Env protein induced proinflammatory microglial responses be neutralized?

Based on the above outlined proinflammatory microglial transformation upon pHERV-W Env protein exposure, it was of interest to investigate whether the observed *in vitro* microglial effects can be pharmaceutically neutralized and hence demonstrate a future therapeutic approach. Microglia were simultaneously exposed to soluble IL-4 and pHERV-W Env protein. IL-4 is well-known to induce a neuro-supportive atmosphere in the CNS, thus promoting a microglial phenotype with predominant neurotrophic features (Park et al., 2005, Gadani et al., 2012, Quarta et al., 2020). Moreover, a study performed in EAE mice exposed to extracellular vesicles containing IL-4, demonstrated a significant decrease of neuroinflammation by reducing clinical signs in EAE mice and upregulation of anti-inflammatory markers (Casella et al., 2018). In another EAE mouse study, performed by Ishihara and colleagues (2020), the mice received a serum albumin (SA)-IL-4 fusion protein systemically every other day, starting on day 8 after immunization to day 13,17 or 34, and thereby could underline its tremendous anti-inflammatory effects by preventing disease development and immune-cell infiltration into the spinal cord. Furthermore, SA-IL-4 fusion protein was shown to efficiently diminish the number of pathologic T helper 17 cells and effectively reduces immune responses to myelin antigen in mice with chronic EAE (Ishihara et al., 2021). Moreover, IL-4 was shown to positively influence the recovery from spinal cord injury (SCI) in rats (Lima et al., 2017) and from cerebral ischemia in mice (Liu et al., 2016) by inducing a microglial/macrophage promoted anti-inflammatory and neuroprotective atmosphere. Although not yet approved for use in humans, therapeutically boosting IL-4 levels within and outside the CNS seems to be a promising approach to overcome neuroinflammation that is not limited solely to neurodegenerative disorders such as MS.

Interestingly, the present *in vitro* study identifies IL-4 as a promising candidate for antagonizing pHERV-W Env protein mediated proinflammatory effects in microglia by decreasing the expression of the proinflammatory genes iNOS and TNFα (Fig. 7 A, B) and increasing transcript levels of neuro-supportive IGF-1 (Fig. 7 C). This effect was further verified by a rising microglial IGF-1 protein release (Fig. 7 D). Remarkably, the obtained IGF-1 release overcomes baseline levels ($crtl_{Env}$+$ctrl_{IL-4}$;

control treated microglia) when cells were exposed to both agents (Fig. 7, D), suggesting an IL-4 induced microglial shift towards a neurotrophic phenotype, hence possibly rescuing Env mediated negative effects. Previous *in vitro* studies demonstrated the onset of proinflammatory effects mediated by pHERV-W Env protein within 2 hours of exposure to microglia monocultures (Sogorski, 2022), so the relatively short observation time of 1d in this present study includes the microglial activation time required for the pHERV-W Env protein. Given that microglia are known to respond differently towards same stimuli, it would be of interest to investigate whether the obtained induced IL-4 response might be maintained for longer periods or whether the reported pHERV-W Env protein induced microglial proinflammatory response is just postponed. Considering that a simultaneous exposure to both agents does not imitate the scenario of MS at all, rescue studies with pHERV-W Env protein pre-stimulated microglia receiving IL-4 preferably at the peak of their proinflammatory response are of major interest for identifying IL-4 as a sufficient rescuing agent. Besides, a translation of the experimental setup to a higher, more realistic environment such as myelinated co-cultures or even organotypic slice cultures exposed to the pHERV-W Env protein can be suggested as a next step to investigate IL-4 dependent rescuing impacts on resident glial cells. Beyond that, with the existing and established pHERV-W Env protein induced mice EAE model (Perron et al., 2013) IL-4 mediated pHERV-W Env protein rescue and long-term effects can be tested to further characterize effects that reflect the *in vivo* situation.

Besides the possible treatment with neuroprotective cytokines like IL-4, it is also a feasible strategy to directly target the negative effector within the system: Env. Temelimab/GNbAC1 was designed to specifically target the subunit of the pHERV-W Env protein, which binds to the TLR4 receptor as well as its full length and features an extraordinary mechanism of action by exerting its anti-pHERV-W Env protein effects without destroying target cells (Curtin et al., 2015). *In vitro* studies reported a temelimab mediated reduction of pHERV-W Env protein induced nitrosative stress in OPCs, which was previously shown to impair their differentiation capacity (Kremer et al., 2015, Förster, 2020). Considering the immense potential of the pHERV-W Env protein to contribute to the pathogenesis of MS and the extreme influence on microglia, as presented in this study, it was of interest to investigate

whether temelimab might diminish the observed pHERV-W Env protein mediated tremendous microglial release of nitrosative stress. Results indicate that microglia seem to be more susceptible towards the pHERV-W Env protein compared to OPCs, as a transfer of the experimental setup from OPCs to microglia failed to reduce microglia's level of induced iNOS. Even a 5-fold increase of the used ratio of temelimab to pHERV-W Env protein (150:1, formerly 30:1) demonstrates no significant reduction of induced iNOS expression in microglia (Fig. 8). It is conceivable that during the preincubation step not all pHERV-W Env protein molecules were successfully ligated by the antibody, and due to the natural behaviour of microglia a minimum amount of unbound pHERV-W Env protein is sufficient for activation. Besides, the antibody-protein complex itself might activate microglia, since they express diverse receptors on their cell surfaces which recognize unfamiliar stimuli (Town et al., 2005, Butovsky and Weiner, 2018). Moreover, an alternative induced pHERV-W Env signalling beyond the already deciphered TLR4 receptor cascade is also possible, since it is not evident that pHERV-W Env protein effects are limited to microglial TLR4 expression. Based on previous studies (Kremer et al., 2013, Förster, 2020) a pHERV-W Env protein induced change of microglial behaviour is assumed predominantly by TLR4 signalling but it is still unclear whether these findings can be translated to microglia in the same way. Hence, future microglial receptor blocking approaches and TLR4 knock out studies are needed to address these issues.

Regarding the methodological aspects, a contamination with bacterial LPS, which is commonly incorporated in the membrane of gram-negative bacteria (Lüderitz et al., 1982) might also be possible, since the used recombinant pHERV-W Env protein is produced in E. coli bacteria. LPS is well-known to activate microglia by TLR4 receptor signalling, which may also have occurred during the conduction of the experimental procedure, as temelimab is not a TLR4 receptor blocker. The aforementioned facts may explain why in the here performed experiments with primary microglia, temelimab was not able to reduce pHERV-W Env protein induced nitrosative stress (Fig. 8). Subsequently, the experimental setup was changed and experiments with the use of PMXB, a sufficient LPS blocking agent (Lynn and Golenbock, 1992) were investigated. Interestingly, a generally reduced pHERV-W Env protein mediated microglial iNOS expression is obtained upon exposure to

PMXB (from 10% to 3%; Fig. 9), which might be ascribed to a certain LPS contamination, but this is rather speculative. Notably, upon co-stimulation with pHERV-W Env protein and temelimab, an increasing trend of iNOS expressing microglia is detected (Fig. 9). A possible explanation for the observed iNOS increasing trend could be the stabilizing effect of PMXB on $P2X_7$ receptors, which are highly expressed on microglia (Ferrari et al., 2007). As the $P2X_7$ receptor is associated with increasing proinflammatory features (Monif et al., 2009, Sidoryk-Węgrzynowicz and Strużyńska, 2021), it may be possible that the combination of the protein-antibody complex and the supplemental exposure to PMXB promotes further microglial activation, thus resulting in an increased iNOS expression. These results highlight once more the importance of future microglial receptor blocking approaches. When considering technical aspects, which might suppress a potential neutralizing effect of temelimab, another approach of this *in vitro* study was designed. Specifically, the supplemented quantity of FCS in microglial cell culture media was reduced from 10% to 0.5%. It was assumed that FCS might interact with the recombinant pHERV-W Env protein, consequently inhibiting a successful binding to temelimab. Results indicate that the amount of supplemented FCS used for microglia cell cultures is not responsible for suppressing the assumed temelimab induced neutralization of pHERV-W Env protein mediated microglial nitrosative stress (Fig. 10).

As iNOS and NO levels are induced by exposure to the pHERV-W Env protein, targeting this axis might seem an additional suitable therapeutic target. However, In the pathogenesis of MS, NO produced by iNOS appears to be a double-edged sword, so complete antagonization is not desirable. On the one hand there are clues that colossal iNOS produced NO triggers the inflammatory, the demyelinating and the neurodegenerative aspects of MS but on the other hand, certain levels of iNOS-based NO seem to exert necessary immunomodulatory functions in MS (Smith and Lassmann, 2002). A study with iNOS knockout mice highlighted its crucial role in regulating neuroinflammation at different phases of EAE with iNOS knockout mice suffering from enhanced inflammation and apoptosis of oligodendrocytes during the priming phase (Sonar and Lal, 2019). Additionally, clinical trials conducted with temelimab in RRMS patients demonstrated that temelimab failed to significantly reduce acute neuroinflammation at the highest administered doses measured by

not diminishing the number of gadolinium-enhancing lesions on brain MRI scans (Hartung et al., 2022). Nevertheless, temelimab was shown to exert regenerative effects and in particular to mediate the decrease of neurodegenerative events, since temelimab receiving RRMS patients displayed less cortical and thalamic brain atrophy and a reduced number of black holes on brain MRI scans as well (Hartung et al., 2022). Moreover, in a recent study (GeNeuro press release, 2022/03/21) temelimab was administered to relapsing MS patients at higher doses and in combination with rituximab, thus highlighting temelimab's immense and unique potential to positively influence neurodegenerative aspects of MS (see introduction, section 1.5). This is of great biomedical importance, since up to today no drug particularly featuring neurodegeneration in MS is available. Additional clinical trials, firstly performed in a larger cohort of relapsing MS patients and secondly especially performed in MS patients suffering from severe forms of the disease such as PPMS and SPMS are needed to emphasize temelimab's unique mechanism of action (Hartung et al., 2022, GeNeuro press release, 2022/03/21). Moreover, if further studies can confirm temelimab's potential of positively modulating neurodegeneration in MS patients, a central influence of the pHERV-W Env protein contributing to the pathogenesis of MS becomes more likely. Regarding the ongoing clinical trials for temelimab in MS patients, the here performed microglial *in vitro* study might reflect the *in vivo* observed failure of temelimab to rescue features of acute neuroinflammation, represented here by not affecting pHERV-W Env protein mediated microglial iNOS expression. The reported neurodegenerative modulating features of temelimab might be better investigated on cellular basis *in vitro* using a myelinated co-culture system, consisting of OLs, astrocytes and myelinated neurons but lacking microglia, which is exposed to the pHERV-W Env protein and applicated microglia, such as published in Kremer et al., 2019. With the help of this experimental setup, it was possible to demonstrate increased expression levels of markers linked to axonal damage, such as neurofilament (NFL), synaptophysin (SYP) and myelin basic protein (MBP) to pHERV-W Env protein exposure (Kremer et al., 2019b). A possible next step could be to examine whether temelimab might positively influence those markers in order to draw conclusions about its effect on promoting anti-neurodegenerative aspects on a cellular basis.

4.5 Limitations and future perspectives

Since the origin and pathogenesis of MS is still obscure, no suitable study design covering all aspects of the disease exists so far. Most importantly, rodent microglia are readily available compared to humans, but multiple species dependent differences in microglial constitution exist which limit the transferability of findings in rodent to human. Regarding the here presented study, human microglia physiologically do not express TLR4 receptors in such high amounts as rodent microglia do (Jurga et al., 2020), probably indicating that human microglia might not become that strongly proinflammatory activated by exposure to the pHERV-W Env protein via induction of the TLR4 receptor signalling pathway. In this way, it seems more feasible that pHERV-W Env effects are not only restricted to TLR4 receptor signalling. Besides, the use of neonatal primary rat microglia constitutes a limitation itself, since the used rat cells have not been already exposed to harmful environmental factors and pathogens as human adult microglia are at the timepoint of manifestation of MS. In this context it is nevertheless remarkable, that the obtained results of this *in vitro* study were shown to be transferable to adult human microglia. Cultivated human microglia were demonstrated to react with increasing release of the proinflammatory molecules TNFα and IL-6 and showed decreasing transcript levels of the also *in vitro* investigated microglial myelin debris clearance regulating genes TREM2 and MertTK after receiving the same applicated pulse of recombinant pHERV-W Env protein (Kremer et al., 2019b). The fact that these studies were performed in human adult microglia *ex vivo* and for a very short period emphasizes the ability of pHERV-W Env protein to mediate microglial proinflammatory effects, but does not portray surely existing long-term effects. Long-term effects could be investigated, for instance, by inducing a microglial endogenous expression of the Env protein via transfection or using a genetically modified animal model expressing the pHERV-W Env protein. Moreover, studies might be conceivable with a human subject group of younger age than the reported average onset of MS, who are incidentally tested positive for the presence of the pHERV-W Env protein in the blood or CSF. Markers of CNS inflammation could be then measured in liquor samples. Additionally, a feasible transformation to one of the already reported pHERV-W Env protein linked diseases might be detectable (Küry

et al., 2018) which might consequently accentuate the contribution of the pHERV-W Env protein to disease onset.

Furthermore, there are reported strong differences of microglial behaviour towards general inflammatory stimuli within the same species depending on exposure *in vivo* or *in vitro* (Jeong et al., 2013). To study the obtained *in vitro* pHERV-W Env protein mediated microglial inflammatory effects in more detail, pHERV-W Env protein expressing mice can be used, which have already been established by other members of the research group and investigations are ongoing. Like this, pHERV-W Env protein mediated long-term effects on all resident CNS cells can be additionally investigated as well.

Since MS is an autoimmune disease which affects especially women, it would be interesting to investigate whether sex dependent differential microglial responses towards the exposure of pHERV-W Env protein are evident and whether females are more susceptible. Based on the here presented study, neonatal rats might be separated into females and males and hereafter isolated microglia are exposed to pHERV-W Env protein *ex vivo*. In parallel, pHERV-W Env protein expressing mice might also be separated into females and males and consequently analysed for pHERV-W Env protein mediated microglial proinflammatory transformations and subsequent possible harmful reactions towards other resident glial cells and axons.

5 Conclusion

The amount of HERV-W DNA measured in MS patients has been reported to correlate with severe disease courses as well as with progressive forms of MS (see introduction, section 1.4) probably exposing HERVS as potential biomarkers for disease severity in the future (Song et al., 2021). These findings are supported by a tremendous microglial potential to mediate pHERV-W Env protein induced effects, which could extremely promote the pathogenesis of MS. To this end, the here presented dissertation clearly identifies microglia as important target cells for the pHERV-W Env protein and further highlights the pHERV-W Env protein mediated microglial-dependent contribution to main issues of MS: inflammatory as well as demyelinating aspects. Microglial activation was shown to result in the activation of

a proinflammatory and anti-neuroprotective phenotype probably negatively influencing the crosstalk to OPCs and astrocytes. Besides, microglial myelin phagocyting properties – essential for proper repair processes facilitating remyelination – were shown to be extremely downregulated upon exposure to the pHERV-W Env protein. The fact that the pHERV-W Env protein promotes microglial-dependent and -mediated axonal damage (Kremer et al., 2019b) underlines itself and microglia as promising targets for future pharmacological approaches. In this context, the monoclonal antibody temelimab surprisingly failed to diminish microglial nitrosative stress induced by pHERV-W Env protein stimulation, possibly explaining the outcome of the current clinical trials, demonstrating no rescue of inflammation in MS patients. Nevertheless, temelimab still constitutes an encouraging candidate for future studies, as it was shown to positively modulate particularly neurodegenerative aspects of MS which so far are not successfully addressed by the current approved DMTs.

6 References

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