Aus der Klinik für Neurologie
der Heinrich-Heine-Universität Düsseldorf
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HERV-W ENV protein leads to a differentiation blockade in oligodendroglial precursor cells via nitrosative stress

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
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2020

Als Inauguraldissertation gedruckt mit Genehmigung der
Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

gez.:
Dekan: Univ.-Prof. Dr. med. N. Klöcker
Erstgutachter: Univ.-Prof. Dr. rer. nat. P. Küry
Zweitgutachter: Univ.-Prof. Dr. med. J. Timm

Per aspera ad astra

Für meine Familie

und L.F.

Parts of this work have been published:

Kremer, D., Schichel, T., Förster, M., Tzekova, N., Bernard, C., van der Valk, P., van Horssen, J., Hartung, H.-P., Perron, H., Küry, P., (2013), Human Endogenous Retrovirus Type W Envelope Protein Inhibits Oligodendroglial Precursor Cell Differentiation. *Annals of Neurology,* (74, no. 5) 721-32.

Kremer, D.*, Förster, M.*, Schichel, T., Göttle, P., Hartung, H.-P., Perron, H., Küry, P., (2015), The Neutralizing Antibody GNbAC1 Abrogates Herv-W Envelope Protein-Mediated Oligodendroglial Maturation Blockade. *Multiple Sclerosis Journal,* (21, no. 9) 1200-03.

Zusammenfassung

Multiple Sklerose (MS) ist eine chronisch-entzündliche Autoimmunerkrankung des zentralen Nervensystems (ZNS) bislang unklarer Ätiologie, bei der die Axon-umhüllenden Myelinscheiden, die von entscheidender Bedeutung für die Informationsfortleitung sind, angegriffen und zerstört werden. Remyelinisierung, also der Ersatz verlorengegangener Myelinscheiden, basiert im ZNS auf der Differenzierung oligodendroglialer Vorläuferzellen (*oligodendroglial precursor cells;* OPCs). Dieser Prozess ist insgesamt limitiert und wird durch zahlreiche extrinsische und intrinsische Faktoren beeinflusst, die die Geschwindigkeit und den Erfolg bestimmen. Dies ist von großer pathophysiologischer Relevanz, da aufgrund einer unzureichenden Remyelinisierung letztlich die Axone selbt degenerieren, was zu Hirnatrophie und progredienten klinischen Beeinträchtigungen führt. Als möglicherweise differenzierungsbeeinflussender Faktor werden sogenannte humane endogene Retroviren (HERVs) diskutiert. HERVs der Familie W (HERV-W) konnten in 53%-100% der Blut- und Liquorproben von MS-Patienten isoliert werden und sind mit einem prinzipiell ungünstigen Krankheitsverlauf im Sinne einer schnelleren Behinderungsprogression und einer höheren Konversionsrate der Patienten hin zum chronisch-progredienten Krankheitsverlauf assoziiert. Im Rahmen der hier vorgestellten Arbeit wird untersucht, inwiefern das Hüllprotein (*envelope protein;* ENV) dieser Viren mit OPCs interagiert und ihre Fähigkeit zur Reifung und Bildung von Myelin beeinflusst. Hierfür wurden *in vitro* ENV-Stimulationsexperimente in OPC Primärkulturen aus der Ratte durchgeführt, die zeigten, dass HERV-W ENV über eine Aktivierung des *Toll-like receptor* 4 (TLR4) zu einer verminderten Expression von Myelinproteinen bei reifenden OPCs führt. Mechanistisch basiert dieser Effekt auf einer Induktion proinflammatorischer Gene wie Tumornekrosefaktor α (TNFα), Interleukin 1 (IL1), Interleukin 6 (IL6) und vor allem der induzierbaren Stickstoffmonoxidsynthase (iNOS), was zu gesteigertem nitrosativen Stress führt. Zudem wurde der Effekt eines ENV-neutralisierenden Antikörpers namens GNbAC1 untersucht, der die ENV-assoziierte Induktion nitrosativen und proinflammatorischen Stresses verhindern kann und protektiv auf die Myelinexpression dieser Zellen wirkt. Die Ergebnisse dieser Arbeit legen nahe, dass HERV-W ENV die Differenzierungskapazität von OPCs vermindert und somit zu der oben beschriebenen unzureichenden Remyelinisierung bei MS beitragen könnte. Eine GNbAC1-Therapie wäre im Stande diese oligodendrogliale Differenzierungsblockade zu neutralisieren und eine Verbesserung der Remyelinisierung bei MS-Patienten herbeizuführen, was letztlich auch eine verminderte axonale Degeneration bewirken könnten.

Abstract

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) of yet unknown etiology. Its main histopathological hallmark is the destruction of the axon-sheathing myelin which is crucial for signal transmission. Remyelination in the CNS, i.e. the replacement of lost myelin sheaths, is based on the differentiation of oligodendroglial precursor cells (OPCs). However, this process is overall limited and influenced by numerous extrinsic and intrinsic factors that determine its speed and success. This is of particular pathophysiological relevance as insufficient remyelination ultimately leads to axonal degeneration manifesting itself in brain atrophy and increasing progressive clinical impairment. So-called human endogenous retroviruses (HERVs) are discussed to be such a differentiation-influencing factor. HERVs of the family W (HERV-W) have been isolated in 53% - 100% of blood and CSF samples of MS patients and are associated with a generally unfavourable disease course in terms of more rapid disability progression and a higher conversion rate to the chronic progressive disease course. The work presented here investigates how the envelope protein ENV of this virus family interacts with OPCs and influences their ability to mature and produce myelin. To this end, *in vitro* HERV-W ENV stimulation experiments were performed in rat OPC primary cultures which showed that HERV-W ENV leads to a reduced expression of myelin proteins in maturing OPCs via activation of Toll-like receptor 4 (TLR4). Mechanistically, this effect is based on the induction of proinflammatory genes such as tumor necrosis factor α (TNFα), interleukin 1 (IL1), interleukin 6 (IL6) and above all inducible nitric oxide synthase (iNOS) which leads to an increase of nitrosative stress. In addition, the effect of an ENV-neutralizing antibody called GNbAC1 was investigated which can prevent the ENV-associated induction of nitrosative and proinflammatory stress. This, in turn, was found to exert a protective effect on myelin expression. The results of this work suggest that HERV-W ENV may decrease the differentiation capacity of OPCs, thus contributing to the inadequate remyelination in MS as described above. Accordingly, treatment with GNbAC1 might be able to neutralize this oligodendroglial differentiation blockade and could improve remyelination in MS patients. As a result, axonal degeneration might be decreased or even prevented.

Abbreviations

°C *Degrees celsius*
3-NT *3-nitrotyrosine*
A2B5 *Monoclonal antibody A2B5*
AEBSF *4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride*
AQP4 *Aquaporin 4*
ATP *Adenosine triphosphate*
BBB *Blood-brain-barrier*
BSA *Bovine serum albumin*
CD14 *Cluster of differentiation 14*
CD20 *Cluster of differentiation 20*
CD4 *Cluster of differentiation 4*
CD52 *Cluster of differentiation 52*
cDNA *Complementary DNA*
CHO *Chinese hamster ovary cells*
CIDP *Chronic inflammatory demyelinating polyradiculopathy*
cm *Centimetre*
CNPase *2,3-cyclic nucleotide-3-phosphohydrolase*
CNS *Central nervous system*
CO2 *Carbon dioxide*
CSF *Cerebrospinal fluid*
CXCL *Chemokine ligand*
CXCR *CXC chemokine receptor*
DAPI *4',6-diamidino-2-phenylindole*
DC *Detergent compatible*, *Dendritic cell*
ddH2O *Double distilled water*
D-medium *Differentiation medium*
DMEM *Dulbecco's modified eagle medium*
DMF *Dimethyl fumarate*
DMSO *Dimethyl sulfoxide*
DMT *Disease-modifying therapy*
DNA *Deoxyribonucleic acid*
D-NAME *Nω-nitro-D-arginine methyl ester hydrochloride*
dNTP *Deoxyribonucleotide triphosphate*
DPBS *Dulbecco's phosphate buffered saline*
DTT *Dithiothreitol*, *Dithiothreitol*
E64 *L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine*
EAE *Experimental autoimmune encephalomyelitis*
EBV *Epstein-Barr virus*
EDTA *Ethylenediaminetetraacetic acid*
EGTA *Egtazic acid*
ENV *Envelope protein*
EU *European units*
FcRγ *Common γ chain of immunoglobulin Fc receptor*
FCS *Fetal calf serum*
GA *Glatiramer acetate*
GAG *Group-associated antigen*
GalC *Galactosylceramidase*
GAPDH *Glyceraldehyde 3-phosphate dehydrogenase*
GNbAC1 *Monoclonal anti-HERV-W ENV antibody*
GT3 *Ganglioside GT3*
GTP *Guanosine-5'-triphosphate*
GWAS *Genome wide association study*
H2O *Water*
HCl *Hydrogen chloride*
HERV *Human endogenous retrovirus*
HERV-W ENV *Envelope protein of the human endogenous retrovirus family W*
Hes *Hairy and enhancer of split*
HHV-6 *Human herpes simplex virus 6*
HLA *Human leukocyte antigen*
Id *Inhibitor of differentiation*
IFN *Interferon*
Ig *Immunoglobulin*
IKK *Inhibitor of NFκB kinase*
IL-1β *Interleukin-1β*
IL-6 *Interleukin-6*
iNOS *Inducible nitric oxide synthase*
IRAK-1/4 *Interleukin-1 receptor-associated-kinase-1/4*
IRF-3 *Interferon regulatory factor-3*
IκB *Inhibitor of NFκB*
kDa *Kilodalton*
kPa *Kilopascal*
L-15 *Leibovitz's medium*
LAL *Limulus amebocyte lysate*
LDS *Lithium dodecyl sulfate*
LINGO-1 *Leucine rich repeat and Immunoglobulin domain-containing Nogo receptor-interacting protein*
L-NAME *Nω-nitro-L-arginine methyl ester hydrochloride*
LPS *Lipopolysaccharide*
LRR *Leucine rich repeat*
LTR *Long terminal repeat region*
M *Molar*
M1 *Proinflammatory microglial cell*
M2 *Protective microglial cell*
MBP *Myelin basic protein*
MCT1 *Monocarbocylate transporter 1*
MEM *Minimum essential medium*, *Minimum essential medium*
MG *Microglial cell*
MHC *Major histocompatibility complex*

min *Minute*
ml *Millilitre*
mM *Millimolar*
MOG *Myelin/oligodendrocyte glycoprotein*
MRI *Magnetic resonance imaging*
mRNA *Messenger RNA*
MS *Multiple sclerosis*
MSRV *Multiple sclerosis-associated retrovirus*
MTR *Magnetic transfer ratio*
MyD88 *Myeloid differentiation response 88*
Na_2EDTA *Disodium ethylenediaminetetraacetate dihydrate*
Na_3VO_4 *Sodium orthovanadate*
NaCl *Sodium chloride*
NAWM *Normal appearing white matter*
NEDA *No evidence of disease activity*
NEMO *NFκB essential modulator*
NF-κB *Nuclear factor κB*
NG2 *neural/glial antigen 2*
NGS *Normal goat serum*
nM *Nanomolar*
NO *Nitric oxide*
NP-40 *Nonyl phenoxypolyethoxylethanol*
ODC *Ornithine decarboxylase*
OL *Oligodendrocyte*
OPC *Oligodendrocyte precursor cell*
P0/1 *Post-partum day 0*/1
p57kip2 *Cyclin-dependent kinase inhibitor 1C*
PBMC *Peripheral blood mononuclear cell*
PBS *Phosphate-buffered saline*
PBST *PBS mixed with 20 mole ethoxylate of sorbitan monolaurate*
PCR *Polymerase chain reaction*
PDGFR-α *Platelet-derived growth factor α-receptor*
PDL *Poly-D-lysine*
PFA *Paraformaldehyde*
PLP *Proteolipid protein*
pmol *Picomolar*
PMS *Progressive multiple sclerosis*
PNS *Peripheral nervous system*
POL *DNA polymerase*
PPMS *Primary progressive multiple sclerosis*
PRR *Pattern recognition receptor*
rev *Revolutions*
RhoA *Ras homolog gene family member A*
RIPA *Radioimmunoprecipitation assay*
RMS *Relapsing multiple sclerosis*
RNA *Ribonucleic acid*
RNS *Reactive nitrogen species*
ROS *Reactive oxygen species*
rpm *Revolutions per minute*
RPMS *Relapsing-progressive multiple sclerosis*
RRMS *Relapsing-remitting multiple sclerosis*
RT *Reverse transcription*
RT qPCR *Real-time quantitave PCR*
RT-PCR *Real-time PCR*
S1P *Sphingosin-1-phosphate*
SDS *Sodium dodecyl sulfate*
SNAP *S-nitroso-N-acetyl-DL-penicillamine*
SPMS *Secondary progressive multiple sclerosis*
T1D *Type 1 diabetes*
T3 *L-thyronine*
T4 *L-thyroxine*
TAK1 *Transforming growth factor beta-activated kinase 1*
TBK1 *TANK-binding kinase 1*
TBS *Tris buffered saline*
TC *Tissue culture*
TGS *Tris-glycine-SDS*
TIR *Toll/interleukin 1 receptor homology domain*
TLR4 *Toll-like receptor 4*
TNFα *Tumor necrosis factor α*
TRAF 6 *TNF associated factor 6*
TRIF *TIR-domain-containing adapter-inducing interferon-β*
Tris *Trisaminomethane*
Tris-HCl *Trisaminomethane hydrochloride*
tRNA *Transfer ribonucleic acid*
Tween 20 *Polyethylene glycol sorbitan monolaurate*
Tween20 *20 mole ethoxylate of sorbitan monolaurate*
U *Units*
V *Volt*
VCAM1 *Vascular adhesion molecule 1*
VLA4 *Very late antigen 4*
VZV *Varicella-zoster virus*
w/v *Weight per volume*
WB *Western blot*
ZETT *Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben*
ΔΔCt *Comparative cycle threshold method*
µg *Microgram*
µm *Micrometer*

1 Introduction

1.1 The structure of the nervous system

The human nervous system is a complex organ composed of many different specialised cell types. It can be divided into the central nervous system (CNS) consisting of the brain and the spinal cord and the peripheral nervous system (PNS) composed of nerves and ganglia that conduct impulses to and from the CNS. From a functional point of view, the CNS can be subdivided in multiple systems such as, for instance, the motor, the sensory and the visual system which receive and process information from all parts of the organism. CNS cells can generally be subdivided into neurons and glia (Kettenmann and Verkhratsky, 2008). Neurons specialise in the integration of information which they receive from other neurons. They transmit signals to effector organs often conducting impulses over very long distances – as it is exemplarily the case for the pyramidal cells of the motor cortex. The glial cells of the CNS, which can be further subclassified into macroglia (astrocytes, oligodendrocytes, ependymal cells, radial glial cells) and microglia, maintain CNS homeostasis in part by protecting and supporting neurons. They were first described by Virchow (Virchow, 1854) and are classically thought of as a fundamental component connecting all nerves – hence their name "glia" derived from the Greek "γλία" meaning glue. With their end foot processes astrocytes form a continuous layer which constitutes a physical and immunological barrier to CNS blood vessels and the cerebrospinal fluid (CSF), named "glia limitans". Additionally, they influence cerebral blood flow by regulating the vascular tone and the water transport via the membranous water channel aquaporin 4 (AQP4). Apart from these functions, astrocytes are involved in potassium buffering, the control of extracellular pH, the clearance of neurotransmitters such as glutamate and γ-aminobutyric acid (Kimelberg, 2010) and the modulation of synapse signaling (Panatier et al., 2011). Oligodendrocytes (OLs), are the myelin-producing cells of the CNS. By establishing lipid rich and electrically insulating myelin sheaths around axons they enable the so-called saltatory nerve conduction thereby accelerating electrical impulse propagation. Moreover, they also provide physical and metabolic support and protect axons (Griffiths et al., 1998). Microglial cells (MGs) which are part of the body's innate immune system, are derived from the embryonic yolk sac and play an important role in the maintenance and immunological surveillance of both the healthy and the diseased CNS. In the healthy brain they protect neuronal integrity by phagocytosis of debris, the modulation of synaptic signaling, the inactivation of pathogens and the neutralization of deleterious agents such as, for instance, free radicals (Aloisi, 2001). Derived from the phenotyping of macrophages, this protective

microglial subtype is classically referred to as “M2” (Kigerl et al., 2009). However, in the inflamed or injured CNS MG can also contribute to neuroarchitectural damage by promoting an inflammatory milieu via the production of pro-inflammatory cytokines leading to cytotoxicity (the so-called “M1” phenotype) which directly harm axons (Kigerl et al., 2009).

1.2 Oligodendrocytes and myelin

Oligodendrocytes (OLs) were first described by the Spanish histologist Pío del Río Hortega (Río Hortega, 1921) and are responsible for the myelination as well as the metabolic and trophic support of axons (Lee et al., 2012). OLs express monocarboxylate transporter 1 (MCT1), a transporter for monocarboxylate metabolites, such as pyruvate and lactate which are key for axonal energy generation. Accordingly, depletion of MCT1 in OLs was found to induce axonal swelling and death (Lee et al., 2012). Additionally, OLs secrete diverse soluble mediators, which positively modulate axonal and dendritic growth as well as synaptic plasticity (Byravan et al., 1994, Wilkins et al., 2001, Wilkins et al., 2003). During animal evolution two strategies have evolved to increase the speed of nerve conduction. The first strategy is to increase axon diameter which results in the generation of very large axons. Such gigantic axons can be found in invertebrates such as squids where diameters can reach enormous relative sizes (Hodes, 1953). The second and more efficient approach is to wrap axons in insulating sheaths of myelin which are organised in sections called internodes separated by so-called nodes of Ranvier (Ranvier, 1871). Their lipid content allows myelin sheaths to efficiently insulate axons preventing voltage loss during signal propagation. In addition, impulse conduction occurs in a saltatory fashion from one node of Ranvier to the next which further accelerates velocity. This is made possible by the fact that the non-insulated nodes of Ranvier are rich in sodium and potassium channels which allows for an amplification and renewal of electric signals (Poliak and Peles, 2003). Of note, every OL extends up to 50 processes which spiral around axons with their myelin containing plasma membranes forming distinct internodes (Matthews and Duncan, 1971). However, before mature OLs can initiate axonal myelination, their precursor cells need to migrate, proliferate, recognise target axons and differentiate into myelinating OLs. Oligodendrocyte precursor cells (OPCs) originate from different regions of the embryonic CNS such as the ventral neuroepithelium of the neural tube and the dorsal spinal cord and hindbrain during later embryonic stages. From there they migrate into specific zones of the developing white matter for further proliferation (Noll and Miller, 1993, Vallstedt et al., 2005, Noble et al., 1990). In these regions some of them exit the cell cycle and undergo

differentiation into myelin-forming OLs, while others remain in a precursor state well into adulthood (Miller, 2002). Differentiation of OPCs, both during development as well as in the adult CNS, involves different stages of increasing morphological complexity (e.g. an increase of the number of cellular branches and total cell diameter) during which specific surface antigens and protein markers are expressed (Baumann and Pham-Dinh, 2001). From a molecular point of view, OPCs prominently express platelet-derived growth factor α-receptor (PDGFR-α), the chondroitin sulfate proteoglycan neural/glial antigen 2 (NG2) and other glycolipids that are still not fully characterised but recognized by the monoclonal antibody A2B5. PDGFR-α is highly expressed in proliferating early OPCs but is downregulated in maturing cells. NG2, is an integral membrane proteoglycan and plays an important role in migration, proliferation, cell adhesion and communication and is co-expressed with PDGFR-α (Stallcup, 2002, Levine and Nishiyama, 1996, Nishiyama et al., 1996). Several gangliosides recognized by A2B5 such as GT3 are expressed in OPCs and disappear during differentiation into mature OLs (Farrer and Quarles, 1999). Mature OLs, on the other hand, express specific markers such as galactosylceramides (GalC), 2,3-cyclic nucleotide-3-phosphohydrolase (CNPase), myelin basic protein (MBP), proteolipid protein (PLP) and myelin/oligodendrocyte glycoprotein (MOG). GalC is a glycolipid marker, that is expressed in early immature stages but also remains expressed in mature myelinating OLs. CNPase represents about 4% of all myelin proteins and constitutes the earliest myelin specific protein in oligodendroglial differentiation. MBP accounts for about 30% of all myelin proteins and is being upregulated 2-3 days later than GalC and CNPase alongside with PLP, leading to the initiation of myelin formation and compaction (Baumann and Pham-Dinh, 2001). OPC differentiation and maturation is regulated by the presence of various inhibitory and promoting components guiding OPC homeostasis and managing terminal differentiation to a post-mitotic myelinating oligodendrocyte (Kremer et al., 2011, Mitew et al., 2014). Further details on the multiple inhibitory and promoting components guiding OPC differentiation are provided in chapter 1.3.5.

1.3 Multiple Sclerosis

1.3.1 History and clinical findings

Multiple sclerosis (MS) is a chronic autoimmune inflammatory demyelinating CNS disease of yet unknown etiology. It is the leading cause of non-traumatic disability in young adults and affects more than two million people worldwide (Baneke et al., 2013). MS was first recognized as a distinct disease entity by the French neurologist Jean Martin Charcot who provided a

detailed clinico-pathological description of the disease (Charcot, 1868, Charcot, 1879). Pathologically, MS is multi-faceted potentially affecting motor, sensory, visual and/or cerebellar functions as well as cognition and the vegetative nerve system. Based on its clinical course, MS can be classified into different subtypes (Lublin and Reingold, 1996). Relapsing-remitting MS (RRMS) is characterised by episodes of acute or subacute neurological worsening (i.e. relapses) followed by full or partial recovery without disease progression in-between. In order to classify such episodes as relapses they must last for at least 24 hours in the absence of fever or other signs of infection. Patients may or may not retain residual deficits upon recovery. Primary-progressive MS (PPMS) presents with a progressive worsening of symptoms with or without plateaus and lacks relapses or recovery. Secondary-progressive MS (SPMS) is a disease subtype where, over time, RRMS develops into a progressive form similar to PPMS. Relapsing-progressive MS (RPMS) features a steady disease progression from onset but with superimposed relapses. Of note, new consensus guidelines have proposed a simplified classification system differentiating only between a relapsing (RMS) and a progressive disease (PMS) course (Lublin et al., 2014).

1.3.2 Epidemiology and genetics

As with many other autoimmune diseases, MS is more frequently observed in females than in males with a ratio exceeding 3:1 in some regions (Alonso and Hernan, 2008, Bove and Chitnis, 2013). The average age of onset is 30 years which explains why it is the third most common cause of disability in people between the ages of 15 to 50 years in the United States (Howard et al., 2016, Baneke et al., 2013, Kingwell et al., 2013). MS is present in every region of the world and in 2013 2.3 million people worldwide were affected by the disease (Baneke et al., 2013). Various environmental risk factors have been identified during the past decades. Studies have shown that MS prevalence is lowest at the equator and increases with proximity to the poles (Limburg, 1950, Kurtzke, 1975a, Kurtzke, 1975b). Immigration studies show that migrants above the age of 15 moving from low- to high-risk areas or vice versa retain their risk of birthplace for developing MS. However, migrants younger than 15 years of age adopt the risk of their new residence, suggesting an influence of environmental factors in disease pathogenesis (Kurtzke, 1993). Based on these observations, ultraviolet radiation and high vitamin D serum levels have been discussed as potentially protective factors (Lucas et al., 2015). By contrast, typical lifestyle factors in industrial nations such as smoking, adolescent obesity or night work seem to increase the risk of MS (Olsson et al., 2017). Moreover, there is epidemiologic data providing evidence that infectious agents might be involved in MS

pathogenesis. This includes reports on infectious mononucleosis caused by Epstein-Barr virus (EBV) (Warner and Carp, 1981, Ascherio and Munger, 2007, Ascherio and Munch, 2000), human herpes simplex virus 6 (HHV-6) (Sola et al., 1993, Soldan et al., 1997), varicella-zoster virus (VZV) (Perez-Cesari et al., 2005, Sotelo et al., 2008) or human endogenous retroviruses (HERVs), although none of these have proven to be causative for MS (Milo and Kahana, 2010, Ascherio and Munger, 2007, Virtanen and Jacobson, 2012). Interestingly, in spite of the above-described geographical disease distributions, there are some ethnic groups living in high risk areas, that are much less susceptible for developing MS, such as Native Americans (Rosati, 2001), Aboriginals (Miller et al., 1990), Norwegian Lapps (Gronlie et al., 2000) or, in general, entire people such as the Chinese or Japanese (Rosati, 2001). On the other hand, a strong genetic factor is suggested by twin and family studies, indicating an almost 17-fold increased risk to develop MS for siblings of MS patients and a concordance rate of 30-50% in monocygotic twins (O'Gorman et al., 2013, Reich et al., 2018). Other data based on genome wide association studies (GWAS) point to specific variations of the human leukocyte antigen (HLA) system encoded on chromosome 6 as the greatest individual risk factor for MS. Within the HLA system the gene variant HLA-DRB1*15:01, encoding for a major histocompatibility complex (MHC) class II protein, has the strongest association with MS among the alleles examined, whereas the variant HLA-A*02:01, encoding for a MHC class I protein, has a protective function (International Multiple Sclerosis Genetics et al., 2011). Aside from that, more than 110 non-HLA gene polymorphisms were found to be associated with MS (Olsson et al., 2017).

1.3.3 Etiology and Pathology

Histopathologically, MS features plaque-like lesions of the white and grey matter which are the focal points of autoimmune inflammatory demyelination and consequent gliosis disseminated throughout the entire CNS (Reich et al., 2018). MS lesions which are most commonly found around small venous CNS vessels are characterized by a breakdown of blood-brain-barrier (BBB) integrity, followed by perivascular inflammation, inflammatory loss of OLs with subsequent demyelination, axonal degeneration and, ultimately, glial scar formation by reactive astrocytes (Dutta and Trapp, 2007). Although traditionally MS is considered as a primarily demyelinating disease, secondary irreversible axonal destruction is the key event driving its clinical course. Inflammation in MS is mainly based on T lymphocytes, triggering the recruitment and activation of both peripheral invading macrophages and brain-resident microglial cells (Brück et al., 1995, Sriram and Rodriguez, 1997). Four different patterns of

immune response and demyelination can be distinguished potentially implying heterogenous pathogenic pathways (Lucchinetti et al., 1996, Lassmann et al., 2001). Pattern I is characterized by macrophage-associated demyelination, probably via toxic products such as tumor necrosis factor α (TNFα) or reactive oxygen species (ROS). Pattern II features antibody-mediated demyelination similar to pattern I with the exception of additional immunoglobulin and complement C9neo antigen depositions at sites of active demyelination. Inflammatory lesions of both patterns typically occur perivenously, i.e. around small veins and venules. Pattern III has been attributed to oligodendrocyte dysfunction. It features distal degeneration of oligodendrocyte processes and cellular apoptosis as well as inflamed small vessels dominated by T lymphocyte infiltrates. This damage pattern is also commonly observed in virus-induced human white matter diseases or the penumbra of ischemic strokes. Pattern IV is characterized by primary oligodendrocyte damage of unclear origin with DNA fragmentation and apoptosis and a nearly complete loss of OLs in the centre of the lesion (Metz et al., 2014, Lucchinetti et al., 2000). Underlying pathological or etiological differences of the four different patterns of demyelination have not been identified yet. Despite that, they could be linked to different proposed pathogenetic mechanisms: a) MS as an autoimmune encephalomyelitis (patterns I and II), b) MS caused by environmental infectious or toxic agents (pattern III) or c) MS caused by metabolic or genetic defects (pattern IV). However and of note, there is evidence pointing to a coexistence of different patterns in the same patient (Barnett and Prineas, 2004). In general, demyelination leading to vulnerable "naked" axons is followed by axonal damage and ultimately degeneration. This is a multi-step process involving axonal swelling, axonal transection and finally neuronal degeneration which leads to persisting clinical deficits (Kornek and Lassmann, 1999). Accordingly, quantification of axonal loss in MS showed axon density reductions ranging from 19% to up to 68% which is supported by magnetic resonance imaging (MRI) of the brain and spinal cord of MS patients (Ganter et al., 1999, Bjartmar et al., 2000, Losseff and Miller, 1998). The exact mechanisms underlying axonal damage in MS remain to be further clarified. However, various studies propose either a direct attack on axons by microglia or macrophages, or nonspecific damage mediated by an overall inflammatory milieu containing proteolytic enzymes, cytokines, ROS or reactive nitrogen species (RNS) as well as free radicals. Moreover, failing axonal integrity and lacking axonal support due to OL malfunction and demise appear to contribute as well (Hohlfeld, 1997, Lee et al., 2012, Trapp et al., 1998).

1.3.4 Current treatment and prognosis

In light of a still unclear pathogenesis, currently no causal therapy for MS is available. Nonetheless during the past 25 years a multitude of immunomodulatory and disease-modifying therapies (DMTs) have been developed to decrease relapse rate and progression of disability. DMTs are a heterogeneous group of agents, including diverse variants of interferon β, monoclonal antibodies or small molecules. Regarding prognosis, 80% of RRMS patients eventually develop SPMS (Baneke et al., 2013) and overall life expectancy is reduced by 7 to 14 years in MS compared to the healthy population (Scalfari et al., 2013). DMTs are effective in reducing the inflammatory activity in the CNS and modulate the natural course of MS often leading to NEDA ("no evidence of disease activity") (Kappos et al., 2016). Being the first DMTs to be approved for the treatment of MS, interferons are the best-investigated MS drugs today. They modulate the immune system in various ways, leading to decreased T cell activation and leukocyte migration through the BBB, increased apoptosis of autoreactive T cells, the induction of regulatory T cells, and the modulation of endogenous cytokine production (Dhib-Jalbut and Marks, 2010). Another DMT, glatiramer acetate (GA), was approved for MS therapy shortly after the introduction of the interferons. A beneficial modulation of antigen-presenting cells as well as an induction of an anti-inflammatory CD4 T cell polarization seem to be involved in its mode of action (Weber et al., 2007). Newer DMTs such as teriflunomide, dimethyl fumarate, fingolimod, and cladribine are administered orally and, compared to the interferons and GA, seem to be more efficient regarding the reduction of relapse rate. Teriflunomide inhibits rapidly proliferating cells such as activated lymphocytes (Bar-Or et al., 2014) but also seems to promote oligodendroglial differentiation and myelination (Gottle et al., 2018). Dimethyl fumarate (DMF) is an α,β-unsaturated carboxylic acid ester whose exact mode of action is still unknown but probably relies on its lymphopenic effect and antioxidative properties (Al-Jaderi and Maghazachi, 2016). Fingolimod is a first-in-class sphingosine-1-phosphate (S1P) receptor modulator, which binds to the G protein-coupled S1P receptors S1P1, S1P3, S1P4, and S1P5 that guide lymphocyte exit out of lymphoid tissues. Interaction of Fingolimod and S1P receptors leads to receptor internalisation which prevents lymphocytes to exit lymph nodes and results in their degradation (Schwab and Cyster, 2007). Cladribine is an adenosine analogon that functions as an antimetabolite in the DNA synthesis of lymphocytes and thereby suppresses the immune system (Baker et al., 2017). The third category of DMTs encompasses the humanized monoclonal antibodies natalizumab, ocrelizumab and alemtuzumab which are all administered intravenously. Natalizumab was the first monoclonal antibody to be approved for MS treatment. It is directed against the α4 subunit of very late antigen 4 (VLA4) required by

leukocytes to bind to vascular cell adhesion molecule 1 (VCAM1) on the surface of vascular endothelial cells (Yu et al., 2013). Via neutralization of VLA4 natalizumab prevents lymphocytes from migrating into the CNS. Alemtuzumab on the other hand, induces antibody- and complement-dependent cytolysis of cluster of differentiation 52 (CD52)-positive B- and T-lymphocytes (Ruck et al., 2015). Finally, ocrelizumab binds to the surface protein cluster of differentiation 20 (CD20) causing depletion of CD20 expressing B-cells and to a minor extent also T-cells (Hohlfeld and Meinl, 2017).

1.3.5 Remyelination and repair

In the adult CNS a limited degree of myelin repair, i.e. remyelination, can be observed (Prineas et al., 1993, Ludwin, 1987). The main source for this process are OPCs which migrate into demyelinated areas where they are attracted to demyelinated but still intact axons, differentiate and then produce new myelin sheaths (Wood and Bunge, 1991, Ludwin, 1979). Although remyelination is a naturally occurring spontaneous CNS process, its efficiency remains overall low and is even further decreased during disease progression. Besides general aspects such as age and sex there appear to be several mechanisms limiting myelin repair capacity. Failure in OPC recruitment, inhibition of OPC differentiation, lack of stimulatory cues and the presence of harmful pathogenic elements are the main factors preventing efficient remyelination (Kremer et al., 2011). Against this backdrop, the identification of inhibitory pathways and stimulatory agents as well as the neutralization of pathogens could constitute novel therapeutic targets in order to improve myelin repair in the inflamed CNS. Several negative regulators of oligodendroglial differentiation have already been identified in human MS lesions (Kremer et al., 2011). Accordingly, clinical and preclinical studies are currently investigating candidate drugs based on their potential for interference with these negative regulatory pathways and their capacity to promote and facilitate remyelination (Kremer et al., 2018). One such candidate drug is opicinumab, an antibody directed against the transmembrane LRR- and Ig domain-containing Nogo receptor-interacting protein (LINGO-1). LINGO-1 negatively regulates myelination by oligodendrocytes (Mi et al., 2005) and its neutralization in experimental autoimmune encephalomyelitis (EAE) was found to reduce disease severity and to enhance remyelination (Rudick et al., 2008, Mi et al., 2007). Mechanistically, LINGO-1 activates the GTPase Ras homolog gene family member A (RhoA) (Mi et al., 2004) which is linked to cytoskeletal modulators inducing OPC process retraction (Miron et al., 2008), impairing neurite outgrowth (McGee and Strittmatter, 2003), and inhibiting oligodendroglial differentiation and myelination (Kremer et al., 2018). Another protein linked

to RhoA is the OPC sphingosine-1-phosphate receptor 5 (S1P5). Upon activation, S1P5 leads to an upregulation of RhoA followed by OPC process retraction and decreased myelination (Jaillard et al., 2005). Both the above-described S1P modulator fingolimod and its more specific successor siponimod target S1P1 and S1P5 which, over time, leads to a downregulation of S1P5 suggesting a potential beneficial effect for remyelination by a decreased RhoA activation (Miron et al., 2008). Other negative regulators of OPC differentiation are the Notch signaling pathway (John et al., 2002, Zhang et al., 2009), the Wnt signaling pathway (Shimizu et al., 2005, Feigenson et al., 2009), transcription factors such as hairy and enhancer of split 1 and 5 (Hes 1 and 5) and Inhibitor of differentiation 2 and 4 (Id2 and 4) as well as cell cycle regulators such as p57kip2 (Kremer et al., 2009). Aside from these, there are extrinsic components such as, for instance, the high molecular weight form of hyaluronan (Back et al., 2005) or MBP debris (Kotter et al., 2006) which have been found to be detrimental for OPC differentiation. On the other hand, OPC differentiation may also be affected by the downregulation or decreased activation of stimulatory receptors on OPCs, such as the adenosine triphosphate (ATP) purinoreceptor P2, the common γ chain of immunoglobulin Fc receptors (FcRγs) or CXC chemokine receptors (CXCR 2, CXCR4 and CXCR7) (Kremer et al., 2011). In this regard, therapeutic approaches could involve the direct application of the respective stimulating ligand and/or the delivery of drugs, increasing the respective ligand intrinsically. In relation to pathogenic factors negatively affecting remyelination in MS retroviruses have recently drawn increasing attention in the field of regenerative neurobiology (Antony et al., 2011). In this context, particularly human endogenous retroviruses (HERVs) such as the multiple sclerosis-associated retrovirus (MSRV) have been investigated (Perron et al., 1991, Perron et al., 1997, Garson et al., 1998) which is also the subject of the work presented here (see chapter 1.4). In conclusion, it is evident that the complex interplay of partly converging inhibitory, stimulatory and pathogenic factors demands further clarification. Above all, it is still largely unknown, whether the respective pathways are acting independently and/or in parallel to each other or whether there might even be a single but yet unknown "master regulator" at the center of pathways controlling remyelination (Kremer et al., 2011).

1.4 The human endogenous retrovirus family and MS

HERVs originate from exogenous retroviral germ-line cell infections of our primate ancestors 30-60 million years ago (Dolei, 2006). As genetic remnants of these infections, they constitute up to 8% of the human genome (Lander et al., 2001) and at least 31 different families of HERVs

have been identified so far (Katzourakis et al., 2005, Tristem, 2000, Belshaw et al., 2005). Genetically, they are classified as so-called transposons which are able to move within the genome through transcription from DNA to RNA and reverse transcription from RNA to copy DNA, which is inserted back into the genome at different sites (Rebollo et al., 2012). Functional HERV elements contain genes encoding the group-associated antigen (GAG), reverse transcriptase and integrase (POL) as well as envelope proteins (ENV) flanked by long terminal repeat regions (LTRs) (Lander et al., 2001, Löwer et al., 1996). Although in most cases HERVs are epigenetically silenced they can be reactivated leading to their re-expression (Cotton, 2001). Almost 30 years ago, Perron and colleagues isolated a leptomeningeal cell line from the CSF of an MS patient (Perron et al., 1989, Perron et al., 1991). These cells displayed reverse transcriptase activity and were found to produce viral particles. Moreover, viral RNA could also be found in the supernatant of peripheral blood mononuclear cells (PBMCs) and retrovirus-like particles were detected in monocyte cultures of other MS patients (Perron et al., 1991, Dolei and Perron, 2009). This new virus which was ultimately identified as a member of the HERV family was then designated as "multiple sclerosis associated retrovirus" (MSRV). The identification of MSRV then led to the establishment of a new HERV family, named HERV-W. This nomenclature is based on the amino acid tryptophan (W) which serves as the ligand for the tRNA functioning as a primer to promote MSRV reverse transcription (Perron et al., 1997, Blond et al., 1999). Molecular epidemiologic studies in the following years provided evidence of a significantly increased MSRV positivity in the blood and CSF of MS patients (53-100%) as compared to healthy controls (7-17.3%) (Arru et al., 2007, Garson et al., 1998). Further studies revealed an association of MS progression and severity with the presence of MSRV in so far as in positive patients a significantly greater rate of non-remitting disability and an increased conversion to SPMS was observed (Sotgiu et al., 2010). Increasing quantities of HERV-W envelope protein (HERV-W ENV) were detected in MS plaques depending on the degree of active inflammation and demyelination (Mameli et al., 2007a). In regard to the pathomechanism of MS, the HERV-W ENV was found to induce the production and release of proinflammatory cytokines In T lymphocytes and monocytes via an activation of the innate immune system pattern recognition receptor (PRR) Toll-like receptor 4 (TLR4) and cluster of differentiation 14 (CD14) (Perron et al., 2001, Rolland et al., 2006). In addition, HERV-W ENV induces dendritic cell (DC) maturation and commits T lymphocytes to Th1 lineage differentiation (Rolland et al., 2006). In order to further evaluate and analyse the potential of this pro-inflammatory effect in vivo, Perron and colleagues performed EAE experiments. They immunized C57B1/6 mice with the myelin peptide MOG35-55 emulsified either with standard mycobacterial lysate containing complete Freund's adjuvant or HERV-W ENV solved in

incomplete Freund's adjuvant (Perron et al., 2013). Analysis of the HERV-W ENV-treated mice indicated a deterioration of symptoms similar to the disease course of standard EAE. In addition, brain and spinal cord histological analyses could confirm the clinical data as they showed focal demyelinating lesions that were not different from the histopathological findings of standard EAE. Furthermore, exposition of mouse DCs to HERV-W ENV led to a TLR4/CD14-dependent proinflammatory cytokine production and immunization with HERV-W ENV in combination with MOG35-55 induced specific T-cell activation followed by γ-interferon production. Taken together, in these experiments Perron and colleagues established a new *in vivo* model in which HERV-W ENV fully replaces traditional mycobacterial lysate to induce autoimmunity and EAE in mice. This, in turn, allowed for the conduction of pre-clinical studies to investigate potential therapeutic agents that target HERV-W ENV. Such studies were of great relevance for MS, as based on its epidemiology and the previously described ENV-mediated pro-inflammatory activation of immune cells, neutralization of HERV-W ENV appeared to be a viable new therapeutic approach for MS. Accordingly, after further experiments a neutralizing monoclonal anti-ENV antibody named GNbAC1 (also known as temelimab) was developed. It is a full-length humanized recombinant monoclonal antibody of the immunoglobulin (Ig) G4/κ subclass, containing human framework regions and complementary determining regions of a parent murine antibody (Curtin et al., 2012). This parent HERV-W ENV-specific mouse monoclonal antibody by the name of mu-GNbAC1 had been obtained by immunization of mice with purified HERV-W ENV. It was selected based on its capability to prevent HERV-W ENV-dependent proinflammatory cytokine production in PBMCs as well as based on its affinity to HERV-W ENV (Curtin et al., 2015). The amino acid sequence of mu-GNbAC1 was then used to generate a humanized version of the antibody. *In vitro* immunoglobulin cytotoxicity assessment of the existing GNbAC1 IgG1 and IgG4 isotypes favoured the latter one as the isotype with the better safety profile. In contrast to the GNbAC1 IgG1 isotype, GNbAC1 IgG4 neither displayed an antibody dependent cell-mediated cytotoxicity nor a complement-dependent cytotoxicity response (Curtin et al., 2015). It selectively binds to the extracellular domain (i.e. surface unit) of the HERV-W ENV (HERV-W ENV-SU) but also to the full length HERV-W ENV (HERV-W ENV-T). Therapeutic efficacy assessment was then conducted in HERV-W ENV-induced mouse EAE and revealed a reversal of the above-described HERV-W ENV-induced clinical deterioration following GNbAC1 administration. First clinical trials in humans (Curtin et al., 2012, Derfuss et al., 2015) could not detect serious adverse drug reactions and only mild to moderate adverse events unrelated to treatment. In a next step, a phase IIb study in RRMS patients (CHANGE-MS, NCT02782858) demonstrated a significant beneficial effect of GNbAC1 on both cortical and thalamic atrophy,

with relative volume loss reductions of 31% and 72%, respectively (Hartung et al., manuscript in preparation). The number of T1 hypointense lesions, a magnetic resonance imaging marker for permanent neuronal loss, was reduced by 63% versus the control group. Furthermore, there was a benefit in magnetic transfer ratio (MTR) in both normal appearing white matter (NAWM) and cerebral cortical bands, suggesting an effect on remyelination (Chen et al., 2008). In summary, this demonstrates that antibody-mediated neutralization of HERV-W ENV by GNbAC1 seems to be a promising therapeutic approach for MS.

1.5 Goals of this thesis

The aim of this study was to investigate the impact of HERV-W ENV on OPC differentiation. The author strove to:

a) replicate previously generated results of his research group pointing to an HERV-W ENV-mediated induction of proinflammatory factors in OPCs,
b) clarify the underlying molecular pathways mediating such an effect,
c) further investigate the impact of HERV-W ENV on OPCs regarding their capacity for remyelination and
d) address the capacity of a neutralizing antibody to rescue oligodendroglial reactions upon exposure to HERV-W ENV.

In order to answer these questions, several *in vitro* methods were applied as elucidated in detail in the "Material and methods" section.

2 Material and methods

2.1 Materials

Primary oligodendroglial cell cultures were prepared from cortices of newborn Wistar rats (P0 and P1). Animals were bred 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). There, they were kept under defined and sterile conditions. Reagents and their respective suppliers for preparation and maintaining oligodendroglial cell culture, substrates and inhibitors for the incubation experiments, reagents needed for reverse transcription and polymerase chain reaction as well as those for immunocytochemistry and western blot are listed below.

2.1.1 Cell culture

Reagent	Supplier
Bovine insulin	Sigma-Aldrich Chemie, Taufkirchen, Germany
Bovine serum albumin (BSA-V)	Carl Roth, Karlsruhe, Germany
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich Chemie, Taufkirchen, Germany
Dulbecco's modified eagle medium (DMEM)	Thermo Fisher Scientific, Waltham, USA
Dulbecco's modified eagle medium high glucose (DMEM D-Glucose 4,5g/L)	Thermo Fisher Scientific, Waltham, USA
Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium	Lonza, Basel, Switzerland
Ethanol	Merck, Darmstadt, Germany
Fetal calf serum (FCS)	Thermo Fisher Scientific, Waltham, USA
Human transferrine	Sigma-Aldrich Chemie, Taufkirchen, Germany
Isofluoran	Baxter, Deerfield, USA
L-cysteine	Sigma-Aldrich Chemie, Taufkirchen, Germany
Leibovitz's medium (L-15)	Thermo Fisher Scientific, Waltham, USA
L-glutamine	Thermo Fisher Scientific, Waltham, USA
L-thyronine (T3)	Sigma-Aldrich Chemie, Taufkirchen, Germany
L-thyroxine (T4)	Sigma-Aldrich Chemie, Taufkirchen, Germany
Minimum essential medium (MEM)	Thermo Fisher Scientific, Waltham, USA
Papain from papaya latex	Sigma-Aldrich Chemie, Taufkirchen, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
Penicillin	Thermo Fisher Scientific, Waltham, USA
Poly-D-lysine hydrobromide	Sigma-Aldrich Chemie, Taufkirchen, Germany
Progesterone	Sigma-Aldrich Chemie, Taufkirchen, Germany
Putrescine	Sigma-Aldrich Chemie, Taufkirchen, Germany
Sodium selenite	Sigma-Aldrich Chemie, Taufkirchen, Germany
Streptomycin	Thermo Fisher Scientific, Waltham, USA
Trypan blue	Lonza, Basel, Switzerland
Trypsin inhibitor from turkey egg white	Sigma-Aldrich Chemie, Taufkirchen, Germany

Substrates and inhibitors	Supplier
Recombinant HERV-W ENV-T	GeNeuro, Plan-les-Ouates, Switzerland and PX'Therapeutics, Grenoble, France
HERV-W ENV-T buffer	GeNeuro, Plan-les-Ouates, Switzerland and

	PX'Therapeutics, Grenoble, France
Anti-HERV-W ENV antibody GNbAC1	GeNeuro, Plan-les-Ouates, Switzerland and Polymun, Klosterneuburg, Austria
GNbAC1 buffer	GeNeuro, Plan-les-Ouates, Switzerland and Polymun, Klosterneuburg, Austria
Anti-TLR4 mouse monoclonal antibody	Abcam, Cambridge, UK
Anti-CD14 rabbit polyclonal antibody	Abbiotec, San Diego, USA
Interleukin-1 receptor-associated-kinase-1/4 inhibitor I (IRAK-1/4 Inhibitor I)	Sigma-Aldrich Chemie, Taufkirchen, Germany
DMSO	Sigma-Aldrich Chemie, Taufkirchen, Germany
TRIF inhibitory peptide (Pepinh-TRIF)	InvivoGen, San Diego, USA
N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME)	Sigma-Aldrich Chemie, Taufkirchen, Germany
N_ω-nitro-D-arginine methyl ester hydrochloride (D-NAME)	Sigma-Aldrich Chemie, Taufkirchen, Germany
S-nitroso-N-acetyl-DL-penicillamine (SNAP)	Sigma-Aldrich Chemie, Taufkirchen, Germany

2.1.2 Reverse transcription and PCR reagents

Reagent	Supplier
β-mercaptoethanol	Sigma-Aldrich Chemie, Taufkirchen, Germany
RNeasy Mini Kit RNeasy Mini Spin Columns Collection Tubes (1.5 ml) Collection Tubes (2 ml) Buffer RLT Buffer RW1 Buffer RPE (concentrate) RNase-Free Water	Qiagen, Hilden, Germany
High capacity cDNA reverse transcription Kit 10x RT Buffer 10x RT Random Primers 25x dNTP Mix (100 mM) MultiScribe Reverse Transcriptase 1 tube (1.0 mL) 2 tubes (0.1 mL) RNase Inhibitor	Thermo Fisher Scientific, Waltham, USA
Power SYBR Green PCR Master Mix SYBR® Green I dye AmpliTaq Gold® DNA Polymerase dNTPs Passive reference dye Optimized buffer components	Thermo Fisher Scientific, Waltham, USA

2.1.3 Immunocytochemistry

Reagent	Supplier
4',6-diamidino-2-phenylindole (DAPI)	Hoffmann-La Roche, Basel, Switzerland
Anti-NF-κB polyclonal rabbit antibody (ab16502)	Abcam, Cambridge, UK
Anti-3-NT monoclonal mouse antibody (ab110282)	Abcam, Cambridge, UK
Anti-MBP monoclonal mouse antibody (836504)	Biolegend, San Diego, USA
Normal goat serum	Sigma-Aldrich Chemie, Taufkirchen, Germany
Alexa goat anti mouse IgG 488	Invitrogen, Carlsbad, USA

Alexa goat anti mouse IgG 594	Invitrogen, Carlsbad, USA
Alexa goat anti rabbit IgG 488	Invitrogen, Carlsbad, USA
Citifluor mounting medium	Citifluor, Hatfield, USA

2.1.4 Western Blot

Reagent	Supplier
Trypsin	Thermo Fisher Scientific, Waltham, USA
1x RIPA buffer Tris-HCl (pH 7.5) 20 mM NaCl 150 mM Na_2EDTA1 mM EGTA 1 mM NP-40 1% Sodium deoxycholate 1% Sodium pyrophosphate 2.5 mM β-glycerophosphate 1 mM Na_3VO_4 1 mM Leupeptin 1 µg/ml	Cell Signaling Technology, Danvers, USA
Halt protease inhibitor cocktail AEBSF 1mM Aprotinin 800nM Bestatin 50µM E64 15µM Leupeptin 20µM Pepstatin A 10µM EDTA 5mM	Thermo Fisher Scientific, Waltham, USA
Ethylenediaminetetraacetic acid (EDTA)	Thermo Fisher Scientific, Waltham, USA
DC Protein Assay Reagent A (alkaline copper tartrate solution) Reagent B (dilute folin reagent) Reagent S	Bio-Rad Laboratories, Hercules, USA
4x Novex NuPAGE LDS sample buffer Tris HCl 106 mM Tris base 141 mM LDS 2% Glycerol 10% EDTA 0.51 mM Serva blue G250 0.22 mM Phenol red 0.175 mM pH 8.5	Thermo Fisher Scientific, Waltham, USA
10x Novex NuPAGE Sample Reducing Agent Dithiothreitol (DTT) 500mM Solvent	Thermo Fisher Scientific, Waltham, USA
RunBlue Precast 4-12% SDS-PAGE gel cassette	Expedeon, San Diego, USA
20x RunBlue Rapid SDS Run Buffer unBlue RAPID SDS Run Buffer 40 mL ddH2O 760 mL	Expedeon, San Diego, USA
MagicMark XP Western Protein Standard	Thermo Fisher Scientific, Waltham, USA
10x RunBlue TGS blot buffer Tris (base) 0,25M Glycin 1,92M SDS 1%	Expedeon, San Diego, USA
RunBlue blot sandwich nitrocellulose (90x85mm)	Expedeon, San Diego, USA

1x Tris buffered saline (TBS) Tris base 20 mM NaCl 150 mM ddH2O pH adjusted to 7.6 using HCl	Sigma-Aldrich Chemie, Taufkirchen, Germany
Startingblock blocking buffers	Thermo Fisher Scientific, Waltham, USA
Anti-iNOS rabbit polyclonal antibody	Abcam, Cambridge, UK
Anti-GAPDH mouse monoclonal antibody	Merck, Darmstadt, Germany
IRDye 800CW donkey anti-rabbit antibody	Li-Cor Biosciences, Lincoln, USA
IRDye 800CW donkey anti-mouse antibody	Li-Cor Biosciences, Lincoln, USA
Polyethylene glycol sorbitan monolaurate (Tween 20)	Sigma-Aldrich Chemie, Taufkirchen, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
Ponceau S staining solution	Sigma-Aldrich Chemie, Taufkirchen, Germany
Novex Sharp Protein Standard	Thermo Fisher Scientific, Waltham, USA

2.1.5 Procedural and technical equipment

Procedural and technical equipment	Supplier
Infinite M200 Pro plate reader	Tecan Group, Männedorf, Switzerland
RunBlue Dual Run&Blot Unit	Expedeon, San Diego, USA
Heraeus Hera hot-air disinfectable gassed incubator BBD 6220	Thermo Fisher Scientific, Waltham, USA
EBA 12R table top centrifuge	Hettich, Tuttlingen, Germany
Centrifuge 5804	Eppendorf, Hamburg, Germany
GSA rotor	Thermo Fisher Scientific, Waltham, USA
New Brunswick Excella E24 incubator	New Brunswick Scientific, Edison, USA
Power Pac200 Power supply	Bio-Rad Laboratories, Hercules, USA
Biometra Trio thermoblock	Analytik Jena, Jena, Germany
Axioplan 2 fluorescence microscope	Carl Zeiss, Oberkochen, Germany
RC-5B Plus Superspeed Centrifuge	Thermo Fisher Scientific, Waltham, USA
Eclipse TE 200 microscope	Nikon Instruments, Melville, USA
Table top centrifuge 5417C	Eppendorf, Hamburg, Germany
ABI 7900 real time PCR system	Thermo Fisher Scientific, Waltham, USA
Sonifier cell disruptor B15	Branson, Danbury, USA
GeneAmp PCR System 9700	Thermo Fisher Scientific, Waltham, USA
Axiovision 4.2 software	Carl Zeiss, Oberkochen, Germany
Biophotometer	Eppendorf, Hamburg, Germany
Odyssey infrared imaging system scanner	Li-Cor Biosciences, Lincoln, USA
Odyssey software	Li-Cor Biosciences, Lincoln, USA
Cell culture flasks 75cm^2 (TC treated)	Greiner bio-one, Gremsmünster, Austria
Falcon Conical centrifuge tube (15mL, 50mL)	Thermo Fisher Scientific, Waltham, USA
Menzel Coverslips (diameter 13mm)	Thermo Fisher Scientific, Waltham, USA
Microcentrifuge tube (0,5mL, 1,5mL, 2mL)	Eppendorf, Hamburg, Germany
Multiwell culture plate (TC treated, 24 wells)	Greiner bio-one, Gremsmünster, Austria
Petri dishes (diameter 6cm, 10cm)	Greiner bio-one, Gremsmünster, Austria

2.2 Production of HERV-W ENV and GNbAC1

2.2.1 Production and purity of HERV-W ENV

Full length HERV-W ENV was produced by PX'Therapeutics, Grenoble (France), on behalf of our collaborator GeNeuro SA, Plan-les-Ouates (Switzerland). HERV-W ENV is a BL21 Escherichia coli strain-produced recombinant protein of 548 amino acids with a molecular weight of 61.44 kDa. The protein sequence is outlined in table 1 using the single-letter amino acid code.

```
MALPYHTFLFTVLLPPFALTAPPPCCCTTSSSPYQEFL
WRTRLPGNIDAPSYRSLSKGNSTFTAHTHMPRNCYNSA
TLCMHANTHYWTGKMINPSCPGGLGATVCWTYFTHTSM
SDGGGIQGQAREKQVKEAISQLTRGHSTPSPYKGLVLS
KLHETLRTHTRLVSLFNTTLTRLHEVSAQNPTNCWMCL
PLHFRPYISIPVPEQWNNFSTEINTTSVLVGPLVSNLE
ITHTSNLTCVKFSNTIDTTSSQCIRWVTPPTRIVCLPS
GIFFVCGTSAYHCLNGSSESMCFLSFLVPPMTIYTEQD
LYNHVVPKPHNKRVPILPFVIRAGVLGRLGTGIGSITT
STQFYYKLSQEINGDMEQVTDSLVTLQDQLNSLAAVVL
QNRRALDLLTAKRGGTCLFLGEERCYYVNQSRIVTEKV
KEIRDRIQCRAEELQNTERWGLLSQWMPWTLPFLGPLA
AIIFLLLFGPCIFNFLVKFVSSRIEAVKLQIVLQMEPQ
MQSMTKIYRGPLDRPARLCSDVNDIEVTPPEEISTAQP
LLHSNSVGSSHHHHHH
```

Table 1. Protein sequence of HERV-W ENV. The HERV-W ENV is composed of 548 amino acids with a molecular weight of 61.44 kDa.

The stock concentration of recombinant HERV-W ENV was 0,5 mg/mL in a buffer composed of Tris-HCl 20 mM pH 7.5, NaCl 150mM, SDS 1.5%, DTT 10mM. This dilution buffer alone was used as a control and is designated as "buffer" hereafter. Sterility and purity were assessed under approved conditions by PX'Therapeutics, Grenoble, France. Endotoxin levels were measured by the limulus amebocyte lysate (LAL) test and found to be below the detection limit of <5EU/ml. Recombinant HERV-W ENV stock solution and the prepared aliquots were stored at -80°C until being used.

2.2.2 Production and purity of GNbAC1

GNbAC1 is a recombinant monoclonal antibody of the G4/κ subclass (IgG_4) produced and purified by Polymun, Klosterneuburg, Austria and provided to us by our collaborator GeNeuro SA, Plan-les-Ouates Switzerland. Recombinant epithelial cell lines from Chinese hamster ovary cells (CHO) were established to recombinantly express GNbAC1. The application and purity of the antibody was validated by the Swiss Agency for Therapeutic Products Swissmedic, Bern, Switzerland, a federal institution of Switzerland and surveillance authority for medicines and

medical devices. The stock concentration of the delivered IgG_4 antibody was 10 mg/mL diluted in buffer containing 20 mM histidine, 5% (w/v) sucrose and 0.01% (w/v) polysorbate 20 at pH 6.0. The dilution buffer alone was used as control using equal dilutions as GNbAC1. It is designated as "buffer GNbAC1" hereafter. Both GNbAC1 stock solution and its dilution buffer were stored at 4°C. GNbAC1 is patented by GeNeuro SA, Geneva (Switzerland) since 14.01.2010 (World Intellectual Property Organization: International Publication Number WO2010/003977, International Application Number PCT/EP2009/058663).

2.3 Cell culture methods

2.3.1 Coating of cell culture dishes

For cultivation of primary rat oligodendroglial cells sterile filtered poly-D-lysine solution (PDL, 0.5 mg/ml in PBS) was used to coat cell culture flasks and dishes over night at 4°C in order to facilitate cell adhesion. Before cell plating, the plastic surfaces of culture dishes were washed three times with PBS in order to eliminate PDL residues and to prevent culture contamination. For cells to be processed for immunofluorescent staining 24-well cell culture multiwell plates were supplemented with ø 13 mm PDL coated microscope cover slips. Before cell plating cell culture dishes were filled with medium and incubated at 37°C, 98 % humidity and 5 % CO_2 for equilibration.

2.3.2 Preparation of primary rat oligodendroglial precursor cells

The generation and purification of primary oligodendroglial precursor cells from early postnatal cerebral rat cortices (P0 and P1) was performed according to McCarthy and de Vellis. All animal procedures were performed in compliance with the experimental guidelines approved by the regional authorities and ZETT. Briefly, P0/1 rat cortices were prepared and purified by laboratory technicians of our research team as follows: Postnatal rats were anesthetised using isoflurane and decapitated. After the cortices were released from the meninges and vessels they were cut into small pieces. Tissue pieces were collected in MEM-Hepes medium and centrifuged for 30 seconds at 1200 rpm. Then, the medium was discarded and replaced with fresh MEM-Hepes medium, containing 30 U/ml papain, 0.24 mg/ml L-cysteine and 40 μg/ml DNAse I type IV following a 45 min incubation step at 37°C, 98 % humidity and 5 % CO_2 in order to gently digest and dissociate tissue. After that, 1ml trypsin inhibitor solution (1 mg/ml ovomucoid trypsin inhibitor, 50 mg/ml BSA V and 40 μg/ml DNAse I

type IV in 1 ml L-15 medium) was added at room temperature for 5 min to stop digestion. The supernatant was then discarded and another 1ml trypsin inhibitor solution was added. After thorough trituration with a glass pipette, 10 ml of DMEM-medium containing 10% fetal calf serum (FCS) were added followed by centrifugation for 10 min at 1.500 rpm. The supernatant was then discarded, and the cell pellet was resuspended in 20 ml of DMEM medium containing 10% FCS, 4mM L-glutamine (Lonza) and 100 U/ml penicillin as well as 0.1 mg/l streptomycin. The cell suspension was added to 75cm^2 tissue-culture-treated (TC-treated) T75 cell culture flasks and incubated for 10 days at 37°C, 98 % humidity and 5 % CO_2 and with medium change at regular intervals twice a week.

2.3.3 Culturing primary rat oligodendroglial precursor cells

After 10 days of cultivation OPCs were harvested by means of a 2-hour shaking step at 250 rev/min for microglial decontamination followed by another 20-hour shaking step at 250 rev/min to achieve OPC detachment from the underlying astrocytic layer. For further purification and microglia elimination, the supernatant of one 75cm^2 TC-treated cell culture flask was transferred to a non-coated 10 cm bacterial culture dish and incubated for 20 min during which time microglia attached to the plastic surface. The OPC containing supernatant was then collected and centrifuged for 10 min at 1500 rpm and the cell pellet was resuspended in 1ml SATO-medium (Table 2 shows the exact composition of SATO-medium). The concentration of OPCs within this cell suspension was determined by means of trypan blue staining using a Neubauer chamber. Cells were further diluted to the required concentrations using SATO medium supplemented with 0.5% FCS (D-medium).

Agent	Final concentration
Dulbecco's modified eagle medium high glucose (DMEM D-Glucose 4,5g/L)	Solvent
Bovine insulin	5 µg/ml
Human transferrin	50 µg/ml
Bovine serum albumin (BSA-V)	100 µg/ml
Progesterone	6.2ng/ml
Putrescine	16 µg/ml
Sodium selenite	5 ng/ml
L-glutamine	4 mM
Penicillin/Streptomycin	5000 U
L-thyronine (T3)	400 ng/ml
L-thyroxine (T4)	400 ng/ml

Table 2. Composition of SATO medium and the final concentrations of the respective ingredients.

2.3.4 Oligodendroglial precursor cell treatments

Stimulation of primary rat OPCs with recombinant HERV-W ENV or buffer was performed using two different schemes: Either OPCs were exposed to surface bound HERV-W ENV or OPCs were stimulated with soluble HERV-W ENV (or buffer) diluted in D-medium to a concentration of 100 ng/ml. For soluble stimulation OPCs were distributed to the multiwell plates at a density of 30,000-50,000 cells/1,9 cm^2 and incubated at 37°C, 98 % humidity and 5 % CO_2 in D-medium. Media were substituted after 24 hours with 500 µl of either D-medium containing 100 ng/ml HERV-W ENV or D-medium with buffer, only. For stimulation experiments using surface bound HERV-W ENV, a further incubation step was added after the initial PDL coating procedure. HERV-W ENV was diluted in PBS at a concentration of 1000 ng/ml and 500 µl of this solution was added to each well for 2 hours at 37°C, 98 % humidity and 5 % CO_2. After that, the culture dishes were washed three times and then cells were plated at a density of 30,000-50,000 cells/1,9 cm^2. The same procedure was performed with buffer in equal dilutions for control purposes. For experiments using heat inactivated HERV-W ENV (and buffer controls) heating was performed prior to protein coating (Figure 2C). To this end, a 15 µl of the HERV-W ENV stock solution and the buffer solution were aseptically transferred to a 2 ml Eppendorf tube, respectively. These tubes were then transferred into a 50 ml falcon tube, which was placed into a commercial steriliser and autoclaved at a temperature of 123°C for 30 min at a pressure of 100 kPa. For neutralizing experiments HERV-W ENV was preincubated with GNbAC1. To provide suitable binding conditions, autoclaved glass beakers were used. 100 µl of sterile filtered FCS was added to each glass beaker and supplemented with a combination of two of the following agents: buffer, GNbAC1 buffer, HERV-W ENV or GNbAC1. To achieve an appropriate neutralization of HERV-W ENV by GNbAC1, we used a ratio of GNbAC1 to HERV-W ENV of 30:1 (based on personal communication with Dr. H. Perron; GeNeuro). First, GNbAC1 was added to the FCS up to a concentration of 600 µg/ml. Then HERV-W ENV was added until a concentration of 20 µg/ml was reached. This reaction solution was repeatedly mixed, and the glass beaker was covered with aluminium foil. For controls, the same concentrations of HERV-W ENV and GNbAC1 were used either complemented with GNbAC1 buffer in case of HERV-W ENV or with buffer in case of GNbAC1. The combination of buffer and GNbAC1 buffer was used as a negative control, resulting in a total of four different conditions. After incubation for 60 min at room temperature the solutions were carefully mixed again. A separate Falcon tube for each condition was prepared and filled with variable volumes of SATO medium based on the number of planned experiments. In order to obtain D-medium with final concentrations of 100 ng/ml HERV-W ENV or 3 µg/ml of GNbAC1 an appropriate volume of each HERV-W

ENV/GNbAC1 preincubated solution was then transferred to corresponding Falcon tubes. Stimulation media were then added to OPC cultures in the course of a medium change after 24 hours of preincubation at 37°C, 98 % humidity and 5 % CO_2.

For antibody and small molecule treatment OPCs were prepared, purified and cultured according to the outlined procedures described in 2.3.2 and 2.3.3. To do so, D-medium was supplemented with the following agents: For blockade of TLR4- or CD14 receptors, either anti-TLR4 antibody alone at a concentration of 10 μg/ml or 15 μg/ml, anti-CD14 antibody alone at a concentration of 10 μg/ml or 15 μg/ml, or both antibodies combined at the same concentrations were added to 30,000 OPCs in a volume of 500 μl immediately after the purification step (see 2.3.2). In a following step tubes were placed in a New Brunswick Excella E24 incubator at 37°C under continuous shaking for 2 hours at 250 rev/min. After that, 30,000 OPCs were dispensed to the each well of the multiwell plate and three wells were used per condition. For inhibition of interleukin-1 receptor–associated kinase-1/-4 (IRAK-1/-4), IRAK-1/-4 inhibitor I was diluted in dimethyl sulfoxide (DMSO) in accordance to the manufacturer's protocol. Following reconstitution, this stock solution was frozen at -20°C. After purification, 150,000 OPC were resuspended in 2 ml D-medium supplemented with IRAK-1/-4 inhibitor at a final concentration of 2400 nM and incubated in a 50 ml Falcon tube for 3 hours at 37°C under continuous shaking in a New Brunswick Excella E24 incubator. The same volume of DMSO was added to 2 ml D-medium in another Falcon tube with 150,000 OPC and served as control. For the blocking experiment of TIR-domain-containing adapter-inducing interferon-b (TRIF), the lyophilized TRIF inhibitory peptide Pepinh-TRIF was mixed with endotoxin-free water to achieve a stock concentration of 3.92 μg/μl and vortexed according to the specification of the manufacturer and then frozen at -20°C. In analogy to the blocking experiments with IRAK-1/-4 inhibitor I, 2 ml D-medium were supplemented with Pepinh-TRIF to a final concentration of 50 μM. In order to inhibit nitric oxide (NO) formation Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was dissolved in endotoxin-free water to reach a stock concentration of 50 mg/ml and immediately used after reconstitution. L-NAME was then added to 2 ml D-medium leading to a final concentration of 100 μM and incubated for 30 min applying the same conditions as mentioned above. Nω-Nitro-D-arginine methyl ester hydrochloride (D-NAME), which is the inactive enantiomer of L-NAME, was used as a negative control and applied the same way. S-Nitroso-N-acetyl-DL-penicillamine (SNAP), a nitrosothiol derivative, was used as an NO-donor and served as a positive control at a concentration of 100 ng/ml in endotoxin-free water. SNAP was immediately used after reconstitution and applied to the cells.

2.3.5 Immunocytochemistry

Immunostaining and analysis of paraformaldehyde(PFA)-fixed cultured OPC was performed as described below. At the time of fixation, multiwell plates were removed from the incubator and OPCs were washed three times with PBS and subsequently fixed at room temperature with 4% PFA. After 10 min, PFA was carefully aspirated and the OPCs were again washed with PBS. PBS supplemented with 10% normal goat serum (NGS) was added to each well to prevent unspecific staining of surface antigens. After 40 min of blocking, the respective primary antibody was added in the corresponding buffer and incubated over night at 4°C under light-protected conditions. Table 3 indicates the primary antibodies and the respective dilutions used in the experiments. After 24h, OPCs were washed again three times with PBS and incubated for 2 hours at room temperature with secondary antibodies diluted in PBS and (see Table 3).

Reagent	Dilution	Supplier
Anti-NF-κB polyclonal rabbit antibody (ab16502)	1:1000	Abcam, Cambridge, UK
Anti-3-NT monoclonal mouse antibody (ab110282)	1:1000	Abcam, Cambridge, UK
Anti-MBP monoclonal mouse antibody (836504)	1:1000	Biolegend, San Diego, USA
Alexa goat anti mouse IgG 488	1:500	Invitrogen, Carlsbad, USA
Alexa goat anti mouse IgG 594	1:500	Invitrogen, Carlsbad, USA
Alexa goat anti rabbit IgG 488	1:500	Invitrogen, Carlsbad, USA

Table 3. Primary and secondary antibodies used in immunocytostainings.

Nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI) and coverslips were mounted using Citifluor mounting medium. An Axioplan 2 fluorescence microscope (Zeiss) with Axiovision 4.2 software (Zeiss) were used to visualize stainings. Cells were analysed for marker expression by counting nine representative fields of each coverslip.

2.4 Molecular biological methods

2.4.1 Isolation of nucleic acids

In order to isolate ribonucleic acids from cultured OPCs, the RNeasy Mini Kit from Qiagen was used in accordance to the manufacturer's protocol. OPC cultures were washed three times with PBS prior to cell lysis. Cells were then lysed with 350 µl RLT lysis buffer supplemented with 0.1 M β-mercaptoethanol (dilution 1:100). In order to obtain a homogeneous cell extract,

the sample volume was transferred to QIAshredder columns and centrifuged for 2 min at 14.000 rpm at room temperature. Of note, all centrifugation steps were done at room temperature. In a next step, 350 µl of 70% ethanol was added to the eluate. Eluates in ethanol were then transferred to RNeasy spin columns and centrifuged for 2 min at 10.000 rpm. During this procedure RNAs are being bound to the RNeasy column and eluates were discarded. After a washing step using 350 µl RW1 buffer followed by another centrifugation for 2 min at 10.000 rpm, genomic DNA was removed from the column by a 15-min incubation step, using 80 µl RNAse free DNAse buffer (10 µl DNAse in 70 µl RDD buffer). Then, 350 µl RW1 buffer was added followed by centrifugation for 2 min at 10.000 rpm. Two further centrifugation steps for 2 min at 10.000 rpm followed, applying 500 µl of RPE buffer each to remove the remaining ethanol. To solve the column-bound RNA, 30 µl of nuclease free ddH2O was applied to the center of the filter and incubated for 5 min. After a final centrifugation step at 10.000 rpm for 2 min, the RNA-containing eluates were stored at -20°C.

2.4.2 Reverse transcription

For reverse transcription of the isolated RNA, the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor was used in accordance with the manufacturer's protocol. Depending on the number of samples, a master mix was prepared according to the manufacturer's protocol. Details of the composition are provided in table 4 indicating the respective volume of each reagent. 15 µl of the reverse transcription master mix were gently mixed with 15 µl of the RNA sample, resulting in a total sample volume of 30 µl. Reverse transcription was then performed according to the following protocol in a thermocycler: First step for 10 min at 25°C, second step for 120 min at 37°C, third step for 5 seconds at 85°C and lastly at 4°C until process termination. Finally, complementary DNA (cDNA) was diluted to a total volume of 100 µl for real-time quantitave PCR (RT qPCR).

Reagent	Volume in µl
10x RT Buffer	3
10x RT Random Primers	3
25x dNTP Mix (100 mM)	1,2
MultiScribe Reverse Transcriptase	1,5
RNase Inhibitor	1,5
Nuclease-free H2O	4,8

Table 4. Reverse transcription master mix. Different components of the reverse transcription master mix and their respective volumes.

2.4.3 Real-time quantitative PCR (Real-Time qPCR)

For Real-Time PCR the ABI 7900 Fast-Real-Time sequence detection system was used in combination with Power SYBRGreen universal master mix according to the manufacturer's protocol. Each primer was used at a final concentration of 0.30 pmol. Primer sequences as listed in Table 5 were designed using the PrimerExpress 2.0 software.

Real-time qPCR primer	Sequence
r-GAPDH (reference)	Fwd: GAA CGG GAA GCT CAC TGG C Rev: GCA TGT CAG ATC CAC AAC GG
r-ODC (reference)	Fwd: GGT TCC AGA GGC CAA ACA TC Rev: GTT GCC ACA TTG ACC GTG AC
r-iNOS	Fwd: CTC AGC ACA GAG GGC TCA AAG Rev: TGC ACC CAA ACA CCA AGG T

Table 5. Oligonucleotide real-time qPCR primer sequences.

The following protocol was applied: 30 µl of cDNA were diluted with 30 µl nuclease-free water. 5 µl of this mixture were combined with 15 µl of the SYBR green master mix and a corresponding volume of forward and reverse primers (1.8 µl fwd and 0.3 µl rev for GAPDH primers and 1.8 µl fwd and 1.8 µl rev for ODC and iNOS) as well as nuclease-free water (7.9 µl for GAPDH and 6.4 µl for ODC and iNOS) resulting in a total volume of 30 µl. We used an amplification profile involving 45 amplification cycles, each consisting of 15 seconds at 95°C and 1 min at 60°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ornithine decarboxylase (ODC) were used as endogenous reference genes. Relative gene expression levels were determined according to the ΔΔCt method and each cDNA sample was measured in duplicates or triplets.

2.4.4 Western blot analysis

For the isolation of proteins 500,000 – 1,200,000 OPCs were seeded on 6 mm dishes, that were either coated with HERV-W ENV or buffer according to the coating procedure further specified above (see 2.3.1). OPC cultures were stimulated with HERV-W ENV for 24 hours and then washed two times with PBS. After that, each dish was incubated with 2 ml 0.05 % trypsin/

ethylenediaminetetraacetic acid (EDTA) at 37°C for 4-5 min until cells were dislodged from the surface. The reaction was stopped with 5 ml FCS medium (10% FCS in DMEM high glucose) per dish and cells were collected in conic tubes for centrifugation at 1500 rpm at room temperature for 5 min. The OPC pellets were washed three times with PBS (once with 20 ml PBS, two times with 10 ml PBS) and each time centrifuged at 1500 rpm at 4°C for 5 min. For cell lysis RIPA buffer supplemented with 1x Halt protease, 1x Halt phosphatase inhibitor cocktails and 1xEDTA was applied for 5-10 min in accordance to the manufacturer's protocol. Subsequently lysates were sonicated in two cycles, each lasting 10 seconds (settings: cycle at 5x (10%), power at 50%). Then lysates were centrifuged for 10 min and 5 µl were taken out and mixed with RIPA buffer at a ratio 1:5. Protein concentrations were determined by means of DC Protein Assay on an Infinite M200 Pro plate reader at 750 nm. The protein samples were then mixed with 1x NuPAGE LDS Sample Buffer (Novex) and 1xNuPAGE Sample Reducing Agent (Novex) followed by heating to 90°C for 5 min. A RunBlue Precast 4-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel cassette was used for protein separation. 3.5 µl MagicMark XP Western Protein Standard and 8 µl Novex Sharp Pre-stained Protein Standard were applied to each gel for protein standard specification. 10 µg – 23 µg of sample proteins were applied per lane. SDS-PAGE gel was inserted into a RunBlue Dual Run&Blot unit filled with 1x RunBlue RAPID SDS run buffer, running at 150V and at constantly 60mA/gel for 70-90 min. The separated proteins were transferred onto nitrocellulose membranes by electroblotting with 20 % methanol at constantly 200 V for 70 min in the RunBlue Dual Run&Blot unit. The success of protein transfer was checked with transient staining of the nitrocellulose membrane by Ponceau S staining solution. Afterwards 2% milk powder in TBS was used to block the membranes for 60 min at room temperature. The primary antibodies anti-iNOS (1:250) and anti-GAPDH (1:1000) used for detection were diluted in 2% milk in 1xPBST (PBS+0,05% tween20). Incubation was performed over night at 4°C. Then membranes were washed four times with 1xPBST under minimal shaking for 5 min. Incubation with secondary antibodies was performed for 2 hours at room temperature with 2% milk and PBST under light protection and both secondary antibodies were used at a dilution of 1:10,000. Membranes were washed again with PBST four times and signals were visualized by the Odyssey infrared imaging system scanner. Odyssey software was used for protein band quantification and the intensity of each band was determined and normalized to the intensity of the GAPDH band of the corresponding probe.

2.5 Statistics

Unless otherwise stated, data is presented as mean values ± standard deviation (SD) and significance was assessed by two-sided Student's t-test, unpaired comparison for means (with GraphPad Prism). A significant difference for the experimental groups with their respective probability value *p* was considered at *p<0.05, **p<0.01, ***p<0.001; ns, not significant. In this regard, *n* represents the number of the conducted independent experiments.

3 Results

3.1 HERV-W ENV induces iNOS expression in OPCs via TLR4 activation

A previous study by Rolland and colleagues indicated that in human PBMCs and DCs HERV-W ENV induces a proinflammatory response by an activation of the PRR Toll-like receptor 4 (TLR4) and its co-receptor, the glycosyl phosphaditylinositol-anchored protein Cluster of differentiation 14 (CD14) (Rolland et al., 2006). PRRs such as TLR4 are involved in the initiation of the innate immune response against microbial pathogens (Trinchieri and Sher, 2007) such as bacterial lipopolysaccharides (LPS) (Poltorak et al., 1998) and viral particles (Figure 1) (Kurt-Jones et al., 2000). Since oligodendroglial TLR4 expression had previously been controversially discussed in the literature (Lehnardt et al., 2002, Taylor et al., 2010, Yao et al., 2010), the author's research group confirmed its expression on rat and human OPCs both via immunocytochemistry and quantitative RT-PCR (Kremer et al., 2013, Schichel, 2014). Furthermore, data generated by the author's research group had demonstrated that HERV-W ENV strongly increased the expression of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and the inducible NO synthase (iNOS) leading to the production of nitric oxide (NO), a harmful reactive nitrogen species (Kremer et al., 2013, Schichel, 2014). The first step of this project was therefore to reproduce the previously obtained results showing an induction of proinflammatory factors by HERV-W ENV. To this end, OPCs were stimulated with either surface-bound ("ENV solid") or soluble ("ENV soluble") HERV-W ENV resulting in a logarithmic increase of iNOS transcription (Figure 2A,B). In order to prove the involvement of the ENV/TLR4 ligand-receptor pair in this proinflammatory response, heat inactivation (i.e. denaturation) of HERV-W ENV and antibody-mediated blockade of TLR4 and CD14, were used respectively (Figure 2C,D). In both approaches iNOS transcription levels were used as a readout for successful neutralization of the HERV-W ENV effect. These experiments demonstrated that heat inactivation of HERV-W ENV (dashed grey bar) prevented iNOS gene induction (Figure 2C). In parallel to that, antibody-mediated blockade of TLR4 (Figure 2D, dashed black bar) also lead to a neutralization of the HERV-W ENV effects. On the other hand, antibody-mediated blockade of TLR4 co-receptor CD14 (Figure 2D, dashed white bar) did not prevent an increase of iNOS transcription levels.

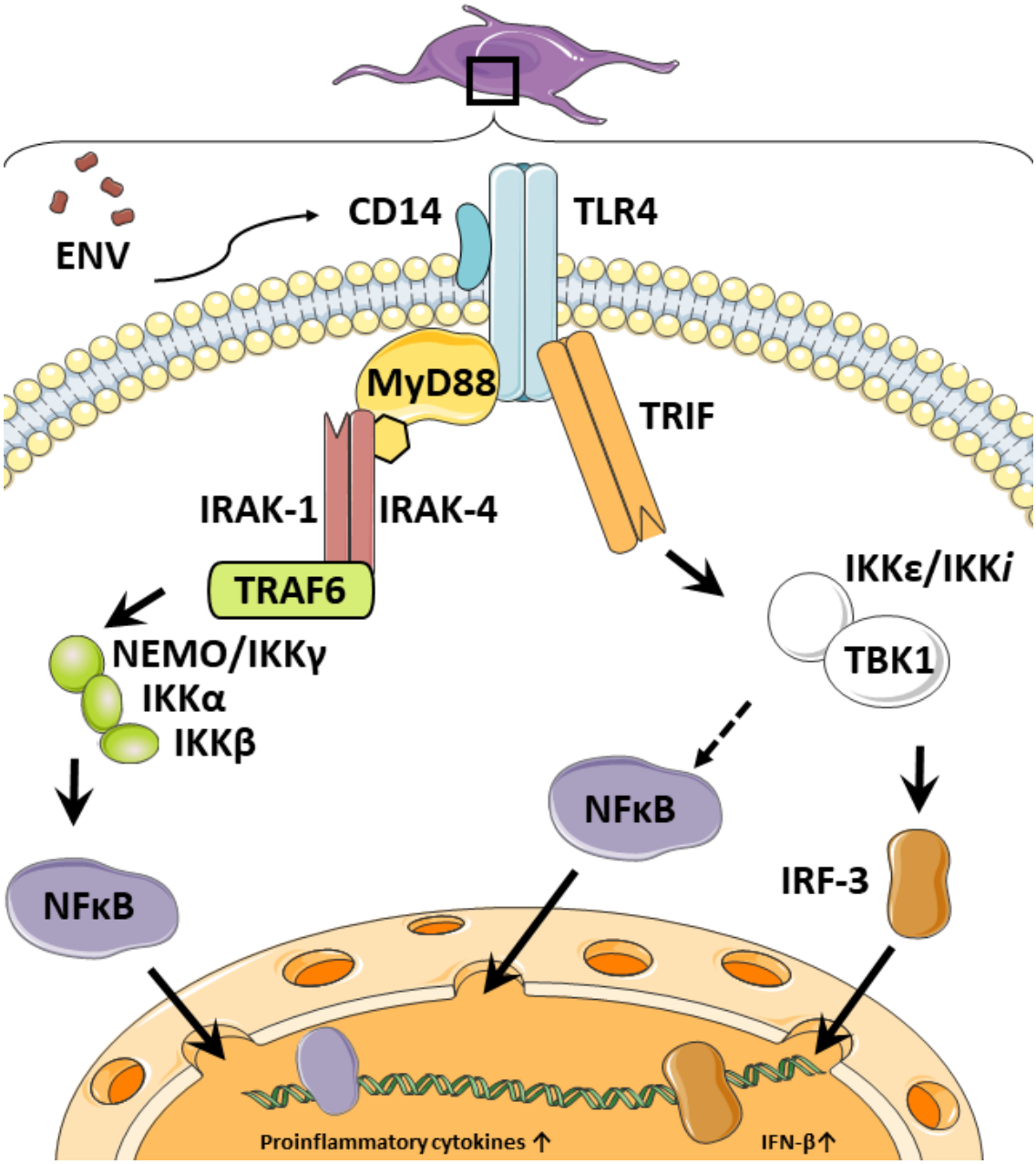


Fig. 1. Toll-like receptor 4 (TLR4) signaling cascade activated by HERV-W ENV. TLR4 is an transmembrane receptor protein with an extracellular domain consisting of a leucine-rich repeat (LRR) domain for ligand recognition and a cytoplasmic Toll/interleukin 1 receptor homology domain (TIR) critical for intracellular signal transduction (Medzhitov et al., 1997). HERV-W ENV binding to TLR4 leads to the activation (Rolland et al., 2006) of two diverging intracellular signaling cascade. The myeloid differentiation response 88 (MyD88)-dependent pathway is common to all TLRs and involves the recruitment of IL-1 receptor-associated kinase (IRAK)-4, IRAK-1 and TNF associated factor 6 (TRAF 6) to the TLR4 cytoplasmic domain. This is followed by a dissociation of the activated IRAK-1 and TRAF 6 complex, an activation of transforming growth factor beta-activated kinase 1 (TAK1) (not shown) and a phosphorylation of the Inhibitor of NFκB (IκB) kinase (IKK) complex, consisting of IKKα, IKKβ, and NEMO/IKKγ. Phosphorylation of this complex promotes the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) resulting in the transcription of proinflammatory cytokines such as inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNFα) and interleukin-1 β (IL-1 β) (Takeda and Akira, 2004). In contrast, the MyD88-independent pathway is mediated by the TIR domain-containing adaptor inducing IFN-β (TRIF), which leads to an activation of interferon regulatory factor-3 (IRF-3) and possibly to a delayed activation of NFκB (dashed arrow) via TANK-binding kinase 1 (TBK1) and IKKε/IKKi (Takeda and Akira, 2004). Intranuclear activated IRF-3 promotes transcription of antiviral IFN-β and IFN-α. This drawing is modelled after Takeda and colleagues (Takeda and Akira, 2004).

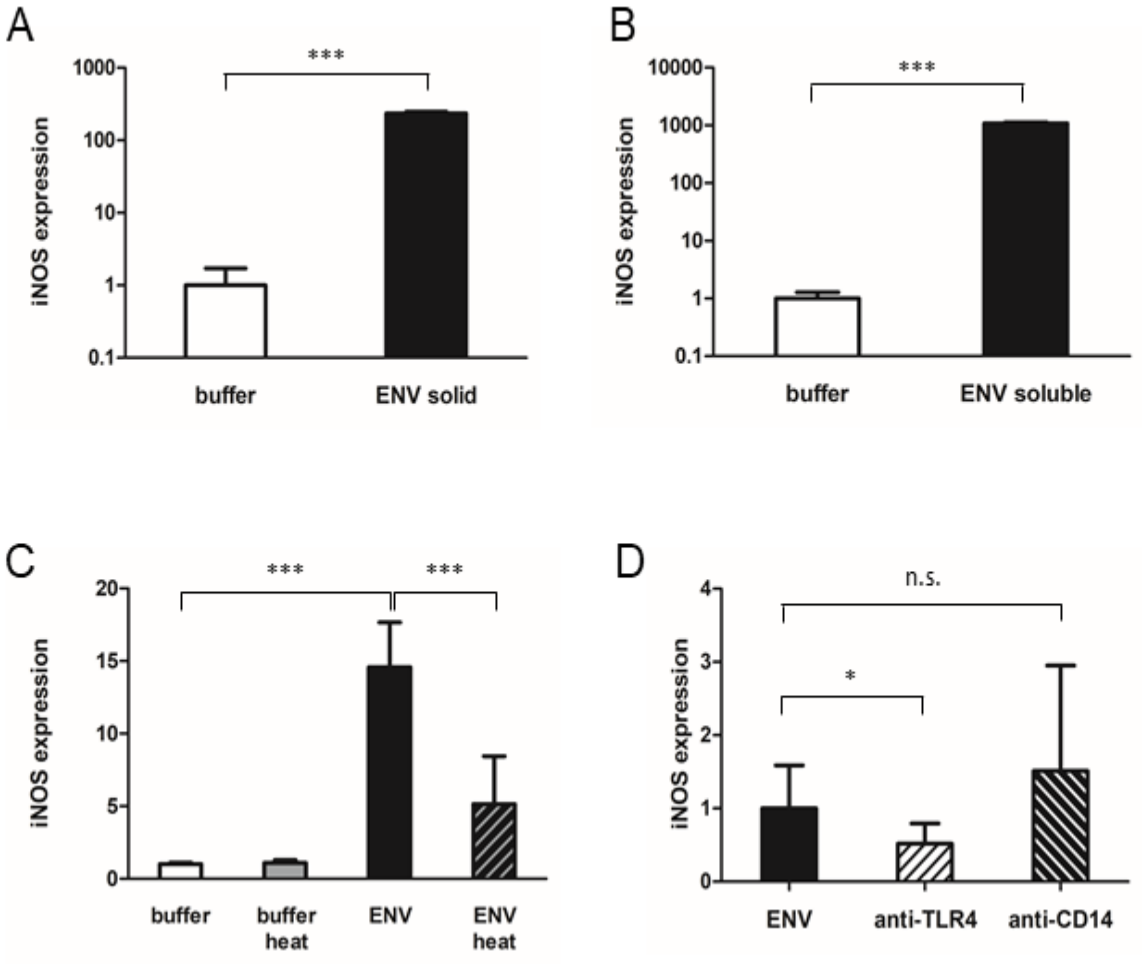


Fig. 2. Stimulation of OPCs with recombinant HERV-W ENV leads to a logarithmic increase of inducible nitric oxide synthase (iNOS) gene expression which can be neutralized by heat inactivation of HERV-W ENV or antibody-mediated TLR4 blockade. (A,B) Stimulation of cultured OPCs with surface-bound ("ENV solid"; A) and soluble HERV-W ENV ("ENV soluble"; B) leads to a strong induction of iNOS gene expression as compared to buffer treated cells. (C) Heat inactivation of HERV-W ENV (dashed grey bar) partially prevents iNOS gene induction. (D) Preincubation of OPCs with anti-TLR4 antibody (dashed black bar) followed by stimulation with surface-bound HERV-W ENV also prevents iNOS gene induction. However, preincubation with anti-CD14 antibody (dashed white bar) cannot prevent HERV-W ENV-mediated iNOS gene induction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as the reference gene in all experiments. Data are shown as mean ± standard deviation and represent one out of eight (A) and four (B) independent experiments, respectively. Data in C and D derive from three independent experiments, respectively. Statistical significance was determined using t-test (*$p < 0.05$, ***$p < 0.001$, n.s. = not significant).

3.2 MyD88-dependent and MyD88-independent pathways are involved in HERV-W ENV/Toll-like receptor 4 signaling

As already described above, TLR4 receptor activation can lead to the activation of two separate downstream pathways converging on intranuclear proinflammatory gene activation (see Figure 1). The myeloid differentiation response 88 (MyD88)-dependent pathway is common to all TLRs and involves a recruitment of IL-1 receptor-associated kinase (IRAK)-4,

IRAK-1 and TNF associated factor 6 (TRAF 6) to the TLR4 cytoplasmic domain. On the other hand, the MyD88-independent pathway is mediated by the TIR domain-containing adaptor inducing IFN-β (TRIF), involving an activation of interferon regulatory factor-3 (IRF-3). In order to identify the TLR4 downstream pathway responsible for the previously observed HERV-W ENV-mediated proinflammatory effects small inhibitory molecules directed against IRAK-1/-4 and TRIF, respectively, were applied, both separately as well as in combination (Figure 3). Again, iNOS transcription levels were used as a readout. Both the single application of IRAK-1/-4 inhibitor and TRIF inhibitor significantly prevents an increase of iNOS gene induction by HERV-W ENV (Figure 3A,B). However, a combined simultaneous blockade of both pathways led to the strongest prevention of iNOS induction (Figure 3C).

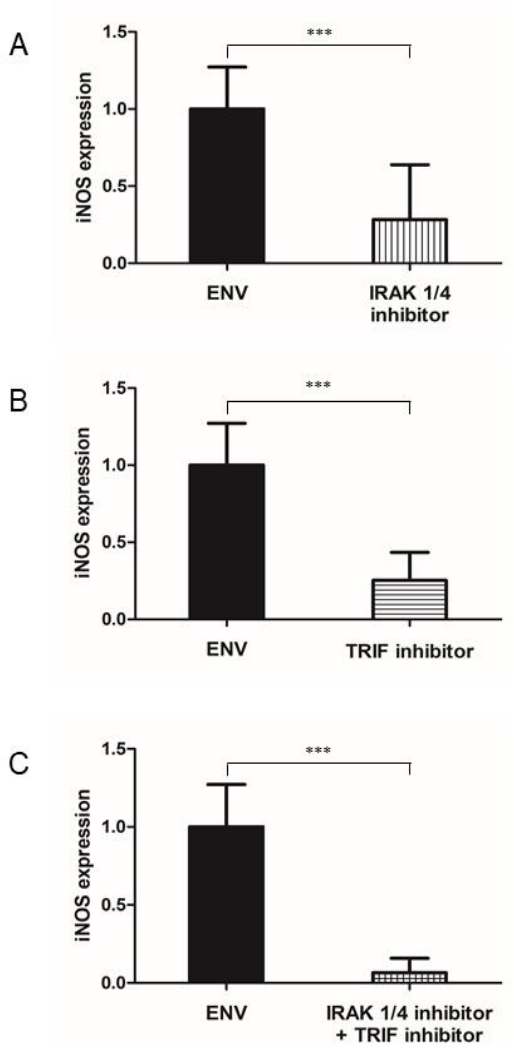


Fig. 3. Blockade of the MyD88-dependent and -independent TLR4 downstream pathways prevents HERV-W ENV-dependent iNOS gene induction. (A) Blockade of the MyD88-dependent pathway by IRAK-1/4 inhibitor as well as (B) a blockade of the MyD88-independent pathway by TRIF inhibitor both prevent iNOS gene induction by HERV-W ENV. (C) Combined inhibition of both pathways results in the most efficient prevention of HERV-W ENV-mediated iNOS gene induction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as the reference gene in all experiments. Data are shown as mean values ± standard deviation and derive from three independent experiments each (n=3). Statistical significance was determined using t-test (***$p < 0.001$).

3.3 HERV-W ENV modulates the nuclear translocation of NFκB

Nuclear translocation of the proinflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is mainly mediated by the MyD88-dependent signaling pathway but is also thought to be part of the MyD88-independent signal transduction pathway as a "delayed response" after activation of TLR4 (see Figure 1). NFκB is known to increase the transcription of proinflammatory genes such as iNOS, the interleukins and TNFα (Barnes and Karin, 1997, Ganster et al., 2001). In order to investigate the HERV-W ENV-dependent subcellular localisation of NFκB immunofluorescent stainings of cultured OPCs stimulated with HERV-W ENV were performed. OPCs in the control group displayed either cytoplasmic, perinuclear or no NFκB signals at all (Figure 4B,B'; arrowheads) while only few cells displayed nuclear NFκB signals. In contrast to that, HERV-W ENV stimulation significantly increased the amount of nuclear NFκB (Figure 4C,C'; arrows) pointing to a connection between this transcription factor and the previously observed HERV-W ENV-mediated induction of proinflammatory cytokines (see Figures 2 and 3).

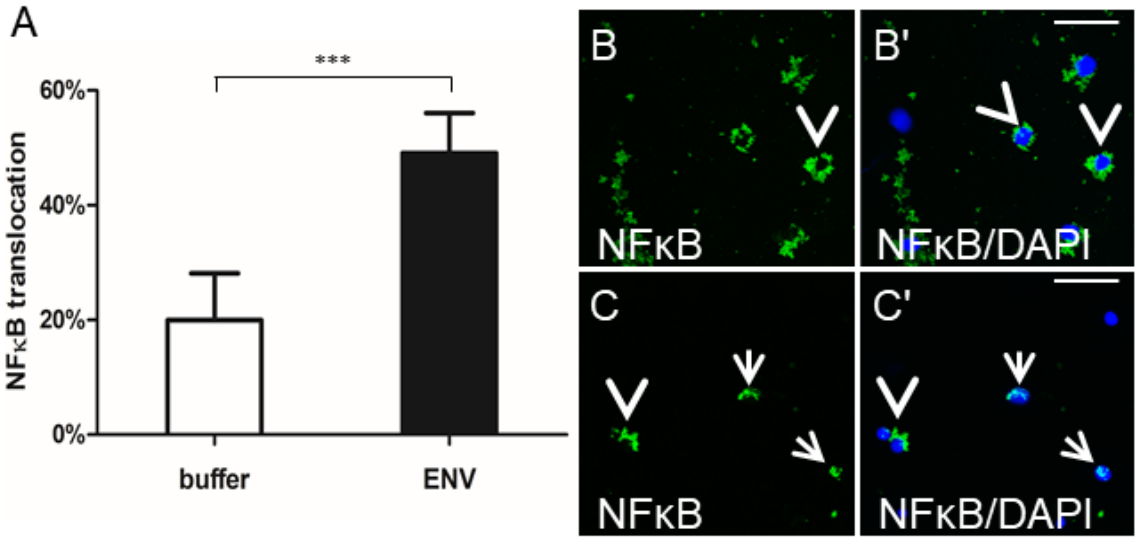


Fig. 4. Intracellular localization of NFκB following HERV-W ENV stimulation. (A) HERV-W ENV leads to an increase of nuclear NFκB localization as compared to controls. (B-C') Representative NFκB immunostainings of controls (B,B') and HERV-W ENV-stimulated OPCs (C,C'). Arrowheads point to OPCs with cytoplasmic localization of NFκB. Arrows indicate OPCs with nuclear localization of NFκB. Scale bars = 30 μm. Data are shown as mean values ± standard deviation and derive from three independent experiments. Statistical significance was determined using t-test (***p < 0.001). DAPI = 4',6-diamidino-2-phenylindole. Images modified after Kremer et al. (Kremer et al., 2013), copyright © 2013 American Neurological Association.

3.4 HERV-W ENV induces iNOS protein synthesis and nitrosative stress

As shown above HERV-W ENV leads to a TLR4-mediated activation of both the MyD88-dependent and MyD88-independent TLR4 downstream pathway inducing NFκB nuclear translocation and iNOS gene induction. In order to investigate the impact of the HERV-W ENV-induced iNOS gene induction on the downstream protein level, Western blot (WB) analysis was performed. Consistent with the observed elevated iNOS mRNA levels, HERV-W ENV stimulation increased iNOS protein levels as showed by qualitative (Figure 5A) and semi-quantitative (Figure 5B) WB analysis. Following HERV-W ENV stimulation, semi-quantitative analysis displayed a 1,71- to 2,35-fold increase in iNOS protein expression as compared to controls.

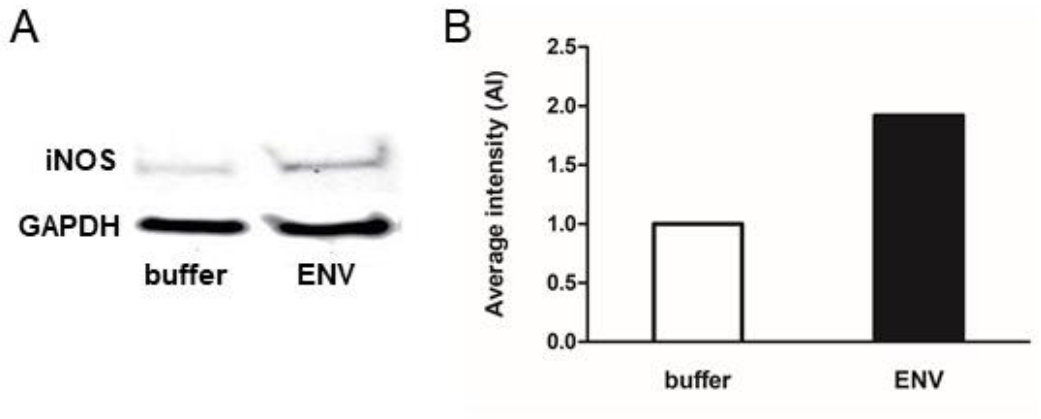


Fig. 5. iNOS protein synthesis following HERV-W ENV stimulation. (A) Qualitative Western blot analysis demonstrates an elevated iNOS protein expression in HERV-W ENV-stimulated OPCs as compared to controls. (B) HERV-W ENV stimulation leads to 1.71- to 2.35-fold increase of iNOS protein as shown by semi-quantitative Western blot analysis of protein band intensities. GAPDH bands were used for normalization of the corresponding iNOS samples. Data derive from one out of five representative experiments. Modified after Kremer et al. (Kremer et al., 2013), copyright © 2013 American Neurological Association.

As a next step, the consequences of an increased HERV-W ENV-induced iNOS protein synthesis were investigated. The enzyme iNOS is known to produce NO, a free radical gas which can diffuse across cell membranes. Under physiological conditions this functions as a defense mechanism targeting pathogen replication by interfering with viral proteases or bacterial DNA (Lowenstein and Padalko, 2004). Previous experiments in OPCs had already shown an HERV-W ENV concentration-dependent increase of NO formation following HERV-W ENV stimulation (Schichel, 2014). However, NO has a short half-life so that it is challenging to measure it by means of spectrometry. However, NO can also react with superoxide radicals, resulting in the formation of toxic peroxynitrite (Szabo et al., 2007). This molecule, in turn, can nitrate protein tyrosine residues leading to the formation of 3-nitrotyrosine (3-NT). 3-NT is a marker of nitrosative stress, inflammation, and cell damage and has been detected in MS lesions as well

as in several other human diseases (Ischiropoulos, 1998). Accordingly, in order to investigate the biological implications of the above-described increased iNOS protein synthesis, 3-NT formation was used as a readout for increased nitrosative stress mediated by HERV-W ENV (Figure 6). HERV-W ENV stimulation significantly increased the number of 3-NT positive OPCs almost 3-fold as compared to controls (Figure 6A, black bar). In order to prove the specific role of iNOS in this context the cell-permeable NOS inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used. Immunofluorescent analysis demonstrated that pre-treatment with L-NAME prevented HERV-W ENV-mediated 3-NT formation (Figure 6A, red bar). On the other hand, its inactive enantiomer D-NAME did not affect HERV-W ENV-mediated 3-NT formation (Figure 6A, dashed white bar). Stimulation with the NO-donor S-Nitroso-N-Acetyl-D,L-penicillamine (SNAP) was performed separately and served as a positive control leading to the highest number of 3-NT positive OPCs (Figure 6A, white bar).

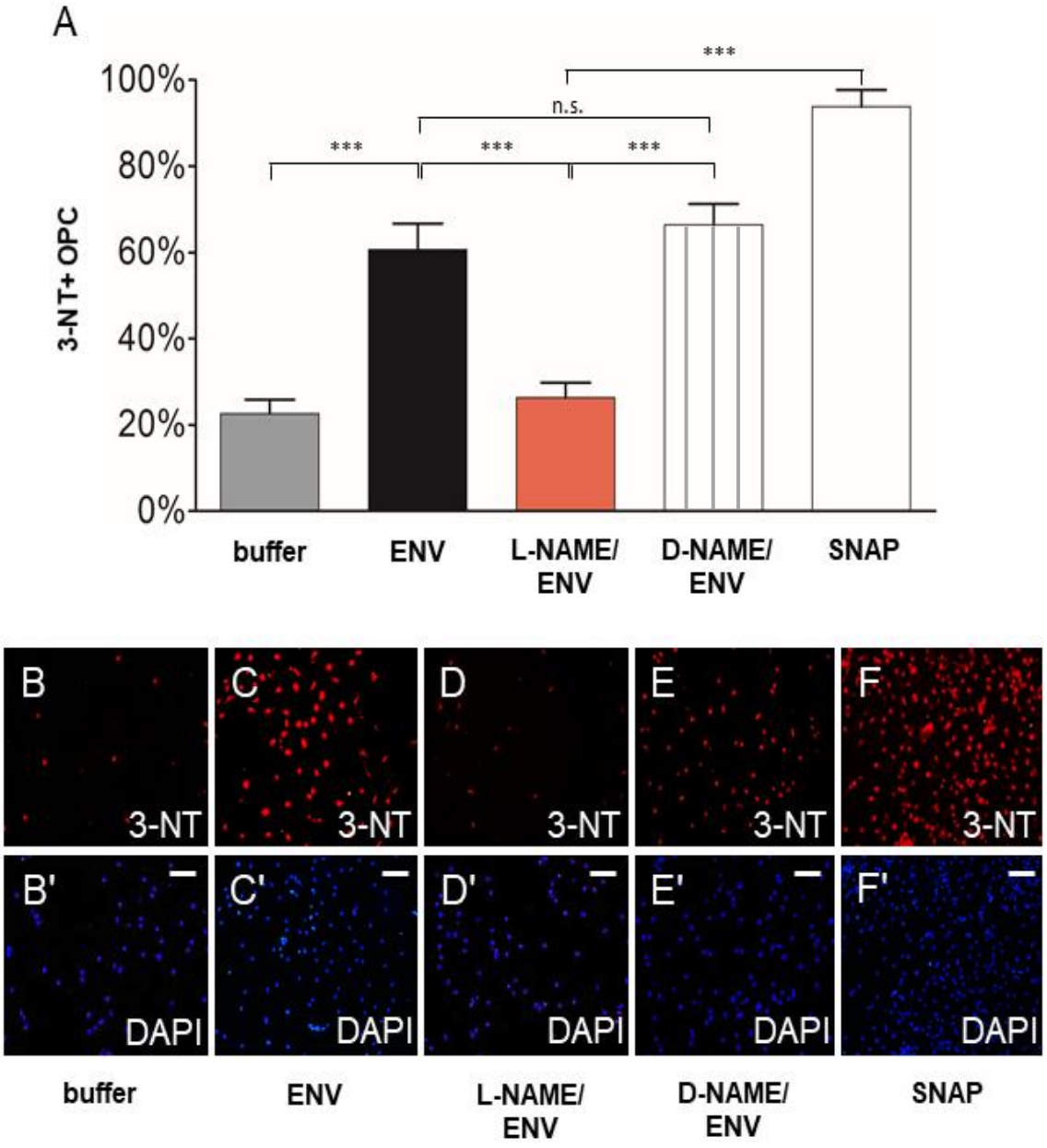


Fig. 6. HERV-W ENV increases nitrotyrosine formation via iNOS. (A) Stimulation with HERV-W ENV significantly increases the number of 3-nitrotyrosine (3-NT) positive OPCs (black bar) as compared to controls (grey bar). Preincubation of OPCs with the iNOS inhibitory molecule N(ω)-nitro-L-arginine methyl ester (L-NAME) prevents the HERV-W ENV-mediated 3-NT formation (red bar). However, preincubation with its inactive enantiomer D-NAME does not prevent HERV-W ENV-dependent 3-NT formation (white dashed bar). S-nitroso-N-acetylpenicillamine (SNAP) is a strong NO donor leading to nitrotyrosination and is used as a positive control (white bar). (B-F') Representative immunofluorescent staining of OPCs after exposure to buffer (B,B'), HERV-W ENV (C,C'), and additional preincubation with L-NAME (D,D') or D-NAME (E,E') prior to HERV-W ENV stimulation. SNAP is used as a positive control (F,F'). Anti-3-NT antibody (B-F, red) and 4',6-diamidino-2-phenylindole (DAPI, B'-F', blue) were used. Scale bars = 50 µm. Data are shown as mean ± standard deviation and represent 1 out of 3 independent experiments (n=3). ***$p < 0.001$ by student's t-test; n.s. = not significant. Modified after Kremer et al. (Kremer et al., 2013), copyright © 2013 American Neurological Association.

3.5 HERV-W ENV affects OPC differentiation

NO and its metabolites are known to negatively affect the cell cycle regulation and differentiation of cells types such as embryonic stem cells (Tejedo et al., 2010), neural stem/progenitor cells (Shen et al., 2013), and human monocytes (Fernandez-Ruiz et al., 2004). However, little is known about the effect of NO and its metabolites on OPC differentiation. Accordingly, the expression of MBP as an indicator for a successful OPC differentiation was analysed following HERV-W ENV stimulation (Figure 7).

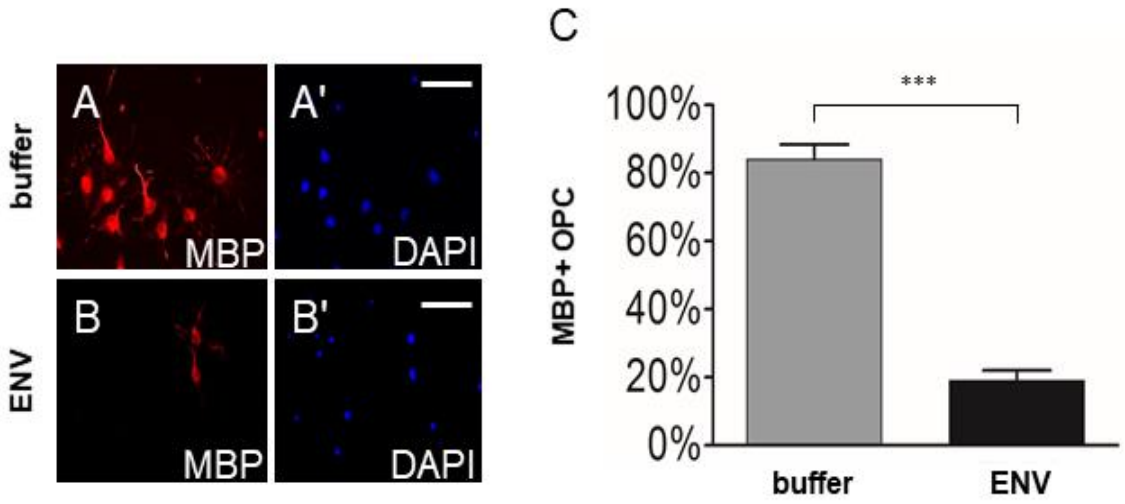


Fig. 7. HERV-W ENV inhibits OPC differentiation. (A-B') HERV-W ENV impairs OPC differentiation (B) compared with controls (A) as demonstrated by representative immunofluorescent staining visualizing the MBP expression. (C) Exposure to HERV-W ENV significantly decreases the number of MBP positive OPCs (black bar) as compared to controls (grey bar) pointing to an inhibited OPC differentiation. Anti-MBP antibody (A,B, red) and 4',6-diamidino-2-phenylindole (DAPI, A',B', blue) were used. Scale bars = 50 µm. Data are shown as mean values ± standard deviation and represent one out of four independent experiments. *** $p < 0.001$ by student's t-test. Modified after Kremer et al. (Kremer et al., 2013), copyright © 2013 American Neurological Association.

HERV-W ENV stimulation led to a significant decrease in MBP production as demonstrated by immunofluorescent stainings (Figure 7C). This points to a relevant negative impact of HERV-W ENV on OPC differentiation. Of note, similar findings were made by the author's research group regarding the production of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) at earlier time points (data not shown). In line with the previous experiments (see Figure 6), HERV-W ENV-induced differentiation blockade could be prevented by the application of the NOS-inhibitory molecule L-NAME (Figure 8D, red bar). Its inactive enantiomer D-NAME was not able to rescue this HERV-W ENV-induced effect (Figure 8D, white bar).

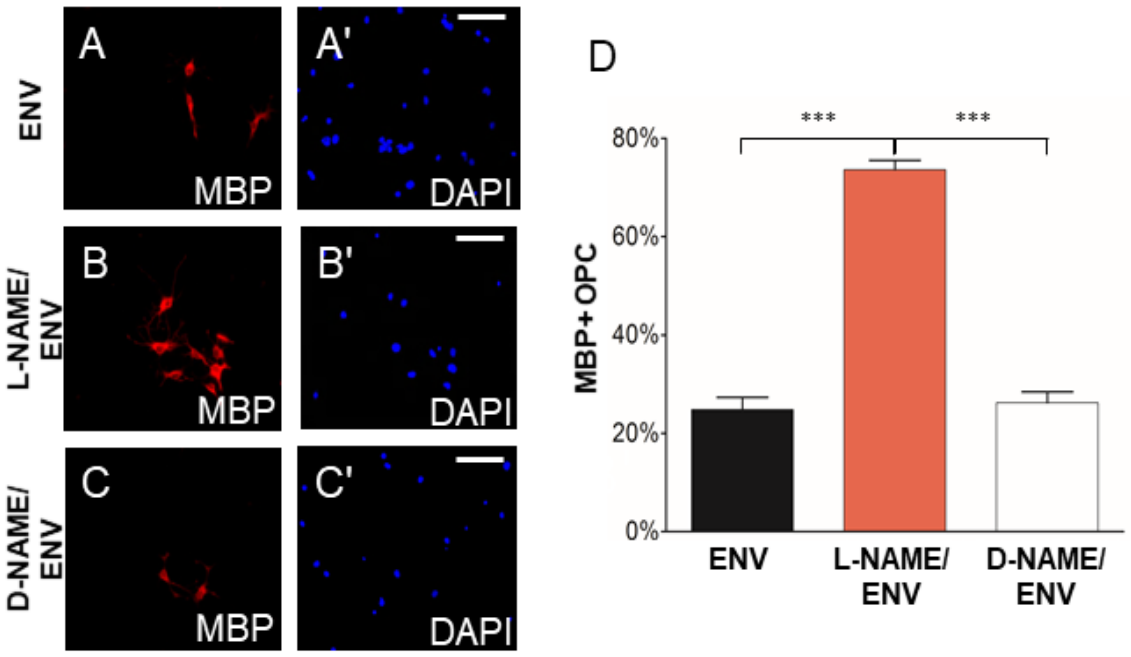


Fig. 8. The HERV-W ENV-mediated differentiation blockade can be rescued by the iNOS inhibitory molecule L-NAME. (A and black bar in (D)) Only few OPCs express MBP following HERV-W ENV stimulation indicating a decreased rate of differentiation. (B and red bar in (D)) Pretreatment with L-NAME prevents this HERV-W ENV-mediated effect. (C and white bar in (D)) Application of the inactive enantiomer of L-NAME, D-NAME does not prevent HERV-W ENV-mediated differentiation blockade. Anti-MBP antibody (A-C, red) and 4',6-diamidino-2-phenylindole (DAPI, A'-C', blue) were used. Scale bars = 50 µm. Data are shown as mean values ± standard deviation and represent one out of four independent experiments. ***$p < 0.001$ by student's t-test. Modified after Kremer et al. (Kremer et al., 2013), copyright © 2013 American Neurological Association.

3.6 HERV-W ENV-mediated iNOS gene induction is prevented by the HERV-W ENV specific antibody GNbAC1

As demonstrated further above HERV-W ENV protein activates TLR4 leading to iNOS gene induction and 3-NT formation which results in an impaired OPC differentiation. The following section focusses on the neutralizing HERV-W ENV-specific antibody GNbAC1 (see chapter 1.4.3). In order to prove its ability to prevent the HERV-W ENV-mediated OPC differentiation blockade GNbAC1, HERV-W ENV, and their respective buffer solutions were applied as previously described (see section 2.3.4) and iNOS transcription levels were used as a readout. As already demonstrated HERV-W ENV stimulation led to a significantly increased iNOS gene induction (Figure 9A, black bar and see Figure 2,A,B). Preincubation with GNbAC1 could significantly diminish this HERV-W ENV-mediated effect as demonstrated by real-time qPCR analysis (Figure 9A, dashed grey bar). Neither the respective buffer solutions of HERV-W ENV or GNbAC1 (Figure 9A, white bar) nor the neutralizing antibody GNbAC1 (Figure 9A, grey bar)

led to an enhanced iNOS gene induction. This effect was also observed after stimulation for twenty-four hours with the respective agents (Figure 9B).

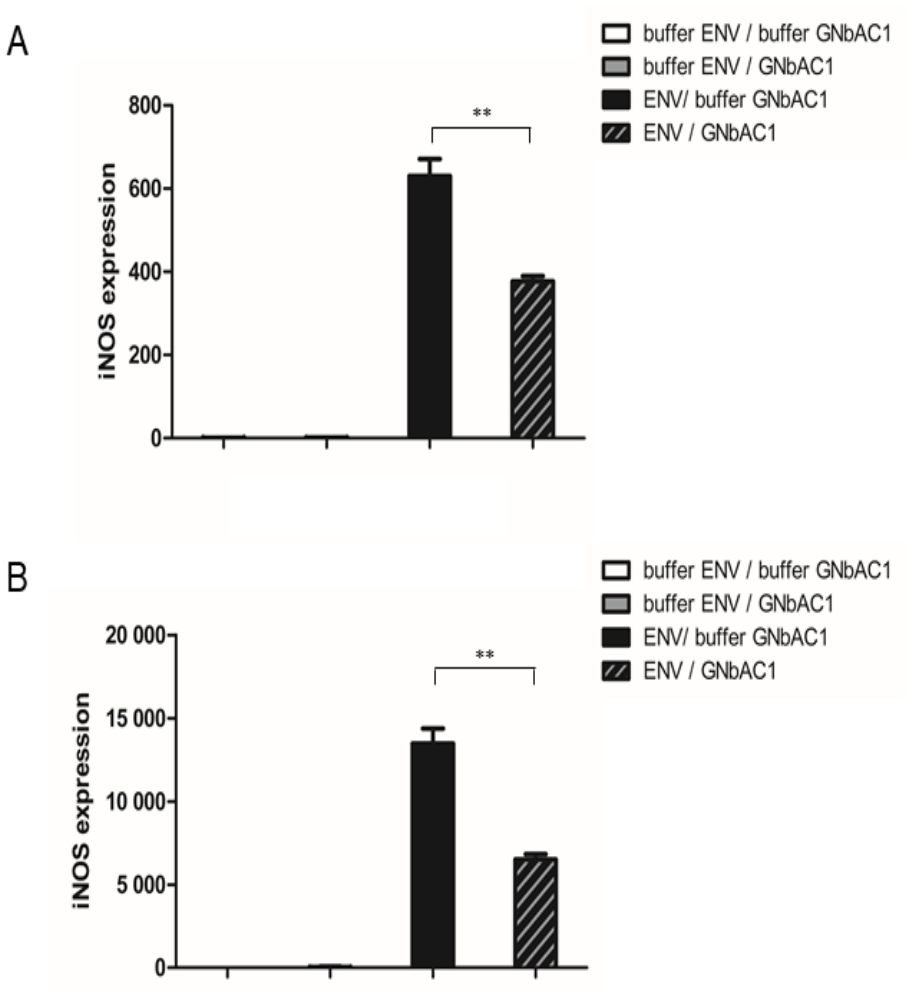


Fig. 9. The neutralizing HERV-W ENV-specific antibody GNbAC1 abrogates HERV-W ENV-mediated iNOS gene induction. (A) Following 8 hours of stimulation HERV-W ENV leads to a strong iNOS gene induction in OPCs (black bar). Preincubation with the HERV-W ENV-specific antibody GNbAC1 can prevent this effect (grey dashed bar). Stimulation with buffer-only (white bar) or GNbAC1 (grey bar) does not affect iNOS gene induction. (B) This effect can also be observed following 24 hours of HERV-W ENV stimulation. GAPDH was used as the reference gene in all experiments. Data are shown as mean values ± standard deviation and derive from one representative out of four independent experiments. t-test: ** $p < 0.01$ by student's t-test.

3.7 GNbAC1 decreases HERV-W ENV-mediated 3-NT formation and protects OPC differentiation

Furthermore, the biological effect of GNbAC1 on the previously observed impact on OPC differentiation was investigated based on 3-NT positivity (Figure 10) and MBP expression (Figure 11), respectively. In parallel to previous experiments, HERV-W ENV stimulation increased the number of 3-NT positive OPCs (Figure 10A, black bar). However, preincubation of

HERV-W ENV with GNbAC1 could significantly reduce this effect leading to an amount of 3-NT positive OPCs comparable to controls (Figure 10A, grey dashed bar). GNbAC1 alone (Figure 10A, grey bar) and the respective buffers (Figure 10A, white bar) did not exert effects on 3-NT positivity and were used as controls.

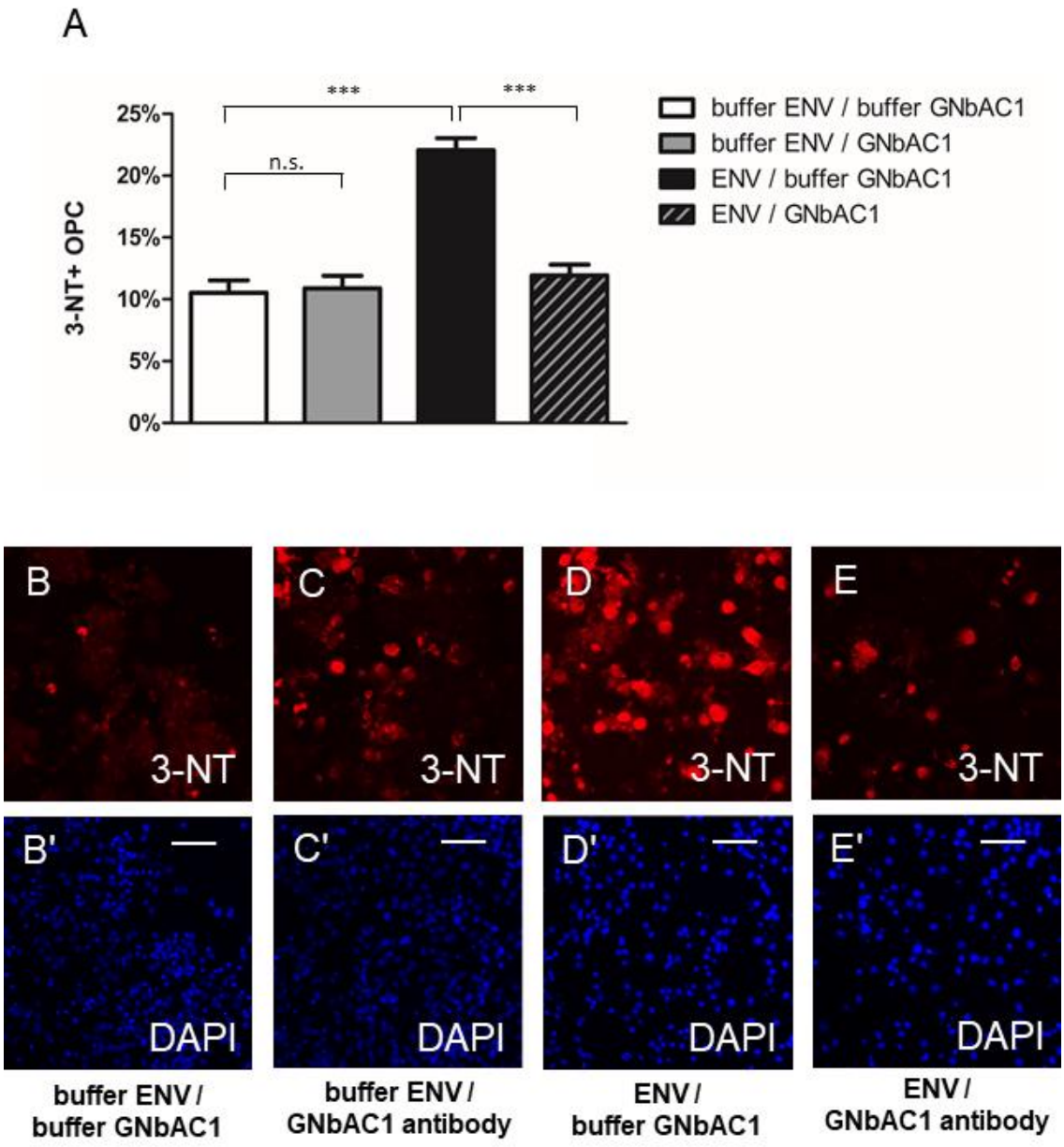
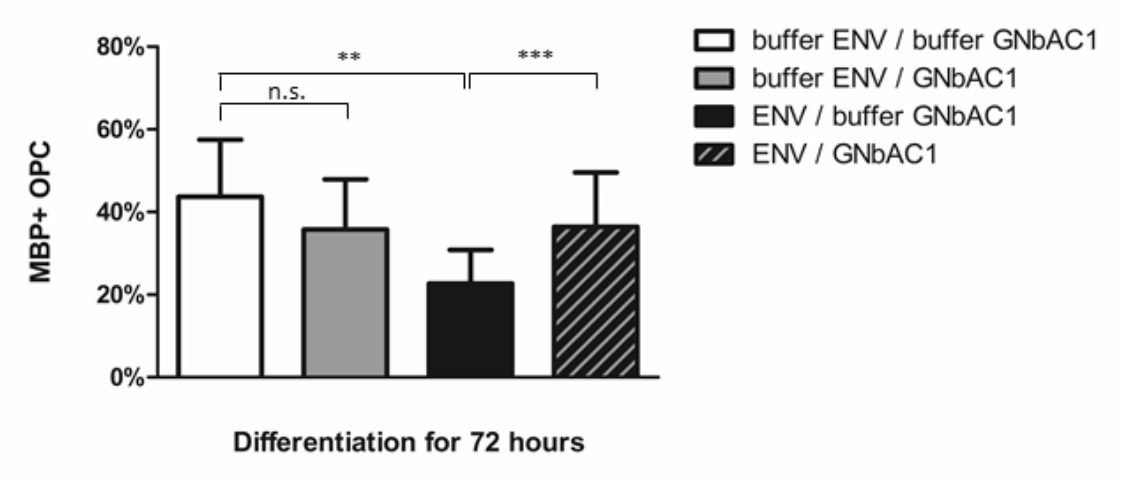


Fig. 10. GNbAC1 reduces HERV-W ENV-mediated 3-NT formation. (A) HERV-W ENV increases the number of 3-NT positive OPCs after 72 hours (black bar). Preincubation with GNbAC1 significantly diminishes this effect and can almost reduce the number of 3-NT-positive OPCs to control levels (grey dashed bar). GNbAC1 alone (grey bar) or the respective buffer solutions (white bar) had no impact on 3-NT formation and were used as a control. (B-E') Representative anti-3-NT immunostainings of OPCs. (E) Pretreatment of HERV-W ENV with GNbAC1, (B) respective buffer solutions, (C) GNbAC1 alone, (D) HERV-W ENV stimulation. Anti-3-NT antibody (A-E, red) and 4',6-diamidino-2-phenylindole (DAPI, A'-E', blue) were used. Scale bars = 100 µm. Data are shown as mean values ± standard deviation and derive from three independent experiments. n.s. = not significant, ***$p < 0.001$ by student's t-test. Modified after Kremer and Förster et al. (Kremer et al., 2015).

Additionally, it could be demonstrated, that GNbAC1 protects oligodendroglial MBP expression against the detrimental impact of HERV-W ENV (Figure 11A, black bar). Preincubation and neutralization of HERV-W ENV with GNbAC1 restored myelin expression to almost baseline level (Figure 11A, dashed grey bar). GNbAC1 alone (Figure 11A, grey bar) and the respective buffer solutions (Figure 11A, white bar) had no effect on MBP expression and were used as controls. These results indicate that the neutralizing HERV-W ENV-specific antibody GNbAC1 can significantly reduce HERV-W ENV-mediated nitrosative stress leading to a rescue of myelin expression.

A



B

Experiment	1	2	3	4	5	6	7	8	9	overall
Relative rescue of MBP expression (%)	41,25	69,09	72,30	79,73	87,16	87,62	87,67	92,22	103,06	**Ø 80,01**

Fig. 11. GNbAC1 prevents the HERV-W ENV-mediated OPC differentiation block and restores MBP expression in OPCs. (A) HERV-W ENV stimulation for 72 hours significantly decreases the number of MBP-positive OPCs (black bar). Preincubation with GNbAC1 neutralizes this differentiation blockade increasing MBP positivity to control levels (dashed grey bar). (B) Relative rescue of MBP expression levels for every conducted experiment and average relative rescue of MBP expressing obtained by GNbAC1 antibody. Data in (A) are shown as mean values ± standard deviation and derive from nine independent experiments. t-test: n.s.=not significant, **p<0,01, ***p < 0.001. Modified after Kremer and Förster et al. (Kremer et al., 2015).

4 Discussion and conclusion

This work demonstrates that the envelope protein (ENV) of human endogenous retrovirus type W (HERV-W) leads to a differentiation blockade in oligodendroglial precursor cells (OPCs). This effect is mediated both by the MyD88-dependent and the MyD88-independent TLR4 signaling pathway which converge on the nuclear translocation of the proinflammatory transcription factor NFκB leading to the induction of genes such as IL1b, TNFα and, above all, iNOS. As an enzyme, iNOS increases the production of nitric oxide (NO), a reactive nitrogen species (RNS) which leads to the nitrosylation of protein tyrosine residues resulting in the formation of 3-nitrotyrosine (3-NT), a marker for so-called nitrosative stress. In the MS brain nitrosative stress leads to mitochondrial dysfunction (Dutta et al., 2006), glutamate-associated excitotoxicity (Rosin et al., 2004, Pitt et al., 2003) and blood-brain-barrier (BBB) dysfunction (Thiel and Audus, 2001) which are key for both the inflammatory and the neurodegenerative aspects of the disease. Accordingly, in models of experimental autoimmune encephalomyelitis (EAE) inflammation could be decreased by specifically targeting NO (Hooper et al., 1997). Probably via the above-described nitrosative stress mechanism HERV-W ENV results in a decreased expression of oligodendroglial maturation markers such as myelin basic protein (MBP). This differentiation blockade can be neutralized by the HERV-W ENV-specific monoclonal antibody GNbAC1. This is of biomedical relevance as inefficient remyelination in MS is mainly based on a diminished capacity of resident OPCs to differentiate into mature cells which can remyelinate demyelinated axons. Methodically, it is remarkable that the author observed substantial differences in the increase of iNOS transcription levels between single qPCR experiments following stimulation of OPCs with HERV-W ENV (compare Figures 2 and 9). Quantitatively, iNOS transcription levels sometimes differed by a factor of 1.000 which could possibly be attributed to the extremely dynamic and sharp gradient of the respective mRNA increases. Accordingly, minimal differences of lysis time points could play a decisive role. Regarding the underlying TLR4-based signaling mechanism responsible for the above-described effects it is important to mention that the exact subcellular localization of this receptor is still unclear. Several studies have demonstrated its presence on the cell surface while others have reported an intracellular localization in cell types such as macrophages (Shibata et al., 2011) or endothelial cells (Dunzendorfer et al., 2004). Of note, irrespective of its exact subcellular localization, the TLR4 blocking experiments described in this study clearly demonstrate that TLR4 mediates nitrosative stress in OPCs via iNOS induction. In contrast to that, the involvement of the TLR4 coreceptor CD14 seems to be of minor relevance in the context of the HERV-W ENV-mediated inflammatory effects given the fact that its neutralization did not lead

to a significant decrease in iNOS transcription levels. At first glance, this suggests that blocking TLR4 could be biomedically beneficial in MS. However, TLR4 signaling is required physiologically to defend against bacteria so that in MS patients its blockade would be likely to result in serious infectious adverse events (Akira, 2000, Weiss et al., 2004). The same rationale applies to a potential inhibition of the downstream TLR4 signaling cascade proteins IRAK-1/4, TRIF and NFκB. Therefore, GNbAC1-mediated neutralization of HERV-W ENV as the ligand of TLR4 constitutes the most promising biomedical approach to prevent the above-described effects (Curtin et al., 2015). In a recently completed clinical phase IIb study in RRMS patients GNbAC1 was well tolerated with almost no serious adverse events (SAEs) and resulted in a significant decrease of brain atrophy and neurodegeneration (CHANGE-MS, Clinical Trial Assessing the HERV-W Env Antagonist GNbAC1 for Efficacy in MS, ClinicalTrials.gov NCT02782858, Hartung et al., manuscript in preparation). Of note, CHANGE-MS missed its primary endpoint, which was a decrease in the number of inflammatory gadolinium-enhancing T1 lesions in the first trial period from week 12 to 24. However, against the backdrop of the numerous available anti-inflammatory therapy options for RRMS, such as the highly effective monoclonal antibodies alemtuzumab or ocrelizumab there is no compelling clinical need for yet another such medication. In contrast to that, in the second trial period from week 24 to 48, treatment with GNbAC1 reduced dose-dependently cortical, thalamic and total brain atrophy as well as the number of T1 hypointense lesions (i.e. so-called “black holes”) which are a marker of permanent neuronal loss, and thus addresses a currently entirely unmet clinical need. However, CHANGE-MS did not study whether this MRI-based anti-neurodegenerative effect also translates to improved cognition which will have to be the subject of future studies. Furthermore, GNbAC1 had a beneficial impact on the MRI readout of magnetization transfer ratio (MTR) consistent with a potential benefit on remyelination or at least myelin protection (Dousset et al., 1998, Chen et al., 2007, Chen et al., 2008). This is a hot topic in that recent clinical trials investigating potentially remyelinating effects of new treatment options still have problems in moving away from established endpoints such as the EDSS or the number of active inflammatory lesions and finding more suitable endpoints, sufficiently sensitive and specific to measure remyelination or neuroprotection (Sormani and Pardini, 2017). In addition to MTR, other promising MRI measurement techniques such as diffusion tensor imaging (DTI), myelin water imaging (MWI) or susceptibility weighted imaging (SWI) are emerging as possible imaging outcome measures of neuroprotection and repair in MS and could provide further insights in this regard (Oh et al., 2019). The work presented here provides a neurobiological rationale for this above-mentioned effect as GNbAC1 treatment led to a rescue of myelin expression in OPCs. Interestingly, CHANGE-MS enrolled exclusively patients with RRMS, an MS

subtype in which neuroinflammation is traditionally assumed to outweigh neurodegeneration. Nevertheless, the study could clearly show that even in RRMS mechanisms of neurodegeneration are highly relevant and can be effectively targeted. This is corroborated by histological studies suggesting that extensive axonal damage is already present in early MS stages (Kuhlmann et al., 2002). In summary, further clinical trials are warranted to investigate the effect of GNbAC1 in patients that rather meet the needs of a remyelinating therapy such as in progressive MS where neurodegeneration is assumed to outweigh neuroinflammation. Future outcome measures should therefore capture the potentially remyelinating or anti-neurodegenerative effect rather than the anti-inflammatory effect - whether through alternative or additional radiological imaging or clinical measurement tools such as for cognitive, emotional or fine motor skill improvement.

Regarding other potential antiviral treatment options, it is worthwhile to discuss the general role of herpes viruses in the etiology of MS. It has long been known that there is a strong association between Epstein-Barr virus (EBV) infection and MS (Ascherio and Munch, 2000) since anti-EBV seropositivity reaches virtually 100% in MS patients (Munch et al., 1997). Furthermore, it has been shown that infectious mononucleosis as a symptomatic EBV infection significantly increases the risk to develop MS (Thacker et al., 2006). This is probably linked to the ability of EBV to immortalize and activate antibody producing cells and to its impact on the T-cell repertoire (Dreyfus et al., 1996, Rickinson et al., 1996, Thacker et al., 2006). Of note, recent studies demonstrated that the membranous EBV glycoprotein gp350 is able to induce HERV-W ENV expression in PBMCs as well as in astrocytes (Mameli et al., 2012) which provides a link between the risk to develop MS and HERV-W ENV. Interestingly, beyond MS other diseases with inflammatory aspects, such as chronic inflammatory demyelinating polyradiculopathy (CIDP) and type 1 diabetes (T1D) have also been linked to HERV-W ENV. In CIDP patients there is an increased seropositivity for HERV-W ENV, its transcription is upregulated in PBMCs (Perron et al., 2012) and it could be detected in nerve biopsies of 71% of affected patients (Faucard et al., 2016). Mechanistically, HERV-W ENV was found to activate TLR4 in Schwann-cells resulting in a significant increase in the production of proinflammatory cytokines such as IL-6 and the chemokine CXCL10. Similar results were obtained in T1D patients where HERV-W ENV was detected in 70% of patient sera and could be detected immunohistochemically in the pancreas (Levet et al., 2017). Pathomechanistically, HERV-W ENV was found to inhibit insulin secretion causing the disease-defining hyperglycemia. This effect is presumably also mediated via TLR4 but definite evidence for a direct HERV-W ENV/TLR4 interaction in β-cells is currently still elusive (Levet et al., 2017). Accordingly, GNbAC1 is also considered as a therapeutic option for these disease entities (Curtin et al.,

2018) which led to the initiation of a phase 2 clinical trial investigating the safety and tolerability of GNbAC1 in patients with recent onset T1D (Clinical Trial Assessing the GNbAC1 in Patients With Onset of Type 1 Diabetes Within 4 Years, RAINBOW-T1D, ClinicalTrials.gov NCT03179423). Apart from this, HERVs in general appear to be also involved in other diseases, such as HERV-K in amyotrophic lateral sclerosis (Arru et al., 2018) and HERV-W in schizophrenia (Karlsson et al., 2004). Of note, however, HERVs are not always pathogenic but can also participate in important physiological processes (Grandi and Tramontano, 2018). In this context it is important to discuss syncytin-1, the envelope protein of another member of the HERV-W family. It is encoded by the gene ERVW-1 which is stably located within the locus ERVWE1 on chromosome 7 and flanked by the proviral domains ERVW-1 GAG and ERVW-1 POL. Its ENV-encoding domain ERVW-1 appears to be fully functional whereas the ERVW-1 GAG and ERVW-1 POL sequences contain non-sense mutations rendering them non-coding. As a product of ERVW-1, the protein syncitin-1 could be best described as a “domesticated” HERV protein based on its physiological expression in the placenta where it is vital for syncytiotrophoblast formation (Mi et al., 2000). Even though syncytin-1 could be found in astrocytes of the MS brain by other research groups (Antony et al., 2007, Mameli et al., 2007b, Roebke et al., 2010), the authors of the respective studies used an antibody known to detect HERV-W ENV and not syncytin-1 calling therefore its specificity into question. As a result, the exact impact of syncytin-1 on the homeostasis of glial cells in the context of MS remains currently unclear.

In summary, this work provides a neurobiological rationale for the myelin-protective effect of anti-HERV-W ENV treatment observed in the CHANGE-MS study. As this trial was carried out in RRMS patients future studies will have to address the potential benefit of GNbAC1 for other MS subtypes, such as PPMS and SPMS for which currently only few medications are available.

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Danksagung

An dieser Stelle richte ich ein ganz besonderes Dankeschön an meinen Doktorvater Prof. Dr. Patrick Küry, der mich über die vergangenen Jahre stets mit großer Motivation, ausreichend Geduld, Gelassenheit, Freude und wissenschaftlicher Präzision begleitet hat und individuell unterstützen konnte. Hierfür bin ich außerordentlich dankbar und weiß dies sehr zu schätzen!

Ebenso gilt mein ganz besonderes Dankeschön meinem Betreuer und Mentor PD Dr. David Kremer, der mich zu jeder Zeit intensiv, professionell und ideal unterstützt und motiviert hat, ganz im Sinne des Sprichwortes „Per aspera ad astra.“. Er hat eine für mich rundum perfekte Betreuung gewährleisten können und ist zu einer durchaus prägenden Person für die vergangenen Jahre meiner Laufbahn geworden – vielen Dank!

Ebenso möchte ich den zahlreichen Mitgliedern der Arbeitsgruppe Küry und des neurochemischen Labors danken, die mir den Einstieg in die wissenschaftliche Arbeit immens erleichtert und das Arbeiten im Labor in jeder Hinsicht bereichert haben. Besonderen Dank möchte ich unter anderem an Dr. Peter Göttle, Dr. Tanja Schichel und Dr. Nevena Tzekova richten. Ebenso möchte ich ein besonderes Dankeschön an Brigida Ziegler für die Unterstützung im Labor richten, aber auch für die wissenschaftliche Penibilität, die ich von ihr lernen durfte.

Ich danke meinen Schwestern Annelie, Josefine und Marie für die regelmäßige Ablenkung, Freude und das Interesse an mir und dieser Arbeit. Ebenso danke ich meinem Vater und meiner Mutter für den Glauben an mich und das Vertrauen, das sie mir entgegengebracht haben sowie für die Unterstützung in jeglicher Hinsicht. Sehr gerne hätte ich den Abschluss dieser Arbeit mit beiden feiern dürfen. Meiner Freundin Laura möchte ich danken, für die Zeit und sehr wichtige Unterstützung in den besonders schwierigen Phasen – Danke! Mein Dank gilt auch Sebastian für den regelmäßigen wissenschaftlichen Diskurs, Austausch und die langjährige bereichernde Begleitung meines Lebens in vielerlei Hinsicht!