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The role of the MSRV envelope protein on oligodendroglial differentiation in the context of demyelinating diseases of the central nervous system

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Zusammenfassung

Oligodendrozyten produzieren die Myelinscheiden, welche zur Isolierung neuronaler Axone im zentralen Nervensystem (ZNS) notwendig sind. Eine intakte Myelinscheide ermöglicht eine beschleunigte Reizweiterleitung. Bei inflammatorischen Erkrankungen des ZNS wie der Multiplen Sklerose (MS) führen der Verlust von Oligodendrozyten und der damit einhergehende Verlust der axonalen Isolierung zu Beeinträchtigungen der betroffenen Nerven und schließlich zu funktionellen Einbußen der Patienten. Es konnte bereits im Vorfeld gezeigt werden, dass im Blut von MS-Patienten eine gegenüber gesunden Kontrollpersonen signifikant höhere Konzentration des Hüllproteins (ENV) des Multiple Sklerose assoziierten Retrovirus (MSRV), eines humanen endogenen Retrovirus des Typ W (HERV-W), vorliegt. In dieser Studie beleuchteten wir nun den Einfluss des ENV-Proteins auf die Homöostase und Differenzierung von oligodendrogliären Vorläuferzellen (OPC).

Mit Hilfe von Immunfärbungen sowie Genexpressionsanalysen untersuchten wir die Expression des vorbekannten ENV-Rezeptors TLR4 auf humanen und Ratten OPCs. Desweiteren wurde durch diese Methoden sowie durch Spektrophotometrie der Einfluss einer Stimulation mit rekombinanten ENV Protein auf kultivierte OPCs beleuchtet. Wir wiesen die Expression von TLR4 sowohl auf humanen als auch auf Ratten OPCs nach. Ferner zeigten wir, dass es unter Stimulation mit ENV zu einer deutlichen Induktion proinflammatorisch wirksamer Zytokine sowie der induzierbaren NO-Synthase (iNOS) kommt. Es fand sich eine vermehrte Bildung von Nitrotyrosin-Gruppen als Zeichen für nitrosativen Stress; die Myelinproduktion war unter ENV-Einfluss supprimiert. Unsere Ergebnisse lassen vermuten, dass der durch Stimulation mit ENV ausgelöste nitrosative Stress negativ auf die Differenzierung von OPCs und damit schlussendlich hemmend auf hirneigene Remyelinisierungsprozesse wirkt. Spezifische gegen ENV gerichtete Antikörper könnten durch Inhibierung dieser schädlichen Prozesse eine interessante Therapieoption in der Therapie der Multiplen Sklerose darstellen.

Abstract

In the central nervous system (CNS) axonal isolation is provided by myelin sheaths, which are produced by oligodendrocytes. To ensure optimal functionality of nerve fibers an intact myelin sheath is mandatory since it allows for accelerated transmission of impulses. The loss of oligodendrocytes leads to loss of axonal isolation and finally to impairment of the affected nerves and consecutive functional losses of the patient, which can be observed in inflammatory diseases of the CNS such as multiple sclerosis (MS). Previous studies have shown that the concentration of the envelope protein (ENV) of multiple sclerosis associated retrovirus (MSRV) in the blood of MS patients is significantly higher in comparison to healthy controls. In this study we illuminate the influence of ENV protein on homeostasis and differentiation of oligodendroglial precursor cells (OPC).

Through use of immunostainings and analysis of gene expression we studied the expression of TLR4 and its co-receptor CD14 both on human and rat OPCs, respectively. Additionally, we illuminated the influence of a stimulation with recombinant ENV protein on cultured OPCs through these methods as well as through spectrophotometry. We established that both TLR4 and its co-receptor CD14 are present on the surface of human as well as on rat OPCs. We were able to show a distinct induction of pro-inflammatory cytokines like TNFα or IL1β and to a special degree inducible NO-synthase (iNOS). The significant induction of iNOS and the consecutive production of nitric oxide (NO) point to nitrosative stress induced by stimulation with ENV. Myelin production on the other hand was significantly suppressed. Our results corroborate the idea that stimulation with ENV and the resulting nitrosative stress inhibit differentiation of OPCs and therefore inhibit remyelination processes. Selective, ENV-binding antibodies might present an interesting option to prevent these detrimental processes through direct elimination of ENV. Ultimately, this might lead to new therapeutic approaches in the therapy of demyelinating diseases of the CNS.

Abbreviations

BSA	bovine serum albumin
BBB	blood brain barrier
DNA	deoxyribonucleic acid
CNPase	2',3'-cyclic nucleotide 3'-phosphohydrolase
CNS	central nervous system
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
ENV	MSRV- envelope protein
FGF	fibroblast growth factor
FCS	fetal calf serum
GalC	galactosylceramidase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
HERV-W	human endogenous retrovirus type W
MBP	myelin basic protein
mM	millimolar
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MSRV	multiple sclerosis associated retrovirus
ODC	ornithine decarboxylase
OPC	oligodendocyte precursor cell

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-AA	platelet derived growth factor AA
PDGFR-α	platelet derived growth factor receptor alpha
PDL	Poly-D-Lysine
PFA	paraformaldehyde
PLP	proteolipid protein
PPMS	primary progressive multiple sclerosis
RNA	ribonucleic acid
RRMS	relapsing remitting multiple sclerosis
RT	reverse transcription
SEM	standard error of the mean
shRNA	small/short hairpin RNA
SPMS	secondary progressive multiple sclerosis
SVZ	subventricular zone
T3	triiodothyronine
T4	thyroxine

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1. Introduction

1.1 Multiple Sclerosis

1.1.1 History and clinical presentation

First described in 1868 by the French neurologist Jean-Martin Charcot (Charcot, 1868), Multiple Sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS) in young adults affecting as many as 2 to 150 in 100.000 individuals depending on geographical location (Rosati, 2001). Charcot, taking previous reports and his own histological studies into account called the disease "*sclerose en plaques*" and established a symptomatic neurological triad, consisting of nystagmus, intention tremor and telegraphic speech, which he found to be typical of MS patients (Clanet, 2008). While several further symptoms have been described in the meantime the above-mentioned deficits known as "Charcot's Triad" are still in clinical use today. Most common at disease onset are different kinds of paresthesias, visual deficiencies caused by optic neuritis, muscle weakness and fatigue. Symptoms like ataxia, aphasia or apraxia can also be observed but are substantially rarer (Compston and Coles, 2008). In terms of disease course four different patterns are described which may follow the initial attack (Lublin and Reingold, 1996). Relapsing remitting MS (RRMS), the most common course presented by 80 to 90% of all patients is characterized by acute exacerbations (relapses) which are followed by total or partial recovery (remissions). In about 40% of all relapsing remitting courses RRMS may eventually transform into secondary progressive MS (SPMS) featuring a constant, often insidious, worsening of symptoms over time. Primary progressive MS (PPMS), on the other hand, shows a steady worsening of symptoms without relapses or remissions right from disease onset. It occurs in approximately 15% of all patients and is most common among patients with a late onset of MS. The fourth and least common form is relapsing progressive MS (RPMS), which presents itself with intermitting acute relapses (Lublin and Reingold, 1996; Compston and Coles, 2008).

1.1.2 Etiology and epidemiology

While the etiology of MS is yet unknown, there are, however, environmental and infectious factors in discussion, which combined with a genetic predisposition might contribute to the initial development of MS (Compston and Coles, 2002).

It is widely accepted that autoimmune inflammatory processes in the CNS are responsible for the degeneration of oligodendrocytes and their myelin sheaths followed by axonal degeneration and reduced velocity of axonal transmission finally leading to glial scar formation by reactive astrocytes (Compston and Coles, 2002; Dutta and Trapp, 2007). Though there is no unitary classification allowing an accurate differentiation between different MS stages yet, four different histological patterns of demyelination can be distinguished (Lucchinetti et al., 2000). These patterns are defined by various factors such as loss of myelin, localization and extension of MS plaques, oligodendrocyte destruction or involvement of the immune system. While patterns I and II resemble T cell–mediated or T cell plus antibody–mediated autoimmune encephalomyelitis, patterns III and IV show oligodendroglial dystrophy similar to virus- or toxin induced demyelination encouraging the concept of a polycausal etiology of MS (Lucchinetti et al., 2000).

Epidemiological data demonstrate a great variety in MS prevalence worldwide. MS follows a north-south gradient in the northern hemisphere and a south-north gradient in the southern hemisphere with lowest prevalence in people living near the equator (Sadovnick and Ebers, 1993). These gradients, which may be caused by environmental factors, cannot, however, explain the fact that in high prevalence regions there are ethnic groups with considerably lower risks of developing MS like the Inuit people of Northern Canada (Milo and Kahana, 2010). Taken together, these facts indicate that a genetic predisposition may also increase MS risk. In addition, migration studies have shown that changing residence influences MS risk. In this regard, it was observed that moving from a low risk to a high risk area may slightly increase an individual's risk of developing MS, while individuals moving from high risk to low risk areas retain a significantly higher risk than people originating from a low risk area (Kurtzke, 1993). In both cases data on age at migration suggest that the MS risk is established largely in the first two decades of life and does not change significantly due to migration at an older age which supports the theory of a

putative infectious agent acquired in the first two decades of life influencing an individual's overall risk of developing MS (Kurtzke, 1993, 2000).

Regarding the genetic predisposition, it was shown that first-degree relatives of MS patients are 30-50 times higher at risk to develop this disease as compared to the general population. This risk decreases for second- and third- degree relatives (Sadovnick et al., 1988; Lindsey, 2005). Polymorphisms of certain MHC alleles such as HLA-DR2, located on chromosome 6 (Barcellos et al., 2003) have been attributed to MS susceptibility, and recently genome wide association studies revealed further loci involved in or related to MS pathology (Sawcer et al., 1996, Sawcer et al., 2011; Hafler et al., 2007; Baranzini et al., 2009). These findings support the notion that MS rather depends on the involvement of several genes contributing only small individual effects than on a few genes or even one gene of major importance as observed in "classical" genetic diseases such as, for instance, hemophilia or cystic fibrosis (Antonarakis et al., 1995; Bobadilla et al., 2002). Similar to most autoimmune disorders MS is more common in women than in men with a ratio of 1.5:1 with a peak age of onset of 27 (Kurtzke, 1993).

1.1.3 Pathology

MS symptoms are based on demyelinating processes which present themselves as so-called MS plaques on a histopathological level. These plaques originate from smaller lesions which can be found around small veins and venules. Forming in multiple perivenous locations they tend to fuse while growing; finally presenting themselves as large demyelinating plaques (Brownell and Hughes, 1962; Adams, 1977). Though there are several preferred localizations, lesions can principally occur everywhere in the CNS without preference of right or left hemisphere, even including myelin poor gray matter. The periventricular white matter appears to be a side of predilection, especially alongside the bodies of the lateral ventricles. Furthermore, the junction of cortex and white matter as well as the optic nerves, the brainstem and the spinal cord show an increased susceptibility for such lesions (Adams, 1977; Dalton et al., 2012). Clinical manifestation of these plaques depends on collateral axonal damage. Opposing to previous opinions axonal damage appears to be partly independent of demyelinating processes, being mediated by macrophages, microglia and CD8- positive T-cells (Bitsch et al., 2000). Additionally, axonal

damage may occur in early disease stages without obvious demyelination occurring. On the other hand, the decay of myelin sheaths is partly due to autoimmune processes. It could be shown that autoreactive T lymphocytes with a specificity for myelin basic protein (MBP) or proteolipid protein (PLP) accumulate in the CNS - especially in the MS lesions - and may even persist in the blood of MS patients for years (Stinissen et al., 1997). It is yet unknown how the production of these autoreactive T cells is triggered, but it is assumed that viral antigens may play a role by presenting myelin related epitopes at their surface (molecular mimicry). Apart from this, the mechanisms preventing the production of auto-antibodies were shown to be defective or at least diminished in MS patients compared to healthy subjects (Stinissen et al., 1997).

1.1.4 Remyelination, repair and role of oligodendrocytes, cellular parameters of redifferentiation

There are three major categories of CNS cells: neuronal cells, macroglial cells such as oligodendrocytes and astrocytes as well as microglial cells. The tasks of glial cells are manifold and include first line defense and phagocytosis in case of microglia as well as support, homeostasis, protection and electrical insulation provided by astrocytes and oligodendrocytes, respectively. The myelinating macroglial cells of the CNS, the oligodendrocytes, produce myelin sheaths which are used to isolate axons and thus provide electrical insulation. Each oligodendrocyte can interact with several (up to 40) adjacent axons (Bjartmar et al., 1994). Adjacent myelin sheaths are interrupted by constrictions at equal distances, the so-called nodes of Ranvier. These nodal regions are enriched of sodium and pottasium channels and present sites where action potentials can occur, this in contrast to internodes (covered by myelin sheaths) which are electrically isolated. This construction enables a saltatory hence accelerated propagation of nerve impulses along the myelinated axon (Sherman and Brophy, 2005). This means that depolarization does not follow the axon in a wavelike manner but initiates a new depolarization at each consecutive node thus in a “jumping” like manner from node to node.

A loss of myelin sheaths due to pathological mechanisms such as described above therefore results in conduction deficiencies which contribute to the previously described clinical manifestations of MS. While mature

oligodendrocytes show only limited abilities to regenerate myelin sheaths and rather degenerate upon inflammatory attacks, resident oligodendroglial precursor cells (OPCs) present a potential source for cell replacement and restoration of myelination (Franklin, 2002). OPCs being of neuroectodermal origin display a widespread distribution in the adult CNS and can be activated and recruited upon injury or disease. Eventually these cells can replace destroyed oligodendrocytes and successfully remyelinate previously demyelinated axons (Zawadzka and Franklin, 2007). However, the efficiency of this repair process is limited and was found to further diminish during the course of demyelinating diseases. Among other reasons, it is currently assumed that this is mostly due to the fact that the glial differentiation process is influenced by extrinsic and intrinsic inhibitory components interfering with successful maturation, myelination and interaction with axons (Kremer et al., 2011).

1.2 MSRV and ENV

1.2.1 Description of MSRV and ENV

Presumably in early stages in the evolution of primates endogenous retroviruses (ERV) have entered the human genome. Though most retroviruses tend to infect somatic cells, occasionally the germ cell line may be infected as well. Being integrated in the germinal DNA the infection can then be passed on (Sverdlov, 2000). Currently, there are over 40000 known copies of Human Endogenous Retrovirus (HERV) which are divided into several classes depending on features such as cross-reactivity or morphology (Nelson et al., 2003). Overall approximately eight percent of the human genome is made up of HERV-DNA (Dolei, 2006). Throughout the course of evolution most of the HERV sequences have been subject to deletions and mutations rendering them incapable of producing proteins. A few, however, have preserved an open reading frame which can be transcribed and eventually may even give rise to viral protein production (Patience et al., 1997; Griffiths, 2001). Activation and reexpression of HERVs can be triggered by different kinds of stimuli such as chemical noxae or other viruses (Rasmussen et al., 1993) and may vary depending on cell type, HERV family or intraindividual methylation pattern.

Since a great variety of HERV sequences have been preserved in the human genome it can be assumed that they might have obtained certain physiological functions. Indeed it could be shown that hosts can benefit from certain retroviral proteins such as for example syncytin, which is expressed during placental development and enables the fusion of fetal trophoblast cells into the placental syncytiotrophoblast (Malassiné et al., 2005; Noorali et al., 2009). On the other hand, HERV sequences can also influence gene transcription of adjacent genes, either activating or silencing transcription. Importantly, the influence of HERVs is not always beneficial as demonstrated by studies suggesting a strong association of HERV activity with several diseases of multifactorial etiology such as schizophrenia, cancer or MS (Bieda et al., 2001; Büscher et al., 2005; Dolei, 2006).

In the context of MS one member of the so-called HERV-W family is of special interest. Since it has been first discovered in MS patients, the retroviral element

has been denominated as Multiple Sclerosis associated retrovirus (MSRV) (Perron et al., 1989, 1991, 1997; Garson et al., 1998). MSRV is an enveloped retrovirus that has been shown to induce production of potent pro-inflammatory responses both *in vivo* and *in vitro* (Rolland et al., 2005; Dougier et al., 2009). Previous studies have also indicated that there is a significant increase of MSRV both in the blood as well as in the cerebrospinal fluid of MS patients (Rolland et al., 2005; Sotgiu et al., 2006). Considering its role as a pathogen the envelope protein (ENV) of MSRV is of particular interest in this context. Being a 543 amino acid sequence with a signal peptide and six N-glycosylation sides the complete ENV protein consists of a transmembrane part (ENV-TM) and a surface unit (ENV-SU) which presumably allows interaction with surrounding structures and - most importantly - activation of immunocompetent cells (Dolei and Perron, 2009).

1.2.2 TLR4 and its signaling pathways

The innate immune response presents the first line of defense against the potential invasion of exogenous microorganisms. It includes phagocytes like macrophages, dendritic cells or neutrophil granulocytes which utilize Toll-like receptors (TLR) to differentiate between self and pathogen. These receptors recognize typical microbial patterns and following activation initiate the production of antimicrobial peptides and cytokines (Warren, 2005).

TLRs are glycoproteins which consist of an extracellular ligand-binding domain and an intracellular toll interleukin receptor (TIR) domain (Mogensen, 2009). Currently more than eleven members of the TLR-family are known in humans (Wong and Wen, 2008). They all belong to the larger superfamily of interleukin 1 receptors sharing significant similarities in their cytoplasmatic TIR-regions while they differ slightly in their signaling pathways activating distinct or partly overlapping downstream cascades (Dunne and O'Neill, 2003). Another distinction is presented by the microbial patterns they discern including nucleic acids, lipids or lipoproteins. While TLR3, for instance, is activated by double-stranded viral RNA (Alexopoulou et al., 2001), TLR5 detects flagellin, a protein forming the helical filament in bacterial flagella (Gewirtz et al., 2001). In regard to the present study TLR4 which is primarily activated by Lipopolysaccharide (LPS), an endotoxin found in the outer membrane of gram-negative bacteria (Triantafilou and Triantafilou, 2002) is of particular interest since previous

studies concerning ENV and its effects on immune cells in the peripheral blood suggest that the cellular response to ENV is mediated through TLR4 (Rolland et al., 2005, 2006).

TLR4 activation depends on its co-receptor, the cell membrane cluster of differentiation (CD) 14 (Triantafilou and Triantafilou, 2002). CD14 is structurally related to the extracellular domain of TLR4, but has no cytoplasmatic part and is therefore unable to mediate intracellular signaling by itself. A further protein, the extracellular LPS-binding protein (LBP), collects free unbound LPS and reduces it to monomers thus facilitating binding to CD14. The LPS/CD14 complex then binds to TLR4 and initiates receptor activation (Ziegler-Heitbrock and Ulevitch, 1993). There are essentially two different signaling pathways that are activated upon receptor stimulation. The first pathway is dependent on TIR-domain containing adapter molecule myeloid differentiation factor 88 (MyD88), while the other is MyD88-independent depending on TIR-domain-containing adapter-inducing interferon-β (TRIF) instead. Regarding the first pathway receptor stimulation induces conformational changes, which lead through binding of TIR-domain containing adaptor protein (TIRAP) to recruitment of MyD88 and eventually to activation of several Interleukin-1 receptor-associated kinases (IRAKs). Subsequently these steps result in an early activation of NF-κB (Kawai and Akira, 2010). In case of the TRIF-depending pathway the TRIF-related adapter molecule (TRAM) assumes the role of TIRAP and activates TRIF. The ensuing activation of IRF3 finally leads to activation of NF-κB as well (Fitzgerald et al., 2003). Both cascades eventually culminate in the induction of pro-inflammatory genes paving the way to the production of cytokines such as IL-1, IL-6 or TNF-α (Takeda and Akira, 2004a).

1.2.3 Relevance in MS and goal of the thesis

Given the likeliness that activation of MSRV and cellular interactions with its envelope protein (ENV) occur in the inflamed CNS, the question evolves as to whether viral particles or surface structures can affect neural cells, such as for example oligodendroglial cells of the adult CNS. As outlined above, activation, migration and differentiation of OPCs as well as their successful interaction with demyelinated axons are a key to myelin repair. An observed impact of MSRV or ENV would thus be of particular interest regarding the brain's endogenous capacity to restore its myelin. This study therefore aims at demonstrating

whether ENV dependent pro-inflammatory effects similar to those observed in peripheral blood cells can also be detected among OPCs and whether this affects their survival, differentiation or homeostasis. To this end stimulation studies with recombinant ENV as well as ENV overexpression approaches were undertaken using primary oligodendroglial precursor cells of rat and human origin. Proving that ENV has indeed an inhibiting effect on remyelination might eventually open up new therapeutic approaches such as the production of anti-ENV neutralizing antibodies which could abrogate the deleterious effects of this surface protein.

2. Material and Methods

2.1 Materials

2.2.1 Cell culture

Recombinant MSRV envelope protein (ENV)	GeNeuro, Geneva, Switzerland
ENV buffer	GeNeuro, Geneva, Switzerland
Bovine Serum Albumin (BSA)	Gibco, Karlsruhe
Fetal Calf Serum (FCS)	Gibco, Karlsruhe
Cover Slips	Menzel- Glaser, Braunschweig
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Karlsruhe
Dulbecco's Modified Eagle Medium + Hepes (DMEM + Hepes)	Gibco, Karlsruhe
Minimum Essential Medium Eagle (MEM)	Gibco, Karlsruhe
L-glutamine	Gibco, Karlsruhe
Papain	Sigma- Aldrich, Taufkirchen
L-cysteine	Sigma- Aldrich, Taufkirchen
DNAse I Type IV	Sigma- Aldrich, Taufkirchen
Trypsin Inhibitor	Sigma- Aldrich, Taufkirchen
Paraformaldehyde (PFA)	Merck, Darmstadt
Penicillin/ Streptomycin	Gibco, Karlsruhe
Phosphate Buffered Saline	PAA, Paschin, Österreich
Poly-D-Lysine (PDL)	Sigma- Aldrich, Taufkirchen
Cell Culture Dishes	Greiner, Frickenhausen
Bovine insulin	Sigma- Aldrich, Taufkirchen

Human transferrin	Sigma- Aldrich, Taufkirchen
Progesterone	Sigma- Aldrich, Taufkirchen
Basic fibroblast growth factor (bFGF)	R&D Systems, Wiesbaden
Platelet- derived growth factor alpha (PDGF-AA)	Peprotech, Hamburg
Putrescine	Sigma- Aldrich, Taufkirchen
Sodium selenite	Sigma- Aldrich, Taufkirchen
Tri- iod- thyronine (T3)	Sigma- Aldrich, Taufkirchen
Thyroxine (T4)	Sigma- Aldrich, Taufkirchen

2.1.2 Transfection and immunostaining reagents

NanoJuice Transfection Reagent Kit	Merck, Darmstadt
Lipofectamine Transfection Reagent	Invitrogen, Karlsruhe
Opti-MEM I Reduced Serum Medium	Gibco, Karlsruhe
pcDNA3-HygB-citrine	Life Technologies, Applied
(vector modified as described in Heinen et al., 2008)	Biosystems, Darmstadt
pV14_ENV	GeNeuro, Geneva, Switzerland
pV14_ctrl	GeNeuro, Geneva, Switzerland
anti-ENV antibody	GeNeuro, Geneva, Switzerland
mouse anti-O4 antibody	Chemicon, Temecula, CA
mouse anti-A2B5 antibody	Chemicon, Temecula, CA
mouse anti-MBP antibody	Sternberger monoclonals, Baltimore, MD
mouse anti-CNPase antibody	Sternberger monoclonals, Baltimore, MD
mouse anti-TLR4 antibody	Abcam, Cambridge, UK
rabbit anti-TLR4 antibody	Abcam, Cambridge, UK

mouse anti-GalC antibody	Millipore, Billerica, MA
rabbit anti-GalC antibody	Millipore, Billerica, MA
mouse anti-nitrotyrosine antibody	Sigma- Aldrich, Taufkirchen
S-nitroso-N-acetyl-penicillamine (SNAP)	Sigma- Aldrich, Taufkirchen
Alexa Fluor 488 antibody	Molecular Probes
Alexa Fluor 594 antibody	Molecular Probes
Citifluor	Citifluor, Leicester, UK
4',6-Diamidin-2'-phenylindoldihydrochlorid (DAPI)	Roche, Mannheim
Normal goat serum (NGS)	Sigma- Aldrich, Taufkirchen
In Situ Cell Death Detection Kit	Roche Applied Science, Mannheim
senescence ß-galactosidase staining kit	Cell Signaling, Danvers, MA

2.1.3 Reverse transcription and polymerase chain reaction reagents

High Capacity cDNA Reverse Transcription Kit	Life Technologies, Applied Biosystems, Darmstadt
RNeasy Mini Kit for RNA purification	Qiagen, Hilden
Power SYBRGreen universal master mix	Life Technologies, Applied Biosystems, Darmstadt
PCR amplification primers	MWG Biotech

2.1.4 Technical equipment

Axioplan 2 fluorescence microscope	Zeiss, Jena
7900 HT Fast-Real-Time PCR System	Life Technologies, Applied Biosystems, Darmstadt
TRIO Block Thermocycler	Biometra, Göttingen
Heraeus Hera Safe incubator	Heraeus, Hanau
Excella E24 Incubator Shaker	New Brunswick Scientific, Nürtingen

2.2 Methods

2.2.1 Oligodendroglial cell isolation and culture

Oligodendrocyte precursor cells (OPCs) were purified and cultivated following the protocol of McCarthy and de Vellis (1980). After collecting the cortices of new born rats in MEM-Hepes medium (Gibco), they were centrifuged for 30 seconds at 1200 rpm. 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. Then the tissue was incubated for 45 min at 37°C. 1 ml of 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 before incubating the solution for 5 min at room temperature. After discarding the old medium another ml of the above described solution was added. After mixing thoroughly with a glass Pasteur pipette 10 ml of DMEM medium containing 10% FCS were added. Following centrifugation for 8 min at 1500 rpm the medium was discarded and replaced with 20 ml of DMEM medium containing 10% FCS, 4 mM L-glutamine and P/S 5000. After resuspending, the cells were cultured on poly-D-lysine (PDL) coated T-75 cell culture flasks. After 10 days of incubation at 37°C in 5% CO_2 containing atmosphere the flasks were shaken at 250 rev/min for two hours to eliminate microglial contamination. A second shaking step for 20 hours detached the OPCs from the underlying astrocyte-layer. Cells were then replated on PDL-coated culture dishes in high-glucose (DMEM+GlutaMAX-1, Gibco) SATO-based medium containing bovine insulin (5 µg/ml), human transferrin (50 µg/ml), BSA V (100 µg/ml), progesterone (6.2 µg/ml), putrescine (16 µg/ml), natrium selenite (5 ng/ml), 4 mM L-glutamine, 5000 units penicillin/streptomycin and both tri- iod- thyronine (T4) and thyroxine (T3) (400 ng/ml each). The medium was changed to either proliferation medium containing both 10 ng/ml fibroblastic growth factor (bFGF) and 10 ng/ml platelet derived growth factor (PDGF) or to differentiation medium containing 0.5% FCS.

2.2.2 ENV production and purity

The MSRV-ENV protein is a bacterial recombinant protein provided as is by our collaborator Geneuro SA, Geneva, Switzerland and has been originally produced by PX-Therapeutics/ France.

To ensure purity and the absence of bacterial endotoxin the Limulus Amebocyte Lysate (LAL) test was performed under regulatory approved conditions (Clean Cells/ France), where endotoxin levels <5 UE/ml were attained. The proteins were dissolved in a Tris 20 mM pH 7.5, NaCl 150 mM, SDS 1.5%, dithiothreitol (DTT) 10 mM buffer.

2.2.3 Immunostaining procedures

After fixing cells with 3.7% paraformaldehyde (PFA, Merck) diluted in PBS (PAA) for 10 min they were washed three times with PBS. Cells were then blocked for 30 min with normal goat serum (NGS, Sigma-Aldrich) before being incubated overnight with primary antibodies at 4°C. The following primary antibodies were used as indicated: mouse anti-O4- (1:100) and mouse anti-A2B5 antibodies (1/200, both Chemicon), mouse anti-MBP- and mouse anti-CNPase antibodies (1/1000 and 1/500, respectively, both Sternberger monoclonals), mouse anti-TLR4 antibody (1:250, Abcam) as well as rabbit anti-GalC antibody (1:500, Millipore). Following overnight incubation the cells were washed three times with PBS and sequentially incubated with secondary fluorescent antibodies for 2h at room temperature. Secondary antibodies were diluted in PBS as follows: Alexa Fluor 488- and Alexa Fluor 594- antibody (both 1:500, Molecular Probes). Consecutively the cells were washed again three times with PBS before staining the nuclei with DAPI (Roche). Finally the cells were mounted in Citifluor (Citifluor).

2.2.4 RNA preparation, cDNA synthesis and RT-qPCR

In order to isolate and purify total RNA from cultured cells the RNeasy mini-kit from Qiagen was used according to following protocol: After being rinsed with PBS cultured cells were lysed with 350 µl of lysis buffer (0.1 M ß-mercaptoethanol diluted in RLT buffer 1:100). The lysate was centrifuged through QIAshredder columns at 14000 rpm for 2 min to homogenize the sample material and remove cell debris and high-molecular-weight cell components. Subsequently 350 µl 70% ethanol was added to the elution which was then transferred onto RNeasy spin columns. After centrifugation at 10000 rpm for 2 min the RNA adhered to the filter in the column and the elution was discarded. To prevent contamination with genomic DNA the RNA was incubated with RNase free DNase for 15 min at room temperature (10 µl DNase was

diluted in 70 µl RDD-buffer). For further purification the RNA was treated several times with RW1- and RPE-buffer and subsequently centrifuged at 10000 rpm for 2 min at room temperature. Finally 30 µl of RNase free ddH_2O were added and the RNA was extracted from the columns through centrifugation at 10000 rpm for 2 min at room temperature. The purified RNA was then stored at -20°C.

Reverse transcription of the isolated RNA was achieved through the high capacity cDNA Reverse Transcription Kit from Life Technologies/Applied Biosystems according to following protocol: 15 µl of master mix consisting of 3 µl 10x RT Buffer, 1.2 µl 25x dNTP Mix (100 mM), 3 µl 10x RT random primers, 1.5 µl MultiScribe Reverse Transciptase, 1.5 µl RNase Inhibitor and 4.8 µl nuclease-free H_2O were mixed thoroughly with 15 µl RNA. The mix was then kept at 25°C for 10 min before being heated to 37°C for 120 min. Inactivation was done for 5 sec at 85°C then the mix was cooled down to 4°C.

Quantitative PCR of cDNA preparations was performed using the 7900 HT Fast-Real-Time sequence detection system (Applied Biosystems) in combination with Power SYBRGreen universal master mix (Applied Biosystems). Primer sequences were designed using the PrimerExpress 2.0 software by Applied Biosystems and subsequently tested for the generation of specific amplicons:

Primer	**forward**	**reverse**
hu-GAPDH	TGGACCTGACCTGCCGTCTA	AGGAGTGGGTGTCGCTGTTG
hu-iNOS	TGAGGAGCAGGTCGAGGACT	TGATAGCGCTTCTGGCTCTTG
r-GAPDH	GAACGGGAAGCTCACTGGC	GCATGTCAGATCCACAACGG
r-Hes1	GGAGAGGCTGCCAAGGTTTT	AAGCAAATTGGCCGTCAGG
r-Hes5	CGCAGAGGGAAGACGATCAC	GGGAAGAAACGCGCAGAA
r-Id2	AGAACCAAACGTCCAGGACG	TGCTGATGTCCGTGTTCAGG
r-Id4	CAGCTGCAGGTCCAGGATGT	AAAGTGGAGATCCTGCAGCAC
r-IL1β	GAAACAGCAATGGTCGGGAC	AAGACACGGGTTCCATGGTG
r-IL6	GTTGTGCAATGGCAATTCTGA	TCTGACAGTGCATCATCGCTG
r-iNOS	CTCAGCACAGAGGGCTCAAAG	TGCACCCAAACACCAAGGT

r-MKP5	AACCATGGAAACCTCTGTGACA	CCACCTCCCGGCACTCT
r-ODC	GGTTCCAGAGGCCAAACATC	GTTGCCACATTGACCGTGAC
r-TLR4	CTGGGTTTCTGCTGTGGACA	AGGTTAGAAGCCTCGTGCTCC
r-TNFα	AGCCCTGGTATGAGCCCATGTA	CCGGACTCCGTGATGTCTAAG
r-TRPC4	CGCTGTCAAGTGAAGACCCTTT	GTTCTTGCAGCTCCCAGCTT
r-CD14	GGCTCAATGGGTAAAAGCCA	CCCGAGGATCAAAATCAGGAG

Table 1. Oligonucleotide primer pairs used for quantitative RT-PCR based amplification. GAPDH and ODC were used as reference genes.

PCRs were performed according to following protocol: 30 µl of cDNA were diluted with the same amount of H_2O LiChrosolv (Merck). 5 µl of this dilution were mixed with 15 µl of SYBR green master mix, a correspondent amount of forward and reverse primer (1.8 fwd and 0.3 µl rev in case of GAPDH and 1.8 µl fwd and 1.8 µl rev in case of every other gene) as well as H_2O LiChrosolv (7.9 µl for GAPDH and 6.4 µl for every other gene) up to a total volume of 30 µl were added. Each sample was measured in duplicate. According to the default amplification procedure the following protocol was applied: 10 min at 95°C, 2 min at 50°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Data was evaluated using Applied Biosystems' ΔC_T method with GAPDH and ODC as reference genes. Shown are mean values with +/- standard errors of the mean. To ensure statistical significance t-test was performed.

2.2.5 Spectrophotometry

Since nitric oxide (NO) is not directly traceable in supernatants due to its short half life, we used the colorimetric nitric oxide assay kit by Calbiochem to detect the NO disintegration products nitrate and nitrite. Spectrophotometric quantification of NO is achieved by using the Griess reagent, which allows for measurement of nitrite. However, depending on experimental circumstances the ratio of nitrate and nitrite concentrations might vary. Therefore, the NADH-dependent nitrate reductase is applied to convert accruing nitrate into nitrite. Thus, an accurate way of measuring total NO production is provided. OPCs were treated with ENV or buffer according to protocol described above. Then nitrate reductase, NADH working solution and standards in different dilutions were prepared according to protocol. After adding 85 µl of each standard to a

96-well plate, 85 µl of OPC supernatant was added to the plate. Then 10 µl of nitrate reductase was added to each well. After addition of 10 µl of 2 mM NADH the plate was shaken for 20 min at room temperature. Interrupted by short shaking 50 µl of both color reagent #1 and #2 were added. Finally the absorbance was determined at 540 nm in a microplate reader. To analyze the data a standard curve was plotted and the sample concentrations were calculated from this curve.

2.2.6 Transfection of oligodendrocyte precursor and U343 glioblastoma cells

OPCs were grown for 24h in proliferation medium containing growth factors bFGF and PDGF as described above. Transfection was performed with NanoJuice reagent (Merck) according to instructions of the manufacturer. To transfect three wells of a 24-well cell culture dish containing 30'000 OPCs in 500 µl medium, 64 µl of serum-free medium were prepared. After adding 0.8 µl of NanoJuice core transfection reagent and 0.8 µl of NanoJuice transfection booster and subsequent thorough mixing the mixture was incubated for 5 min at room temperature. Thereafter 0.25 µg of DNA at a concentration of 0.5-1 µg/µl were added before incubating the mixture for 15 min at room temperature. Used were either a TLR4 suppression vector, an empty control vector or a scrambled control vector (OriGene). These vectors contain DNA sequences which are arranged in a tight hairpin turn resulting upon transcription in small hairpin RNAs (shRNA). While the actual suppression vector prevents expression of TLR4, the empty vector contains no information and the scrambled vector contains a scrambled RNA sequence enabling both to serve as negative controls. For visualization each of these vectors contains pGFP by default, which allows for expression of GFP upon successful transfection. Finally 20 µl of the transfection mixture were added to each well. Depending on the following experiment the cells were incubated at 37°C till further usage.

Transfection of U343 glioblastoma cells was accomplished utilizing Lipofectamine (Life Technologies/Invitrogen) according to manufacturer's protocol. One day prior to transfection 500'000 U343 cells were plated in proliferation medium in a 24-well cell culture dish. On the day of transfection, 500 ng of plasmid DNA were diluted in 100 µl Opti-MEM I reduced serum medium (Gibco) for each transfection sample and mixed gently. To this end

citrine containing plasmids were mixed with pV14_ENV and pV14_ctrl plasmids (supplied by Geneuro SA, Switzerland) in a ratio of 1:5. Subsequently 1.25 µl of Lipofectamine LTX reagent were added to the diluted DNA and incubated at room temperature for 30 min. Finally, 100 µl of transfection mixture was added to the wells containing the previously plated U343 cells. The success of transfection was verified by assessment of citrine expression under fluorescence microscopy.

2.2.7 Senescence

To detect a possible influence of ENV on the natural senescence process of OPCs the senescence ß-galactosidase staining kit (Cell Signaling) was used. Acid ß-galactosidase is only present in senescent cells and cannot be found in presenescent cells. OPCs were plated in 35 mm wells of a 6-well plate. According to the protocol provided by the manufacturer a fixative solution, a staining solution and an X-gal stock solution were prepared. After rinsing with PBS cells were fixed for 15 min using the fixative solution. Subsequently cells were rinsed two times with PBS. After adding the staining solution, OPCs were incubated overnight at 37°C in a dry CO_2-free incubator. While still in staining solution cells were checked for development of blue color and furthermore quantitatively evaluated after 24h.

2.2.8 Apoptosis

The in situ cell death detection kit (Roche) was used according to the manufacturer's protocol to determine whether ENV affects cell death rates through apoptotic or rather necrotic mechanisms. First the OPCs were fixed using 3.7% PFA at room temperature for 20 min. Subsequently cells were rinsed two times with PBS and then permeabilized for two min on ice. Following another washing step the cells were treated with TUNEL reaction mixture containing both the enzyme solution and the labeling solution. Then they were incubated for 60 min at 37°C before being rinsed again with PBS. Finally OPCs were mounted in Citifluor and analyzed via fluorescence microscopy.

2.2.9 Morphology

To determine the effects of ENV on cell morphology 24-well cell culture dishes were either coated with 1000ng/ml ENV or corresponding buffer concentration

as a control. OPCs were then plated in proliferation medium as described above. After 24h of incubation at 37°C cells were transfected with a citrine expression vector according to the NanoJuice transfection protocol. Citrine-positive OPCs were subsequently fixed with 3.7% PFA and mounted in Citifluor. The morphological differentiation degree was determined by means of fluorescence microscopy utilizing a designed morphology assessment key (Kremer et al., 2009) taking into account cell diameter and the degree of process branching.

3. Results

3.1 TLR4 expression during OPC differentiation

3.1.1 Immunostaining reveals expression of TLR4 on OPC surface

Previous studies have postulated that the effects observed under ENV stimulation are mediated via TLR4 and its co-receptor CD14 (Rolland et al., 2006). Since its expression on OPCs has been discussed controversially in literature (Lehnardt et al., 2002; Taylor et al., 2010) it was necessary to verify if OPCs actually express TLR4. To this end, we strived to detect receptor expression via immunocytochemistry using antibodies against TLR4 and CD14 both on rat and human oligodendroglial precursor cells. Figures 1 (B-E') and 4 (B) show that OPCs of both species express the TLR4/CD14 complex on their surface. In order to prove the presence of the receptor complex on young as well as mature oligodendrocytes we co-stained the cells for both galactocerebroside (GalC), after three days of oligodendroglial differentiation in culture and myelin basic protein (MBP), a marker for more mature oligodendrocytes after six days, respectively. Interestingly, this staining revealed that more mature OPCs express only minute amounts of TLR4 or CD14, respectively, while less differentiated cells show generally stronger expression levels. As a next step we performed quantitative RT-PCR on cultured primary OPCs during spontaneous differentiation to determine the expression of TLR4 and CD14 over time. While being strongly expressed after one day of differentiation in culture TLR4 expression was significantly downregulated over time, with CD14 following after three days of differentiation (compare fig. 1 A).

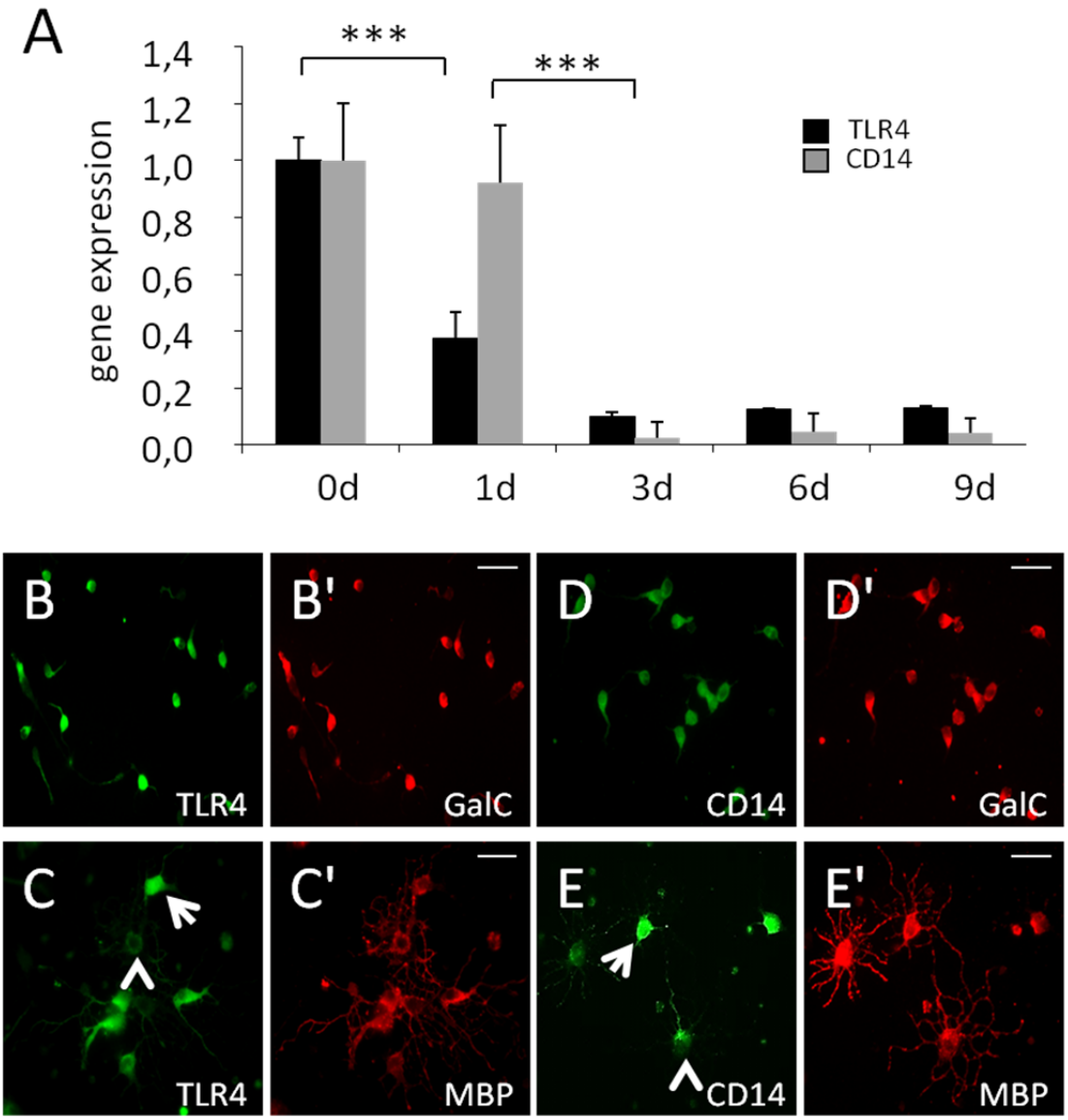

Figure 1. Quantitative RT-PCR reveals a downregulation of TLR4 and CD14 expression during spontaneous differentiation of OPCs (A) for up to 9 days in culture; GAPDH was used as reference gene (n=2). B-B‘ and D-D‘ show representative examples of TLR4/GalC and CD14/GalC immunostainings, respectively, after three days of spontaneous differentiation. After six days OPCs were double-stained with mature oligodendrocyte marker MBP and TLR4 or CD14, respectively (C-C‘, E-E‘). Arrows point towards cells with strong expression while arrowheads mark cells with only weak expression levels (n=3). Scale bars: 50µm.

3.1.2 Expression of TLR4/CD14 in spinal cord white matter

Following the observations of spontaneous TLR4 regulation in culture, we turned to examining TLR4 expression in the diseased spinal cord using MOG-induced experimental autoimmune encephalomyelitis (MOG-EAE) in rats as an inflammatory animal model (MOG-EAE tissue sections were provided by Prof.

S. Jander, Department of Neurology, Düsseldorf). To instigate an inflammatory reaction, mice were injected with CNS proteins such as myelin basic protein (MBP) or - in our case - myelin oligodendrocyte glycoprotein (MOG) which have been shown to be a main target of the immune system. This treatment ultimately causes small disseminated lesions throughout the CNS which show certain similarities to MS lesions. Using this material tissue samples were stained for TLR4 in order to screen for receptor expression in the diseased spinal cords of MOG-EAE rats. Double-immunostainings for TLR4 and APC (CC-1), which is an established marker for mature oligodendrocytes (McTigue et al., 2001; Kitada and Rowitch, 2006) and for TLR4 and platelet-derived growth factor receptor-α (PDGFR-α), which is used to detect early stages of OPC differentiation (Hall et al., 1996; Li et al., 2002a), confirmed oligodendroglial expression of TLR4 in diseased spinal cord white matter (Fig. 2).

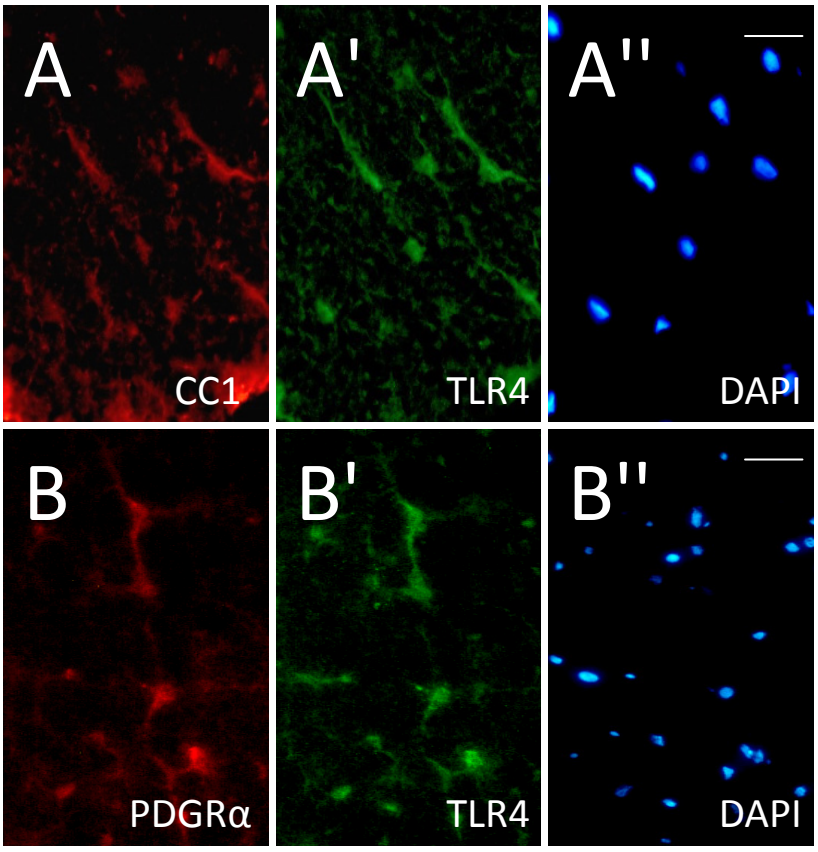

Figure 2. Double immunostainings for CC1 and TLR4 (A,A') and for PDGFRα and TLR4 (B,B') indicate that within the diseased spinal cord white matter, oligodendrocytes and OPCs express TLR4, respectively (n=1). Scale bars: 50µm.

3.1.3 TLR4 suppression via transfection

After demonstrating the presence of TLR4 on OPCs we searched for possibilities to specifically modulate receptor expression in order to confirm our staining results. One way to alter expression patterns is via suppression of TLR4 using small hairpin RNA (shRNA) encoding gene suppression vectors. OPCs were transfected in parallel with a TLR4 suppression vector, a scrambled vector and an empty control vector and all of which were co-transfected with an expression vector encoding citrine to mark transfected cells. The success of transfection was controlled through fluorescence microscopy. Neither the scrambled nor the empty control vector led to a change in TLR4 expression, while OPCs transfected with TLR4 suppression vector were found to be devoid of TLR4 expression as revealed by immunocytochemistry proving the specificity of the previously used anti-TLR4 antibody (Fig. 3C,C').

3.1.4 Blocking of TLR4

Another option to address functionally the oligodendroglial response to ENV exposition is given by antibody-mediated blocking of TLR4. To this end, OPCs were incubated with 20 µl/ml anti-TLR4-antibody for one hour before being stimulated either with 1000 ng/ml ENV or corresponding buffer concentration. Quantitative RT-PCR was performed to monitor inflammatory response. However, there was no significant reduction in the induction of inflammatory genes being observed (Fig. 3A,B). Additionally, the antibody seemed to cause minor receptor activation as revealed by quantitative RT-PCR.

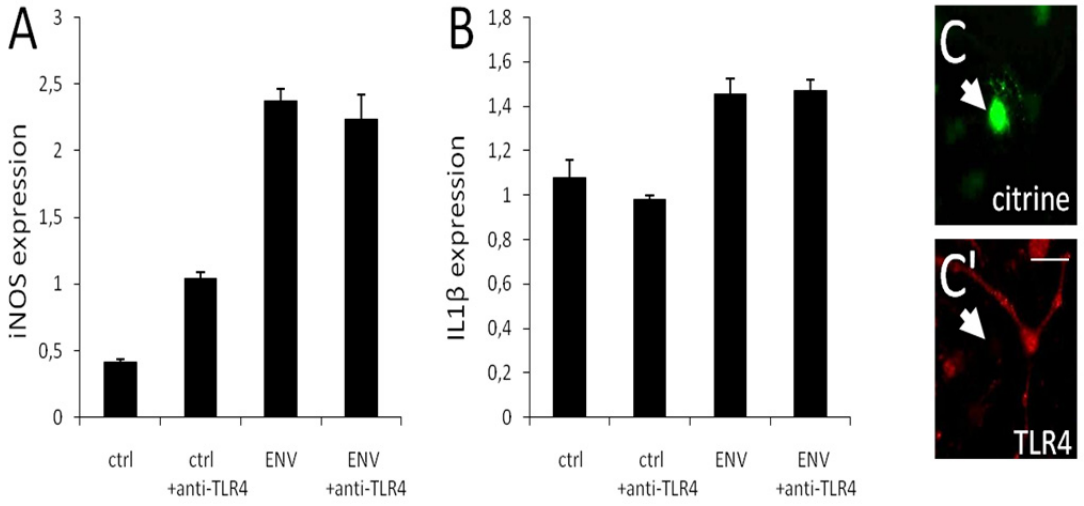

Figure 3. (A,B) Treatment of OPCs with anti-TLR4 antibody prior to stimulation with soluble ENV does not inhibit induction of proinflammatory genes such as iNOS or IL1β (n=7). (C,C') Upon suppression of TLR4 receptor expression, immunostaining for TLR4 showed that transfected OPCs did not express this receptor (arrowhead) thus proving the antibody's specifity. Co-transfection with a citrine expression vector was used to mark transfected cells (n=5).

3.2 ENV stimulation

3.2.1 Soluble recombinant ENV

There are several options to determine the effects of MSRV envelope protein on OPCs in terms of possible modes of protein exposure to cells. Firstly, ENV can be added in its soluble form to the medium of cultured OPCs. To this end, cells were plated according to protocol before being allowed to settle and adhere at 37°C for two hours. Subsequently, they were treated with different concentrations of soluble recombinant ENV ranging from 10 ng/ml to 100 ng/ml and a maximum of 1000 ng/ml, which is diluted in a buffer consisting of Tris 20mM pH7.5, sodium dodecyl sulfate (SDS) 1.5%, dithiothreitol (DTT) 10mM and NaCl 150 mM. Buffer without ENV protein served as negative control and was added in corresponding dilutions.

This treatment allowed the cells to settle down in an environment adapted to their needs before being exposed to the ENV stimulus. However, this setting does not resemble the *in vivo* situation and therefore permits only an incomplete statement about the effects of ENV. Besides, the buffer in use may possess

toxic properties due to the detergent SDS, which can also be used for cell lysis or protein denaturation.

3.2.2 Surface-bound recombinant ENV

A second approach of treating OPCs with ENV is provided by surface coating of cell culture wells with recombinant ENV protein prior to plating cells. To this end, a PDL-coated plate was rinsed with PBS and subsequently incubated with recombinant ENV for 2h at 37°C at a concentration of 1000 ng/ml. Similar to the stimulation with soluble ENV, buffer without ENV protein served as negative control and was used in a matching dilution. Following incubation the plate was rinsed again several times with PBS before OPCs were seeded.

Of note, this experimental setup allows to minimize toxic effects induced by the buffer control allowing for an assessment of the ENV effect without interference. Additionally, it also bears more resemblance to the *in vivo* situation as compared to the treatment with soluble ENV as OPCs have to settle in an environment already containing the potentially noxious ENV protein thus paralleling the migration of OPCs to potentially ENV containing lesion areas in the CNS. Despite its advantages it still is an *in vitro* setting and does not take the various interactions between different cell types into account that exist *in vivo*. In addition the OPCs are not allowed to settle down in a stress-free environment after plating since the wells themselves are coated with ENV. On the other hand, a surface presentation of ENV might reflect more realistically interactions between OPCs and ENV expressing and presenting cells considering how various immunocompetent cells such as macrophages and microglia have been shown to express ENV on their surface (Perron et al., 2005).

3.2.3 ENV overexpression in U-343 glioblastoma cells

Overexpression of ENV through U-343 glioblastoma cells was used as the third option in order to expose OPCs to ENV. For this purpose, U-343 cells were plated on 24-well cell culture dishes and transfected either with pV14_ENV or pV14_ctrl expression vectors. OPCs were seeded on top of the U-343 layer and thereby exposed to ENV, which was expressed and presented by on the surface of the transfected U-343 cells.

Originating from a Caucasian male's glioblastoma this cell line has several

merits commending it for our purposes. It shows a flat monolayer growth pattern which facilitates the seeding of OPCs on top of it. Being of human origin U-343 cells do not influence analyses based on RT-PCR with rat-specific amplification primers, when using rat OPCs. And finally being descended from astrocytes these cells bear closer resemblance to the *in vivo* situation in that they can mimic ENV expressing cells which are in contact to OPCs. Nevertheless, it is an immortalized cell line and may as such not react physiologically. Additionally, the expression of ENV on their surface may not only harm the OPCs but the U-343 cells as well inducing an autocrine inflammatory response.

3.3 ENV dependent molecular reactions

3.3.1 Analysis of gene expression reveals an up-regulation of pro-inflammatory genes

To evaluate the ability of ENV to induce cytokine production in rat OPCs we performed quantitative RT-PCR utilizing the previously described methods of ENV stimulation. OPCs were treated either with soluble or surface-bound ENV or plated on pV14_ENV transfected U-343 cells. In the first two cases buffer without protein served as control, in the third setup, U-343 were transfected with pV14_ctrl vector, which is the expression vector without insert (open reading frame). After 24 h incubation the OPCs were lysed and gene expression was analyzed using quantitative RT-PCR. As illustrated in Fig. 4 a significant induction of pro-inflammatory genes could be observed with a particularly strong induction of the inducible NO synthase (iNOS). In summary, both the presentation of membrane-bound ENV expressed on transfected U-343 cells and the stimulation with either soluble or surface-bound ENV lead to a significant increase in iNOS expression (Fig. 4A-A''). Furthermore, interleukins-1β and -6 (IL1β and IL6) as well as TNF-α showed a significant increase in transcription levels (Fig. 4E-G). Other pro-inflammatory genes such as IFNγ were not significantly induced (data not shown). In order to translate our observations to human cells, we conducted analogous experiments with human OPCs (hOPCs) treating them with surface-bound ENV. Analysis for iNOS induction via quantitative RT-PCR showed a strong up-regulation after stimulation with ENV as compared to buffer control (Fig. 5C).

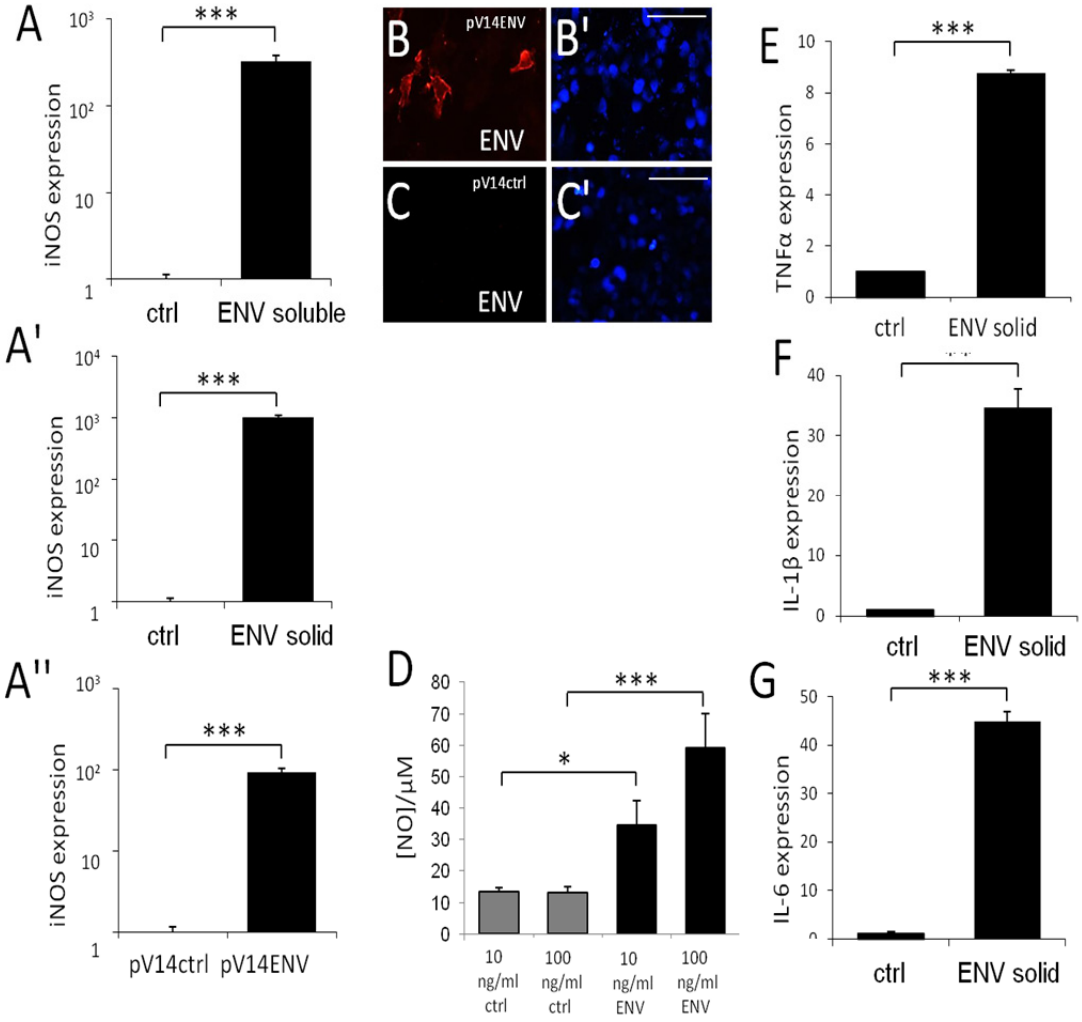

Figure 4. Stimulation of OPCs either by soluble ENV (n=21) or by ENV bound to the surface of cell culture dishes (=solid) (n=17) lead to a strong induction of iNOS (A,A‘) compared to control cells. GAPDH was used as reference gene. Furthermore, ENV expressed on the surface of pV14-transfected cells of the U343 line (B-C‘) leads to a strong iNOS induction in OPCs, as well (A‘‘) (n=8). Besides iNOS several proinflammatory cytokines were also significantly upregulated (E-G). GAPDH and ODC were used as reference genes.

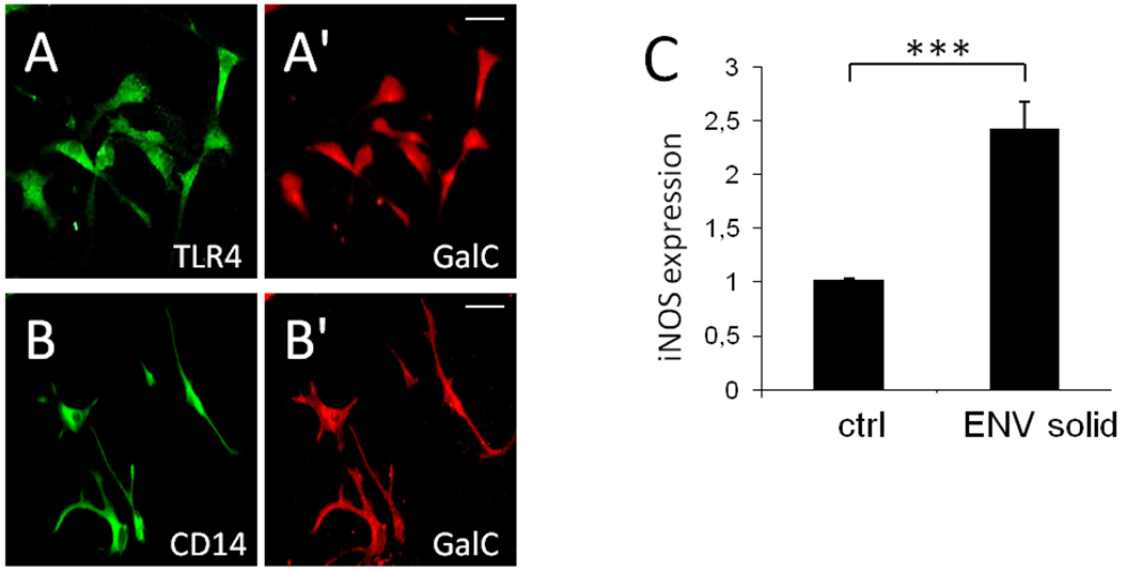

Figure 5. (A-B‘) show representative examples of human OPCs, which were double-stained for TLR4/GalC and CD14/GalC, respectively, after one day of spontaneous differentiation in culture. Scale bar: 50µm. Analogous to rat OPCs human OPCs show a significant upregulation of iNOS upon stimulation with ENV (C). GAPDH was used as reference gene (n=4).

3.3.2 Spectrophotometry shows increase in NOS activity

Nitric oxide or nitrogen monoxide (NO) is an important cellular signaling molecule. It is a radical gas with a short half life which is able to diffuse freely across cell membranes making it ideal for paracrine and autocrine signaling. NO, also known as endothelium-derived relaxing factor (EDRF), causes vascular dilatation and muscle relaxation via the soluble guanylate cyclase but is also part of the human immune response being produced by phagocytes such as monocytes or neutrophil granulocytes, thus functioning not only as a messenger molecule but showing direct antimicrobial activity.

Due to its short half life period it is, however, difficult to detect NO production directly. Spectrophotometry therefore represents an elegant method to circumvent this difficulty. By measuring the absorption and thereby deducing the concentration of the NO disintegration product nitrite at a defined wavelength of light, we were able to conclude indirectly the amount of NO secreted into the medium. OPCs were treated with increasing doses of soluble ENV (Fig. 4D) and NO production was subsequently evaluated photometrically after 24 h and after 48 h. As illustrated in Fig. 4D ENV stimulation leads to a dose-dependent increase of NO secretion into medium.

3.3.3 3-nitrotyrosine formation

It is commonly accepted that the exposition to noxious influences leads inter alia to the production of pro-inflammatory cytokines such as NO. NO, being one of the main members of the so-called reactive nitrogen species (RNS) has been shown to initiate stress reactions in a variety of biochemical contexts (Eu et al., 2000; Klatt and Lamas, 2000), namely causing nitrosative stress. The strong upregulation of iNOS after stimulation with ENV as shown in Fig. 3A'' suggests that ENV might induce nitrosative stress as well. One approach to verify this hypothesis is presented by staining for 3-nitrotyrosine. As mentioned above it is difficult to directly measure the accruing NO which is why it was necessary to make use of the byproducts and decomposition products. Peroxinitrite (ONOO-) is a potent oxidant resulting from the reaction between NO and superoxide (O_2^-). It can react with a wide array of molecules including proteins, nucleic acids and lipids causing significant cellular damage. For our purposes, especially the nitration of the amino acid tyrosine is of interest. Superoxide dismutase catalyzes the dismutation of peroxinitrite into nitronium-like reactive species which react with tyrosine to form 3-nitrotyrosine. The amount of accruing 3-nitrotyrosine therefore adequately reflects the production of NO, making indirect visualization of NO through immunostaining possible and subsequently allowing for an assessment of nitrosative stress levels.

OPCs were plated on ENV-coated 24-well cell culture dishes. After 24 h the cells were fixed and stained for 3-nitrotyrosine. Buffer treated cells served as negative controls while SNAP treated cells were used as positive controls. SNAP, (S-nitroso-N-acetylpenicillamine), being an NO donor, releases NO under physiological conditions. It could be demonstrated that upon ENV stimulation significantly more OPCs stained positive for the nitrosative stress marker 3-nitrotyrosine as compared to negative control cells whereas SNAP treatment leads to 3-nitrotyrosine positivity in almost all cells (Fig. 6).

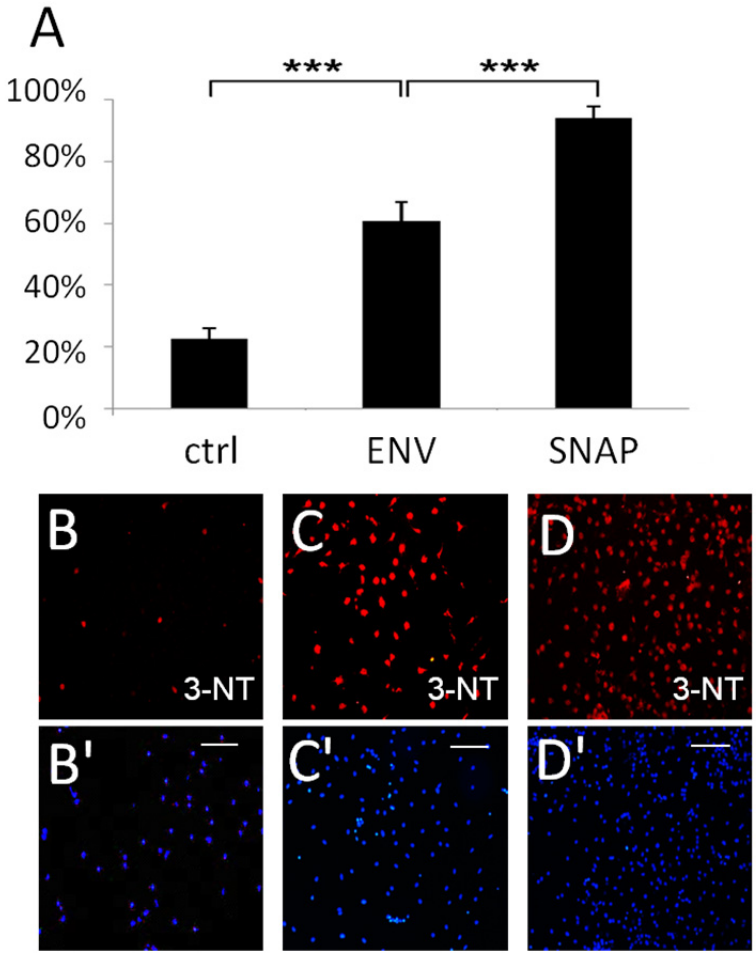

Figure 6. Induction of nitrosative stress marker nitrotyrosine (3-NT) (A). Following 24h of stimulation with surface bound recombinant ENV, significantly more OPCs expressed the NO-dependent nitrosative stress marker 3-NT as compared to control cells. As a positive control S-Nitroso-N-acetylpenicillamine (SNAP), a strong NO donor, was used causing massive 3-NT formation. (B-D') Representative anti-3-NT stainings of control cells (B,B‘), ENV stimulated OPCs (C,C') and OPCs exposed to SNAP (D,D‘) (n=6). Scale bars: 100µm.

3.3.4 Calcium dependency

Recent studies suggested that intracellular calcium concentration plays an important role in TLR4 signaling. As previously mentioned, there are two main pathways downstream of TLR4 activation: the MyD88-dependent and MyD88-independent pathway. It has been shown that, being dependent on IRF3, the MyD88-independent pathway indirectly depends on intracellular calcium levels (Chiang, 2007), while there is no clear evidence for a calcium dependency of the MyD88-dependent pathway.

To determine the effect of calcium depletion on pro-inflammatory reactions in OPCs caused by ENV stimulation, gene expression analysis using quantitative RT-PCR was performed. To this end, cells were either treated with soluble ENV

or corresponding buffer concentration after being plated either in calcium-free or standard calcium-containing differentiation medium. Compared to control cells ENV led to a strong induction of iNOS both in calcium-free and in standard differentiation medium. However, the absence of calcium caused a significant attenuation of iNOS induction compared to ENV treated OPCs in standard medium (Fig. 7A). Although gene expression analysis revealed a strong induction under stimulation with ENV compared to control cells, the aforementioned effect could not be observed in proinflammatory cytokines such as IL6 (Fig. 7B).or IL1β and TNFα (data not shown).

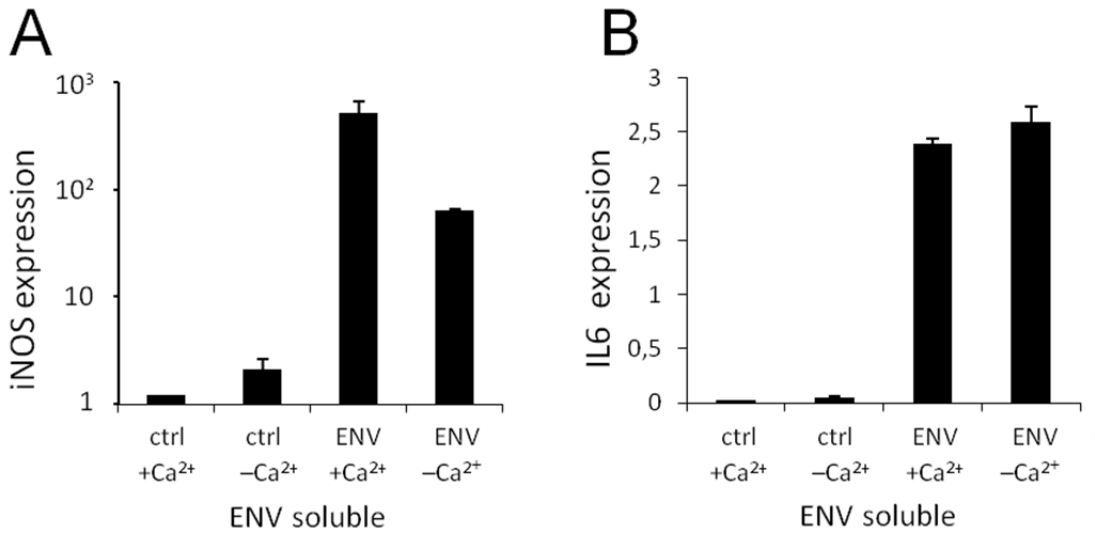

Figure 7. Stimulation with soluble ENV leads to iNOS gene induction regardless of calcium concentration in medium (A,B). Significantly reduced induction of iNOS under depletion of calcium compared to standard differentiation medium (A). ENV dependent IL6 proinflammatory cytokine induction was not affected by the calcium depletion (B) (n=8).

3.4 ENV mediated cellular reactions

3.4.1 Myelin expression and morphological maturation

Observing the induction of inflammatory genes following stimulation with ENV the question arose whether ENV treatment and the subsequent inflammatory reaction might affect oligodendroglial maturation processes. To monitor the differentition of OPCs over time cells were plated on ENV-coated 24-well cell culture dishes and fixed for immunostaining after three and six days. After three days the cells were stained for the early oligodendroglial myelin marker 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) while after six days myelin basic protein (MBP) was used to visualize myelin expression. As early as day three ENV-treated OPCs were found to express significantly less CNPase as compared to buffer-treated control cells (Fig. 8A-A'''). Correspondingly, the degree of MBP expressing OPCs was strongly decreased after six days (Fig. 8 B-B'''), thus suggesting an inhibition of myelin expression hence OPC differentiation through ENV.

Based on the observed differences in myelin expression we also studied possible morphological alteration of OPCs upon ENV stimulation. Since differentiation of OPCs in culture is not a synchronized process various maturity levels at each time point featuring OPCs with varying degrees of branches can be observed. To categorize maturity levels the cells were classified into six groups depending on the number and complexity of their branches, ranging from "very low" differentiated progenitors over "low", "medium" and "high" differentiated cells to "very high" differentiated mature oligodendrocytes and even so-called "sheaths" bearing cells of complex, flattened appearance (Kremer et al., 2009). After plating OPCs on either ENV- or buffer-coated surfaces they were transfected with a citrine expression vector to visualize cell morphology (Heinen et al., 2008). After three days the cells were fixed and analyzed via fluorescence microscopy. Interestingly, no significant deviations concerning morphological progress could be determined in comparison to control cells (Fig. 8G). This notion is further supported by the observation that morphology of O4 stained OPCs appeared to be unchanged (Fig. 8E-F').

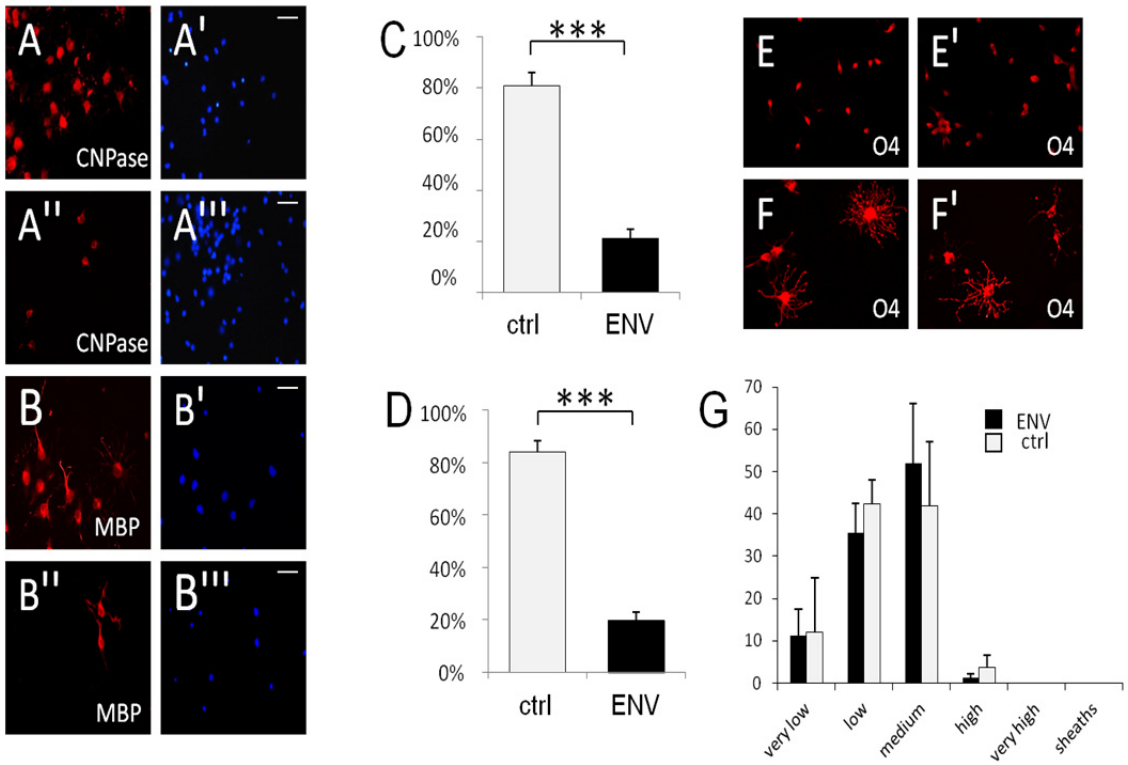

Figure 8. Impact of ENV on oligodendroglial differentiation and morphology (C,D) ENV stimulation leads to an significant decrease in myelin marker expression following one day (C) and three days (D) of differentiation. (A-B''') Representative stainings of OPCs for early myelin marker CNPase after one day differentiation following stimulation with ENV (A''-A''') and for late myelin marker MBP after three days differentiation (B''-B''') reveal a significant decrease in expression compared to control cells (A-A' and B-B'). (E-F') Despite ENV's strong impact on gene induction and myelin production OPC staining for O4 revealed no alteration of OPC morphology after one day (E=control, E'=ENV) and after three days (F=control, F'= ENV) of differentiation. Scale bar: 50µm. (G) Analysis of OPCs based on morphological maturation stages comparing ENV to buffer-treated control cells (in %). No significant differences could be determined (n=14).

3.4.2 Cellular senescence

As established in the prior experiments exposure to ENV induces nitrosative stress and subsequently an inflammatory response in OPCs. As a next step we investigated whether this response affects not only maturation but also natural senescence processes featuring induction of acid β-galactosidase. To this end, OPCs were plated in proliferation medium before adding soluble ENV and corresponding buffer. OPCs in proliferation medium without ENV or buffer added were used as negative control, while OPCs, which were additionally treated with 15% FCS, served as positive control. FCS was used because of its senescence promoting properties (Duggal and Brinchmann, 2011). After three and five days cells were fixed and quantitatively evaluated. While almost 50% of

the OPCs in proliferation medium and nearly 80% of the OPCs in proliferation medium containing 15% FCS stained positive for acid β-galactosidase, we found that only about 30% of both ENV- and buffer-treated cells express this enzyme. Considering the almost identical staining results for acid β-galactosidase there appears to be no significant acceleration of senescence processes under ENV stimulation compared to treatment with buffer only (Fig. 9A).

3.4.3 Survival and apoptosis

Although senescence is not significantly affected by ENV stimulation another point of interest is whether ENV can modulate programmed cell death decisions. Apoptosis can be initiated by a variety of intrinsic or extrinsic factors; stress in general being an important one of them (Buttke and Sandstrom, 1994). Previous studies suggested that nitrosative stress sensitizes cells to cytokine-induced apoptosis (Marshall and Stamler, 2002), raising the question if this also applies to ENV induced nitrosative stress in OPCs.

To determine whether ENV treatment leads to initiation of apoptosis, OPCs were treated either with ENV or buffer before being prepared for TUNEL treatment. TUNEL is an established method to reveal fragmentation of DNA occurring in the course of apoptosis. The terminal deoxynucleotidyl transferase (TdT) catalyzes the addition of dUTP, which are labeled with a fluorescent marker, to exposed hydroxyl groups at DNA breakage points. This technique allows for a sensitive and fast method able to distinguish between necrosis and apoptosis (Sgonc et al., 1994). Analysis by fluorescence microscopy did, however, not reveal statistically significant differences between ENV treated OPCs and control cells, thus not allowing the presumption of ENV leading to a premature induction of apoptosis in OPCs (Fig. 9B).

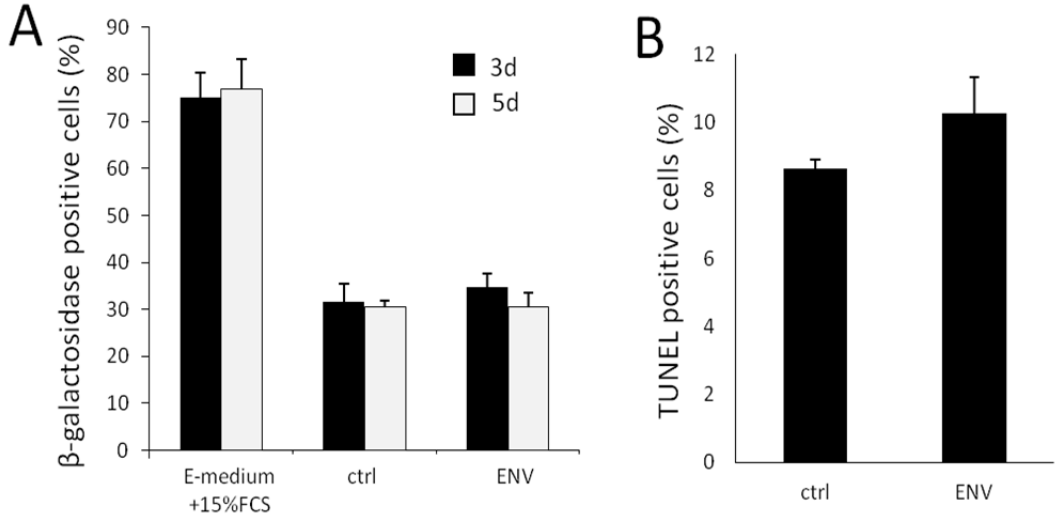

Figure 9. (A) Staining of OPCs for acid β-galactosidase shows strong expression in proliferation medium (E-medium) containing 15% of fetal calf serum (FCS) with only slight differences between days 3 and 5. No significant differences between ENV- and buffer-treated (ctrl) cells concerning the expression of acid β-galactosidase (n=6). (B) TUNEL staining of OPCs after 24h of incubation revealed no significant increase of apoptotic cells compared to buffer-treated control cells (t-test 0,762) (n=6).

3.4.4 OPC inhibitory gene expression

After observing inhibitory effects of ENV on oligodendroglial differentiation in immunostainings for myelin proteins, in a next step we evaluated the induction of differentiation inhibiting genes upon ENV stimulation. Over the past years many studies conducted genome-wide screens for differentiation regulators, identifying a vast collection of potential differentiation inhibiting candidate genes. Nuclear helix-loop-helix transcription factors like hairy and enhancer of split 1 and 5 (Hes1 and Hes5) or inhibitor of differentiation 2 and 4 (Id2 and Id4), intracellular signaling proteins like β-Catenin or p57kip2 and intracellular tyrosine phosphatases like MAP kinase phospahatase 5 (MKP5) or protein tyrosine phosphatase receptor-type R (PTPRR) have been found to inhibit oligodendroglial differentiation (Kondo and Raff, 2000a; Kremer et al., 2009, 2011; Li et al., 2009; Pescini Gobert et al., 2009).

To determine the effect of ENV on inhibitory gene induction in OPCs gene expression analysis using quantitative RT-PCR was performed. OPCs were plated on either ENV or buffer coated 24-well cell culture dishes and lysed after 24 h or 48 h, respectively. This analysis revealed a very selective induction of some inhibitory genes. While Hes1 and MKP5 were found to be significantly up-

regulated under ENV contact, transient receptor potential cation channel (TRPC4) (Zechel et al., 2007) was significantly down-regulated (Fig. 10). Other inhibitory genes such as Id2, Id4, p57kip2, Hes5, Notch-1, which encodes a transmembrane receptor, tumor suppressor gene TIP30, small GTPase encoding RhoA, PTPRR or protein kinase C encoding PRKCA were screened but found not to be significantly affected (data not shown).

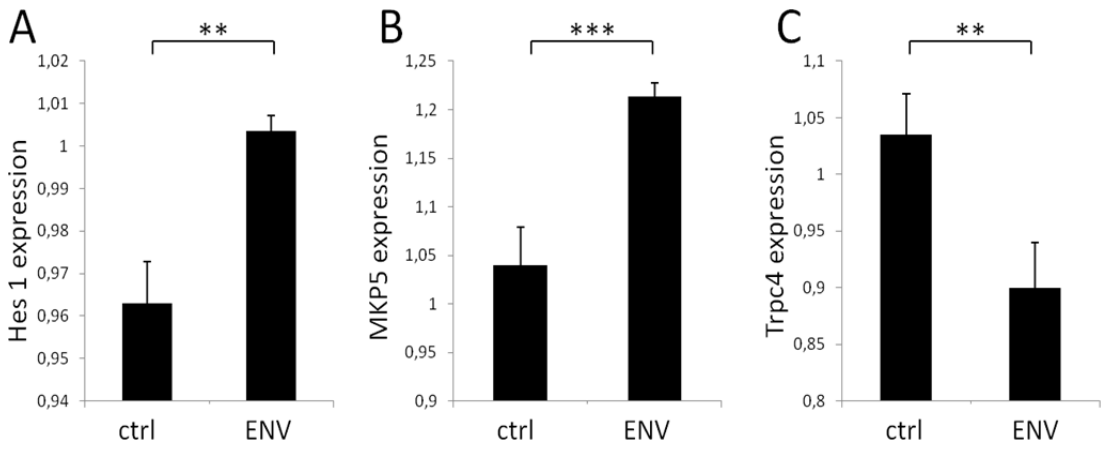

Figure 10. Regulation of differentiation inhibiting genes following stimulation with ENV (surface-bound). (A,B) Hes 1 and MKP5 were substantially upregulated after 24h while the cation channel Trpc4 showed a significantly decreased expression (C). GAPDH was used as reference gene (n=10).

4. Discussion

4.1 ENV mediated effects on homeostasis of oligodendroglial precursor cells

4.1.2 ENV mediates its effects on OPCs through the TLR4/CD14 receptor complex

Considering how insufficient differentiation of oligodendroglial precursor cells (OPCs) leads to inadequate remyelination and eventually clinical impairment it is important to shed a light on the mechanisms which negatively influence differentiation. In this study the influence of MSRV envelope protein (ENV) on differentiation and homeostasis of oligodendroglial precursor cells (OPCs) was investigated. Given the complexity of these processes it was necessary to study them on different levels of cell homeostasis including molecular and cellular reactions as well as morphological changes. Interestingly, the observed pro-inflammatory effect of ENV on OPCs seems to be independent from the experimental setting, be it stimulation with soluble, surface-bound or membrane-bound ENV (compare Fig. 4A-A''). Furthermore, we were able to demonstrate for the first time the expression of TLR4 and its co-receptor CD14 on OPCs. ENV interaction with these receptors has already been shown to mediate proinflammatory effects on peripheral blood mononuclear cells (PBMCs) (Rolland et al., 2006). In contrast to previous studies denying the expression of TLR4 on oligodendroglial cells we were able to not only demonstrate an extensive expression of both TLR4 and CD14 on OPCs but also – to a lesser degree – on more mature oligodendrocytes both in culture and in diseased tissue. The observed downregulation of the assumed ENV receptor during spontaneous cell differentiation correlates well with our observation that precursor cells show a stronger reaction to stimulation with ENV than mature cells. This finding implies a higher vulnerability of younger and immature cells while matured cells or myelinating oligodendrocytes appear to be more resistant to the deleterious effects of ENV. It has already been shown that ENV is indeed significantly expressed in MS lesions mostly by macrophages and microglia (Perron et al., 2005). Since adequate remyelination

in the CNS depends on an intact pool of oligodendroglial precursor cells (Franklin and ffrench-Constant, 2008) the observed sensitivity towards ENV of these cells indicates that ENV could interfere with successful remyelination of demyelinated areas in the brain. In order to modulate the detrimental effect of ENV on oligodendroglial differentiation we suppressed the expression of TLR4 via shRNAs. Although cell transfection provides an effective method to interfere with receptor expression, one has to be aware that the procedure itself can negatively affect OPC homeostasis and survival. Transfection agents can lead to considerable - chemically induced - stress reactions and cell death, which finally leads to diminished cell numbers. Another option to alter oligodendroglial responses is given by antibody-mediated blocking of TLR4. We could, however, not determine a significant reduction in induction of pro-inflammatory genes upon application of anti-TLR4 antibody treatment prior and during ENV stimulation. Additionally, gene expression analysis revealed a slight increase of expression of those genes that have previously been detected upon ENV stimulation only, suggesting minor receptor activation caused by the antibody itself (Fig. 3A,B). This might be due to specific antibody properties and needs to be further addressed in future experiments. There are, however, further possibilities of influencing receptor signaling. Specific inhibiting agents directed against TLR4 dependent messenger proteins such as IRAK or TRIF could allow for a more nuanced attribution of ENV effects and will be addressed in a future studies. Another option to disable the ENV induced effects on OPCs could be heat denaturation of the ENV protein in order to directly assign the observed cellular effects to ENV protein structure and function. Therefore, recombinant ENV protein was incubated at 100°C for 15 minutes prior to coating of 24-well cell culture dishes. Both, dishes coated with non-inactivated ENV as well as buffer coated dishes served as controls. OPCs were plated on these surfaces and gene expression analysis performed. However, this assay did not reveal significant differences in the induction of proinflammatory genes (data not shown) compared to normal ENV treatment, which suggests that the ENV protein was not effectively inactivated. This could be due to a claimed heat resistance of ENV, which would thus require higher temperatures for inactivation (personal communication H. Perron, Geneuro SA, Geneva).

4.1.2 ENV dependent gene expression

To determine the influence of ENV stimulation on OPC gene expression we performed quantitative RT-PCR. As depicted in Figure 3A,E-G stimulation with ENV induces pro-inflammatory genes. Interestingly, only few genes were significantly induced such as iNOS, TNFα, IL1β or IL6, whereas other pro-inflammatory genes such as IFNγ were not affected. This supports the theory of ENV leading to a targeted immune response rather than to an unspecific one as suggested earlier (Rolland et al., 2006). Stimulation with ENV significantly increased the expression of several important cytokines, namely interleukin 1β, interleukin 6 and tumor necrosis factor α (IL1β, IL6 and TNF-α), all of which are known to play an important part in inflammatory processes. Being primarily produced by microglia but also by a number of other cells IL1β, for instance, induces the production of other cytokines such as IL6 and TNFα and triggers the production of NO as well (Chung and Benveniste, 1990; Aloisi et al., 1992; Hewett et al., 1993; Basu et al., 2004). IL1β has been shown to support proliferation and activation of macrophages and astrocytes *in vitro*, thus being generally associated with the exacerbation of inflammatory situations (Dinarello, 1988; Brough et al., 2011). IL6, which is also known as B-cell stimulating factor-2 or hepatocyte stimulating factor, is produced by a wide range of different cell types including monocytes, activated T-cells or fibroblasts. Its various names reflect the diversity of functions performed by this cytokine. Notably, it can stimulate B-cell differentiation, promote T-cell proliferation and it plays an important part in the production of acute-phase proteins in hepatocytes, a class of proteins whose blood levels increase during inflammation such as the antimicrobial C-reactive protein (CRP), the iron-binding ferritin or plasminogen, which inhibits blood clotting (Akira et al., 1990; Kishimoto, 2010). Primarily produced by activated macrophages TNF-α exerts a wide range of functions including induction of other pro-inflammatory cytokines, regulation of vascular permeability or stimulation of glial cells after brain injury or ischemia (Selmaj et al., 1990; Arvin et al., 1996). Additionally it has been shown to be associated with MS disease progression (Sharief and Hentges, 1991). While previous studies have already shown an ENV dependent induction of pro-inflammatory cytokines in PBMC (Rolland et al., 2005; Saresella et al., 2009), our results now indicate an upregulation of these genes in resident glial precursor cells. In

addition to cytokines the inducible NO synthase (iNOS) was particularly strong upregulated. Three isoforms of NO synthase have “emerged” during evolution, each located on a different chromosome (Marsden et al., 1993; Chartrain et al., 1994). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are expressed constitutively thus called constitutional NOS (cNOS) as opposed to the inducible NOS (iNOS). This inducible form was found in high concentrations in inflammatory lesions of demyelinating diseases such as MS, being expressed by a variety of cells including macrophages and astrocytes. There is, indeed, evidence for a distinctive upregulation of NO derivatives in cerebrospinal fluid and blood of MS patients (Giovannoni, 1998). Moreover, NO has been shown to increase permeability of the blood-brain-barrier in the context of neuroinflammatory diseases (Boje, 1996; Mayhan, 2000). On the other hand, NO plays an important part in the immune system both as messenger molecule and as agent exerting both antiviral and antimicrobial effects (Nathan and Hibbs, 1991; Croen, 1993; Weller, 2009; Mehta et al., 2012). Besides the strong induction of iNOS at gene expression level we were able to show a significant increase of NO in medium of cultured OPCs upon stimulation with ENV both directly through spectrophotometry and indirectly through staining for 3-nitrotyrosine. These findings corroborate previous studies claiming ENV to be a relevant factor in maintaining, supporting and exacerbating inflammatory processes especially in the CNS and suggest ENV to be a relevant cause for nitrosative stress in OPCs (Johnston et al., 2001; Sotgiu et al., 2004). While the activity of cNOS is regulated by the intracellular calcium concentration with higher concentrations resulting in binding of intracellular messenger protein calmodulin, iNOS is generally believed to be independent of intracellular calcium concentrations (Iadecola et al., 1995). However, we were able to show a significant attenuation of iNOS induction under ENV treatment upon depletion of calcium in cell culture medium (Fig. 7A). To investigate this unexpected observation in detail, additional experiments will be carried out in the future. Such mechanistic investigations are, however, out of scope for this thesis.

4.1.3 Cellular reactions following stimulation with ENV

We were able to show a distinct pro-inflammatory impact of ENV on OPCs, which led to the question in how far these deleterious effects could interfere with oligodendroglial differentiation. Therefore, we analyzed the expression of myelin

proteins following stimulation with ENV. As shown in Figure 7C,D exposure to ENV significantly reduced expression of the early myelin marker CNPase after three days of differentiation as well as late myelin marker MBP after six days thus suggesting a relevant blockade of myelin formation in OPCs. Since maturation and differentiation of OPCs are accompanied by distinct changes in form and size, morphological appearance represents an important differentiation associated parameter. We were, however, not able to determine noticeable changes in OPC morphology (Fig. 8E,F). This observation indicates that ENV-mediated effects do not primarily manifest themselves in an altered cytoskeletal structure but rather in an influence on the myelin production process itself. However, not only morphology but also oligodendroglial senescence remains virtually unaltered as ascertained through staining for acid β-galactosidase (Fig. 9A). Additionally, neither TUNEL experiments nor quantitative DAPI-based cell counts revealed an increased cell death (Fig. 9B) therefore suggesting that ENV has no effect on apoptosis and cell survival. Taken together these observations corroborate the idea of ENV acting as a potent interference factor of OPC function and homeostasis but rather not as a toxic agent.

We were also able to verify differentiation inhibiting effects on a molecular level. Recently, a few genome-wide studies identifying various differentiation inhibiting genes located on different chromosomes were conducted. In our experiments we found that especially hairy and enhancer of split 5 (Hes5) and MAP kinase phospahatase 5 (MKP5) are significantly induced upon ENV stimulation while transient receptor potential cation channel (TRPC4) shows a distinct downregulation (Fig. 10). These are, however, subtle regulations and future experiments with time course stimulation schemes need to be performed to confirm these findings.

Multiple studies support the role of Hes5 as a potent inhibitor of myelin gene expression showing a strong upregulation in demyelinating diseases which are associated with impaired remyelination. Furthermore, expression of Hes5 has been shown to progressively decrease during OPC differentiation which is accompanied by an increasing myelin production, stressing its importance in developing brains (Kondo and Raff, 2000b; Liu et al., 2006). MKP5 on the other hand appears to play a part both in the regulation of myelin production and in T-

cell proliferation and therefore – through increased levels of IFNγ and TNFα - in immune reactions (Gobert et al., 2009). Furthermore a MKP5 deficiency seems to promote a certain protection from demyelinating disorders including EAE, which may be partly due to enhanced remyelination as shown in MKP5 knockout animals (Zhang et al., 2004; Gobert et al., 2009). In summary, an upregulation of both Hes5 and MKP5 can be associated with a considerable detrimental effect on the differentiation processes of glial cells concerning myelin production, remyelination processes and induction of proinflammatory cytokines. These assumptions correlate well with our own observations regarding the influence of ENV on OPCs.

Interestingly, the cation channel TRPC4 - being significantly downregulated - shows therefore the opposite reaction after stimulation with ENV. Being part of a family of calcium channels, TRPC4 activity depends on intracellular calcium though it seems to be independent of calcium concentrations in intracellular stores (Schaefer et al., 2000), suggesting - as already mentioned above - a connection between intracellular calcium concentrations and cellular reaction to ENV although the exact mechanisms remains yet to be determined. Immunohistochemical analyses revealed a preferred expression of TRPC4 in septal, hippocampal, cortical, and cerebellar regions of the developing brain with an absence in regions of higher proliferation such as the ventricular zone (Zechel et al., 2007). Although being generally downregulated during development, TRPC4 expression persists in the adult brain, supporting the thesis of it being involved in cell to cell signaling. Indeed, it could be shown to play a role in regulating calcium homeostasis, modulating neuronal growth and axon guidance and maintaining cellular excitability (Clapham et al., 2001; Li et al., 2005; Fowler et al., 2007). An ENV induced downregulation of TRPC4 could therefore cause significant interference in basic cell to cell interactions, thus supporting the deleterious effects of ENV on oligodendroglial differentiation.

4.2 ENV in the context of MS, therapeutic approaches and future studies

Considering all these findings one has to keep in mind that they derive primarily from observations made on primary cultures of rat oligodendroglial precursor cells. To assess the value of our findings in the context of inflammatory diseases of the CNS such as multiple sclerosis it will be necessary to verify their transferability both to human cells and to *in vivo* systems in the form of suitable animal models. We were able to both show the detrimental effects of ENV on rat OPCs in culture and the expression of presumed ENV receptor TLR4 on the surface of OPCs. We were, however, not able to significantly interfere with receptor activation neither through antibody blocking nor through heat inactivation of ENV prior to use on OPCs. The failure to effectively block activation through use of antibodies and the observed slight increase of pro-inflammatory cytokines in antibody treated cells might be due to specific antibody properties as mentioned above (Fig. 3A,B). There are, however, other possibilities to interfere with TLR4-signaling which allow for a more precise observation of intracellular signaling pathways. As mentioned above intracellular signaling upon TLR4 receptor activation follows either the MyD88-dependent or -independent pathway or - in most cases - both pathways to a different degree (Akira, 2003; Takeda and Akira, 2004a). To determine which pathway is preferably activated upon stimulation with ENV it would be interesting to specifically block one pathway and assess then the regulation of pro-inflammatory genes. Taking previous studies into account especially the serine/threonine kinase IRAK-4 appears to be a promising candidate to assess the MyD88-dependent pathway. It has been shown that IRAK-4 knockout mice lack a normal response to TLR4-activator LPS with an impaired production of pro-inflammatory cytokines (Takeda and Akira, 2004b). Additionally, IRAK-4, which causes subsequent recruitment and activation of IRAK-1, is directly activated by MyD88 and therefore situated on top of the signaling cascade preventing the interference of other molecules in downstream signaling (Li et al., 2002b; Akira, 2003). In regard to the MyD88-independent pathway this central role is played by adaptor protein TRIF. TRIF is essential for the activation of transcription factor IRF3, which finally leads to the activation of NF-

κB and the induction of pro-inflammatory genes (Yamamoto et al., 2003; Covert et al., 2005). Blocking of TRIF could thus show to what extent the MyD88-independent pathway is involved in intracellular signaling following stimulation with ENV. Corresponding experiments using specific inhibiting agents directed against IRAK1, IRAK4 and TRIF have already been initiated by our research group and are subject of future studies and theses. Of note, if blocking of either pathway leads indeed to a significant reduction of pro-inflammatory cytokine production after exposition to ENV, these findings might - in the context of inflammatory diseases of the CNS - present a new therapeutic approach in order to promote endogenous remyelination processes.

Regarding the role of ENV as a putative inflammatory and nitrosative stress inducing agent in patients with MS, it is - as a next step - necessary to transfer these findings both to the human organism in general and the diseased organism of MS patients in particular. To do so will require a considerable amount of bioptical material of healthy probands in order to isolate human OPCs. Obviously, it is highly unlikely to find healthy probands willing to participate in brain biopsy in order to provide material for medical research. There are, however, alternatives to gain the required cells. As mentioned before one could purchase oligodendroglial precursor cells, which are acquired from aborted fetal tissue (see also Fig. 5). Another option is presented by bioptical material received from patients who have undergone brain biopsy for medical reasons. Eventually, this material would allow for experimental settings similar to those presented in this study in order to determine whether our observations concerning rat OPCs apply to human OPCs as well. Quantitative RT-PCR would provide the opportunity to screen both for pro-inflammatory genes as well as differentiation inhibitors upon stimulation with ENV. Bioptical material gathered from MS patient in different stages of disease progression could allow for an immunohistochemical evaluation. Through use of antibodies against TLR4 and ENV, respectively, one could not only show the expression of the presumed ENV receptor but also the spatial relations between expressed ENV and resident oligodendroglial precursor cells in MS lesions. To provide relevant data we investigated commercially available human oligodendroglial precursor cells derived from aborted fetal CNS tissue. Using these rare and precious cells we were able to confirm that cultured human OPCs indeed express both TLR4

and CD14 (Fig. 5A-B'). Furthermore gene expression analyses showed that stimulation with ENV can induce an upregulation of iNOS supporting the assumption that human OPCs react in a similar manner to ENV as primarily shown for rodent cells (Fig. 5C). Although these results are promising and encourage further investigation one has to be aware of several difficulties regarding acquisition and quality of material. Gaining sufficient amounts of material from healthy probands both for cell culture and as negative control for immunohistochemical techniques will prove difficult since there are strict indications for brain biopsy. However, this problem would be the least difficult to solve since there are various medical centers specializing in procedures such as surgical epilepsy treatment for instance, which could grant access to an abundance of tissue samples. There is, however, a relevant restriction one has to take into consideration. Most patients undergoing this procedures are likely to suffer from various neurological diseases which may present a relevant experimental confounder interacting with cell homeostasis and differentiation in unknown ways. Considering material gathered from MS patients yet another major problem presents itself. It is highly unlikely to find MS patients who have not undergone medical treatment. Most drugs used in MS therapy are potent agents interfering with cell/cell interactions in complex ways which could bias observations. Apart from interacting with TLR4 and its intracellular pathways to block ENV by using specific antibodies against ENV present a more direct approach. In this respect a monoclonal antibody (mAb), which shows an especially high affinity to ENV's extracellular domain has been successfully tested in a phase I study in healthy male volunteers (Curtin et al., 2012). The humanized antibody with the designation GNbAC1 is an IgG antibody comprised of a human framework and a murine antigen binding site selected for its high affinity to ENV. Curtin et al. were able to show that the presumed therapeutic dosage of GNbAC1 was well tolerated by the study participants while its pharmacokinetic properties allow for monthly administration. As a next step it will be necessary to determine whether GNbAC1 has any actual effect in patients (phase II study). Therefore the drug will be applied to MS patients and evaluated in regards to efficacy and side effects depending on dosage. Usually these studies are performed on groups consisting of several hundred people. If GNbAC1 can convince during these phase II studies its clinical value has to be

determined. In this regard, GNbAC1 will be compared to placebos, against which it should obviously demonstrate superiority and/or other already established drugs, against which it should at least prove equality. For this purpose, a large collective of several thousand patients has to be monitored for up to several years to gather data on side effects and long-term treatment. Generally, multiple independent phase III studies, which are usually designed as randomized clinical trials, have to confirm a drug's efficacy before it can be approved by the responsible regulatory agencies.

Over the last decades various studies examined the relation between autoimmune inflammatory processes occurring in diseases such as for example MS and disruption and consecutive breakdown of the blood-brain barrier (BBB) (Minagar and Alexander, 2003; Holman et al., 2011; Lassmann, 2011). In the healthy organism the BBB is a practically impermeable safeguard between blood circulation and CNS consisting of tight junctions, which are formed by cerebral endothelial cells on the one hand and selective transporter molecules and channels on the other hand (Abbott et al., 2010). It exists to maintain homeostasis in the brain and controls the exchange of substances between the extra- and intracerebral milieu. Inflammation, however, leads to release of cytokines, infiltration of leukocytes and eventually leakage and breakdown of the BBB (Minagar and Alexander, 2003; Holman et al., 2011). Now, several drugs used in the treatment of MS intervene at this point. Natalizumab, a humanized anti-α_4 integrin antibody, directly blocks the α_4 integrin mediated attachment of – in this case – T-cells to the BBB thus inhibiting leukocyte migration through the barrier (Coisne et al., 2009). Other drugs such as interferon β or glatiramer acetate act as immunomodulators through inhibition of proinflammatory and induction of anti-inflammatory cytokines (Burger et al., 2009; Dhib-Jalbut and Marks, 2009), although the exact underlying mechanisms still remain largely unclear. GNbAC1, however, is supposed to pass the due to inflammatory processes permeabilized BBB and neutralize the circulating ENV both in the blood as well as in active MS lesions. As shown in this study ENV activates TLR4 on OPCs thus leading to induction of both proinflammatory cytokines and iNOS on the one hand and myelin inhibiting genes on the other hand. This way, a situation promoting inflammation is maintained, while remyelination – and therefore clinical remission – is inhibited. Applying an

antibody against ENV during a relapse might limit the inflammatory response by blocking the induction and expression of proinflammatory cytokines in OPCs. As a consequence, the migration of immunocompetent cells such as leukocytes through the BBB would be prevented as well, which would inter alia limit the autoimmune triggered destruction of myelin. In the course of disease this would mean an earlier induction of remission and possibly less severe neurological deficits.

Furthermore, the neutralization of ENV in the blood through application of the antibody during phases of remission might facilitate remyelination. Without the inhibitory effect of ENV on myelin production, OPCs, which migrate into lesion areas, could more efficiently remyelinate axons, which have lost myelin sheaths during a relapse. Clinically, that would mean a chance at regredience of neurological deficits and subsequently a better disease outcome. GNbAC1 has yet to show its efficacy in clinical studies, but if it does, an interesting new approach to therapy of MS might be at hand.

4.3 Multiple sclerosis and viruses

Over the last years numerous studies have claimed a linkage between multiple sclerosis and different viruses. There is, for instance, evidence that respiratory infections act as a triggering event for exacerbations in MS patients. Indeed, several respiratory viruses such as respiratory syncytial virus (RSV), influenza A and B or adenovirus have been linked to MS in this context (Andersen et al., 1993; Panitch, 1994; Oikonen et al., 2011). There is strong evidence pointing towards a significant relation between an undergone respiratory infection and a via MRI verified exacerbation of MS (Edwards et al., 1998). So the idea of an exogenous factor influencing MS pathogenesis is not new, although the underlying mechanisms are yet unknown. Beside the influence of respiratory infections on MS an association with Epstein-Barr-Virus (EBV) has been long-since established (Ascherio and Munch, 2000; Haahr and Höllsberg, 2006). Ubiquitous EBV is - as part of the herpes-virus family - very well adapted to the human host. Infection may occur at a young age showing only mild symptoms or even an asymptomatic clinical course. Should the virus first be acquired during adolescence the infection may take the more severe course of manifested mononucleosis with fever, sore throat, splenomegaly and lymphadenopathy. Various studies found a significantly increased prevalence of EBV in the blood of MS patients compared to healthy controls corroborating the assumption of an association between the virus and the disease although the exact relation between EBV and pathogenesis of MS remains unclear. There is, however, evidence for an increase of EBV in the blood on the onset of clinical symptoms during a relapse suggesting a re-activation of the virus in direct relation to disease progression (Lindsey et al., 2009). Additionally, there is evidence that primary infection with EBV actually may increase the risk to develop MS as observed in a large prospective study among military staff (Levin et al., 2010). Taken together a linkage between EBV and MS appears to be very likely, even though it has yet to be seen if EBV simply increases the risk to develop MS in the first place or if infection might actually alter the MS course after disease onset. Similar conditions seem to apply to human herpes virus 6 (HHV-6). Infection with HHV-6, which has been initially isolated from PBMCs of AIDS patients, seems to be connected to various diseases including AIDS-associated encephalomyelitis or exanthema subitum in infants. Like EBV HHV-6

is ubiquitous with infection occurring during early childhood. Several studies found an increase of viral DNA both in the CSF as well as in the blood of MS patients consequently concluding an association between virus and disease (Soldan et al., 1997; Pietiläinen et al., 2010). Similar to EBV, it was, however, not possible to ascertain whether HHV-6 indeed is involved in MS etiology or if it is but another exacerbating agent (Swanborg et al., 2003). It has been postulated that high serum levels of anti-HHV-6 IgG concur with a significantly increased occurrence of relapses in RRMS, so that an affiliation between reactivation of virus and progression of disease seems probable (Behzad-Behbahani et al., 2011; Simpson et al., 2012). Interestingly, both EBV and HHV-6 are acquired during early childhood, which matches well with the theory of exposure to an exogenous pathogen during childhood as risk factor for developing MS (Kurtzke, 1993).

This study enqueues in a line of studies, which illuminate the effects of the MSRV envelope protein – in this case on OPCs. Therefore MSRV has to be mentioned in the context of viral agents in MS as well. As early as in the late 1980s a retroviral agent, which would later be denominated as MSRV, was isolated from leptomeningal cells in the CSF of MS patients (Perron et al., 1989). Sequencing of this new element finally related it to the family of human endogenous retroviruses (HERV) (Blond et al., 1999). Since then the subfamily of HERV-W and its founder member MSRV have been intensively investigated. As suggested by its name there is strong evidence linking MSRV to inflammatory reactions in general and to MS in particular (Garson et al., 1998; Perron et al., 2001, 2012; Rolland et al., 2005). It has been shown that the MSRV envelope protein induces pro-inflammatory responses in blood cells and promotes a Th1 immune response in dendritic cells (Rolland et al., 2006). In this study we were able to observe similar reactions in OPCs both *in vitro* and *in vivo*, a strong interference in OPC differentiation and subsequent remyelination thus further corroborating the idea of a significant connection between MSRV and MS.

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Eidesstattliche Versicherung

Ich versichere an Eides statt, dass die Dissertation selbstständig und ohne unzulässige fremde Hilfe erstellt worden ist und die hier vorgelegte Dissertation nicht von einer anderen Medizinischen Fakultät abgelehnt worden ist.

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