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TNF receptor 2 protects oligodendrocyte progenitor cells against oxidative stress

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ABSTRACT

The neuroprotective role of TNF receptor 2 (TNFR2) has been shown in various studies. However, a direct role of TNFR2 in oligodendrocyte function has not yet been demonstrated. Using primary oligodendrocytes of transgenic mice expressing human TNFR2, we show here that TNFR2 is primarily expressed on oligodendrocyte progenitor cells. Interestingly, preconditioning with a TNFR2 agonist protects these cells from oxidative stress, presumably by increasing the gene expression of distinct anti-apoptotic and detoxifying proteins, thereby providing a potential mechanism for the neuroprotective role of TNFR2 in oligodendrocyte progenitor cells.

1. Introduction

Multiple sclerosis (MS), the most common neurodegenerative disease of young adults, is characterized by demyelination in the central nervous system (CNS). Hallmark of MS is an autoimmune attack on oligodendrocytes (OLGs), the myelinating cells of the CNS, resulting in focal demyelinated lesions and ultimately in axonal degeneration and neuronal cell death. Virtually all components of the immune system have been implicated in this disease. In particular, tumor necrosis factor (TNF), a key regulator of the immune system [1], is elevated in MS lesions and disease severity has been correlated with elevated TNF levels [2,3]. Accordingly, anti-TNF treatment has been evaluated as a potential therapy in MS. However, clinical trials with TNF neutralizing reagents in MS patients failed to ameliorate the disease and even led to disease exacerbation [4].

In recent years a dual, context dependent role of TNF in the CNS has been revealed. In many cases, neuroprotective activity has been associated with TNF receptor (TNFR) 2, whereas TNFR1, directly and indirectly, promotes neurotoxicity [5,6]. TNFR2 was shown to protect neurons against toxic insults *in vitro* [7–9] and to promote neuronal survival and OLG regeneration after ischemic and neurotoxic insults, respectively, *in vivo* [10,11]. In contrast, TNFR1 exacerbates axonal and neuronal damage through its potent pro-inflammatory effects, which became particularly obvious during chronic inflammation [12]. Recently it has been shown that a dominant-negative TNF, which specifically inhibits soluble TNF and thereby predominantly TNFR1 [13], ameliorates neurological symptoms in experimental autoimmune encephalomyelitis (EAE), the animal model of MS [14,15].

In vitro TNF interferes with OLG differentiation and causes OLG cell death [16–19]. These detrimental effects of TNF have been attributed to TNFR1 [19–21]. In contrast, although TNFR2 is expressed in OLG [22,23], still little is known about the impact of TNFR2 activation on OLG function, survival or differentiation.

The functional role of TNFR2 was analyzed in OLGs from human TNFR2 (huTNFR2)-transgenic mice (tgE1335 [24]) using a membrane-mimetic TNF-mutein, which is specific for human TNFR2 [9]. We found that TNFR2 is predominantly expressed in OLG progenitor cells (OPCs) and that TNFR2 activation protects these cells from hydrogen peroxide induced oxidative stress.

2. Materials

A membrane-TNF mimetic TNFR2-specific mutein consisting of the trimerization domain of tenascin C (TNC) and a mutated single chain TNF specific for human TNFR2 (TNC-scTNF$_{R2}$) was produced in HEK293 cells as described [9]. Monoclonal antibodies against the epitopes O1 and O4 were produced in hybridoma cells. The antibody against human TNFR2 (HP9003) was from Hycult Biotech

Abbreviations: CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; OLG, oligodendrocyte; OPC, oligodendrocyte progenitor cell; PLL, poly-L-lysine; PLP, proteolipid protein; scTNF$_{R2}$, single chain TNF specific for TNFR2; SOD, superoxide dismutase; TNC, tenascin C; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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(Uden, The Netherlands). The antibody against myelin basic protein (MBP) was from QED Bioscience (San Diego, CA) and the antibodies against A_2B_5 and CNP were from Calbiochem (Darmstadt, Germany). Fluorescently labeled secondary antibodies were from Invitrogen (Karlsruhe, Germany). Apoptotic cells were detected with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit from Roche (Mannheim, Germany). DNase I and all components used for cultivating primary oligodendrocytes were from Sigma–Aldrich (Steinheim, Germany).

3. Methods

3.1. Isolation and cultivation of primary cells

Glial cells were isolated from forebrains of neonatal huTNFR2-transgenic tgE1335 mice expressing low to moderate levels of the transgene in brain tissues [24]. Forebrains of wild type littermates were used as controls. For genotyping of tgE1335 mice, tail ends were digested and analyzed by PCR (5 prime, Hamburg, Germany) for the presence of genomic huTNFR2 using specific primers (ThermoFisher, Schwerte, Germany; see Table 1). After removing the meninges, forebrains were cut into small pieces and further dissected by incubation in 0.05% trypsin–EDTA (Invitrogen), 0.1% DNase I in isolation buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25.5 mM $NaHCO_3$, 14 mM Glucose, 0.03% $MgSO_4$, 0.3% BSA) for 15 min at 37 °C. Trypsin was inactivated by addition of FCS (PAN Biotech, Aidenbach, Germany) and the tissue was homogenized mechanically with a pipette. Homogenates were plated on poly-D-lysine (PDL, 10 μg/ml; Sigma–Aldrich) coated cultures dishes at a density of 100,000 cells/cm^2 in DMEM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). OPCs formed small clusters, which can be collected by flushing them off with a pipette. After removing microglia by differential adhesion, OPCs were collected by centrifugation at 800 rpm for 10 min and cultivated on PLL-coated culture dishes at a density of 50,000 cells/cm^2. OPCs were cultivated in a modified SATO-medium ([25]; OPC-medium) consisting of (final concentrations): DMEM/F12 medium, glucose (10 mg/ml), insulin (5 μg/ml), human apo-transferrin (100 μg/ml), bovine serum albumin (100 μg/ml), progesterone (0.06 ng/ml), putrescine (16 μg/ml) and sodium selenite (40 ng/ml). To promote cell differentiation the thyroid hormones thyroxin (40 ng/ml) and tri-iodothyronine (30 ng/ml) were added (differentiation medium).

Table 1
Primers used in this study.

gDNA		
huTNFR2	fwd	CTCCTCCTCCAGCTGTAACG
	rev	CGTGGGCTCTCAGTAAAACC
cDNA		
PDGFRα	fwd	GGGGAGAGTGAAGTGAGCTG
	rev	CTCCCGTTATTGTGCAAGGT
CNP	fwd	GACAGCGTGGCGACTAGACT
	rev	CACCTGGAGGTCTCTTTCCA
MBP	fwd	TACCCTGGCTAAAGCAGAGC
	rev	GAGGTGGTGTTCGAGGTGTC
PLP	fwd	GCATCACCTATGCCCTGACT
	rev	TGCAGATGGACAGAAGGTTG
Bcl-2	fwd	CATGCTGGGGCCATATAGTT
	rev	AAGCTGTCACAGAGGGGCTA
BCL_{XL}	fwd	TGTTCCCGTAGAGATCCACA
	Rev	TGGTGGTCGACTTTCTCTCC
cIAP1	fwd	CTCCTGACCCTTCATCCGTA
	rev	TATGTCAGAGCACCGCAGAC
XIAP	fwd	GAACAGCATGCGAAGTGGTA
	rev	CGCCTTAGCTGCTCTTCAGT
SOD1	fwd	GCCAATGTGTCCATTGAAGA
	rev	GTTTACTGCGCAATCCCAAT
SOD2	fwd	CCGAGGAGAAGTACCACGAG
	rev	GCTTGATAGCCTCCAGCAAC
GAPDH	fwd	GTGGCAAAGTGGAGATTGTTG
	rev	GATGATGACCCGTTTGGCTCC

3.2. Fluorescence microscopy

OPCs were cultivated on PLL-coated 8-well permanox LabTek chamber slides (Nunc, Wiesbaden, Germany). For staining of surface markers, cells were fixed with 4% paraformaldehyde for 30 min and unspecific binding was blocked with 4% BSA for 30 min. Cells were incubated with primary antibodies for 60 min followed by incubation with fluorescently labeled secondary antibodies and DAPI for 45 min in 2% BSA and mounting with Fluoromount G (Southern Biotech, Birmingham, AL). For staining of cytoplasmic proteins, cells were permeabilized with 0.1% Triton X-100 for 10 min before blocking with 4% BSA and staining with primary and secondary antibodies. Cells were analyzed by wide field fluorescence microscopy (CellObserver, Carl Zeiss, Jena, Germany).

3.3. Treatment of cells with TNC-scTNF$_{R2}$ and H_2O_2

After cultivation in OPC-medium for 24 h, the OPCs were incubated for 1 h with TNC-scTNF$_{R2}$ followed by addition of H_2O_2 (Sigma–Aldrich). After 20 h the cells were fixed with 4% paraformaldehyde. To determine the amount of apoptotic OPCs, the cells were first incubated with antibodies against A_2B_5, followed by anti-mouse IgG coupled to Alexa546. Thereafter cells were permeabilized with 0.1% Triton X-100 and incubated with TUNEL labeling mixture containing UDP–FITC.

3.4. Gene expression studies

OPCs were cultivated on PLL-coated culture dishes (Greiner, Frickenhausen, Germany). RNA was isolated with the RNeasy Micro Kit (Qiagen, Hilden, Germany). Reverse transcriptase PCR (RT-PCR) was performed in the presence of oligo(dT)$_{18}$ primers and dNTPs with M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany) using standard procedures. The obtained cDNA was used to determine gene expression of OLG differentiation markers and anti-apoptotic proteins by quantitative real time PCR (CFX96, Biorad) using specific primers (ThermoFisher, Table 1) and the KAPA SYBR Fast Universal kit from Peqlab (Erlangen, Germany).

3.5. Statistics

To determine the amount of apoptotic cells the percentage of TUNEL-positive nuclei of cells with detectable A_2B_5-staining obtained under the different treatments was determined. Mean values of three experiments ± SD are shown. Statistical significance was determined by *t*-test.

The expression of distinct genes was quantified by quantitative PCR using the ΔΔCt method. The house-keeping gene control was *gapdh*. Mean values of two or three experiments ± SD are shown.

4. Results

4.1. Differentiation of primary mouse oligodendrocytes

To obtain primary OLGs we cultivated homogenates from forebrains of neonatal mice until clusters of small cells formed on top of the astrocyte layer (Fig. 1A). These cells could be easily flushed off the astrocyte layer resulting in a purified population of OPCs, as assessed by specific monoclonal antibodies, which bind to differentiation-regulated surface antigens of these cells, with very low astrocyte contamination (<4% of total cells)

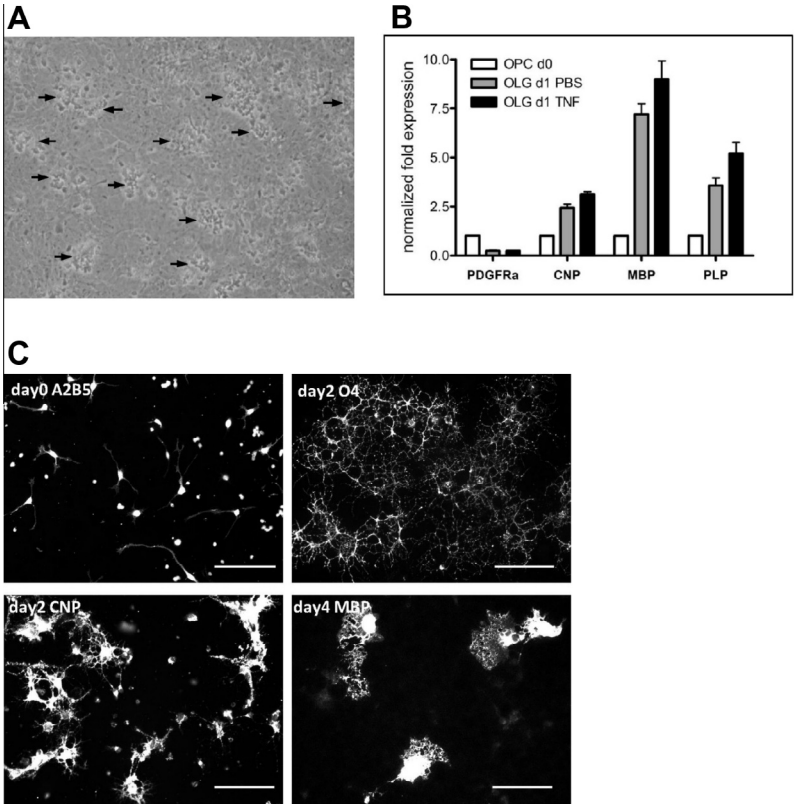

Fig. 1. Cultivation and differentiation of mouse oligodendrocytes. (A) Isolated brain cells were cultivated on PDL-coated culture dishes. After approximately 7 days clusters of OPCs (arrows) grow on top of the astrocyte monolayer. (B) OPCs were cultivated on PLL-coated culture dishes in OPC-medium (OPC, d0) before changing to OLG differentiation medium with or without TNC-scTNF$_{R2}$ for 24 h (OLG, d1). RNA was isolated, converted into cDNA and gene expression was determined by quantitative PCR ($n = 2$). (C) OPCs were cultivated on PLL-coated chamber slides in OPC-medium (d0) or OLG differentiation medium for the indicated time periods. Non-permeabilized cells were labeled with antibodies specific for the cell surface markers A_2B_5 or O4. For staining of the OLG proteins CNP and MBP cells were permeabilized prior to labeling with specific antibodies (scale bar 50 μm).

To analyze the potential of the obtained OPCs to develop into mature OLGs, the cells were cultivated in differentiation medium, i.e. serum free medium containing thyroid hormones. After 24 h of differentiation, gene expression of the OLG-specific proteins CNP, MBP and PLP was significantly increased (Fig. 1B). Concomitantly, expression of PDGFRα, which is predominantly expressed by OPCs, decreased, strongly suggesting the ability of the obtained cells to develop into mature OLGs.

For further verification, we determined expression and localization of typical OLG lipids and proteins as well as the morphological differentiation of the cells, i.e. the outgrowth of multiple cell processes and formation of myelin-like membrane sheets. As differentiation markers we used A_2B_5 (OPC), O4 (early immature OLG), CNP (late immature OLG) and MBP (mature, myelinating OLG).

When grown in OPC medium the cells were strongly stained for A_2B_5 (Fig. 1C) but negative for later markers of OLG differentiation indicating that they are indeed OPCs. After 1 day of differentiation branching of the cells increased and the cells were positive for A_2B_5 and O4 (data not shown). After 2 days of differentiation cells had formed a highly branched network of processes which were positive for O4 and CNP demonstrating differentiation into the OLG lineage (Fig. 1C). Importantly, after 4 days of differentiation the cells synthesized MBP and developed myelin-like membrane sheets indicative of morphological differentiation into mature OLGs (Fig. 1C).

4.2. Expression of huTNFR2 in oligodendrocytes

To analyze the effect of TNFR2 activation on OLG function we made use of huTNFR2-transgenic mice (tgE1335 mice; [24]), since available TNFR2 selective agonists are specific for huTNFR2. No differences in the differentiation of OPCs obtained from transgenic and wild type mice were observed (data not shown).

We first determined at which developmental stage(s) huTNFR2 is detectable at the plasma membrane of OLGs. Surface labeling with an antibody specific for the ectodomain of huTNFR2 revealed that this receptor is localized at the cell surface of A_2B_5-positive OPCs (Fig. 2). Staining for cell surface huTNFR2 declined to barely detectable levels after differentiation had been induced. OPCs from wild type littermates served as negative controls. Subsequent studies on potential impact of TNFR2 activation on OLG functions were therefore performed with undifferentiated OPCs from TNFR2 transgenic mice.

4.3. Physiological effects of TNFR2 activation on oligodendrocyte progenitor cells

To study the role of TNFR2 activation OPCs were treated with the membrane-TNF mimetic TNC-scTNF$_{R2}$, a specific huTNFR2 agonist [9,26]. Incubation with 100 ng/ml TNC-scTNF$_{R2}$ during differentiation resulted in a trend towards increased myelin gene expression (Fig. 1A) indicating that TNFR2 stimulation may promote differentiation.

We then determined whether TNFR2 stimulation can protect OPCs from superoxide induced stress. OPCs were incubated with 100 ng/ml TNC-scTNF$_{R2}$ for 1 h before addition of 5 μM hydrogen peroxide (H_2O_2), a concentration, which caused cell death of the majority of cells within a subsequent 20 h culture. Cells were fixed after 20 h and the amount of apoptotic cells was determined by TUNEL. To restrict analysis to OPCs, we correlated TUNEL-positive cells with recognizable A_2B_5 staining (Fig. 3A). Whereas 5 μM H_2O_2

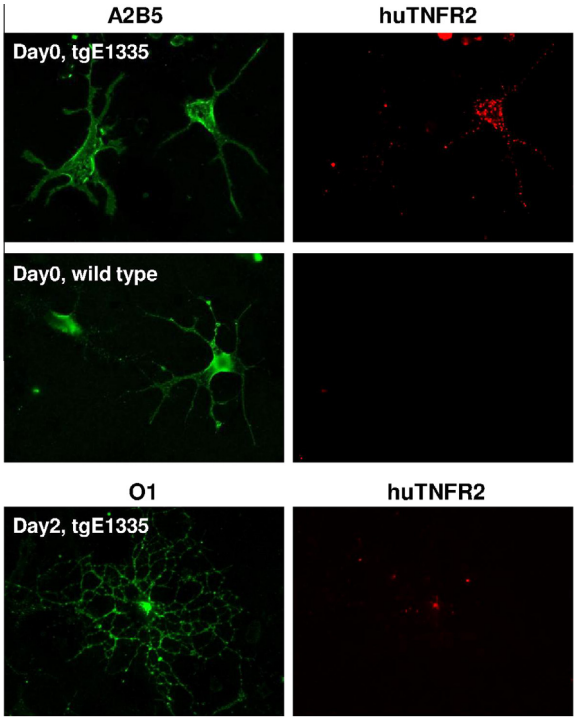

Fig. 2. Human TNFR2 is expressed in OPCs from tgE1335 mice. OPCs from tgE1335 or wild type mice were cultivated on PLL-coated chamber slides. Cell surface was labeled with antibodies specific for A_2B_5 and huTNFR2. To analyze huTNFR2 expression in differentiating OLGs, cells were cultivated for 2 days in differentiation medium and huTNFR2 surface localization was determined in O1-positive cells.

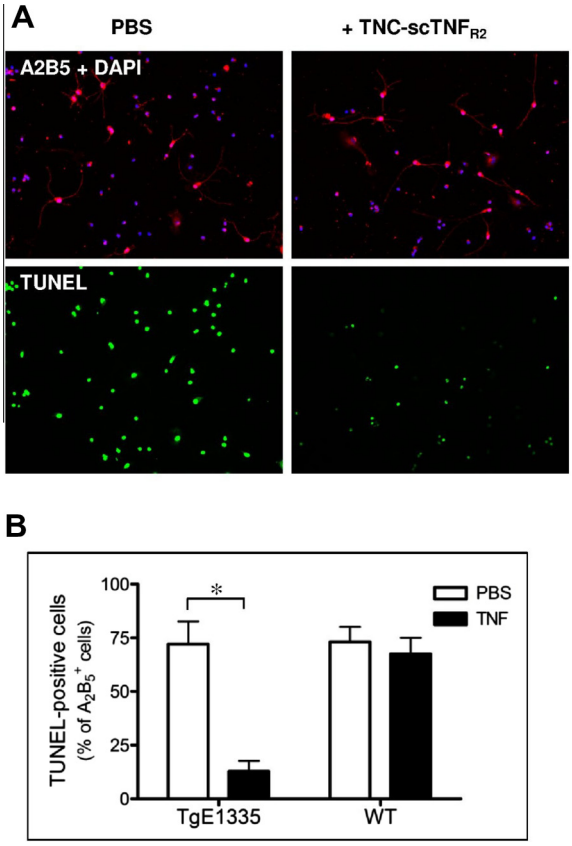

Fig. 3. TNFR2 stimulation protects OPCs from H_2O_2-induced apoptosis. OPCs from tgE1335 or wild type littermates were cultivated on PLL-coated chamber slides. (A) Cells were incubated with or without 100 ng/ml TNC-scTNF$_{R2}$ for 1 h before addition of 5 μM H_2O_2 for 20 h. Cells were fixed and stained with antibodies against A_2B_5 and anti-mouse Alexa-546, followed by permeabilization and staining of apoptotic cells by TUNEL using FITC-labeled UTP. (B) TUNEL-positive nuclei of A_2B_5-positive cells were counted and correlated to the total number of A_2B_5-positive cells ($n = 3$; $^*p < 0.5$).

was sufficient to drive approximately 70% of the OPCs into apoptosis within 20 h, specific activation of TNFR2 1 h before exposure to oxidative stress resulted in survival of almost all cells demonstrating that TNFR2 has a protective effect on OPCs (Fig. 3B). As expected from the strict species specificity of TNC-scTNF$_{R2}$, no protection of wild type OPCs was observed upon H_2O_2 exposure (Fig. 3B).

4.4. TNFR2 activation induced the expression of anti-apoptotic genes

To assess how TNFR2 activation promotes OPC survival we determined the expression of anti-apoptotic genes in OPCs treated for 2 h or 6 h with TNC-scTNF$_{R2}$. We chose members of the IAP-family, namely cIAP1 and XIAP, the BCL-2 family members BCL-2 and BCL$_{XL}$ and the superoxide dismutases (SOD) copper/zinc SOD (SOD1) and manganese SOD (SOD2).

We could not detect a specific signal for BCL$_{XL}$. All other analyzed genes were expressed in OPCs. Treatment for 2 h with TNC-scTNF$_{R2}$ was not sufficient to increase gene expression (data not shown). After TNC-scTNF$_{R2}$ treatment for 6 h, a significant increase in the expression of BCL-2 and SOD2 was observed, whereas the other genes were not significantly upregulated (Fig. 4).

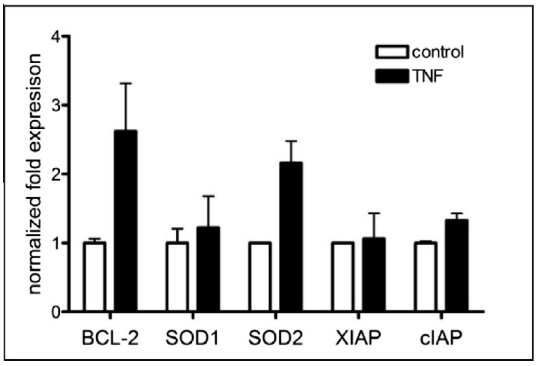

Fig. 4. TNFR2 stimulation induces the gene expression of anti-apoptotic proteins. OPCs were cultivated with or without 100 ng/ml TNC-scTNF$_{R2}$ for 6 h. RNA was isolated, converted into cDNA and gene expression determined by qPCR ($n = 3$).

5. Discussion

In this study we show that TNFR2 is predominantly expressed on primary OPCs compared to differentiated OLGs and that TNFR2 directly protects OPCs from oxidative stress. TNFR2 mediated protection is accompanied by upregulation of anti-apoptotic genes.

We also describe a simple method to isolate and cultivate OLGs from forebrains of newborn mice. Compared with the shake-off method generally used to isolate OPCs from mixed brain cultures from rat forebrains (20 h at 240 rpm; [27]), the method is less time consuming and may be less stressful for the cells. However, the low amount of isolated OPCs precludes extensive studies on protein biochemistry. In general, the differentiation of the cells is similar to rat OPCs as shown by the sequential expression and localization of myelin proteins and lipids as well as the outgrowth of multiple processes and formation of myelin-like membrane sheets.

To determine the effect of TNFR2 activation on OPC physiology we focused on the protection against oxidative stress since reactive

oxygen species play a major role in neurodegenerative diseases [28] and several studies have implicated TNFR2 in cell survival. The protective role of TNFR2 has been shown *in vivo* in disease models of neurodegeneration and myocardial infarction, in which TNFR2 KO-mice have either a more severe phenotype or a reduced regenerative capability compared to wild type mice [10,11,29]. We have recently demonstrated that TNFR2 stimulation can rescue cultivated neurons from cell death induced by oxidative stress [9]. These data and the TNFR2-dependent protection of OPCs in a model of superoxide induced cell death shown here, indicate that TNFR2 signaling is important for neuroprotection. Lack of such a protective mechanism may have contributed to the delayed and reduced remyelination observed in cuprizone-treated TNFR2 KO-mice [11]. In this study, absence of TNFR2 caused a reduced amount of proliferating OPCs compared to wild type mice. Using BrdU-incorporation or staining for Ki-67 we could not observe increased OPC proliferation upon TNFR2-activation (data not shown). One explanation may be that the effect of TNFR2 after cuprizone treatment is due to increased survival of proliferating OPCs. Alternatively, TNFR2 may promote OPC proliferation indirectly, e.g. by microglia, which increase the expression of anti-inflammatory and neuroprotective proteins upon TNFR2 stimulation [30].

TNFR2 stimulation increased the expression of anti-apoptotic and detoxifying proteins, namely BCL-2 and SOD2. Both proteins can protect against apoptosis induced by oxidative stress. SOD2 inhibits apoptosis by stabilization of the mitochondrial membrane [31] whereas BCL-2 inhibits the pro-apoptotic proteins Bax and Bak, mediators of the intrinsic apoptotic pathway [32]. In OPCs, downregulation of BCL-2 has been correlated to increased apoptosis [33] whereas upregulation of SOD2 promotes OPC survival [34].

Mechanistically, TNFR2 could enhance the expression of BCL-2 and SOD2 via two signaling pathways, the PI3-kinase-Akt pathway and the NFkB pathway [7–9,35]. Both pathways have been implicated in OPC survival [36–38] and have been linked to TNFR2 mediated protection of neuronal cells [7]. In conclusion, our results indicate that TNFR2 can protect OPCs from oxidative stress *in vitro* by expression of anti-apoptotic proteins and further underscore the potential of TNFR2 in neuroregenerative processes *in vivo*.

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