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# TNF receptor 2 protects oligodendrocyte progenitor cells against oxidative stress

Olaf Maier [a,*], Roman Fischer [a], Cristina Agresti [b], Klaus Pfizenmaier [a]

[a] Institute of Cell Biology and Immunology, Stuttgart University, Allmandring 31, 70569 Stuttgart, Germany
[b] Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

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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.

* Corresponding author. Fax: +49 711 685 67484.
*E-mail address:* olaf.maier@izi.uni-stuttgart.de (O. Maier).

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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)