

# Essential protective role of tumor necrosis factor receptor 2 in neurodegeneration

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Despite the recognized role of tumor necrosis factor (TNF) in inflammation and neuronal degeneration, anti-TNF therapeutics failed to treat neurodegenerative diseases. Animal disease models had revealed the antithetic effects of the two TNF receptors (TNFR) in the central nervous system, whereby TNFR1 has been associated with inflammatory degeneration and TNFR2 with neuroprotection. We here show the therapeutic potential of selective inhibition of TNFR1 and activation of TNFR2 by ATROSAB, a TNFR1-selective antagonistic antibody, and EHD2-scTNFR<sub>2</sub>, an agonistic TNFR2-selective TNF, respectively, in a mouse model of NMDA-induced acute neurodegeneration. Coadministration of either ATROSAB or EHD2-scTNFR<sub>2</sub> into the magnocellular nucleus basalis significantly protected cholinergic neurons and their cortical projections against cell death, and reverted the neurodegeneration-associated memory impairment in a passive avoidance paradigm. Simultaneous blocking of TNFR1 and TNFR2 signaling, however, abrogated the therapeutic effect. Our results uncover an essential role of TNFR2 in neuroprotection. Accordingly, the therapeutic activity of ATROSAB is mediated by shifting the balance of the antithetic activity of endogenous TNF toward TNFR2, which appears essential for neuroprotection. Our data also explain earlier results showing that complete blocking of TNF activity by anti-TNF drugs was detrimental rather than protective and argue for the use of next-generation TNFR-selective TNF therapeutics as an effective approach in treating neurodegenerative diseases.

TNF | TNFR1 | TNFR2 | neuroprotection | neurodegeneration

Tumor necrosis factor (TNF) is a master proinflammatory cytokine that plays an important role in the initiation and orchestration of immunity and inflammation (1, 2). Elevated TNF levels have been associated with different autoimmune diseases, and deregulation of TNF expression and signaling can lead to chronic inflammation and tissue damage (3–6). Therefore, several anti-TNF therapeutics are clinically approved and successfully used to treat autoimmune diseases, such as rheumatoid arthritis, psoriasis, or inflammatory bowel disease. Up-regulated TNF expression has also been associated with different neurodegenerative diseases, e.g., Alzheimer's disease, Parkinson's disease, stroke, and multiple sclerosis (MS) (7). However, an anti-TNF therapeutic failed in a phase II randomized, multicenter, placebo-controlled study for the treatment of relapsing remitting MS (Lenercept study) because symptoms of Lenercept-treated patients were significantly increased compared with patients receiving placebo, and neurologic deficits tended to be more severe in the Lenercept treatment groups (8). Next to induction or aggravation of demyelinating diseases, all anti-TNF therapeutics may induce severe side effects such as serious infections, including reactivation of tuberculosis and invasive fungal and other opportunistic infections. An increased susceptibility to develop additional autoimmune diseases and lymphomas has also been reported (3).

The negative results of the Lenercept study and the observed severe side effects of anti-TNF therapeutics in approved indications

might be explained by the pleiotropic actions of TNF, including both pro- and antiinflammatory functions and other immune regulatory as well as regenerative activities. Blocking all effects of TNF therefore might be counterproductive. Because most of the proinflammatory actions of TNF are mediated by TNFR1, a more effective therapeutic approach could be the selective blocking of TNFR1 signaling; this would spare TNFR2 signaling, which has been implicated in various protective and regenerative responses, particularly in the central nervous system: TNFR2 signaling was shown to promote neuronal survival and oligodendrocyte regeneration in vivo models of ischemic and neurotoxic insults (9, 10), respectively. Specific activation of TNFR2 rescues neurons (11) and oligodendrocytes (12) from oxidative stress and promotes oligodendrocyte differentiation and myelination (13, 14). In addition, TNFR2 signaling protects neurons from glutamate-induced excitotoxicity in vitro (15, 16).

Glutamate, a key neurotransmitter, can interact with the ionotropic AMPA and NMDA receptors. Exacerbated activation of

## Significance

TNF is known to play an important role in various neurodegenerative diseases. However, anti-TNF therapeutics failed in clinical trials of neurodegenerative diseases. This failure is most likely due to antithetic effects of the TNF receptors in the central nervous system, whereby TNFR1 promotes inflammatory degeneration and TNFR2 neuroprotection. Here we show that novel TNFR-selective therapeutics, i.e., a TNFR1 antagonist and a TNFR2 agonist, block neuroinflammation and promote neuronal survival in a mouse model of neurodegeneration related to Alzheimer disease as well as other neurodegenerative diseases. Most important, neuroprotection mediated by the TNFR1 antagonist is abrogated by simultaneous blockade of TNFR2 activation, revealing that neuroprotection requires TNFR2 signaling and uncover why anti-TNF drugs failed in treatment of neurodegenerative diseases.

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Conflict of interest statement: A.H., K.P., and R.E.K. are named inventors of patents covering the ATROSAB technology. A.H. is owner of Baliopharm, which is developing ATROSAB for clinical use. E.G. is an employee of Baliopharm. R.E.K. is a named inventor covering the EHD2 technology.

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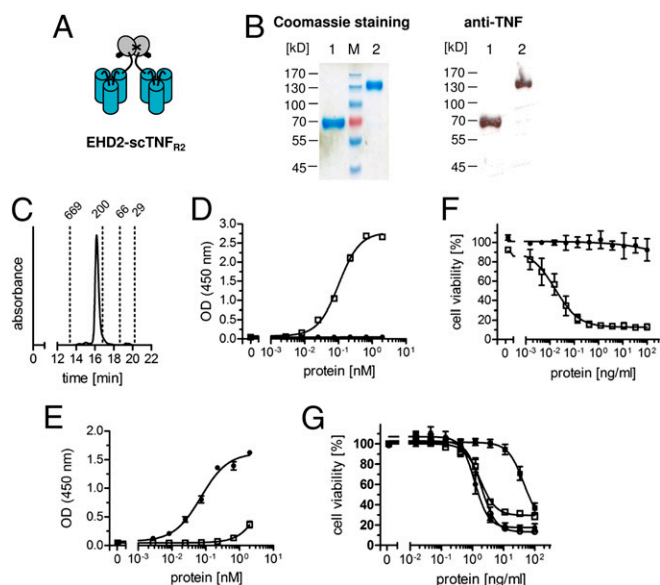
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**Fig. 1.** Genetic engineering and bioactivity of EHD2-scTNFR<sub>2</sub>. (A) Schematic representation of EHD2-scTNFR<sub>2</sub>. (B) Coomassie staining and immunoblot of EHD2-scTNFR<sub>2</sub> under reducing (column 1) and nonreducing (column 2) conditions. (C) HPLC-SEC analysis of EHD2-scTNFR<sub>2</sub> using a BioSep-Sec-2000 column. Peak positions of relevant standard proteins are indicated. (D and E) Binding of soluble huTNF (□) and membrane-TNF mimetic EHD2-scTNFR<sub>2</sub> (●) to huTNFR1 (huTNFR1-Fc) or huTNFR2 (Etanercept; huTNFR2-Fc) was determined by ELISA ( $n = 3$ ;  $\pm$ SEM). (F) L929 cells were cultivated with titrations of recombinant human TNF (huTNF) or EHD2-scTNFR<sub>2</sub>. Cell viability was determined by crystal violet staining ( $n = 4$ ; mean  $\pm$  SEM). (G) Kym-1 cells were stimulated with (open symbols) or without (closed symbol) the TNFR2 cross-linking mAb 80M2 for 30 min at 37 °C. Then titrations of scTNFR<sub>2</sub> (squares) or EHD2-scTNFR<sub>2</sub> (circles) were added and cells were incubated for 24 h. Cell viability was determined by crystal violet staining ( $n = 4-6$ ; mean  $\pm$  SEM).

these glutamate receptors may lead to progressive neuronal cell death, which is a hallmark of acute and chronic neurological diseases (17). Consequently, NMDA receptor antagonists such as dizocilpine (MK-801) or memantine were developed and revealed neuroprotective effects against ischemia-induced neuronal death in vitro and ischemic brain damage in vivo (18, 19). However, inhibitors of NMDA receptors largely failed in clinical studies (20).

Because TNFR2 signaling protects neurons from glutamate-induced excitatory cell death in vitro, ligands promoting TNFR2 signaling might be superior to NMDA antagonists because they do not completely inhibit glutamate-induced signal transmission, but buffer excitotoxicity likely by acquisition of a resistant state of affected cells. Because of the antithetic action of TNF via its two receptors, we reasoned that both an inhibition of TNFR1 signaling and a selective activation of TNFR2 signaling, respectively, could shift the balance of endogenous TNF activity toward an overall neuroprotective/regenerative response. We here report on the therapeutic activity of the human TNFR1-selective antagonist ATROSAB (21, 22) and a human TNFR2 selective agonist (EHD2-scTNFR<sub>2</sub>) in in vitro and in vivo models of excitotoxic brain damage (23, 24) using humanized TNFR1 and TNFR2 knock-in mice.

## Results

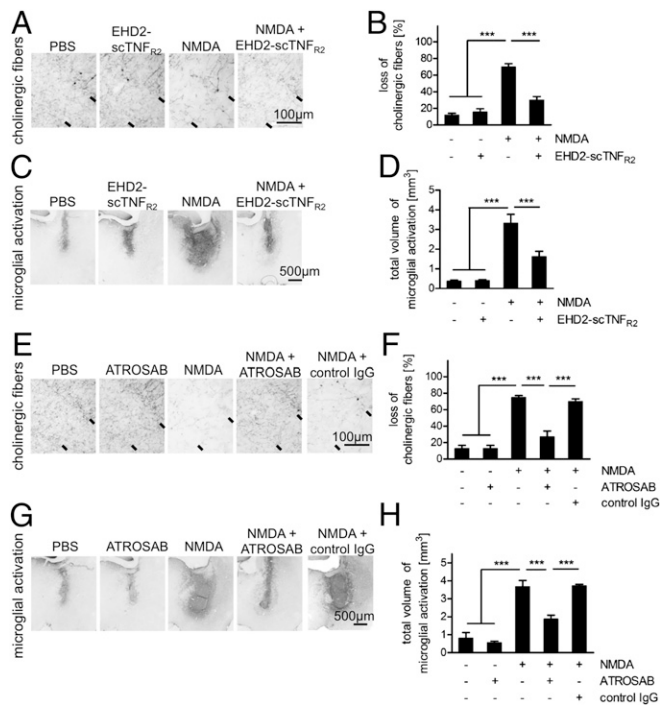
**Generation and Characterization of a TNFR2 Selective Agonist.** Efficient activation of TNFR2 requires receptor oligomerization by the membrane form of TNF (tmTNF) or oligomerized soluble forms of tmTNF mimicking receptor activation via tmTNF (11, 25) such as a TNFR2-selective TNF mutein, fused to the trimerization domain tenascin C (TNC-scTNFR<sub>2</sub>) recently developed by the authors (11). We here describe a TNFR2-selective TNF mutein, which consists of a covalently stabilized human TNFR2-selective (D143N/

A145R) (25) single-chain TNF (scTNFR<sub>2</sub>) fused to the dimerization domain EHD2 derived from the heavy chain domain CH2 of IgE (26), constituting a disulfide bonded dimer that is, with respect to TNF domains, hexameric (EHD2-scTNFR<sub>2</sub>; Fig. 1A). The purity of the recombinant protein was confirmed by SDS/PAGE and Coomassie staining (Fig. 1B). Under reducing conditions, the TNF variant exhibited an apparent molecular mass of  $\sim$ 70 kDa, matching the calculated molecular mass of 68.35 kDa. Under nonreducing conditions the expected dimer of  $\sim$ 130 kDa was observed (Fig. 1B). The oligomerization state of EHD2-scTNFR<sub>2</sub> was further characterized by size exclusion chromatography (SEC; Fig. 1C). EHD2-scTNFR<sub>2</sub> eluted as a single major peak, indicating high purity.

TNF receptor selectivity of EHD2-scTNFR<sub>2</sub> was analyzed by binding studies with immobilized huTNFR1-Fc and huTNFR2-Fc fusion proteins. Whereas EHD2-scTNFR<sub>2</sub> did not interact with huTNFR1, the fusion protein efficiently bound to huTNFR2 (Fig. 1D). In contrast, soluble human TNF (huTNF) efficiently bound to huTNFR1, whereas it just weakly interacted with huTNFR2 (Fig. 1D). Furthermore, EHD2-scTNFR<sub>2</sub> did not activate TNFR1-dependent cell death in L929 (Fig. 1E), verifying that EHD2-scTNFR<sub>2</sub> had lost affinity for TNFR1 due to the mutations D143N/A145R. In contrast, EHD2-scTNFR<sub>2</sub> efficiently induced cell death in Kym-1 cells, which endogenously express both TNF receptors and are highly sensitive to endogenous TNF-induced TNFR1 mediated cytotoxicity (27) (Fig. 1G). Interestingly, induction of cell death was not enhanced by addition of the cross-linking TNFR2-specific monoclonal antibody 80M2, which in concert with soluble TNF is able to mimic the action of tmTNF (11, 28). Differently, the bioactivity of scTNFR<sub>2</sub>, a covalently stabilized huTNFR2-selective TNF trimer, was strongly enhanced in the presence of 80M2 (Fig. 1G), indicating that dimerization of scTNFR<sub>2</sub> via EHD2 is necessary and sufficient to fully activate huTNFR2.

**ATROSAB and EHD2-scTNFR<sub>2</sub> Are Neuroprotective in Vivo.** To assess both EHD2-scTNFR<sub>2</sub> and ATROSAB in an in vivo model of neurodegeneration, hu/mTNFR-knock-in mice (Figs. S1–S4) were generated and used in the nucleus basalis lesion model. The hu/mTNFR knock-in (k/i) mice express a chimeric TNFR, where the extracellular part of the human receptor is fused to the transmembrane- and intracellular region of the mouse TNFR. This chimeric hu/mTNFR was introduced into the germline of C57BL/6 mice by knock-in technology, replacing the endogenous mouse TNFR, thereby using the regulatory elements from the wild-type mouse TNFR; this should lead to an expression pattern of the hu/mTNFR comparable to the wild-type mouse TNFR. The expression and functionality of the chimeric hu/mTNFR in hu/mTNFR-k/i mice was investigated using primary cells isolated from different tissues and in vivo experiments. Using primary immune cells, neurons, and mouse embryonic fibroblasts (MEFs), we demonstrated that expression of chimeric hu/mTNFR1 and hu/mTNFR2 resemble the expression pattern of the wild-type TNFRs (Figs. S5 and S6). Furthermore, we demonstrated the functionality of the chimeric hu/mTNFRs both using in vitro experiments with primary MEFs and thymocytes as well as in vivo experiments showing TNF sensitivity of hu/mTNFR1-k/i mice (Fig. S7).

By stereotactic NMDA injection, lesions in the nucleus basalis magnocellularis (NBM) were generated. Lesioning of the NBM resulted in a decrease of body weight (Fig. S8), reduction of cholinergic fibers of the parietal cortex (Fig. 2B and F), and macrophage/microglial activation within the NBM (Fig. 2D and H and Fig. S9). These symptoms can be assessed to determine the lesion size. Both EHD2-scTNFR<sub>2</sub> and ATROSAB show a strong protective effect in this in vivo model when simultaneously injected with NMDA into the NBM (Fig. 2B, D, F, and H and Fig. S9). Injection of a control IgG (anti-huEGFR), however, did not significantly alter NMDA-mediated neurodegeneration (Fig. 2F and H).



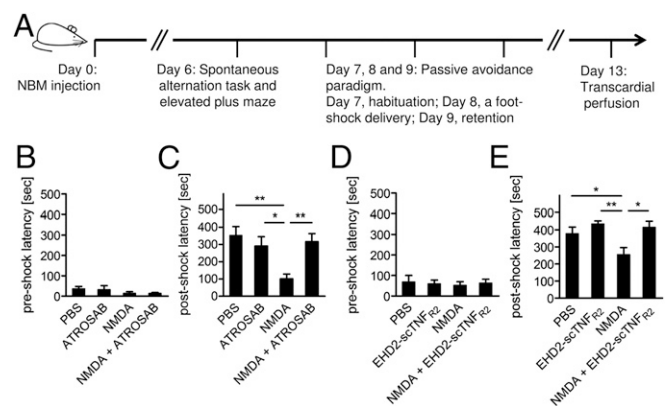
**Fig. 2.** EHD2-scTNFR<sub>2</sub> and ATROSAB prevent NMDA-induced NBM lesion and macrophage/microglia activation. (A–D) hu/mTNFR2-k/i and (E–H) hu/mTNFR1-k/i mice were used. (A and E) Representative images show choline acetyltransferase (ChAT)-positive cholinergic innervations in the somatosensory cortex. NMDA injected into the NBM induced an extensive cholinergic fiber loss in the layer V of somatosensory cortex compared with the control group. However, EHD2-scTNFR<sub>2</sub> or ATROSAB treatment attenuated fiber loss. Parallel bars indicate the layer V of the somatosensory cortex in which quantitative measurements were performed. (B and F) Quantification of cholinergic fiber density in layer V of the somatosensory cortex. Fiber density was measured in eight sections per mouse,  $n = 7$  mice per group. All data in bar charts represent means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ , one-way ANOVA with post hoc comparisons Tukey. (C and G) Representative images show CD11B-positive activated macrophage/microglia in magnocellular nucleus basalis. NMDA injected into the NBM induced a massive volume of macrophage/microglial activation, compared with those in both control groups. However, EHD2-scTNFR<sub>2</sub> or ATROSAB treatment significantly reduced macrophage/microglial activation induced by NMDA. (D and H) Quantification of total extent of activated macrophage/microglia around the injections. Macrophage/microglial activation was measured in a series of sections with macrophage/microglial activation,  $n = 7$  mice per group. All data in bar charts represent means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ , one-way ANOVA with post hoc comparisons Tukey.

**ATROSAB and EHD2-scTNFR<sub>2</sub> Improve Memory Performance in the NBM Lesion Model.** Memory was evaluated as parameter for the protective effects of EHD2-scTNFR<sub>2</sub> in the hu/mTNFR2-k/i mice and ATROSAB in the hu/mTNFR1-k/i mice against NMDA-induced neurotoxicity in the nucleus basalis and subsequent cholinergic fiber loss in parietal cortex. The sequence of memory tests performed in hu/mTNFR2-k/i and hu/mTNFR1-k/i mice are depicted in Fig. 3A and in Figs. S10 and S11. The NMDA-induced NBM lesion and the compounds tested had no significant effect on short-term memory performance in the spontaneous alternation task (Figs. S10B and S11B) or number of entries recorded (Figs. S10C and S11C). Anxiety-like behavior assessed by the elevated plus maze showed no significant changes in the total number of entries (Figs. S10D and S11D), entries into the open arms (Figs. S10E and S11E), and time spent in the dark and light arms and center of the maze (Figs. S10F and S11F) in all of the mice tested. Results from the passive avoidance paradigm showed that NMDA, EHD2-scTNFR<sub>2</sub>, and ATROSAB did not result in differences of the pre-shock latency to enter the

dark compartment in the hu/mTNFR2-k/i mice (Fig. 3B) and hu/mTNFR1-k/i mice (Fig. 3D). However, NBM injection of NMDA caused a significant impairment in the postshock latency in all of the mice tested (Fig. 3C and E). Both EHD2-scTNFR<sub>2</sub> and ATROSAB obliterated NMDA-mediated memory impairment (Fig. 3C and E). EHD2-scTNFR<sub>2</sub> and ATROSAB when given without NMDA had no effect on postshock latency (Fig. 3C and E). These results indicate the lack of neurotoxicity of EHD2-scTNFR<sub>2</sub> and ATROSAB and accentuate their protective effects against NMDA toxicity.

**Emergence of Neuroprotective TNFR2 Signaling in Vitro and in Vivo upon ATROSAB-Mediated Inhibition of TNFR1.** Previously we have shown that the PI3K-PKB/Akt pathway mediates TNFR2-promoted neuroprotection from excitotoxic cell death (10, 15, 16). To investigate the molecular pathways underlying the neuroprotective effects of ATROSAB, we isolated primary neurons from hu/mTNFR1-k/i mice and investigated TNF-induced phosphorylation of PKB/Akt (Ser473). As expected, stimulation with a nonreceptor-selective wild-type mouse variant of EHD2-scTNFR<sub>2</sub>, with tmTNF-mimetic activity (EHD2-sc-mTNF), induced phosphorylation of PKB/Akt. Interestingly, phosphorylation was enhanced in the presence of the TNFR1 antagonist ATROSAB (Fig. 4A), suggesting that PKB/Akt activation occurs via TNFR2, and concomitant TNFR1 signaling interferes with this pathway. In addition, TNF was shown to protect primary neurons from glutamate-induced cell death in a TNFR2-dependent manner (15). Similarly, we could show that the mouse TNFR2-specific variant (D135N/A137R; 13) of the human TNFR2-selective EHD2-scTNFR<sub>2</sub> (EHD2-sc-mTNFR<sub>2</sub>) protects primary hu/mTNFR1-transgenic neurons from excitotoxic cell death (Fig. 4B). In accordance with ATROSAB-mediated increase of PKB/Akt phosphorylation, ATROSAB enhanced the neuroprotective effect of EHD-sc-mTNF at lower concentrations (10 ng/mL; Fig. 4B) in this in vitro cell model, too.

To prove the essential role of TNFR2 signaling in the in vivo NBM lesion model, we simultaneously injected NMDA, ATROSAB, and the mouse TNFR2-specific antagonist MAB426 (Fig. 4C–F) into hu/mTNFR1-k/i mice. Under these conditions, the protective effect of ATROSAB on both cholinergic innervation and macrophage/microglial activation was completely reverted.



**Fig. 3.** EHD2-scTNFR<sub>2</sub> and ATROSAB attenuate NMDA-induced memory impairment. (A) A graphical description for the sequence of behavioral and memory assessments in hu/mTNFR1-k/i (B and C) and hu/mTNFR2-k/i (D and E) mice. Assessment of long-term memory with the passive avoidance paradigm showed no significant differences in (B and D) pre-shock latency between the experimental groups. NBM injection of NMDA caused a significant impairment of long-term memory, measured as postshock latency, which was obliterated by cotreatment of (C) EHD2 B and C scTNFR<sub>2</sub> or (D) ATROSAB. Error bars indicate  $\pm$ SEM. \* $P < 0.05$ , \*\*\* $P < 0.01$ , one-way ANOVA with post hoc comparisons Tukey.

## Discussion

TNF can exert opposite effects via its two receptors, TNFR1 and TNFR2. This dual role of TNF signaling becomes particularly obvious in the central nervous system (5, 6). Using the cuprizone-induced mouse model of demyelination, it was shown that TNFR2 is critical for oligodendrocyte regeneration, whereas TNF signaling via TNFR1 promoted nerve demyelination (9). Similar, investigations in a mouse model of retinal ischemia revealed that TNFR1-deficient animals were protected from ischemic lesions. In contrast, TNFR2<sup>-/-</sup> mice showed enhanced neuronal loss and a more severe pathology compared with wild-type animals, indicating an antagonistic function of the two TNFRs. In the retinal ischemia model, TNFR2's neuroprotective activity in vivo was associated with PI3K-PKB/Akt activation, which was counterbalanced by the neurodegenerative action of TNFR1 (10). The same principle applied to cortical neurons, where TNFR2 mediated in vitro protection from glutamate-induced cell death in a PI3K-PKB/Akt-dependent manner (15, 16). We here show in an in vivo model of NMDA-induced cellular degeneration and loss of neuronal functions that both a TNFR2 selective agonist (EHD2-scTNFR<sub>2</sub>) and a TNFR1 antagonistic antibody (ATROSAB) protect from loss of cholinergic fibers and associated neurologic deficits.

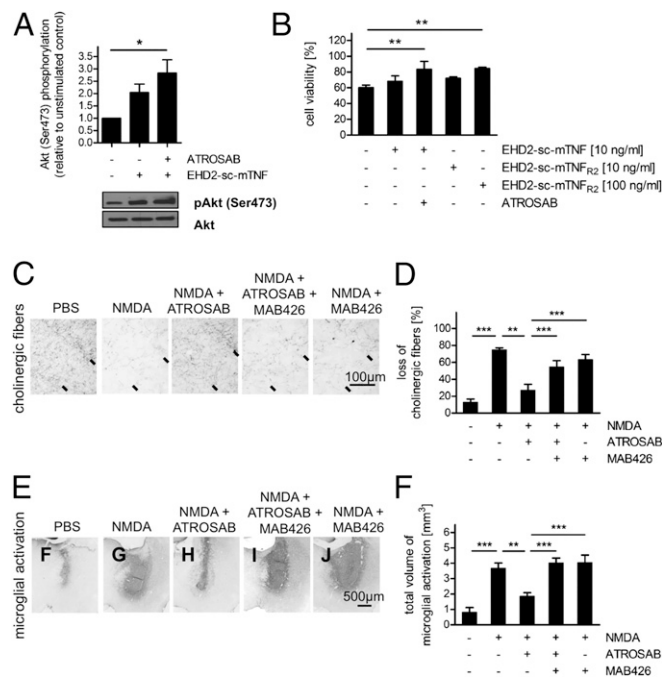
Exacerbated activation of glutamate receptor-coupled calcium channels and subsequent increase in intracellular calcium concentrations ( $[Ca^{2+}]_i$ ), followed by sustained disturbances in the  $[Ca^{2+}]_i$  homeostasis, are established hallmarks of neuronal cell death in acute and chronic neurological diseases (29, 30). Interestingly, activation of potassium intermediate/small conductance calcium-activated channel  $K_{Ca2}$  prevented  $[Ca^{2+}]_i$  deregulation and reduced neuronal death following glutamate toxicity and cerebral ischemia (31). We previously demonstrated that the neuroprotective effect of TNFR2 against a glutamate challenge was associated with an increased expression of  $K_{Ca2.2}$  channels (16), outlining a potential molecular mechanism of TNFR2-mediated protection of neurons from death during exposure of a priori excitotoxic stimuli.

Studies on lovastatin actions further support TNFR2 involvement in neuroprotection. Lovastatin is a cholesterol-lowering drug with reported neuroprotective properties that can reduce the incidence of stroke and progression of Alzheimer's disease. Lovastatin increased the expression of TNFR2 in cortical neurons in vitro (32) and was neuroprotective in TNFR1<sup>-/-</sup> neurons, whereas lovastatin's protection was lost in neurons from TNFR2<sup>-/-</sup> mice (32). Furthermore, lovastatin-mediated neuroprotection led to an increase in PI3K-dependent PKB/Akt phosphorylation, whereas inhibition of PKB/Akt activation entirely abolished lovastatin-induced neuroprotection. This finding is in line with previous findings that TNFR2-mediated neuroprotection is dependent on the PI3K-PKB/Akt pathway (15), and suggests that lovastatin-induced neuroprotection is dependent on TNFR2 signaling. Administration of lovastatin protected cholinergic neurons and their cortical projections against NMDA-induced excitotoxic damage in vivo (23). Furthermore, treatment with the PI3K inhibitor LY294002 to block activation of PKB/Akt resulted in a strong reduction of lovastatin-mediated neuroprotection (23).

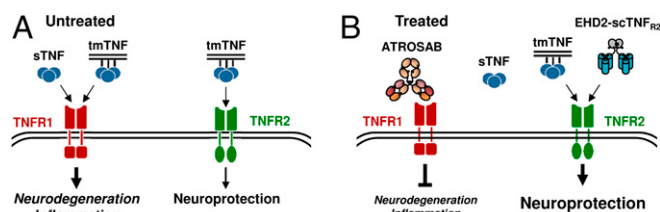
In the present report, we evaluated the therapeutic potential of the TNFR2 agonist EHD2-scTNFR<sub>2</sub> and the TNFR1 antagonistic antibody ATROSAB in this mouse model of NMDA-induced neurodegeneration. Similar to lovastatin, both EHD2-scTNFR<sub>2</sub> and ATROSAB reduced the extent of areas of macrophage/microglia activation at the site of the lesion and protected cholinergic neurons and the neocortical innervations against NMDA-induced excitotoxic damage. Functional consequences of NMDA-induced lesions and the therapeutic activity of TNFR targeting reagents became phenotypically apparent in behavioral performance studies: damage to the NBM selectively affected neocortical cholinergic denervation and its memory functions, while leaving particular hippocampal

innervation and functions unaffected. Treatment of such animals with either EHD2-scTNFR<sub>2</sub> or ATROSAB fully restored the affected cholinergic memory function.

ATROSAB-induced protection from excitotoxicity induced neurodegeneration was found to be linked to an enhancement of TNFR2 signaling leading to PKB/Akt activation; this is evident from abrogation of neuroprotection in vivo upon cotreatment with TNFR2 blockers and from in vitro studies showing increased phosphorylation of PKB/Akt as well as enhanced resistance of primary cortical neurons toward excitotoxic insult by a tmTNF mimetic TNF in the presence of a TNFR1 blockade. We propose that the neuroprotective activity of ATROSAB is accomplished by simultaneous action on two different cellular targets: (i) as a consequence of the excitotoxic insult, activated



**Fig. 4.** ATROSAB is neuroprotective via enhanced TNFR2 signaling. (A) Primary neurons, isolated from hu/mTNFR1-k1 mice, were stimulated with or without ATROSAB (100  $\mu$ g/mL) for 30 min followed by addition of wild-type EHD2-sc-mTNF (10 ng/mL). Then cells were incubated for 24 h and lysed, and phosphorylation of PKB/Akt was quantified by Western blot ( $n = 4$ ,  $\pm$ SEM). (B) Primary neurons, isolated from hu/mTNFR1-k1 mice, were stimulated with or without ATROSAB (100  $\mu$ g/mL) for 30 min followed by addition of non-TNFR-selective EHD2-sc-mTNF or mouse TNFR2-selective EHD2-sc-mTNFR<sub>2</sub>. After 24 h, glutamate (5  $\mu$ M) was added and cells were incubated for an additional hour. Then medium was exchanged to remove glutamate and cells were incubated for 23 h. Cell viability was determined by MTT assay. Data are shown as percentage of MTT signal of untreated control cells ( $n = 3$ ;  $\pm$ SEM). Representative images show (C) ChAT-positive cholinergic innervations in the somatosensory cortex or (E) CD11B-positive activated macrophage/microglia in magnocellular nucleus basalis. NMDA injected into the NBM induced an extensive cholinergic fiber loss in the layer V of somatosensory cortex and a massive volume of macrophage/microglial activation compared with the control group. However, ATROSAB treatment attenuated fiber loss and significantly reduced macrophage/microglial activation induced by NMDA. ATROSAB neuroprotection against fiber loss was prevented by TNFR2 antagonistic MAB426. However, MAB426 alone did not significantly alter NMDA-induced NBM lesion. Parallel bars indicated the layer V of the somatosensory cortex in which quantitative measurements were performed. (D) Quantification of cholinergic fiber density in layer V of the somatosensory cortex. Fiber density was measured in eight sections per mouse. (F) Quantification of total extent of activated macrophage/microglia around the injections. Macrophage/microglial activation was measured in a series of sections with macrophage/microglial activation.  $n = 7$  mice/group. All data in bar charts represent means  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ , one-way ANOVA with post hoc comparisons Tukey.



**Fig. 5.** Mechanism of TNFR1 antagonist ATROSAB and TNFR2 agonist EHD2-scTNFR<sub>2</sub>-mediated neuroprotection. (A) TNFR1 signaling induces neurodegeneration and inflammation, whereas TNFR2 signaling promotes neuroprotection. (B) ATROSAB, an antagonistic TNFR1-selective antibody, can block TNFR1 signaling, leading to prevention of neurodegeneration and inflammation. More importantly, in the presence of ATROSAB, tmTNF stays free to activate neuroprotective signaling via TNFR2. Our data indicate that, by blocking TNFR1, ATROSAB shifts the endogenous tmTNF signaling toward neuroprotective TNFR2 signaling. Similarly, the TNFR2 agonist EHD2-scTNFR<sub>2</sub> can directly activate TNFR2 and thereby induce neuroprotection.

macrophage/microglia promote, via soluble TNF production, the expansion of the degenerative tissue response in an autocrine TNFR1-dependent way; and (ii) inhibition of TNFR1 limits this process both at the level of microglia/macrophage activation and at the level of neurons of TNFR1-mediated degenerative signaling. Further, for neuronal cells, it was shown previously that proper TNFR2 activation induces protection from excitotoxicity in vitro (15, 16); in the in vivo model analyzed here, in neurons in the vicinity of the acute insult and not immediately succumbing to excitotoxic death, competition of TNFRs for limiting amounts of endogenously produced membrane form of TNF exists, which results in suboptimal activation of TNFR2 of this cell population under a nontreatment condition. Moreover, at the level of intracellular signaling, it is known that signal cross-talk between TNFR1 and 2 exists in terms of competition for common signal transducers such as TRAF2 (33–35), with TNFR1 outperforming TNFR2 pathways in the case of abundance of soluble TNF, which triggers exclusively TNFR1 (3, 6). In the presence of ATROSAB, neuronal response is shifted toward neuroprotective TNFR2 signaling because of blocked neuronal TNFR1, allowing the induction of a resistant state by endogenous tmTNF (Fig. 5). The therapeutic activity of TNFR2 activation by treatment with EHD2-scTNFR<sub>2</sub> without interfering with TNFR1 signaling supports the view of limited TNFR2 activation by endogenous TNF. Accordingly, TNFR2's role as an important neuroprotective pathway emerges when TNFR1 ligand binding is stalled and/or when TNFR2 activation is optimized by application of exogenous TNFR2-selective ligands.

Importantly, initial studies with anti-TNF therapeutics showed that TNF blocking drugs cannot be used to treat neurodegenerative diseases such as MS. In support of this finding, we here provide compelling evidence in an in vivo model of NMDA-induced acute neuronal lesions that abrogation of complete TNF signaling by blocking both TNFRs is not protective, because mounting of a neuroprotective TNFR2-dependent response is prevented. However, preclinical studies in the experimental autoimmune encephalomyelitis (EAE) model (36–38) or a mouse model of spinal cord injury (39) revealed that selective neutralization of sTNF/TNFR1 signaling is neuroprotective. Of note, in the EAE model, systemic application of TNFR1-blocking antibodies proved to be therapeutically effective (40).

Accordingly, TNFR1 antagonists such as ATROSAB should be superior to conventional anti-TNF drugs in the treatment of neurodegenerative diseases, as they spare TNFR2 and even enhance TNFR2 signaling but still block detrimental signals transmitted via TNFR1. In addition to the potential therapeutic use of TNFR1-specific antagonistic antibodies, TNFR2-selective agonists seem to be particularly suitable to treat inflammatory, demyelinating diseases, because next to the direct neuroprotective effects shown in this report, data from different laboratories outline that TNFR2 is also involved in immune suppression via expansion and stabilization of regulatory T cells (41–47), and induces remyelination (9, 13, 14). Thus, like TNFR1 antagonists, TNFR2 agonists might promote therapeutic effects via multiple cellular targets. Limiting for the treatment of neurodegenerative diseases is the blood–brain–barrier (BBB), which restricts the transport of therapeutics into the CNS. Under physiologic conditions, antibodies, e.g., ATROSAB, do not cross the BBB. However, of relevance, the BBB undergoes rapid changes in permeability in response to CNS insults, e.g., after spinal cord injury or during MS, potentially enabling therapeutics to reach the CNS parenchyma without specific transport mechanisms. Indeed, the reported therapeutic activity of ATROSAB in EAE models (40) supports this reasoning. Whether alterations of the BBB permeability in different neurodegenerative diseases are sufficient to reach therapeutic effective concentrations in the CNS is presently unclear. Therefore, strategies are now available to overcome limits of BBB passage. Thus, antibodies engineered to be actively transported via BBB have been developed that were shown to be beneficial for the treatment of Alzheimer's disease (48, 49). Cytokines including TNF can cross the BBB (50). By analogy, we assume that the engineered TNF mutein EHD2-scTNFR<sub>2</sub> could be able to penetrate the BBB, too.

In summary, in an acute neurodegenerative disease model, we provide proof of concept that both the TNFR2-selective TNF variant EHD2-scTNFR<sub>2</sub> and the antagonistic TNFR1-selective antibody ATROSAB protect from neurological deficits due to excitotoxic cell death induced by excessive glutamate exposure in vivo. Our data provide a rational base for previous failure of clinical studies with anti-TNF drugs in neurodegenerative diseases and highlight the essential protective role of TNFR2 in the central nervous system. Further investigations in additional neurodegenerative diseases models on the therapeutic potential of the TNFR2 agonist EHD2-scTNFR<sub>2</sub> and the TNFR1 antagonist ATROSAB are warranted and will reveal whether and for which indications these or functionally similar proteins can be successfully applied.

## Materials and Methods

Detailed methods can be found in *SI Materials and Methods*. The TNFR2 agonist EHD2-scTNFR<sub>2</sub> and the tmTNF-mimetic TNF mutein EDH2-scTNF are described in this work. The transgenic hu/mTNFR-k<sub>i</sub> mice were generated as contracted by Ozgene Pty Ltd. Animal care and treatment were carried out in accordance with Committee Groningen (Dierexperimenten Commissie Van de Rijksuniversiteit Groningen) and Committee Stuttgart (Regierungspräsidium Stuttgart) guidelines on the use of experimental animals at the University of Groningen (permit no. DEC6523) and the University of Stuttgart (permit no. 35-9815.81-0350), respectively.

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