Exogenous activation of tumor necrosis factor receptor 2 promotes recovery from sensory and motor disease in a model of multiple sclerosis

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ABSTRACT
Tumor necrosis factor receptor 2 (TNFR2) is a transmembrane receptor that promotes immune modulation and tissue regeneration and is recognized as a potential therapeutic target for multiple sclerosis (MS). However, TNFR2 also contributes to T effector cell function and macrophage-TNFR2 recently was shown to promote disease development in the experimental autoimmune encephalomyelitis (EAE) model of MS. We here demonstrate that systemic administration of a TNFR2 agonist alleviates peripheral and central inflammation, and reduces demyelination and neurodegeneration, indicating that protective signals induced by TNFR2 exceed potential pathogenic TNFR2-dependent responses. Our behavioral data show that systemic treatment of female EAE mice with a TNFR2 agonist is therapeutic on motor symptoms and promotes long-term recovery from neuropathic pain. Mechanistically, our data indicate that TNFR2 agonist treatment follows a dual mode of action and promotes both suppression of CNS autoimmunity and remyelination. Strategies based on the concept of exogenous activation of TNFR2 therefore hold great promise as a new therapeutic approach to treat motor and sensory disease in MS as well as other inflammatory diseases or neuropathic pain conditions.

1. Introduction
Tumor necrosis factor (TNF) is a master regulator of the immune system that initiates and orchestrates inflammation (Aggarwal, 2003). 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 (Fischer and Maier, 2015). Therefore, anti-TNF therapeutics are clinically approved and successfully used to treat autoimmune diseases, such as rheumatoid arthritis, psoriasis, or inflammatory bowel disease (Monaco et al., 2015). Multiple sclerosis (MS) patients also show elevated levels of TNF at the site of active lesions and TNF levels correlate with the severity of the lesion (Hofman et al., 1989; Sharief and Hentges, 1991). However, two patients treated in an open-labeled phase I safety trial showed a transiently increased immune activation and disease activity after infusion of the anti-TNF infliximab (van Oosten et al., 1996). In addition, the “Lenercept” anti-TNF study, a phase II, randomized, multicenter, placebo-controlled study for the treatment of relapsing remitting MS failed (Lenercept Study Group, 1999). 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, indicating that TNF inhibition is contraindicated in MS. In addition, due to the unspecific immune inhibition, all approved anti-TNF therapeutics may induce severe side-effects such...
as increased susceptibility to serious infections, development of additional autoimmune or demyelinating diseases (Fischer et al., 2015; Monaco et al., 2015).

The severe side-effects of anti-TNF therapeutics in approved indications and the failure of the MS studies might be explained by the pleiotropic actions of TNF, including both pro- and anti-inflammatory functions as well as other immunoregulatory and regenerative activities. Recent research has revealed that TNF induces antagonistic biological responses via its two receptors, TNF receptor (TNFR) 1 and TNFR2. Whereas TNFR1 signaling promotes inflammation and tissue degeneration, TNFR2 contributes to immune suppression as well as tissue homeostasis and neuroprotection (Fischer et al., 2015; Probert, 2015).

In particular, TNFR2 promotes expansion and stability of regulatory T cells (Tregs) (Chen et al., 2007; Chen et al., 2013), a subpopulation of lymphocytes with immune suppressive activity that control the development of autoimmune diseases. Indeed, deletion of Treg-TNFR2 was shown to exacerbate motor disease in experimental autoimmune encephalomyelitis (EAE) mice, indicating a role of Treg-TNFR2 in suppressing CNS autoimmunity (Atretkhany et al., 2018). TNFR2 signaling was also shown to induce protective and regenerative responses in the CNS, i.e. neuronal survival in an in vivo model of retinal ischemia (Fontaine et al., 2002) and remyelination in the cuprizone model of toxic demyelination and spontaneous remyelination (Arnett et al., 2001). Further, TNFR2-/- mice develop more severe EAE motor disease than wild type mice (Suvannavej et al., 2000; Williams et al., 2014), and it was demonstrated that oligodendroglial-TNFR2 mediates remyelination in EAE (Madsen et al., 2016) and that genetic ablation of microglial TNFR2 leads to disease exacerbation (Gao et al., 2017). Therefore, in recent years, TNFR2 has emerged as a novel therapeutic target for MS.

However, next to its potential therapeutic effects via Tregs or CNS cells, TNFR2 can be expressed on activated T effector cells (Tefs) and was shown to act co-stimulatory for TCR-mediated T cell activation and effector function (Mehta et al., 2016; Ye et al., 2018). Other studies showed that monocyte/macrophage-TNFR2 contributes to EAE development (Gao et al., 2017). Therefore, in this study, we investigated the systemic administration of a TNFR2 agonist alleviated sensory and motor deficits in EAE, indicating that protective responses via Tregs and/or macrophages exceed potential pathogenic responses via Tefs or macrophages.

2. Materials and methods

2.1. The materials

The production and characterization of the TNFR2 agonist EHD2-sc-mTNFβ2 was described previously (Fischer et al., 2018). Fluorescence-labeled antibodies against CD3, CD25, CD68, MHC class II, and FoxP3 were from Miltenyi Biotech (Bergisch-Gladbach, Germany). Other antibodies included APP (Novus Biologicals, Littleton, CO), Iba1 (Wako Pure Chemicals, Osaka, Japan), CD3 (Novus Biologicals, Littleton, CO), FoxP3 (Abcam, Cambridge, UK), Human IgE (Novus Biologicals, NB7456) and TNF (HP8001, Hycult Biotech, The Netherlands). All other chemicals were of analytical grade.

3. Animals

Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a virus/antigen free facility with a 12-hour light/dark cycle and unlimited access to water and food. All animal experiments were performed according to guidelines of the Institutional Animal Care and Use Committee (IACUC) of Drexel University under protocol numbers 20271/20617. All studies were conducted in accordance with the United States Public Health Service’s Policy on Humane Care and Use of Laboratory Animals. In all behavior studies animals were randomly assigned to groups. Behavior, tissue and cellular analysis were performed double-blinded with investigators being blinded to treatment and experimental groups.

4. Induction of experimental autoimmune encephalomyelitis and pharmacological treatment

Active EAE was induced with MOG35–55 peptide (BioSynthesis Inc.) as previously described (Brambilla et al., 2011). Clinical symptoms of EAE were assessed daily using a standard scale of 0–5 as follows: 0, no clinical signs; 1, loss of tail tone; 2, fully flaccid tail; 3, complete hind limb paralysis; 4, complete forelimb paralysis; 5, moribund or dead. Saline or EHD2-sc-mTNFβ2 (10 mg/kg body weight) were administered i.p. 6, 9, and 12 (treatment onset of pain) or 16, 19, and 22 (treatment onset of motor disease) days post immunization (dpi). Onset of pain (6 dpi) was defined as the first day, where CFA-induced inflammatory pain was distinguishable from CFA/MOG-induced neuropathic pain. Onset of motor disease (16 dpi) was defined as the first day, when more than 50% of the immunized mice had reached a score of above 1.5 for at least one day.

5. Mechanical allodynia

Mechanical allodynia was determined using the Von Frey test as described previously with standardized Von Frey filaments ranging from 0.02 to 2 g (Dellarole et al., 2014). Investigations were performed by experimenters blinded to the surgery and the genotype or treatment. Mice were handled daily for at least 1 week prior to any behavioral analysis. To evaluate tactile sensitivity each mouse was placed individually on a wire mesh beneath a transparent plastic jar and habituated for 30 min before starting the behavioral test. Von Frey filaments (Touch-Test Sensory Evaluator) were applied to the plantar surface of each hind paw in a series of ascending forces (ranging between 0.02 g and 2 g) starting from the 0.4 g filament. A single trial of stimuli consisted of 5 applications of Von Frey filaments every minute perpendicularly to the plantar surface of the paw for about 4–5 s. To qualify a response to tactile stimulation as being painful, the mouse had to show cognitive awareness of pain. Therefore, at least one of the following cognitive responses had to coincide with a withdrawal response to a “painful” stimulation before it was considered a painful response and not a reflexive reaction: 1) avoidance behavior, 2) protecting the injured paw and 3) licking and/or looking at the injured paw. Depending on the positive or negative response, subsequent filaments were applied in order of descending or ascending intensity, respectively. If paw withdrawal occurred in response to the minimum 0.02 g filament, the next trial was performed with the same 0.02 g, cut-off filament. In case the maximum 2 g filament was reached with no response, the next trial was performed with the same 2 g, cut-off filament. Each test consisted of 6 trials. Paw movements associated with locomotion or weight-shifting were not counted as a response. The withdrawal threshold was expressed as the tolerance level in grams.

6. Flow cytometry

Mice were sacrificed at the times indicated in the figure legends, spleens were extracted, dissociated through a 40 µm cell strainer and collected in 10 ml MACS buffer (PBS, 0.5% BSA, 2 mM EDTA). Splenocytes were harvested by centrifugation (300 g, 5 min) and washed once with 10 ml MACS buffer. Then, expression of different immune cell markers was investigated by flow cytometry. Therefore, 10⁶ splenocytes per staining were incubated for 20 min with fluorescence-labeled antibodies against surface antigens. Then cells were washed with MACS buffer by centrifugation (300 g, 5 min) and incubated for 30 min in fixation/permeabilization buffer (Miltenyi Biotech, Bergisch-Gladbach). Cells were washed with MACS buffer by centrifugation (300...
g, 5 min), followed by another washing step with permeabilization buffer (Miltenyi Biotech, 300 g, 5 min) and incubation with fluorescence-labeled antibodies against intracellular antigens for 30 min. Cells were washed in permeabilization buffer (300 g, 5 min) and collected in MACS buffer. Data were acquired using a BD FACS Canto (BD Biosciences, San Jose, CA) and analyzed with FlowJo (TreeStar, Ashland, OR).

7. Tissue preparation for immunohistochemistry

Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The lumbar part of the spinal cord was dehydrated with the Tissue-Tek VIP (Sakura, Nagano, Japan) and stored in paraffin. Paraffinized tissue was cut into 10 µm thick transverse sections with a rotary microtome (Microm HM325, BioTherm). 10 serial sections, taken at regular intervals to cover the whole spinal cord, were used per staining.

8. Luxol Fast Blue (LFB) staining

Tissue sections were deparaffinized and rehydrated to 95% ethanol with the Gemini AS (Thermo Scientific). Sections were incubated in the Luxol Fast Blue (LFB) staining solution overnight at 56 °C in a water bath. Then sections were differentiated by immersion in lithium carbonate solution, dehydrated in ethanol and xylene. Sections were imbedded in paraffin and 4-µm thick sections were cut. Sections were stained with a mixture of 50% chloroform and 50% absolute ethanol. Sections were incubated in the LFB staining solution overnight at 56 °C in a water bath. Then sections were differentiated (10 min), washed in permeabilization buffer (5 min) and collected in MACS buffer.

9. Immunohistochemistry (IHC) antibody staining

Tissue sections were deparaffinized and rehydrated with the Gemini AS (Thermo Scientific). Sections were incubated in 10 mM citric acid buffer for 20 min at 85 °C in a water bath for antigen retrieval, washed three times for 5 min with TBS and blocked for 1 h at room temperature (RT) with blocking buffer (0.05% BSA, 0.03% Triton-X100, TBS). Sections were incubated with primary antibodies diluted in blocking buffer and 5% NGS (normal goat serum) or NHS (normal horse serum) in a humidified chamber overnight at 4 °C. The next day, sections were washed with TBS, incubated with secondary antibodies in blocking buffer and 5% NGS or NHS respectively (VECTASTAIN Elite ABC-HRP Kit) for 1 h at RT and washed with TBS again. Thereafter, sections were incubated with ABC solution (Vector Laboratories) for 1 h at RT, washed with TBS and then incubated with DAB or VIP solution (Vector Laboratories) for 15 min at RT. Sections were washed with tap water and dehydrated using the Gemini AS (Thermo Scientific). Sections were imaged using a light microscope and quantified using ImageJ Fiji (Schindelin et al., 2015).

10. RNA Isolation, cDNA synthesis

Mice were sacrificed as indicated in the figure’s legend, perfused with PBS and the lumbar spinal cord was dissected. Total RNA was extracted using TRizol Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions (Thermo Fisher Scientific). The A260/A280 ratio of the RNA was determined using a UV spectrophotometer (Nanodrop, Thermo Fisher Scientific). Equal concentrations of RNA samples with an A260/A280 ratio ranging from 1.8 to 2.0 were used for cDNA synthesis. RNA was retro-transcribed into cDNA with the AffinityScript QPCR cDNA Synthesis Kit and oligo(dT) primers (Agilent, Santa Clara, CA). The cDNA was preserved at −80 °C until further use.

11. Quantitative real-time PCR

Gene expression was quantified by real-time PCR (qPCR; Rotor-Gene Q, Qiagen) using specific primers (ThermoFisher, Table 1) and the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent). To determine the expression of distinct genes ΔΔCt values were determined by correlating the obtained values to the house-keeping gene GAPDH. Data are presented relative to naïve controls as normalized fold expression.

12. Mouse T cell isolation, culture and flow cytometry

Splenocytes from C57BL/6 mice were isolated as previously described (Fischer et al., 2018). CD3+ T cells were isolated using the FACS Aria III. Purified T cells were activated using plate-bound αCD3 (5 µg/ml, 6 h at 4 °C) in combination with soluble αCD28 (2 µg/ml)
IL-2 (100 U/ml) in 96-well (U form) plates with or without EHD2-sc-mTNFα2 in TexMACS medium (Miltenyi Biotec). After 5 days in culture, CD25+FoxP3+ Tregs were quantified by flow cytometry according to manufacturer’s instructions (Miltenyi Biotec, Bergisch-Gladbach). Data were acquired using a MACSQuant Analyzer 10 (Miltenyi) and analyzed with FlowJo (TreeStar, Ashland, OR).

13. EHD2-sc-mTNFα2-specific ELISA

ELISA plates (Nunc MaxiSorp) were coated with goat anti-Human IgE (Novus Biologicals, NB7456) at 1 µg/ml in PBS and incubated at 4 ºC overnight. Residual binding sites were blocked with 2% skim milk powder in PBS at RT for 2 h. Tissue homogenates were diluted in 2% skim milk powder in PBS and incubated for 1 h at RT. Purified EHD2-sc-mTNFα2 was diluted in 2% skim milk powder and used as a standard. Bound EHD2-sc-mTNFα2 was detected with rabbit polyclonal antibodies to mouse TNF (HP8001; 1 µg/ml; incubation for 1 h at RT) and HRP-conjugated anti-rabbit IgG antibodies (diluted 1:10000; incubation for 1 h at RT). Between each step, non-bound proteins were removed by washing 4 times with 0.005% Tween-20 in PBS.

Then, wells were incubated with TMB substrate solution and reaction was stopped by addition of 1 M H2SO4 and the absorbance at 450 nm was determined with an absorbance reader (Multiskan FC, Thermo Scientific, Karlsruhe, Germany) and data were analyzed using the software Microsoft Excel and GraphPad Prism (GraphPad, La Jolla, CA).

14. Multiplex inflammation assay

Blood was withdrawn from mice via heart puncture at the experimental end point described in the figure’s legend. Blood clotting was allowed for 30 min at room temperature and serum was separated by centrifugation for 20 min at 1000 g. Serum was stored at −80 ºC until use. Concentration of 12 different cytokines was determined using the LEGENDplex mouse inflammation Panel (Biolegend) according to the manufacturer’s instructions. Samples were acquired using a BD FACS Canto (BD Biosciences) and analyzed using the LEGENDplex Software V8.0. Concentrations are presented relative to naïve controls.

15. Primary oligodendrocyte cultures

Cortices from postnatal day 2–3 pups were isolated and dissociated into single cell suspensions with Papain Neural Tissue Dissociation Kit (Miltenyi Biotec). Platelet-derived growth factor receptor α-positive (PDGFRα+) oligodendrocyte progenitor cells (OPCs) were isolated by MACS separation using MS columns (Miltenyi Biotec) after incubation with CD140 (PDGFRα) magnetic microbeads (Miltenyi Biotec). PDGFRα+ cells were cultured on poly-D-lysine/laminin-coated 60 mm dishes in OPC medium consisting of DMEM/F12 supplemented with 1% N2, 2% B27, 0.01% BSA, 2.1 g/l NaHCO3, 0.1% D-glucose, 20 ng/ml PDGF-AA, and 40 ng/ml FGF2. Purity of cultures was greater than 97%, as determined by staining for PDGFRα.

16. OPC proliferation assay

OPCs were seeded onto poly-D-lysine/laminin-coated coverslips (Neuvitro) in a 24-well plate (19,000 cells/well) and stimulated with saline or EHD2-sc-mTNFα2 (200 ng/ml). Then, 5-ethyl-2′-deoxyuridine (EdU) was added to the cultures for 8 h and cells were fixed in 4% paraformaldehyde (PFA) for 15 min. EdU was detected using the click-it EdU imaging kit (Thermo Fisher). Nuclei were visualized using Hoechst staining.

17. Oligodendrocyte differentiation assay

OPCs were seeded onto poly-D-lysine/laminin-coated coverslips (Neuvitro) in a 24-well plate (19,000 cells/well) and cultured for 48 h before switching to oligodendrocyte differentiation medium consisting of DMEM/F12 supplemented with 1% N2, 2% B27, 0.01% BSA, 2.1 g/l NaHCO3, 50 µg/ml insulin and 40 ng/ml triiodothyronine (T3). Cells were differentiated in presence of saline or EHD2-sc-mTNFα2 (200 ng/ml) to myelinating oligodendrocytes for 7 days before fixation in 4% PFA for 30 min. Myelination was assessed by staining with mouse anti-CC1 (1:500, Calbiochem) and rat anti-MBP (1:100, Millipore). Immunoreactivity was visualized with secondary species-specific fluorescent antibodies (Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse, 1:500, Life Technologies). Nuclei were visualized using Hoechst staining. Oligodendrocyte differentiation was analyzed by assessing the percentage of myelinating MBP+ cells within the CC1+ population.

18. Statistics

Data are presented as mean ± standard error of the mean (SEM) of n animals. Normal distribution was analyzed by Shapiro-Wilk normality test. Statistical analyses were performed by Student’s t-test or analysis of variance, followed by a post hoc Tukey’s range test. A value of p < 0.05 was considered statistically significant.

19. Results

19.1. Exogenous activation of TNFR2 reduces motor and sensory disease

We and others have shown that female TNFR2−/− mice develop more severe EA motor symptoms than wild type mice (Suvannavej et al., 2000; Williams et al., 2014), indicating that TNFR2 contributes to suppression of EA development. We therefore here investigated, whether exogenous activation of TNFR2 is therapeutic in female EA mice. In this study, we focused on female mice, since in humans, females are approximately 3-times more likely to develop MS and chronic neuropathic pain is more prevalent in women than men (Murphy et al., 2017). In addition to motor disease, approximately 60% of MS patients report the experience of chronic neuropathic pain (CNP), a chronic pain condition that is caused by a lesion of the somatosensory nervous system and dramatically reduces the quality of life of MS patients. CNP attacks often precede motor relapses and can be used as a treatment indication for MS patients (Murphy et al., 2017). We therefore used onset of neuropathic pain and motor disease as clinical indicators to initiate treatment. First, we compared pain sensitivity of mice immunized with CFA to immunization with MOG emulsified in CFA to distinguish between CFA-induced inflammatory and CFA/MOG-induced neuropathic pain. Mice immunized with CFA had significantly recovered 6 days post immunization (dpi), whereas MOG/CFA immunization resulted in chronic pain over the observation period of 25 dpi (Fig. 1A). We next treated mice immunized with CFA/MOG at 6, 9, and 12 dpi with saline or the TNFR2 agonist EHD2-sc-mTNFα2 using a dose (10 mg/kg) that was therapeutic in experimental arthritis (Fischer et al., 2018). Motor disease was scored, and disease-associated neuropathic pain was determined using the Von Frey test for mechanical allodynia over a period of 25 dpi. EHD2-sc-mTNFα2 treatment significantly ameliorated development of motor disease (Fig. 1B, Table 2), prevented weight loss after onset of motor symptoms (Fig. 1C) and delayed the day of onset of motor deficits (Fig. 1D). Further, treatment with the TNFR2 agonist significantly increased the paw withdrawal threshold in the Von Frey test, indicating TNFR2 agonist-mediated pain resolution (Fig. 1E). We then delayed EHD2-sc-mTNFα2 treatment until after onset of motor deficits (16, 19, and 22 dpi) and evaluated motor disease and disease-associated neuropathic pain. Again, TNFR2 agonist treatment significantly alleviated motor disease progression (Fig. 2A, Table 2) but had no impact on weight loss (Fig. 2B) and disease-associated pain (Fig. 2C).
20. TNFR2 agonist administration expands peripheral Treg levels, but does not impact macrophage activation

It has recently been shown that microglia and macrophage-TNFR2 have opposing functions in the pathogenesis of EAE. Whereas microglia-TNFR2 is neuroprotective, genetic deletion of macrophage-TNFR2 resulted in alleviated development of EAE motor disease, indicating that macrophage-TNFR2 contributes to disease development (Gao et al., 2017). In our studies we observed an immunization-dependent increase in the percentage of splenic CD68+ macrophages 14 dpi after both saline and EHD2-sc-mTNFR2 administration (Fig. 3A). Further, macrophage activation, measured by MHC class II expression, was not altered following administration of the TNFR2 agonist (Fig. 3A and B), indicating that systemic application of a TNFR2 agonist does not interfere with macrophage activity.

We and others have previously shown that administration of a TNFR2 agonist induces peripheral Treg expansion (Chopra et al., 2016; Fischer et al., 2018). Indeed, we detected an increased percentage of splenic CD25+FoxP3+ T cells in mice treated with EHD2-sc-mTNFR2 (Fig. 3A and C). The increase in peripheral immunosuppressive Tregs was accompanied by a decrease in expansion of splenic CD3+ T cells 25 dpi after treatment starting at onset of pain (6 dpi) and onset of motor deficits (16 dpi) (Fig. 3A, and C), supporting that TNFR2 agonism suppresses autoimmunity. We then cultivated isolated CD3+ T cells with or without EHD2-sc-mTNFR2 and determined that TNFR2 agonism induces proliferative expansion of Tregs.

21. TNFR2 agonist administration reduces peripheral and central inflammation

Having demonstrated that TNFR2 agonism induces immunosuppressive signals in the periphery, we characterized peripheral inflammation and measured the concentration of various pro-inflammatory cytokines in the serum 25 dpi. Compared to saline-treated mice, TNFR2 agonist treatment reduced upregulation of pro-inflammatory cytokines in the serum of mice, indicating that exogenous TNFR2 activation induces anti-inflammatory responses in the immune system. Effects were stronger, when EHD2-sc-mTNFR2 was administered at onset of pain (6 dpi) compared to onset of motor symptoms (16 dpi), representing the differences in therapeutic activity observed in the

Table 2
Clinical Parameters of EAE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence (%)</th>
<th>Day of onset, mean ± SEM</th>
<th>Cumulative Score</th>
<th>AUC (clinical score)</th>
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<td>Treatment 6 dpi</td>
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<tr>
<td>saline</td>
<td>92.6% (25 of 27)</td>
<td>15.48 ± 2.06</td>
<td>24.21 ± 12.42</td>
<td>23.03 ± 2.83</td>
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<tr>
<td>EHD2-sc-mTNFR2</td>
<td>28.6% (8 of 28)</td>
<td>17.75 ± 4.45 (*)</td>
<td>7.68 ± 12.60 (****)</td>
<td>7.344 ± 2.65 (****)</td>
</tr>
<tr>
<td>Treatment 16 dpi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline</td>
<td>94.12% (16 of 17)</td>
<td>16.25 ± 2.52</td>
<td>24.54 ± 10.88</td>
<td>23.21 ± 2.84</td>
</tr>
<tr>
<td>EHD2-sc-mTNFR2</td>
<td>75.0% (12 of 16)</td>
<td>16.23 ± 2.92</td>
<td>17.58 ± 12.39 (*)</td>
<td>16.73 ± 2.86 (****)</td>
</tr>
</tbody>
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behavioral studies (Fig. 5A).

Next, we analyzed the impact of TNFR2 agonist treatment on neuroinflammation. We profiled the gene expression of pro-inflammatory and anti-inflammatory mediators in the lumbar spinal cord by qPCR. Similar to the effects observed in the serum samples, EHD2-sc-mTNFR$_{R2}$ treatment resulted in decreased expression of pro-inflammatory genes (Fig. 5B). We then analyzed microglia activation / macrophage infiltration into the lumbar spinal cord by Iba1 immunohistochemistry staining. Treatment with EHD2-sc-mTNFR$_{R2}$ reduced the number of Iba1$^+$ cells when started at either 6 dpi or 16 dpi (Fig. 5C-I).

22. TNFR2 agonist administration reduces T cell infiltration only if treatment is started at onset of pain

Since T cell infiltration into the CNS is recognized as a major driver of EAE/MS pathology that contributes to both development of motor disease as well as neuropathic pain (Hohlfeld and Steinman, 2017), we next investigated the impact of TNFR2 agonist treatment on infiltration of CD3$^+$ T cells into the lumbar spinal cord. Infiltration of T cells into the spinal cord peaked at the onset of motor disease, evident by the highest numbers observed at 14 dpi (Fig. 6A, B, G). Whereas TNFR2 agonist treatment reduced the number of infiltrating CD3$^+$ T cells if started at onset of pain (6 dpi), no effect was observed if treatment was delayed until after onset of motor deficits (16 dpi) (Fig. 6C-G). Since we observed an increase in splenic Tregs after TNFR2 agonist treatment, we next quantified infiltration of Tregs into the lumbar spinal cord. Interestingly, we did not observe differences in the percentage of infiltrating FoxP3$^+$ Tregs relative to the total number of infiltrating CD3$^+$ T cells, between saline and EHD2-sc-mTNFR$_{R2}$-treated mice (Fig. 6H–N).

23. TNFR2 agonist administration reduces neurodegeneration and demyelination

Two major pathological hallmarks of EAE are demyelination and axonal damage. Therefore, we compared the histopathology of mice treated with EHD2-sc-mTNFR$_{R2}$ to mice treated with saline at 14 dpi and 25 dpi. Neurodegeneration was visualized by accumulation of amyloid-beta precursor protein (APP), a marker of axonal injury that is only detectable in axons with impaired axonal transport (Bjartmar and Trapp, 2003). Indeed, EHD2-sc-mTNFR$_{R2}$ administration 6 dpi resulted in a reduced number of APP$^+$ axonal profiles compared to saline treated mice, indicating that TNFR2 agonist treatment reduced neurodegeneration. If treatment was started 16 dpi, a non-significant trend towards reduced neurodegeneration in the TNFR2 agonist treatment group was observed (Fig. 7A–G). Further, TNFR2 agonist treatment started 6 dpi and 16 dpi resulted in significantly reduced spinal cord demyelination, as determined by analysis of LFB staining (Fig. 7H–N).

24. Selective stimulation of TNFR2 promotes OPC proliferation and differentiation

It was shown that TNFR2 has a protective role in EAE via microglia (Gao et al., 2017) and oligodendrocytes (Madsen et al., 2016) and directly protects neurons from cell death (Fischer et al., 2011). We therefore next analyzed whether EHD2-sc-mTNFR$_{R2}$ was transported into the CNS to promote neuroprotection via CNS-resident cells. Indeed, EHD2-sc-mTNFR$_{R2}$ was detected in the spinal cord after treatment start (16 dpi). An increased average concentration of EHD2-sc-mTNFR$_{R2}$ was observed if treatment was started 16 dpi, a time when the blood-spinal cord-barrier is impaired (Fig. 8A), indicating that EHD2-sc-mTNFR$_{R2}$ can activate neuroprotective responses in CNS-resident cells if administered systemically after onset of motor deficits. A previous report showed that OPCs isolated from TNFR2$^{-/-}$ mice showed impaired differentiation into myelinating oligodendrocytes compared to WT OPCs. However, no impact of endogenous TNFR2 signaling was observed on OPC proliferation in this study (Madsen et al., 2016). In contrast to the previous study, we here demonstrate that exogenous activation of TNFR2 via EHD2-sc-mTNFR$_{R2}$ induces OPC proliferation (Fig. 8B) and increases differentiation of myelinating oligodendrocytes (Fig. 8C), indicating that systemically administered TNFR2 agonist therapy may induce remyelination.

25. Discussion

Our study demonstrates that pharmacological activation of TNFR2 alleviates motor disease and disease-associated neuropathic pain in the EAE model of MS. While it is well established that TNFR2 plays an important role for Treg function and neuroregeneration, other data indicate that TNFR2 signaling contributes to Teff function and macrophage-TNFFR2 promotes EAE development. We here show that systemic application of a TNFR2 agonist at onset of pain (6 dpi) or at onset of motor deficits (16 dpi) reduces levels of pro-inflammatory cytokines and chemokines in the blood and the spinal cord. Further, both treatment strategies alleviate motor deficits and early treatment after onset of pain is also therapeutic on disease-associated neuropathic pain, indicating that protective responses induced by TNFR2 exceed potential pathogenic responses. This study emphasizes the important role of TNFR2 for immune modulation and tissue regeneration and highlights, why anti-TNF therapy might have failed in MS studies. We here further provide clinically important evidence that systemic administration of a TNFR2 agonist suppresses CNS autoimmunity and that TNFR2 agonists hold great promise as a novel drug class for MS.

TNFR2 promotes Treg activity and stability in the inflammatory environment (Chen et al., 2013; Chopra et al., 2016; Okubo et al., 2013) and expression levels of TNFR2 are correlated with the suppressive activity of Tregs (Chen et al., 2007, 2010). Indeed, deletion of Treg-
Fig. 3. Exogenous TNFR2 activation does not impact macrophage activity but increased splenic Tregs. Mice were immunized with MOG emulsified in CFA and treated (T) with saline or EHD2-sc-mTNFR2 (10 mg/kg) after onset of pain (6, 9 and 12 dpi) or onset of motor deficits (16, 19, 22 dpi). Mice were sacrificed, and immunity was analyzed (A) 14 dpi or 25 dpi. Splenocytes were isolated and percentage of CD68+ macrophages, CD68+ MHC II+ activated macrophages, CD3+ T cells and CD3+ FoxP3+ Tregs were quantified by flow cytometry. (C) Shown are (A,B) the quantification of the flow cytometry data, and (C) representative dot plots and histograms for the experiment T: 6 dpi, A: 25 dpi (mean ± SEM, T: 6 dpi, A: 14 dpi: CFA: n = 6, saline/EHD2-sc-mTNFR2: n = 8 each; T: 6 dpi, A: 25 dpi: CFA: n = 6, saline: n = 6, EHD2-sc-mTNFR2: n = 8 each; T: 16 dpi, A: 25 dpi: CFA: n = 6, saline/EHD2-sc-mTNFR2: n = 7 each) *p < 0.05, **p < 0.001, ***p < 0.0001, ns = not significant.
TNFR2 was shown to exacerbate EAE motor disease, indicating that Treg-TNFR2 controls CNS autoimmunity (Atrekhany et al., 2018). In healthy individuals, Tregs can control potentially pathogenic auto-reactive T cells, while Tregs in MS patients show insufficient regulatory abilities thereby allowing autoreactive T cells to become pathogenic (Kleinewietfeld and Hafler, 2014). Our data indicate that TNFR2 agonism expands Tregs in the periphery, whereas we did not see a difference in the percentage of Tregs infiltrating the spinal cord. This indicates that peripherally expanded Tregs might suppress autoimmunity, resulting in reduced infiltration of immune cells into the CNS and reduced neuroinflammation. Further T cell-intrinsic TNFR2 was shown to inhibit Th17 differentiation (Miller et al., 2015), indicating that TNFR2 agonism might interfere with Th17 cell differentiation. In line with this, we detected reduced levels of the Th17 cytokine IL-17A in the blood and spinal cord of EHD2-sc-mTNFR2-treated mice.

Next to its immunomodulatory role via Tregs, TNFR2 was shown to act co-stimulatory for TCR-mediated T cell activation, and survival and proliferative expansion of pathogenic Teff cells (Chen et al., 2016; Mehta et al., 2016; Ye et al., 2018), indicating that TNFR2 on Teffs may promote autoimmunity. It was further shown that genetic deletion of monocyte/macrophage-TNFR2 resulted in alleviated EAE motor disease, indicating that TNFR2 on monocytes/macrophages promotes EAE development (Gao et al., 2017). Our study shows that TNFR2 agonist administration at different disease stages does not promote detrimental Teff and/or macrophage activity. In contrast, impaired expansion of peripheral T lymphocytes, reduced infiltration of immune cells into the CNS, alleviated microglia activation, as well as reduced levels of pro-inflammatory cytokines in serum and spinal cord were detected after TNFR2 agonist treatment. This indicates that the TNFR2-mediated beneficial responses, e.g. via Tregs, neurons or glial cells, exceed potential pathogenic effects via Teffs and/or macrophages.

Next to its immunomodulatory role, TNFR2 was shown to induce protective and regenerative responses in the CNS. Particularly, specific activation of TNFR2 rescued neurons (Fischer et al., 2011) and oligodendrocytes (Maier et al., 2013) from oxidative stress-induced cell death and protected primary neurons from glutamate-induced excitotoxicity (Marchetti et al., 2004). Further, TNFR2 signaling promoted neuronal survival in an in vivo model of retinal ischemia (Fontaine et al., 2002). Interestingly, TNFR2-/- mice showed impaired remyelination in the cuprizone model of toxic demyelination and spontaneous remyelination (Arnett et al., 2001), indicating a critical role of TNFR2 for remyelination. Continuing studies demonstrated that astrocyte-TNFR2 contributes to myelination by inducing the secretion of neurotrophic factors that promote oligodendrocyte proliferation and differentiation (Fischer et al., 2014; Patel et al., 2012). Recently, it was demonstrated that oligodendroglial-TNFR2 contributes to remyelination in EAE (Madsen et al., 2016), indicating that intrinsic TNFR2 signaling in oligodendrocytes is also important for remyelination. Our study demonstrates that systemic TNFR2 agonist treatment at onset of pain (6 dpi) and onset of motor deficits (16 dpi) results in significantly reduced motor disease. Treatment at onset of pain (6 dpi) results in a strong reduction of the disease incidence (saline: 92.6%, EHD2-sc-mTNFR2: 28.6%) and delayed treatment after onset of motor deficits (16 dpi) still significantly alleviated motor disease. Histological analysis showed that both treatments (start at 6 dpi and 16 dpi) resulted in reduced demyelination. Interestingly, Tregs were recently shown to promote myelin regeneration in the CNS (Dombrowski et al., 2017). Future analysis will determine, if systemic TNFR2 agonist application may promote the remyelinating potential of Tregs. Further, our data indicate that TNFR2 agonist reaches the CNS after systemic administration and may promote remyelination via local TNFR2 activation on glial cells. Indeed, in vitro studies using TNFR2-OPCs showed that TNFR2 contributes to differentiation but not proliferation (Madsen et al., 2016). Here, we show that exogenous TNFR2 activation via EHD2-sc-mTNFR2 promotes both OPC proliferation as well as differentiation. We further recently demonstrated that local administration of a TNFR2 agonist prevents memory-loss in a mouse model of Alzheimer’s disease (Dong et al., 2016) and other studies have suggested a direct neuroprotective role of TNFR2 (Fischer and Maier, 2015; Orti-Casan et al., 2019; Pegoretti et al., 2018). Altogether, these data outline the high potential of TNFR2 agonists to promote regeneration in the CNS.

Furthermore, we identified an analgesic role of TNFR2, indicating that TNFR2 agonists could impact both sensory and motor impairments in MS patients. It is now recognized that neuropathic pain is a serious concomitant of MS, with approximately 60% of the patients experiencing chronic pain symptoms. Even though the pathophysiology of neuropathic pain in EAE mice and MS patients still needs further investigation, the literature indicates that inflammation, demyelination and neurodegeneration are contributing factors (Murphy et al., 2017). Further, peripheral neuropathy is reported in both in MS patients and EAE mice and is suggested to be involved in neuropathic pain pathology. Many glycoproteins, such as MBP are expressed both in the CNS and PNS, indicating that autoimmunity attacks peripheral nerves (Itoyama et al., 1983; Wang et al., 2017). Indeed, T cell infiltration, glial activation and demyelination in peripheral nerves have been observed in EAE models (Duffy et al., 2016; Thorburn et al., 2016; Wang et al., 2017). TNFR2 agonist treatment at onset of pain alleviated peripheral and central inflammation and reduced demyelination as well as neurodegeneration indicating the possibility of multiple cellular and molecular mechanisms. Interestingly, only treatment starting at onset of pain (6 dpi) resulted in alleviation of CNP, whereas delayed treatment at onset of motor disease (16 dpi) did not impact pain sensitivity. A critical observation was that in contrast to treatment at onset of pain, delayed treatment did not reduce infiltration of pathogenic T cells into the spinal cord. It was previously shown that T cell infiltration into the spinal cord is major contributor to neuropathic pain-like hypersensitivity (Costigan et al., 2009), indicating that abrogation of pathogenic T
cell activity in the spinal cord is critical for the analgesic effect of TNFR2 agonism. Next to its protective effects in the spinal cord, TNFR2 agonism may also impact inflammation and demyelination of peripheral nerves. Therefore, it is possible that potential neuroprotective activities of TNFR2 in the PNS contribute to its analgesic effects. Further, a recent publication has shown that Tregs reduce pain in EAE via alleviation of neuroinflammation (Duffy et al., 2019). This is in line with our observation that TNFR2 agonism expands Tregs and may indicate that Treg-mediated effects contribute to the analgesic effect of EHD2-sc-mTNFR2.

In a model of peripheral nerve-injury mediated neuropathic pain, different immune cells mediated mechanical pain hypersensitivity in male and female mice. Whereas microglia were required for pain development in male mice, adaptive immune cells, like T cells mediated hypersensitivity in females (Sorge et al., 2015). Similar, a recent study proposed that tactile hypersensitivity in female EAE mice may be more immune-driven, whereas pain in male mice with EAE may rely more heavily on neurodegenerative and plasticity-related mechanisms (Catuneanu et al., 2019). This is in line with our data indicating that blocking T cell mediated neuroinflammation is therapeutic on female pain. It is still to be determined, whether a TNFR2 agonist is therapeutic in male EAE mice. Due to the pleiotropic neuroprotective actions of TNFR2, including anti-inflammatory responses via microglia (Gao et al., 2017; Veroni et al., 2010), we hypothesize that exogenous TNFR2 activation alleviates male pain as well.

Despite successful pre-clinical evaluation in rodent models of MS, unspecific anti-TNF therapy failed in clinical trials (Lenercept Study Group, 1999; van Oosten et al., 1996). Since then, subsequent pre-clinical studies demonstrated that only inhibition of sTNF/TNFR1 signaling is therapeutic in EAE (Brambilla et al., 2011; Taoufik et al., 2011; Williams et al., 2018; Williams et al., 2014). Interestingly, the therapeutic effect of sTNF neutralization in EAE were dependent on...
Fig. 6. Exogenous TNFR2 activation alleviates T cell infiltration into the spinal cord. Mice were immunized with MOG emulsified in CFA and treated (T:) with saline or EHD2-sc-mTNFR2 (10 mg/kg) at onset of pain (6, 9, 12 dpi) or onset of motor disease (16, 19 and 22 dpi). Mice were sacrificed and analyzed (A:) 14 dpi or 25 dpi. (A-G) Numbers of infiltrating CD3+ T cells in the lumbar spinal cord were quantified by immunohistochemistry. Shown are (A-F) representative pictures and (G) quantified data presented as number of positive cells per section. (H-N) CNS-infiltrating FoxP3+ Tregs were quantified by immunohistochemistry. (H-M) Black arrows highlight representative FoxP3+ cells. (N) Quantification is shown as the percentage of FoxP3+ Tregs relative to the total number of CNS-infiltrating CD3+ T cells (mean ± SEM, n = 5 per group/treatment). *p < 0.05, ns = not significant.
Fig. 7. Exogenous TNFR2 activation reduces neuroinflammation and demyelination. Mice were immunized with MOG emulsified in CFA and treated (T:) with saline or EHD2-sc-mTNFR2 (10 mg/kg) after onset of pain (6, 9, 12 dpi) or onset of motor disease (16, 19 and 22 dpi). Mice were sacrificed and analyzed (A:) 14 dpi or 25 dpi. (A-G) Neurodegeneration was quantified using APP immunohistochemistry staining. Shown are (A-F) representative pictures and (G) quantified data presented as positive cells per section. (H-N) Demyelination was quantified using Luxol Fast Blue (LFB) staining. Shown are (H-M) representative pictures and (N) quantified data (mean ± SEM, n = 5 per group/treatment) *p < 0.05, **p < 0.01.
presence of oligodendroglial TNFR2 (Madsen et al., 2016). Similar, using a model of Alzheimer’s disease, we recently showed that the therapeutic effect of TNFR1 neutralization is dependent on functional TNFR2 signaling (Dong et al., 2016). These data clearly indicate an essential neuroprotective role of TNFR2. Here, we confirm the safety of systemic TNFR2 activation in the inflammatory EAE model. Altogether our novel data and the published data on the opposing roles of TNFR1 and TNFR2 for neuroinflammation strengthen the development of novel therapeutic approaches to block either sTNF/TNFR1 signaling or to activate TNFR2 signaling and suggest that selective modulation of TNFR signaling has superior clinical potential than conventional anti-TNF drugs.

In summary, our data demonstrate that TNFR2-mediated beneficial responses exceed potential pathogenic responses after exogenous TNFR2 agonist treatment. Importantly, TNFR2 agonists may follow a dual mode of action and induce autoimmune suppression via Tregs, as well as remyelination in the CNS. Our data support the neuroprotective role of TNFR2 and demonstrate the safety profile of TNFR2 agonists in EAE. We propose that TNFR2 agonists have great therapeutic potential for MS, may improve both motor disease and disease associated CNP and therefore advocate for future clinical trial with TNFR2 agonists in MS patients.

Author contributions

R.F., T.P., V.B.R., K.M., G.F.M., N.D., N.E., and M.S. conducted experiments. V.B.R., M.S., R.D., R.J.S., U.L.M.E., R.E.K. and K.P. designed parts of the experimental work, analyzed results and contributed to writing the manuscript. R.F. and J.R.B. designed and supervised the project, analyzed the results and wrote the manuscript.

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Competing Interest

R.F., V.B.R. and J.R.B. are named inventors on patent applications covering the use of TNFR2 agonists for neuropathic pain. R.F., R.E.K. and K.P. are named inventors on patent applications covering the TNFR2 agonists technology.

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