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Tumor necrosis factor receptor 2 activation elicits sex-specific effects on cortical myelin proteins and functional recovery in a model of multiple sclerosis

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Abstract

Multiple sclerosis (MS), a demyelinating autoimmune disease of the central nervous system (CNS), predominately affects females compared to males. Tumor necrosis factor (TNF), a pro-inflammatory cytokine, signaling through TNF receptor 1 contributes to inflammatory disease pathogenesis. In contrast, TNF receptor 2 signaling is neuroprotective. Current anti-TNF MS therapies are shown to be detrimental to patients due to pleiotropic effects on both pro- and anti-inflammatory functions. Using a non-pertussis toxin (nPTX) experimental autoimmune encephalomyelitis (EAE) model in C57BL/6 mice, we systemically administered a TNFR2 agonist (p53-sc-mTNF$_{R2}$) to investigate behavioral and pathophysiological changes in both female and male mice. Our data shows that TNFR2 activation alleviates motor and sensory symptoms in females. However, in males, the agonist only alleviates sensory symptoms and not motor. nPTX EAE induction in TNFR2 global knockout mice caused exacerbated motor symptoms in females along with an earlier day of onset, but not in males. Our data demonstrates that TNFR2 agonist efficacy is sex-specific for alleviation of motor symptoms, however, it effectively reduces mechanical hypersensitivity in both females and males. Altogether, these data support

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CRediT authorship contribution statement

Kayla L. Nguyen: Writing – original draft/reviewing & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ishaan J. Bhatt:** Data curation. **Shruti Gupta:** Writing – review & editing, Data curation. **Nazaf Showkat:** Data curation. **Roman Fischer:** Writing – review & editing, Resources. **Roland E. Konter-mann:** Writing – review & editing, Resources. **Klaus Pfizenmaier:** Writing – review & editing, Resources. **Valerie Bracchi-Ricard:** Writing – review & editing, Supervision, Methodology, Conceptualization. **John R. Bethea:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

None to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2024.110885.

the therapeutic promise TNFR2 agonism holds as an MS therapeutic and, more broadly, to treat central neuropathic pain.

Keywords

Multiple sclerosis (MS); Experimental autoimmune encephalomyelitis (EAE); Sex differences; Tumor necrosis factor (TNF); TNF receptor 2 (TNFR2)

1. Introduction

Tumor necrosis factor alpha (TNF) is a pleiotropic cytokine that can exert its effects on most cell types. In the central nervous system (CNS), TNF is essential in maintaining controlled numbers of synapses through synaptic pruning and scaling under normal physiological conditions (Steinmetz and Turrigiano, 2010; Stellwagen and Malenka, 2006). However, in disease and injury TNF is detrimental to synaptic survival by exacerbating inflammation (Mandolesi et al., 2015). TNF is recognized as a critical pro-inflammatory mediator and dysregulation of TNF signaling contributes to pathogenesis of inflammatory diseases including rheumatoid arthritis, Crohn's disease, and multiple sclerosis (MS) (Monaco et al., 2015). Specifically, TNF can activate microglia, astrocytes, and recruit B cells which, once activated, produce further TNF that results in myelin, oligodendrocyte, and axonal damage (Steinman and Zamvil, 2003).

Previously, anti-TNF therapeutics (e.g. infliximab, etanercept, and adalimumab) were developed and clinically approved to treat inflammatory and autoimmune conditions (Fischer et al., 2019a; Fromont et al., 2009; Monaco et al., 2015). While these therapeutics are highly effective with short-term use, extended administration is shown to leave patients vulnerable to increased incidences of infection or the development of secondary demyelinating diseases (Fischer et al., 2019a, 2015; Monaco et al., 2015). In MS patients, treatment with the anti-TNF therapeutic Lenercept was shown to significantly worsen symptoms compared to placebo treatments (TNF, 1999). These detrimental sides effects associated with anti-therapeutics can be attribute to their non-selective nature targeting both pro-inflammatory and neuroprotective functions of TNF (Sedger and McDermott, 2014; Wajant and Siegmund, 2019; Yang et al., 2019).

TNF exists in two biologically active forms. TNF receptor 1 (TNFR1) binds both soluble TNF (solTNF) and transmembrane TNF (tmTNF). TNF receptor 2 (TNFR2) preferentially binds tmTNF creating a stable ligand-receptor complex, whereas solTNF binding to TNFR2 leads to a transient unstable complex (Fischer et al., 2019a; Grell et al., 1995; Horiuchi et al., 2010). TNFR1 is expressed ubiquitously on most cell types, but TNFR2 expression is more restricted and found on cell types such as myeloid cells, B cell subsets, regulatory T cells (Tregs), glial cells, and fibroblasts (Medler and Wajant, 2019; Wajant and Siegmund, 2019). In the CNS, TNFR1 activation is associated with pathological TNF function, such as inflammation and tissue degeneration, whereas, TNFR2 activation is linked to neuroprotection, immune suppression, and remyelination (Bradley, 2008; Fischer et al., 2019b; Probert, 2015).

In neuroinflammatory disease models in mice, selective activation of TNFR2 is shown to ameliorate neuropathology, improve cognitive function, and promote Treg accumulation in the CNS (Ortí-Casañ et al., 2023, 2022; Pegoretti et al., 2023). More specifically, our lab has previously shown that selective activation of TNFR2 using an agonist reduced motor symptoms in a mouse model of MS, experimental autoimmune encephalomyelitis (EAE), using female mice (Fischer et al., 2019a). In addition, activation of TNFR2 alleviated chronic neuropathic pain (CNP), a common symptom of various autoimmune disorders and injury, in both sexes following a peripheral nerve injury (Fischer et al., 2019a, 2019b). These data, together with genetic studies demonstrating exacerbated EAE progression in female TNFR2$^{-/-}$ mice (Suvannavejh et al., 2000; Williams et al., 2014), suggest a novel mechanism for treatment of EAE/MS. For this study we used a non-pertussis toxin EAE (nPTX EAE) induction method, as previously used in (Murphy et al., 2020), since studies have shown that pertussis toxin alone reduces regulatory T-cell numbers and inhibits G protein-coupled receptor signaling (Cassan et al., 2006; Mangmool and Kurose, 2011). In addition, PTX in EAE has been shown to mask presentation of cortical pathology and override genetic checkpoints associated with disease etiology/progression in a sex specific manner (Blankenhorn et al., 2000). Therefore, we sought to dissect the efficacy of the next generation p53-sc- mTNF$_{R2}$ TNFR2 agonist using a nPTX EAE model on motor and sensory symptoms in female and male mice.

2. Methods

2.1. Mice

Adult wild-type/naïve (WT) C57BL/6 J (#000664) and TNFR2$^{-/-}$(homozygous; B6.129S2-Tnfrsf1b^{tm1Mwm}/J; #002620) female and male mice (10–12 weeks old) were purchased from The Jackson Laboratory. Littermate controls were used for genetic studies. Mice were acclimated to the animal housing facility for 1 week prior to experiments. Mice were housed in groups of 5 under 12 h day/night cycling and given access to water and food ad libitum. All animal-use experiments were approved by Drexel University's Institutional Animal Care and Use Committee (IACUC) under protocol #20627.

2.2. nPTX EAE induction and pharmacological treatment

Mice were injected with a subcutaneous immunization of an emulsion containing 100 μg of MOG$_{35–55}$ peptide and complete Freund's adjuvant (CFA; InvivoGen, San Diego, California) supplemented with 200 μg of heat-inactivated *Mycobacterium tuberculosis* H37Ra in CFA as previously described (Krementsov et al., 2014; Murphy et al., 2020). Injections were given in the posterior right and left flanks 50 μL each. Mice received a booster containing the same emulsion 1 week following the first injection. Drugs [vehicle (saline); TNFR2 agonist (p53-sc-mTNF$_{R2}$ (Fischer et al., 2017); 10 mg/kg body weight)] were administered intraperitoneally (i.p.) at 10-, 13, and 16-days post immunization (DPI). For the delayed treatment study in males, drugs were administered at 16, 19, and 22 DPI.

2.3. Motor scoring

Mice were evaluated on a daily basis for both body weight changes and signs of motor deficits using the following scale: 0 – no motor deficits, 1 – loss of tail tone, 2 – fully flaccid

tail, 3 – hind-limb paralysis, 4 – forelimb paralysis, 5 – moribund, 6 – death (Brambilla et al., 2011; Murphy et al., 2020).

2.4. Pain assessment (von Frey)

Von Frey testing was used to assess mechanical hypersensitivity as previously described (Dellarole et al., 2014; Murphy et al., 2020). Monofilaments were briefly applied to the hindpaw of each mouse to assess sensitivity to a mechanical stimulus. Each mouse was placed in an individual clear plexiglass chamber elevated on a mesh-wire platform and allowed to acclimate for 30 min. The von Frey hair filaments were applied to the plantar surface of each hindpaw using the up-down method of analysis as previously described (Chaplan et al., 1994). If no reaction was elicited by the administered monofilament, one of slightly greater diameter is administered. This is done for a series of 5 trials following the first positive pain response. Paw withdrawal threshold is then measured in grams (g) of force and was only considered a pain response of paired with one or more of the following actions, as previously described, indicative of cognitive awareness of the stimulus as being painful: looking at the hindpaw, stimulus avoidance behavior, tucking the tail under stimulated hindpaw, and/or licking the stimulated hindpaw (Murphy et al., 2019). A week prior to EAE induction, mice were habituated to the von Frey apparatus for 1 h and baseline testing was performed.

2.5. Whole tissue protein extract

EAE mice were deeply anesthetized at 30 dpi with Ketamine (215 mg/kg) and Xylazine (43 mg/kg) and subsequently perfused with phosphate buffered saline (PBS). Sensorimotor cortex and whole lumbar spinal cord (SC) were extracted and immediately stored on dry ice. Tissue was then homogenized in RIPA buffer (10 mM sodium phosphate buffer pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Santa Cruz, Dallas, TX) and phosphatase inhibitors (Biovision, Milpitas, CA) and incubated at 4 °C for 30 min on a rocker. The samples were then centrifuged at 4 °C for 15 min at 13,200RPM. The resulting supernatant was transferred to a separate tube from which protein concentrations were determined using the DC™ protein assay (Biorad, Hercules, CA).

2.6. Western blotting

Protein extracts were resolved using sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 8–15% gels, transferred to nitrocellulose (Turbo blot, Biorad), and blocked for 2 h in 5% bovine serum albumin (BSA) in 1x TBS-T (10 mM tris-HCL, pH7.5, 150 mM NaCl, 0.1% Tween-20). The following primary antibodies were diluted in blocking solution and incubated at 4 °C overnight: tumor necrosis factor receptor 1 (TNFR1, mouse, 1:1000 Santa Cruz); tumor necrosis factor receptor 2 (TNFR2, rabbit, 1:1000, ProteinTech, Rosemont, IL); myelin basic protein (MBP, rat, 1:15,000, Millipore, Burlington, MA); 2’3’ cyclic nucleotide 3’ phosphodiesterase (CNPase, rabbit, 1:1000, Cell Signaling, Danvers, MA). Primary antibody incubation was followed by horseradish peroxidase-conjugated species-specific secondary antibodies. Proteins were visualized with enhanced chemiluminescence (ECL, Pierce, Waltham, MA) and band intensity was quantified using Quantity One software (Biorad). Data were normalized for each sample to

the total amount of protein loaded via visualization with Ponceau S solution (Sigma) and expressed as percent of naïve group as previously described (Murphy et al., 2020).

2.7. Immunohistochemistry (IHC)

Animals were perfused using 4% paraformaldehyde in 0.1 M phosphate-buffered saline at 30 dpi. The brain and lumbar part of the SC were cryoprotected in 15% sucrose in PBS. Tissue samples were embedded in O.C.T. (Optimal cutting temperature, Tissue-Tek®) and frozen. Frozen serial sections (20 μm thick) were cut using on a cryostat with 5 sections per slide and later stored at – 20 °C. Prior to staining, slides were acclimated to room temperature (RT) for 10 mins, then placed in ice cold acetone for 3 mins followed by ice cold methanol for 7 mins. A pap pen was used to create a hydrophobic barrier around the tissue sections and sections were covered in 0.1% Triton-X100 (Tx) in diH_2O for 10 mins at RT. Sections were then blocked in 1% BSA/1% normal goat serum (NGS)/0.3% Tx-PBS for 45 mins. Sections were incubated in the following primary antibodies diluted in blocking buffer overnight at 4 °C: TMEM10/Opalin (1:500 guinea pig, Synaptic Systems); MBP (1:100, rat, Millipore). The following day, sections were washed 3 times for 5 min each in 1x PBS. Secondary antibodies (Invitrogen) were added at 1:500 in 1% NGS/PBS for 1 h at RT as follows: Alexa488 and Alexa594. Washes in 1x PBS were repeated and Hoechst diluted at 1:10,000 was added for 10 mins at RT. Slides underwent another set of washes and were then mounted with coverslips (Electron Microscopy, Fluoro-Gel with TES Buffer).

2.8. Statistical analysis

All analyses were performed using GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, California USA). Statistical analysis of EAE motor scores were conducted using two-way ANOVA and per day analysis using post-hoc Bonferroni's test. Cumulative disease index was analyzed as area under the curve followed by a Mann-Whitney test. Two-way ANOVA was used to assess changes in withdrawal thresholds. Western blots were analyzed using one-way ANOVA and post-hoc Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant. All data where a significant effect was seen following drug treatment based on sex or CNS location we performed a two-way ANOVA with a 2 factorial design to quantitatively derive the interactions between Sex and Treatment or Genotype.

3. Results

3.1. TNFR2 activation delays and reduces motor symptoms in a sexually dimorphic manner

Motor impairments, such as deficits in gait and balance, and chronic neuropathic pain (CNP) are some of the more common symptoms in MS (Murphy et al., 2017; Wajda and Sosnoff, 2015). Despite MS predominately afflicting females, over 60% of those that suffer from this disease experience CNP regardless of sex (Murphy et al., 2020, 2017; O'Connor et al., 2008). These are phenotypically recapitulated in EAE as ascending paralysis, characterized by impaired gait, and mechanical hypersensitivity (Khan and Smith, 2014; Murphy et al., 2020). The role of soluble TNF (sTNF)/TNFR1 signaling was previously shown to influence severity of MS and EAE (Eugster et al., 1999). Inhibition of sTNF in EAE reduces severity

of motor symptoms through preservation of axons and remyelination in the spinal cord (Brambilla et al., 2011). However, we have previously demonstrated in a model of peripheral nerve injury that sTNF inhibition does not alleviate pain in females (del Rivero et al., 2019). In contrast, TNFR2 activation promotes recovery from both motor and sensory deficits in female EAE (Fischer et al., 2019a). Since TNFR2 is essential for tissue regeneration and neuroprotection, we sought to first determine if expression differs between females and males in the cortex and lumbar spinal cord which undergo pathological changes critical to pain and motor symptoms in MS/EAE (Murphy et al., 2020; Potter et al., 2016). Under naïve conditions lacking disease or injury, females and males display comparable TNFR2 expression in both the cortex and lumbar spinal cord (Fig. 1A,B).

Following our determination that baseline TNFR2 expression in our regions of interest are similar between sexes prior to disease, we investigated the role of TNFR2 activation in EAE motor and sensory impairments with sex specificity using p53-sc-mTNF$_{R2}$ (a TNFR2 agonist). The p53-sc- mTNF$_{R2}$ agonist is a tetrameric structure of covalently stabilized scTNF$_{R2}$ oligomerized to a p53 domain. The p53-scmTNF$_{R2}$ agonist, which directly binds to TNFR2s, presents with greater oligomerization and improved specific activity to that of its EHD2-sc- mTNF$_{R2}$ predecessor as described by Fischer et al. (Fischer et al., 2017). Systemic administration of the agonist at 10 mg/kg body weight was delivered at 10, 13, and 16 DPI to correspond with the onset of EAE-induced mechanical hypersensitivity as we previously established in Murphy et al., 2020 and at the same dose used for previous EAE studies in the lab using the EHD2-sc- mTNF$_{R2}$ agonist (Fischer et al., 2019a; Murphy et al., 2020). While mechanical hypersensitivity was alleviated in both females and males treated with p53-sc- mTNF$_{R2}$ (Fig. 2A,B), motor symptoms and cumulative disease severity were reduced in females only (Fig. 3A,B, Table 1, Supplemental Table 3). Furthermore, p53-sc-mTNF$_{R2}$ treated females developed motor symptoms significantly later compared to vehicle treated controls (Table 1). Together, these data demonstrate that p53-sc- mTNF$_{R2}$ reduces mechanical hypersensitivity in both sexes; however, only females experience attenuated severity and delayed onset for motor symptoms.

3.2. Delayed treatment in males causes loss of therapeutic efficacy for mechanical hypersensitivity

Our previous experiments (Murphy et al., 2020), as well as our findings in Table 1, have established that males develop motor symptoms significantly later than females. Therefore, we sought to determine if the inability of p53-sc-mTNF$_{R2}$ to reduce male motor symptoms was due to not administering drugs closer to the motor disease onset. Thus, we repeated the experiment in EAE administering p53-sc-mTNF$_{R2}$ at 16, 19, and 22 DPI in males. Not only did p53-sc-mTNF$_{R2}$ remain ineffective in treating motor symptoms, but efficacy was also lost for mechanical hypersensitivity (Fig. 4A,B). These data indicate that the window for TNFR2 activation is critical to therapeutic efficacy in CNP but may be limited to a treatment window that is prior to onset of motor disease.

3.3. Motor symptoms are more severe in TNFR2$^{-/-}$ females

Increased severity of motor symptoms in classical EAE are known to be exacerbated in female TNFR2$^{-/-}$ mice (Suvannavejh et al., 2000; Williams et al., 2014). To determine if

TNFR2 activity is sexually dimorphic in its control of motor symptoms, we used a genetic approach to corroborate our p53-sc-mTNF$_{R2}$ pharmacological findings. We induced EAE in both female and male TNFR2$^{-/-}$ mice and calculated the cumulative disease index (CDI) to assess severity and day of onset for motor symptoms. Female EAE TNFR2$^{-/-}$ mice presented with both earlier onset and increased severity of motor symptoms compared to EAE WT females (Fig. 5A; Supplemental Table 1). However, there were no significant difference in motor disease between TNFR2$^{-/-}$ and WT EAE males (Fig. 5B; Supplemental Table 2). These data demonstrate that, together, global knockout of TNFR2 and the severity of EAE are sex dependent (Supplemental Table 4). In addition, mechanical hypersensitivity remained prevalent and equivalent between EAE TNFR2$^{-/-}$ and EAE WT mice for both sexes (Fig. 5C,D). Overall, global TNFR2 is critically involved in severity of female motor disease.

3.4. TNFR2 activation is associated with a more robust expression of myelin associated proteins

Demyelination of the cortex and lumbar SC due to inflammation are critically linked to cognitive impairment and motor disease progression in MS (Lucchinetti et al., 2011; Popescu et al., 2011). To determine if p53-sc-mTNF$_{R2}$ protects myelin integrity or promotes remyelination, we measure changes in the cortex and lumbar SC for MBP and CNPase using western blot analysis, integral structural myelin proteins that are reduced during demyelination EAE (Murphy et al., 2020). In the SC, MBP expression in females and males is reduced in both vehicle and p53-sc-mTNF$_{R2}$ treated EAE mice (Fig. 6A,B,E,F). In addition, CNPase is reduced in the male SC; however, no changes are observed in females (Fig. 6C,D). In the female cortex, there is a more robust expression for both MBP and CNPase in p53-sc-mTNF$_{R2}$ treated mice (Fig. 7A,C,E); however, in males there are no changes observed in cortical MBP while CNPase remains decreased (Fig. 7B,D,F). The effect of p53-sc-mTNF$_{R2}$ treatment on CNPase expression was sex-dependent (Supplemental Table 5). These data indicate that in both sexes, myelin associated proteins in the lumbar spinal cord remain reduced following EAE regardless of treatment group. However, female cortical expression of these proteins is more robust with no effect in males.

3.5. Cortical microglial activation is reduced in both sexes following TNFR2 activation

Reactive microglia and macrophages in MS are known to contribute to chronic inflammation through overproduction of glutamate, proteases, and inflammatory cytokines (Haase and Linker, 2021; Kamma et al., 2022; Takeuchi et al., 2006). Chronic inflammation is known to contribute to demyelination and cell death among myelin producing cells, oligodendrocytes (Cudrici et al., 2006; Kalafatakis and Karagogeos, 2021). In previous studies, we observed an increase in cortical and SC microglial/macrophage activation (Murphy et al., 2020). To determine if p53-sc-mTNF$_{R2}$ reduces microglial/macrophage activation, we measured IBA1 expression. We found that while SC IBA1 expression remains upregulated in both sexes, cortical IBA1 is significantly reduced in p53-sc-mTNF$_{R2}$ treated females and males (Fig. 8A–D, Supplemental Table 6). These data indicated that only TNFR2 activation selectively attenuates cortical, but not spinal microglial activity without sex specificity.

4. Discussion

The role of TNF as a critical mediator of neuroinflammation associated with disease and injury is one that is well established (Bradley, 2008; Monaco et al., 2015; Probert, 2015; Wang and Shuaib, 2002). In pathological settings, TNF signaling can have oppositional roles depending on which TNF ligand is binding to its appropriate receptor. sTNF/TNFR1 signaling is associated with the deleterious effects of TNF activity while tmTNF/TNFR2 signaling is neuroprotective and immunosuppressive (Brambilla et al., 2011; del Rivero et al., 2019; Dellarole et al., 2014; Desu et al., 2021; Fischer et al., 2019b, 2019a; Gao et al., 2017; Horiuchi et al., 2010; Probert, 2015; Sedger and McDermott, 2014; Wajant and Siegmund, 2019; Yang et al., 2019, 2018). While previous work has demonstrated that sTNF/TNFR1 inhibition reduces CNP selectively in males, the potential for sex specificity in tmTNF/TNFR2 signaling remains unclear. We demonstrate that severity of motor disease in females, but not males is reduced following TNFR2 activation. Furthermore, either elevated or maintained cortical presence of myelin associated proteins may, in part, contribute to dampened female motor symptoms. However, TNFR2 activation alleviates CNP in both sexes which may be a result of reduced cortical microglial activity. Altogether, these data indicate that TNFR2 activation is a promising therapeutic target through which CNP can be alleviated regardless of sex while also reducing motor symptoms among the predominant patient population, females.

4.1. Therapeutic efficacy of TNFR2 activation is critical in female EAE motor disease and promotes maintenance/integrity of the myelin sheath

Impaired locomotor function is linked to underlying demyelination pathology in both MS and EAE (Bjartmar and Trapp, 2001; Compston and Coles, 2008; Murphy et al., 2017). Chronic CNS inflammation consequentially leads to oligodendrocyte death and loss of this cell population is attributed to progressive loss of remyelinating capability among patients (Cudrici et al., 2006; Kalafatakis and Karagogeos, 2021; Ruffini et al., 2004). tmTNF/TNFR2 signaling on oligodendrocytes is critical in promoting oligodendrocyte differentiation and remyelination (Desu et al., 2021; Madsen et al., 2016). In both EAE and a cuprizone model of demyelination, TNFR2 signaling is shown to elicit regenerative responses in the CNS via OPC proliferation and remyelination (Arnett et al., 2001; Gao et al., 2017). Our data demonstrate that activation of TNFR2 pathways via p53-sc-mTNF$_{R2}$ are critical to female-specific amelioration of motor symptoms. This is achieved, in part, by TNFR2 activation promoting cortical myelin integrity/production. However, underlying mechanisms still need further investigation.

In contrast, enhanced demyelination and cytokine production are known to occur in TNFR2 deficient females following EAE induction (Eugster et al., 1999; Suvannavejh et al., 2000). When TNFR2 is ablated on oligodendrocytes in female mice, EAE motor disease occurs earlier with higher peak scores (Madsen et al., 2020). Similarly, our findings demonstrate genetic ablation of TNFR2 leads to earlier and more severe motor in females; however, our data also indicate that there are no changes in male disease progression. These data support, in a converse manner, the p53-sc- mTNF$_{R2}$ agonistic behavioral results; however, the caveat

remains that our TNFR2 deficient mice are from a germline knockout which may have unforeseen developmental consequences at the molecular level.

4.2. TNFR2 activation attenuates CNP and cortical microglial/macrophage activity in both females and males

CNP afflicts 50–86% of female and male MS patients dramatically decreasing quality of life and this pain is reflected in our previously described nPTX EAE paradigm (Krementsov et al., 2014; Murphy et al., 2020, 2017; Truini et al., 2013). Antidepressants, anti-inflammatories, anticonvulsants, or cannabinoids are often prescribed to treat MS-induced pain; however, there is no treatment that provides more than 50% of pain relief (Khan and Smith, 2014; Murphy et al., 2017; Racke et al., 2022). Previous studies demonstrate that TNFR2 agonism suppresses CNS autoimmunity in EAE and phenotypically reduces mechanical hypersensitivity and motor symptoms (Fischer et al., 2019a). Specifically, TNFR2 agonism promotes peripheral Treg expansion, which reduces neuroinflammation and immune cell infiltration through autoimmune suppression (Fischer et al., 2019a). Our data indicates that TNFR2 agonism significantly attenuates CNP regardless of sex when administered at onset of EAE-induced pain. However, there is a limited therapeutic window during which TNFR2 activation can exert this effect.

Chronic inflammation, characteristic of both MS and EAE, contributes to both the initiation and maintenance of CNP (Ellis and Bennett, 2013; Haase and Linker, 2021). In the CNS, microglia are key regulators of CNP and neuroinflammation and are known to advance the pathogenesis of MS (Chen et al., 2018; Crotti and Ransohoff, 2016). In a model of peripheral nerve injury, microglial ablation prevents CNP development (Inoue and Tsuda, 2018; Liu et al., 2017). Despite an abundance of literature establishing a pertinent role for spinal microglia in CNP, only recently has evidence begun to accumulate that supraspinal microglia become activated following injury despite their distance from the injury site (Inoue and Tsuda, 2018; Liu et al., 2017; Ni et al., 2016; Taylor et al., 2017). We previously established that both spinal and cortical microglial/macrophage activation is present at peak motor disease in nPTX EAE, as indicated by increased IBA1 expression (Murphy et al., 2020). Our current findings indicate that following treatment with p53-sc-mTNF$_{R2}$ cortical, not spinal, microglial/macrophage activity is selectively attenuated in both sexes. Activation of microglial TNFR2 is known to promote anti-inflammatory and neuroprotective processes (Gao et al., 2017; Veroni et al., 2010). Reduction of cortical microglial/macrophage activity regardless of sex may be correlative with observed CNP attenuation following TNFR2 agonism. However, further studies are needed to determine if this correlative change is mechanistically causative.

5. Conclusions

This study is the first to demonstrate that TNFR2 agonism via p53-scmTNF$_{R2}$ effectively alleviates nPTX EAE-induced CNP in both females and males; however, amelioration of motor symptom sex specific to females (Table 2). Our lab has previously demonstrated that TNFR2 agonist administration, following classical EAE induction, attenuates both sensory and motor symptoms in female mice (Fischer et al., 2019a). Together, these data

suggest that TNFR2 agonism is neuroprotective in a neuro-compromised environment by promoting myelin integrity and dampening cortical microglial/macrophage activity (Table 2). The majority of pain management options are often ineffective among MS patients and for females as a whole (Loyd and Murphy, 2014; Murphy et al., 2017; Packiasabapathy and Sadhasivam, 2018; Walker and Carmody, 1998); however, our findings demonstrate the potential for TNFR2 agonism as a therapeutic avenue through which we can effectively alleviate pain without sex-specific efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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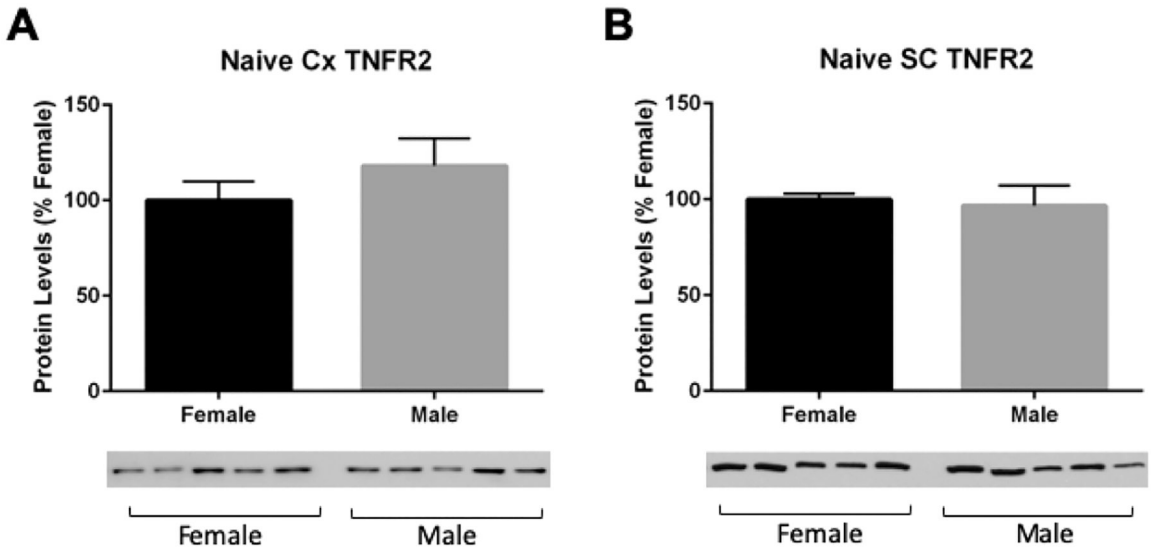

Fig. 1.

TNFR2 expression does not differ between female and male cortex and lumbar spinal cord. Western blot analysis comparing (A) cortical and (B) lumbar spinal TNFR2 expression in naïve WT female and male mice (n = 5 per group; Data represent mean ± SEM).

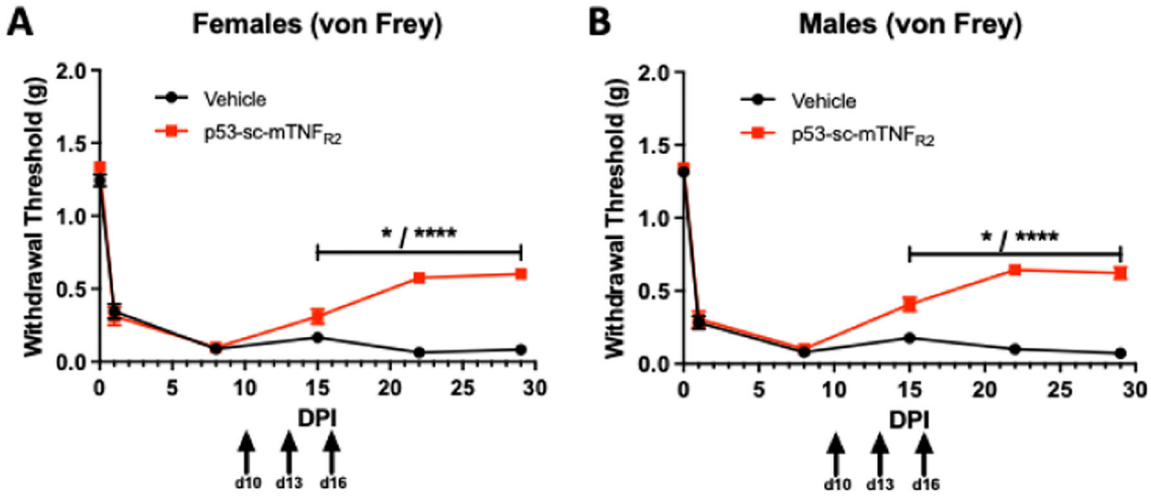

Fig. 2.

TNFR2 agonism alleviates sensory deficits in both females and males. p53-sc-mTNF$_{R2}$ treated females (A) and males (B) demonstrate significantly elevated hindpaw withdrawal thresholds compared to vehicle treated controls (Female vehicle n = 20, p53-sc-mTNF$_{R2}$ n = 20; Male vehicle n = 20, p53-sc-mTNF$_{R2}$ n = 20; *p < 0.05, ****p < 0.0001; Data represent mean ± SEM).

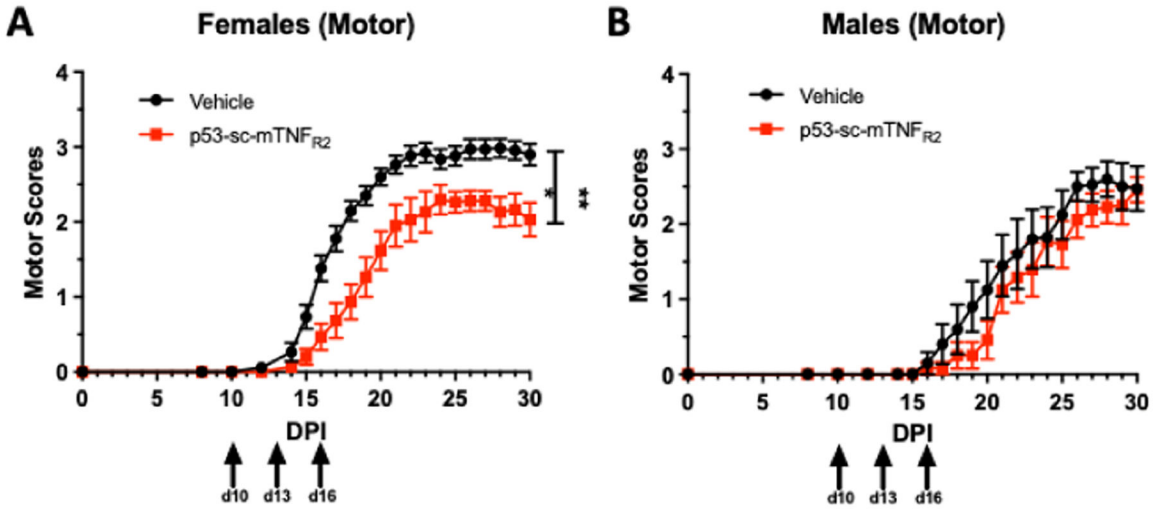

Fig. 3.

TNFR2 agonism selectively reduces EAE motor deficits in females and not males. (A) p53-sc-mTNF$_{R2}$ administered at 10, 13, and 16 DPI (black arrows) significantly reduces female motor scores compared to vehicle treated controls. (B) There are no differences in male motor scores between vehicle or p53-sc-mTNF$_{R2}$ treated groups (Female vehicle n = 17, p53-sc-mTNF$_{R2}$ n = 15; Male vehicle n = 10, p53-sc-mTNF$_{R2}$ n = 12; ***p < 0.001; Data represent mean ± SEM).

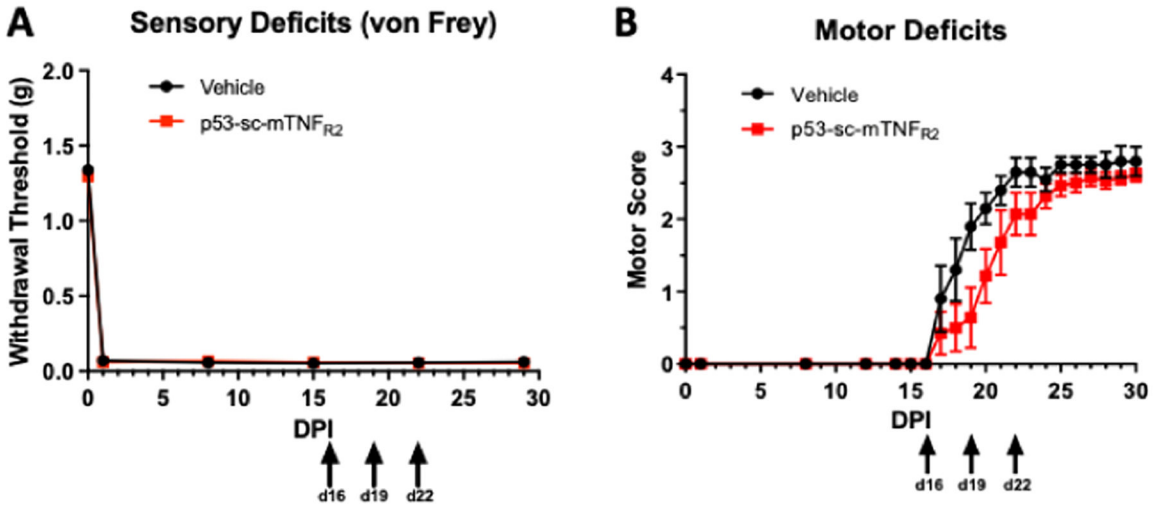

Fig. 4.

Delayed TNFR2 agonist causes loss of therapeutic efficacy for sensory deficits and provides no functional motor recovery in males. (A) Withdrawal thresholds are similarly reduced in both vehicle and p53-sc-mTNF$_{R2}$ treated males. (B) Motor deficits progress with the same severity in both p53-sc-mTNF$_{R2}$ treated males (Sensory vehicle n = 10, p53-sc-mTNF$_{R2}$ n = 10; Motor vehicle n = 5, p53-sc-mTNF$_{R2}$ n = 7; Data represent mean ± SEM).

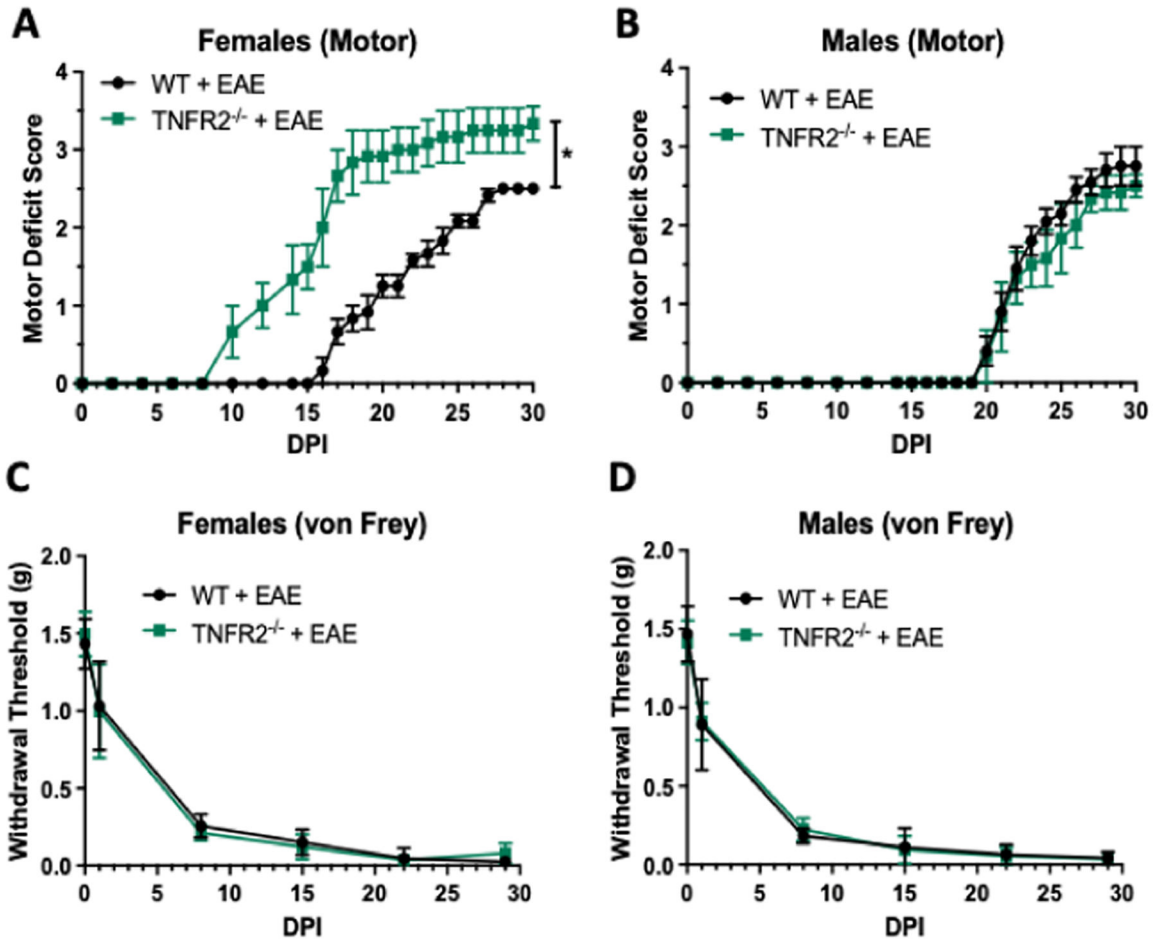

Fig. 5.

Female TNFR2$^{-/-}$ EAE mice have increased severity of motor deficits, but sensory deficits remain prevalent in both sexes. (A) Female TNFR2$^{-/-}$ mice develop significantly more severe motor deficits compared to EAE WT controls. (B) There are no significant differences in motor deficit severity between male TNFR2$^{-/-}$ mice and WT controls. There are no significant differences in withdrawal thresholds following EAE induction in (C) female or (D) male TNFR2$^{-/-}$ mice compared to WT controls (Females WT n = 5, TNFR2$^{-/-}$ n = 5; Males WT n = 5, TNFR2$^{-/-}$ n = 5; *p < 0.05; Data represent mean ± SEM).

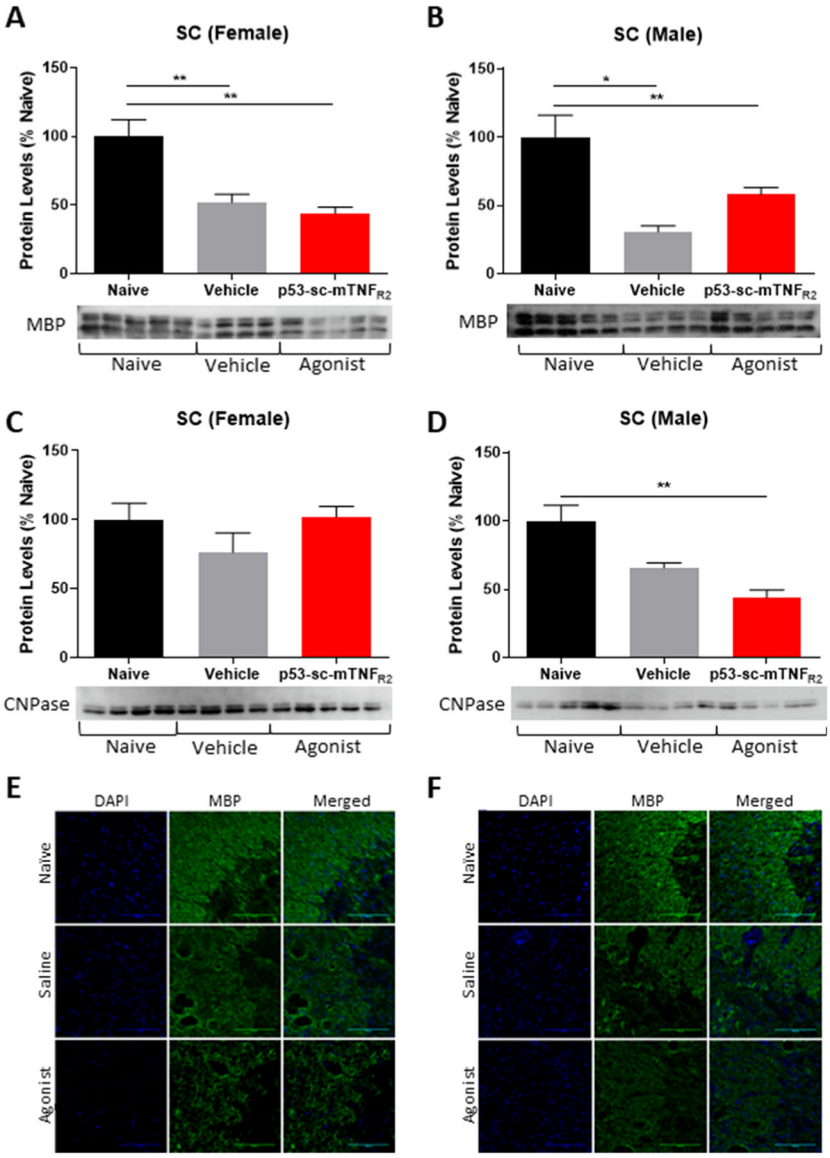

Fig. 6.

Myelin associated proteins remain reduced in the lumbar spinal cord (SC). Western blot analysis of MBP in the (A) female and (B) male lumbar cord shows reduced protein expression in vehicle and p53-sc-mTNF$_{R2}$ treated mice compared to naïve controls. Analysis of CNPase in the lumbar cord shows no changes in (C) females; however, CNPase is reduced in the (D) p53-sc-mTNF$_{R2}$ treated males compared to naïve controls (Naïve n = 4/5, vehicle n = 4, p53-sc-mTNF$_{R2}$ n = 5; *$p < 0.05$, **$p < 0.01$; Data represent mean ± SEM). Representative histological sections stained for nuclei with Hoechst (blue) and MBP (GFP) showing reduced signaling in the (E) female and (F) male ventral lumbar (L3/L4) spinal cord in both saline and p53-sc-mTNF$_{R2}$ groups (Scale Bar = 100 μm; n = 3/group).

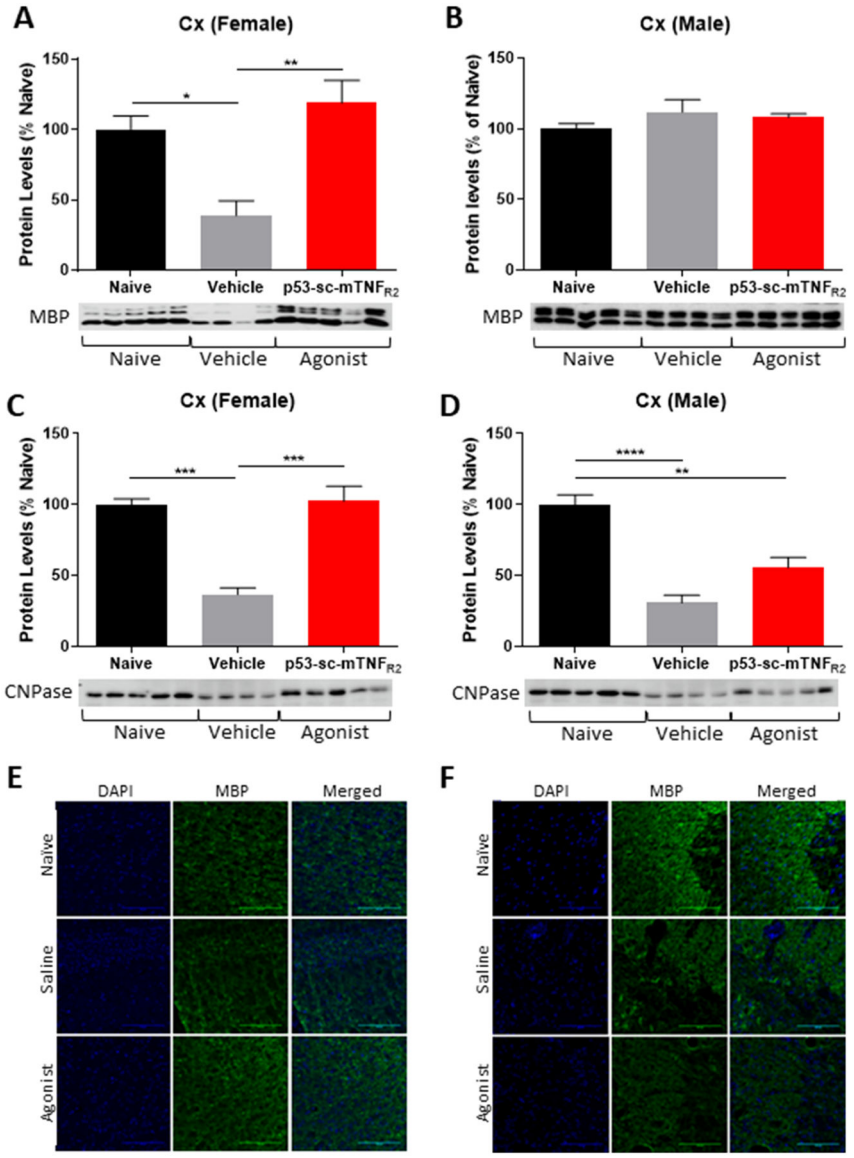

Fig. 7.

Myelin associated proteins are elevated in females following TNFR2 agonism, but not in males. Western blot analysis of cortical (Cx) MBP shows significantly elevated protein expression in p53-sc- $mTNF_{R2}$ treated (A) females compared to vehicle treated controls, but no changes are observed in (B) males. Analysis of cortical (Cx) CNPase shows significantly elevated protein expression in p53-sc- $mTNF_{R2}$ treated (C) females compared to vehicle treated controls, but remains significantly reduced in (D) males (Naïve n = 5, vehicle n = 4, p53-sc-$mTNF_{R2}$ n = 5; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Data represent mean ± SEM). Representative histological sections stained for nuclei with Hoechst (blue) and MBP (GFP) showing more robust signaling in the (E) female somatosensory cortex p53-sc- $mTNF_{R2}$ group and (F) no differences for males (Scale Bar = 100 μm; n = 3/group).

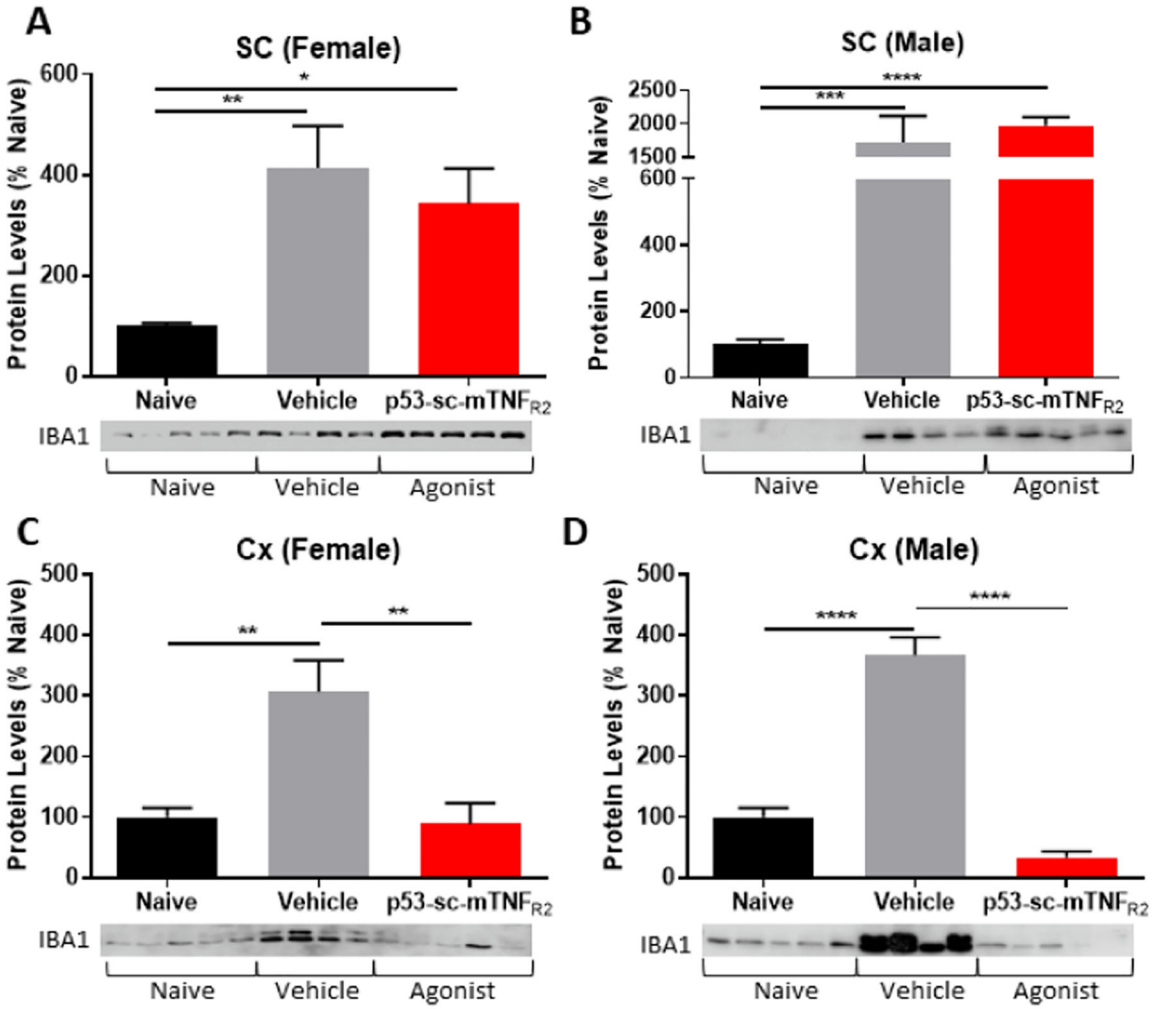

Fig. 8.

Cortical microglial activity is reduced following TNFR2 agonism in both sexes. Western blot analysis of IBA1 in the lumbar spinal cord (SC) shows significantly elevated protein expression in both vehicle and p53-sc-mTNF$_{R2}$ treated (A) females and (B) males. However, cortical IBA1 is significantly reduced in both (C) female and (D) male p53-sc-mTNF$_{R2}$ mice compared to vehicle treated controls (Naïve n = 5, vehicle n = 4, p53-sc-mTNF$_{R2}$ n = 5; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Data represent mean ± SEM).

Table 1

Clinical parameters of TNFR2 agonism in EAE females indicate reduced cumulative motor disease and delayed onset. The day of motor deficit onset was defined as the first day when a mouse scored at least 2 for two consecutive days. The cumulative disease index (CDI), which was calculated as the sum of clinical scores between days 10 and 30, represents a measure for disease severity over time.

	Females			**Males**		
	Vehicle	**p53-sc-mTNF$_{R2}$**	**Sign.**	**Vehicle**	**p53-sc-mTNF$_{R2}$**	**Sign.**
CDI	40.25	26.25	* **p < 0.001	20.88	21.88	n.s.
	n = 17	n = 15		n = 10	n = 12	
Day of Onset	15.00	18.00	* *p < 0.01	19.50	21.00	n.s.
	n = 17	n = 15		n = 10	n = 12	

Table 2

Summation of key behavioral and biochemical changes. Summary of significant behavioral and biochemical changes per sex following administration of p53-scmTNF$_{R2}$ in EAE mice at days 10, 13, and 16 post-immunization.

	Females	**Males**
Mechanical Hypersensitivity	↓ Hypersensitivity	↓ Hypersensitivity
Motor Disease	↓ Severity + Delayed Onset	Same as control
Myelin Associated Proteins	Robust expression in cortex	Remains reduced
Microglial Activity	Cortical attenuation	Cortical attenuation