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TNFR2 promotes Treg-mediated recovery from neuropathic pain across sexes

Roman Fischer,a,1 Maksim Sendetskiia, Tania del Riveroa, George F. Martinezb, Valérie Bracchi-Ricardi, Kathryn A. Swansona, Elizabeth K. Pruinzkyb, Niky Delguercioi, Michael J. Rosalinob, Tanja Padutsch, Roland E. Kontermannbc, Klaus Pfizennmaierbc, and John R. Betheaa,1

*Department of Biology, Drexel University, Philadelphia, PA 19104; ^Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany; and ¶Stuttgart Research Center Systems Biology, University of Stuttgart, 70569 Stuttgart, Germany

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Tumor necrosis factor receptor 2 (TNFR2) is a transmembrane receptor that is linked to immune modulation and tissue regeneration. Here, we show that TNFR2 essentially promotes long-term pain resolution independently of sex. Genetic deletion of TNFR2 resulted in impaired neuronal regeneration and chronic nonresolving pain after chronic constriction injury (CCI). Further, pharmacological activation of TNFR2 using the TNFR2 agonist EHD2-sc-mTNFR2 in mice with chronic neuropathic pain promoted long-lasting pain recovery. TNFR2 agonist treatment reduced neuronal injury, alleviated peripheral and central inflammation, and promoted polarization of central nervous system (CNS)-infiltrating myeloid cells into an antiinflammatory/reparative phenotype. Depletion of regulatory T cells (Tregs) delayed spontaneous pain recovery and abolished the therapeutic effect of EHD2-sc-mTNFR2. This study therefore reveals a function of TNFR2 in neuropathic pain recovery and demonstrates that both TNFR2 signaling and Tregs are essential for pain recovery after CCI. Therefore, therapeutic strategies based on the concept of enhancing TNFR2 signaling could be developed into a nonopioid therapy for the treatment of chronic neuropathic pain.

Significance

Tumor necrosis factor (TNF) is a cytokine that induces signaling via two receptors, TNFR1 and TNFR2. TNF signaling via TNFR1 contributes to development and maintenance of neuropathic pain. Here, we show that TNFR2 is essential for recovery from neuropathic pain across sexes. Treatment of male and female neuropathic mice with a TNFR2 agonist resulted in long-lasting recovery from neuropathic pain. We identified Tregs as the cellular mediator of the analgesic effect of TNFR2. Indeed, TNFR2 agonist administration alleviated peripheral and central inflammation and promoted neuroprotection in a Treg-dependent manner, indicating that TNFR2-dependent modulation of immunity is neuroprotective. We therefore argue that TNFR2 agonists might be a class of nonopioid drugs that can promote long-lasting pain recovery in males and females.

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Conflict of interest statement: R.F. 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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1To whom correspondence may be addressed. Email: rf428@drexel.edu or jrb445@drexel.edu.
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development of mechanical hypersensitivity (18) in male animals. Due to the different pathways underlying pain development in males and females, we analyzed the role of Tregs in spontaneous pain recovery in both sexes. First, we confirmed that male and female (SI Appendix, Fig. S1) WT mice start recovering from chronic constriction injury (CCI)-mediated mechanical allodynia 8 wk after peripheral nerve injury. Then, we depleted Tregs in injured male and female mice during the recovery phase starting 5 wk postinjury (wpi) using the anti-CD25 antibody PC-61, which depletes more than 75% of Tregs through immune-dependent mechanisms and is widely used to interrogate Treg function (21). In male and female mice, depletion of Tregs with PC-61 delayed recovery for ~3 wk compared to control IgG-treated mice (Fig. 1A), with the effect of Treg depletion being stronger in males than in females (SI Appendix, Fig. S2). Whereas the total percentage of splenic CD3+ T cells was not altered (Fig. 1B), we observed a significant reduction of FoxP3+ Tregs 12 wpi (Fig. 1C). Further, the levels of Tregs were correlated with the recovery of both male and female mice from both treatment groups (r = 0.6729, **P = 0.0011, Fig. 1D and SI Appendix, Fig. S2C), indicating that Tregs are important for resolution of mechanical allodynia in males and females. No changes in the percentage of other immune cell subsets were observed between control IgG and PC-61-treated animals (Fig. 1E).

TNFR2 Is Essential for Recovery from Mechanical Allodynia. Due to the well-documented role of TNFR2 in neuronal recovery (7) and Treg function (22), we next compared pain recovery of WT and TNF2−/− mice after CCI. Interestingly, male and female TNF2−/− animals developed chronic nonresolving pain and did not recover from mechanical allodynia (Fig. 2A and SI Appendix, Fig. S3). Whereas no changes in the percentage of total splenic CD3+ T cells were observed (Fig. 2B), the percentage of CD25+ FoxP3+ Tregs was significantly decreased in injured TNF2−/− mice, compared to injured WT and noninjured TNF2−/− mice (Fig. 2C). No differences in the percentages of other immune cells were observed between injured WT and TNF2−/− mice (Fig. 2D). Further, no significant changes were observed in the percentage of all analyzed immune cell subsets between naive noninjured WT and TNF2−/− mice (Fig. 2B and C and SI Appendix, Fig. S3). Interestingly, whereas levels of neurofilament 200 (NF200), a marker for large myelinated Aβ fibers, were increased in WT mice, TNF2−/− mice showed chronic loss of NF200 12 wpi in the spinal cord (Fig. 2F). Similarly, whereas levels of Growth Associated Protein 43 (GAP43), a protein indicative of neuronal repair, were increased in WT mice, TNF2−/− mice show decreased levels of GAP43 (Fig. 2F), suggesting impaired neurorepair in TNF2−/− mice following CCI. Levels of C68 (SI Appendix, Fig. S5A), a protein highly expressed by macrophages, were up-regulated in both WT and TNF2−/− mice. Similar expression of calcitonin gene-related peptide (CGRP) was up-regulated after injury in both groups (SI Appendix, Fig. S5B). CGRP is a neuropeptide that is primarily released from sensory neurons and is up-regulated after nerve damage and neuroinflammation (23). Up-regulated CGRP levels have been implicated in pain pathways (24). These data indicate that the expression level of C68 and CGRP is not influenced by endogenous TNF2 signaling (SI Appendix, Fig. S5C). Baseline expression levels of all analyzed proteins in naive WT and TNF2−/− mice were similar (SI Appendix, Fig. S5D).

TNFR2 Agonism Is Therapeutic for Neuropathic Pain. TNF2 was shown to be expressed on Tregs, activated T cells, myeloid cells, and other immune cells (25). We here confirmed that Tregs and myeloid cells expressed the highest levels of TNF2. TNF2 expression was increased on almost all analyzed immune cell subsets, besides peripheral neutrophils and spinal cord macrophages after CCI (SI Appendix, Figs. S6 and S7). We next investigated whether exogenous activation of TNF2 is therapeutic in the CCI model. To selectively activate TNF2, we used the mouse TNFR2 agonist EHD2-sc-mTNF2 (26, 27). Treatment with

Fig. 1. Treg depletion delays recovery after CCI. (A) WT mice underwent CCI at week 0. Mechanical allodynia was determined using the Von Frey test over a period of 12 wk (mean ± SEM, WT: n = 12, TNF2−/−: n = 20). **P < 0.01 WT vs. TNF2−/− ipsilateral paw. (B–D) Percentage of splenic CD3+ T cells (B) and CD25+ FoxP3+ Tregs (C) (population: CD3+ T cells) and other immune cell subsets (D) was quantified by flow cytometry 12 wpi. Shown are scatterplots from representative experiments. Values in each quadrant are the percentage of positive cells. Quantification shows the mean ± SEM, WT: n = 8, TNF2−/−: n = 10. Expression of NF200 (E) and GAP43 (F) in the spinal cord of naive and injured WT and TNF2−/− mice was quantified by Western blot analysis 12 wpi. Shown are representative blots and the mean ± SEM (technical density of each protein normalized to ß-tubulin), percentage of protein expression of naive mice (n = 6 naive, n = 8 CCI each group). *P < 0.05; **P < 0.01; ****P < 0.0001; ns, not significant.

Fig. 2. TNF2−/− mice have chronic nonresolving pain. (A) WT and TNF2−/− mice underwent CCI at week 0. Mechanical allodynia was determined using the Von Frey test over a period of 12 wk (mean ± SEM, WT: n = 12, TNF2−/−: n = 20). **P < 0.01 WT vs. TNF2−/− ipsilateral paw. (B–D) Percentage of splenic CD3+ T cells (B) and CD25+ FoxP3+ Tregs (C) (population: CD3+ T cells) and other immune cell subsets (D) was quantified by flow cytometry 12 wpi. Shown are scatterplots from representative experiments. Values in each quadrant are the percentage of positive cells. Quantification shows the mean ± SEM, WT: n = 8, TNF2−/−: n = 10. Expression of NF200 (E) and GAP43 (F) in the spinal cord of naive and injured WT and TNF2−/− mice was quantified by Western blot analysis 12 wpi. Shown are representative blots and the mean ± SEM (technical density of each protein normalized to ß-tubulin), percentage of protein expression of naive mice (n = 6 naive, n = 8 CCI each group). *P < 0.05; **P < 0.01; ****P < 0.0001; ns, not significant.
TNR2 Agonism Reduces Central and Peripheral Inflammation. In addition to down-regulating CCR7 expression in the spinal cord, EHD2-sc-mTNFR2 dramatically decreased the number of CGRP neurons in the sciatic nerve (Fig. 4A), indicating decreased neuronal injury and pain signaling in the peripheral nervous system. One of the first clinical signs of peripheral nerve injury is infiltration of immune cells into the peripheral nerve. Concurrently with the reduced pain sensitivity of EHD2-sc-mTNFR2-treated mice, the expression of the great majority of inflammatory markers in the sciatic nerve was down-regulated in TNFR2 agonist-treated mice, whereas expression of BDNF was up-regulated after TNFR2 agonist treatment (Fig. 4B). Compared to saline treatment, we observed reduced infiltration of CD3+ T cells (Fig. 4C and D), but increased infiltration of FoxP3+ Tregs (Fig. 4C and D) into the peripheral nerve. Similarly, FoxP3 expression was increased in the spinal cord (Fig. 4E) of mice treated with EHD2-sc-mTNFR2 after injury, whereas CD4 and CD8A gene expression levels in the spinal cord were decreased in EHD2-sc-mTNFR2–treated mice (Fig. 4E). Further, we observed that compared to saline administration, mice treated with EHD2-sc-mTNFR2 showed reduced expression of TNF and NOS2 and increased expression of IL10, MRC1, and ARG1 in the spinal cord 2 wpi was quantified by quantitative real-time PCR (mean ± SEM, saline: n = 4, EHD2-sc-mTNFR2: n = 6). *P < 0.05; **P < 0.01; ns, not significant. (Scale bars, 50 μm.)
CD8<sup>+</sup> T cells were detected 5 wpi (Fig. 6 and SI Appendix, Fig. S104). Interestingly, we observed a significant reduction in the percentage of splenic CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs 2 wpi in the saline-treated group compared to sham injured mice. However, no differences in the percentage of Tregs was observed in EHD2-sc-mTNF<sub>R2</sub>-treated mice (Fig. 6C). This is in line with previous observations that TNFR2 agonism increases Treg proliferation (27, 29). However, we cannot exclude that other mechanisms contribute to the increased Treg fractions, such as increased stability/survival or altered biodistribution. No differences between the groups were observed 5 wpi (Fig. 6C). Further, EHD2-sc-mTNF<sub>R2</sub> administration prevented injury-induced reduction in the percentage of splenic CD11<sup>b</sup>CD115<sup>+</sup>MHCII<sup>+</sup> neutrophils and CD68<sup>+</sup> macrophages and increased the percentage of CD11c<sup>+</sup>MHCII<sup>+</sup> dendritic cells 2 wpi. Comparable to T lymphocytes, no changes in the percentages of neutrophils, macrophages, and dendritic cells were observed 5 wpi. Percentages of CD45R<sub>B</sub> B cells were not changed 2 and 5 wpi (SI Appendix, Fig. S10). Since both Tregs and TNFR2 on central nervous system (CNS)-resident cells, such as microglia, have been shown to reduce neuroinflammation and to promote neuroprotection (15, 30, 31), we investigated whether EHD2-sc-mTNF<sub>R2</sub> was transported into the spinal cord. Indeed, we were able to detect EHD2-sc-mTNF<sub>R2</sub> after 2 and 5 wk in the spinal cord (SI Appendix, Fig. S11).

TNFR2 Agonism Promotes Pain Recovery via Tregs. We next investigated whether the therapeutic effect of EHD2-sc-mTNF<sub>R2</sub> is mediated via Tregs. Beginning 7 d after injury, male and female mice were first administered a control IgG or PC-61 for 2 wpi and then on days 9, 12, and 15, mice were treated with EHD2-sc-mTNF<sub>R2</sub>. Whereas mice treated with control IgG showed TNFR2 agonist-dependent recovery, mice that received the combination of PC-61 and EHD2-sc-mTNF<sub>R2</sub> did not recover from mechanical allodynia (Fig. 7A and SI Appendix, Fig. S12). While EHD2-sc-mTNF<sub>R2</sub> treatment reduced the injury-mediated increase in splenic CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells in control IgG-treated mice 2 wpi, no significant reduction was observed in mice treated with PC-61 and EHD2-sc-mTNF<sub>R2</sub> (Fig. 7B and SI Appendix, Fig. S13), indicating that EHD2-sc-mTNF<sub>R2</sub>-mediated Treg activity is responsible for suppression of T cell expansion. As expected, Treg levels were significantly decreased in PC-61-treated mice 2 and 5 wpi (Fig. 7B and SI Appendix, Fig. S13). In contrast, PC-61-alkalized depletion of Tregs did not abolish the increase in splenic CD11b<sup>+</sup>CD115<sup>+</sup>Ly6G<sup>+</sup> neutrophils, CD68<sup>+</sup> macrophages/macrophages, and CD11c<sup>+</sup>MHCII<sup>+</sup> dendritic cells percentages observed after treatment with EHD2-sc-mTNF<sub>R2</sub> (SI Appendix, Fig. S13), suggesting that these effects do not contribute to the therapeutic effect of EHD2-sc-mTNF<sub>R2</sub> in the CCI model. Besides a reduction in the percentage of Tregs (Fig. 7B), no major changes were observed 5 wpi (SI Appendix, Fig. S15). Finally, depletion of Tregs abolished the neuroprotective effects of EHD2-sc-mTNF<sub>R2</sub> treatment, i.e., animals that received the combination of PC-61 and EHD2-sc-mTNF<sub>R2</sub> did show a decrease in NF200 levels and no increase in GAP43 levels compared to EHD2-sc-mTNF<sub>R2</sub> treatment alone (Fig. 7C).

Discussion

We recently showed that TNFR1 signaling plays an essential role for pain development in males, but not females. Accordingly, pharmacological inhibition of TNFR1 was only therapeutic in males but had no effect on females (10). Here, we demonstrated that pharmacological activation of TNFR2 induces long-lasting recovery from neuropathic pain across sexes, indicating that the neuroprotective effect of TNFR2 works via a sex-independent pathway. We further provide genetic evidence showing that pain recovery after CCI is abolished in male and female TNFR2<sup>−/−</sup> mice, uncovering a role of TNFR2 for pain recovery. We further show that exogenous TNFR2 activation by EHD-sc-mTNF<sub>R2</sub> loses its therapeutic activity when Tregs are depleted before treatment start. The important role of Tregs for functional recovery after CCI is supported by further data showing that pharmacological depletion of Tregs in the chronic phase of the disease delays recovery. This study therefore provides a basis for a better understanding of the role of Tregs for functional recovery of neuropathic pain and suggests that TNFR2 agonists could be developed into a novel nonopioid therapy for the treatment of neuropathic pain.

It is well documented, that TNF acts as a neuromodulator to increase inflammation and neuropathic pain after injury (32). Endoneurial TNF application into the nervous system results in behavioral and electrophysiological changes associated with induction of thermal hyperalgesia and mechanical allodynia, whereas antagonism of TNF has the opposite effect (33, 34). Further, there is a correlation between TNF expression levels and development of neuropathic pain (33), suggesting an important...
role of TNF in the initiation of pain. We and others have shown that TNFR1 signaling promotes mechanical allodynia (8) and thermal hyperalgesia (9). In contrast, the role of TNFR2 for pain is less well characterized. Using a model of inflammatory pain, it was shown that after intraplantar injection of complete Freund’s adjuvant, heat hyperalgesia was decreased in the early phase in TNFR2−/− mice but reduced in both early and later phases in TNFR1−/− mice (14). While these studies demonstrate that TNFR1 plays a role for both development of neuropathic pain as well as maintenance of pain, they also suggest that TNFR2 is important for the early phase of pain development. In contrast, we here show that TNFR2 plays an essential role for the resolution of pain via immune-dependent mechanisms. Peripheral immune cells were shown to play a pivotal role for the induction and maintenance of neuropathic pain. Whereas cytokines and neutrophils have been found to play an important role during the early stages of acute pain, T lymphocytes appear to play a central role in chronic neuropathic pain (35). In addition, T cells were shown to play a role in pain resolution as well. In particular, chemotherapy-induced mechanical allodynia was significantly prolonged in T cell-deficient mice (36), while adoptive transfer of T cells via intrathecal injection relieved tactile allodynia after chemotherapy (37). Further, after peripheral nerve injury, microglial cells become activated and change their phenotype to produce inflammatory cytokines and chemokines that facilitate recruitment of leukocytes into the brain (38). Inhibition of microglia activation was shown to alleviate mechanical allodynia and hyperalgesia following spinal nerve ligation (39), and a role for microglia in long-term maintenance of allodynia was postulated (40). Interestingly, recent studies advance the hypothesis that there is a difference between the sexes in the initiation and maintenance of neuroinflammation and development of neuropathic pain. In particular, there appears to be a greater involvement of microglia in males, whereas in females neuroinflammation appears to be primarily driven by adaptive immune cells (41). Our data demonstrate that TNFR2 signaling promotes pain recovery in both males and females, indicating that different pain pathways may be targeted by TNFR2 agonism. We show that exogenous TNFR2 activation reduced expansion of T effector cells in the periphery and infiltration into the nerve and spinal cord. We further observed increased Treg levels in the periphery and the spinal cord after TNFR2 agonist administration. Tregs were previously shown to infiltrate the nerve and spinal cord and to interfere with development of neuropathic pain after peripheral nerve injury (18, 19). We here show that Tregs play a pro-TNF role for pain recovery in peripheral nerve injury-mediated neuropathic pain. Next to immunosuppression, Tregs contribute to tissue regeneration in the periphery and the CNS (42) and may solely account for the therapeutic effect observed after TNFR2 agonist treatment. However, TNFR2 is also expressed on other cells, such as activated T cells, myeloid cells or microglia and TNFR2 signaling in those cells was shown to alleviate neuroinflammation (31, 43, 44). We therefore cannot exclude that TNFR2 signaling in non-Treg cells contributes to its therapeutic effect. Next to reduced immune cell infiltration, we observed a repolarization of CNS-infiltrating myeloid cells into an antiinflammatory phenotype after TNFR2 agonist treatment. In particular, antiinflammatory/reparative macrophages have been indicated to play a role for repair processes after nerve injury (45).

After CCI, neurodegenerative changes have been observed in the spinal cord (8). In our experiments, we observed a transient decrease in NF200 levels of the spinal cord after injury, indicating a loss of large myelinated Aβ fibers. Importantly, TNFR2−/− mice with chronic nonresolving allodynia showed prolonged NF200 loss, whereas TNFR2 agonist treatment either prevented NF200 loss or promoted NF200 recovery after injury. This is supported by our data that TNFR2−/− mice showed reduced spinal GAP43 levels after injury and that TNFR2 agonist treatment increased GAP43 levels in the spinal cord. GAP43 is expressed at high levels in neuronal growth cones and is recognized as a supporting factor for axonal growth and synaptic remodeling (46). The chronic loss of GAP43 in TNFR2−/− mice after CCI and the increased levels of GAP43 after TNFR2 agonist treatment therefore indicate that TNFR2 signaling may promote neuroprotection and/or neuronal remodeling. In support of this potential neuroreparative function of TNFR2 agonism, we found decreased levels of proBDNF and increased levels of mBDNF in the spinal cord of EHD2-sc-mTNFα-treated mice. However, next to its role for neurorepair, BDNF signaling via the tropomysin receptor kinase B (TrkB) also has a well-documented pronociceptive role (28, 47). Therefore, further studies are necessary to validate the potential neuroreparative role of TNFR2 in the CCI model. Interestingly, the TNFR2 agonist-dependent increase in NF200 and GAP43 levels was abolished when Tregs were depleted prior to EHD2-sc-mTNFα treatment. This suggests that Tregs contribute to the observed neuroprotective responses. Indeed, Tregs were shown to promote neuroprotective activities and infiltration into the nerve and spinal cord.
(48). However, it needs to be determined, whether this is a direct effect of Tregs on neurons or indirect via Treg-mediated changes in glial cells or other immune cells.

In summary, our data demonstrate that exogenous TNFR2 activation is therapeutic for neuropathic pain via Treg-dependent responses. TNFR2 agonism after CCI results in reduced recruitment of myeloid cells and T cells and increased infiltration of Tregs into the nervous system. Further, CNS-infiltrating myeloid cells switched to an anti-inflammatory/regenerative phenotype after systemic TNFR2 agonist treatment. These changes ultimately result in reduced neuropathology and long-term recovery from neuropathic pain (Fig. 8). We propose that TNFR2 plays an essential role for pain recovery via Tregs and that Treg therapies, such as TNFR2 agonists, may hold great promise as nonopioid analgesic treatments to reduce neuropathic pain.

Materials and Methods

Detailed methods can be found in SI Appendix, SI Materials and Methods. The TNFR2 agonist EHD2-scTNFR2 was described previously (27). Animal care and treatment were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Drexel University under protocol numbers 20295/20628. Male and female mice were analyzed separately. Since behavioral and biological responses in males and females followed the same trend, figures within the manuscript summarize combined data obtained in male and female mice. Behavioral data are shown separated for males and females in the supplementary data.

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