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Pathological Contribution of Spinal Macrophage Migration Inhibitory Factor to Neuropathic Hypersensitivity through Interacting with TNF-α in the Rat

Maria L. Bolick [*,1], Qingsong Zhao [†,1], Shiqin Xu [‡,1], Mary K. Pathak [*], Aili Sunny [*], Fuzhou Wang [*,‡,Δ]

BACKGROUND Our previous data demonstrated that the proinflammatory cytokine macrophage migration inhibitory factor (MIF), a pleiotropic cytokine produced mainly by nonneuronal tissue, has been implicated in the pathogenesis of inflammatory and neuropathic hypersensitivity, whereas the precise underlying mechanisms are not totally elucidated. The aim of this study was to examine the interacting role for spinal MIF with TNF-α in neuropathic pain.

METHODS After approval by the institutional Animal Care and Use Committee, the randomized Sprague-Dawley rats underwent prophylactic intrathecal administration of recombinant MIF (rMIF), TNF-α, MIF mAb, TNF-α mAb, or in combination prior to the spared nerve injury (SNI). Thermal hyperalgesia with hot plate and tactile allodynia using von Frey filaments were assessed after different interventions. Spinal cord levels of MIF and TNF-α were measured using Western Blotting and immunocytochemistry.

RESULTS Exogenous rMIF potentiated SNI-induced nociceptive behavior that were not evoked by single use of rMIF without SNI, and this potential effect could be blocked by MIF antibody in part. After giving rMIF combined with TNF-α to SNI animals, the perception of thermal and tactile stimuli was maximized. Spinal MIF mAb inhibited TNF-α expression, and vise versa for TNF-α Ab on MIF expression after SNI. rMIF or TNF-α combined to SNI produced more significant effect on the levels of MIF and TNF-α than SNI alone, and this effect could be furthered by administering rMIF and TNF-α together.

CONCLUSION These data demonstrate that proinflammatory cytokine MIF is involved in the peripheral nerve injury-induced hypersensitivity through potentiating spinal TNF-α signaling.■

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NOXIOUS challenges can evoke a large number of cytokine productions to prevent and eliminate the original causes. However, a series of debilitating pathological and behavioral events like hyperalgesia is often linked to the immune activation (1). Tumor necrosis factor-α (TNF-α), one of the early proinflammatory cytokines, is released in response to pain evoking conditions (2). TNF-α signaling involves in the sensitization of both primary afferent and spinal cord neurons, and plays essential role primarily in formalin-induced pain condition by inducing peripheral edema and sensitization (3, 4). Meanwhile, peripheral injection of TNF-α can mimic and evoke neural excitability and hyperalgesia (5), and can result in ectopic discharges in primary afferent fibers and rat dorsal horn neurons (6).

Macrophage migration inhibitory factor (MIF), a multi-functional molecule, has been found to be involved in the regulation of the inflammatory responses and other enzymatic activities (7), and strongly associated with some inflammation-based pathology (8-11). Our previous data disclosed the fundamental role of MIF in contributing to the pathogenesis of inflammatory (12) and neuropathic hypersensitivity (13). Interestingly, TNF-α alone can induce local and systemic up-regulation of MIF (14, 15), and high levels of MIF can also induce macrophage TNF-α secretion (15). Accordingly, we surmised that MIF may contribute to the sensitization of afferent fibers and further discharges on dorsal horn neurons by potentiating the TNF-α signaling in peripheral nerve injury-associated hypersensitivity.

MATERIALS AND METHODS

Animals

The animals used in this study and the animal care were presented in our previous studies in detail (12). In brief, after approval by the Institutional Committee of Animal Care and Use, adult male Sprague-Dawley rats weighting 250-350 g were housed in pairs to a plastic cage with soft bedding on a reverse 12:12 h dark/light cycle with lights on at 8:00 AM with free access to food and water throughout the experiment for at least one week before the experiments. Test sessions took place during the light phase between 10:00 AM and 6:00 PM in a quiet room maintained at 22-24 ℃. No food or water was available to the rats during the experiment. Each animal was used only once and was euthanized at the end of the experiment by administrating a lethal dose of pentobarbital for tissue collection.

Procedures

Rats were randomly assigned to one of ten groups with16 each, of which four groups got Sham, spared nerve injury (SNI) (see detailed description below), sole intrathecal (i.t.) injection of recombinant MIF (rMIF, 150 μg/kg) or TNF-α (50 μg/kg), respectively; and another three groups received respective prophylactic i.t. injection of MIF mAb (100 μg/kg), TNF-α mAb (100 μg/kg) and MIF mAb+TNF-α mAb and another three groups got i.t. injection of rMIF, TNF-α, or rMIF+TNF-α prior SNI surgery, respectively. All i.t. drugs were administered 4 days consecutively with once each day before nerve injury.

After the first group allocation, rats in each group were redivided into two subgroups according to a second-step stratified sequence number produced by computer for receiving thermal and tactile nociceptive test separately. Possible effects of repeated testing were minimized via measuring the threshold to respond to noxious stimuli on all tests.

Intrathecal catheterization

Rats were implanted with intrathecal catheter (ALZET Osmotic Pumps, Cupertino, CA, USA) for drug delivery as described previously (16). In brief, after shaving and sterilizing the cephalic-cervical area, a midline incision was made followed by dissection of the paravertebral muscles from the spinous processes. The dura was slit and the catheter was inserted for 7.0 cm caudal from the dural slit and then it was fixed with a drop of tissue glue (B. Braun, Tuttingen, Germany) and was further secured on the fascia of paravertebral muscle. Finally, sodium penicillin 10,000 IU (Shanghai AoBopharmtech, Shanghai, China) was given intramuscularly against infection. The rats would be excluded (~10%) if neurological deficits were exhibited after catheterization. The intrathecal catheter was not removed until the day of sacrifice to prevent

evoking unexpected behavioral stress and spinal cord injury.

Spared nerve injury model and behavior test

The spared nerve injury (SNI) model was used as described in our previous work (16). In brief, animals were anesthetized with isoflurane, and the tibial and common peroneal branches of the sciatic nerve were ligated and sectioned distally, but the sural nerve was left intact. For sham surgeries, the sciatic nerve was merely exposed but not ligated or dissected.

Allodynia measurement

Pain thresholds were assessed using the von Frey filaments (Stoelting Co., Wood Dale, IL) prior to surgical procedures and again at different time points thereafter. The testing protocol has been described in our previous study (13, 16). In brief, the filaments were applied to the central surface of the hind paw plantar for a maximal of 10 s to determine the threshold of the stimulus through evoking a withdrawal response. The increment of stimulus was based on the response of the rat to the current filament, if the paw withdrew, the same hair was again used 60 s later; but if not, the next stronger hair was presented. If the rat withdrew its paw in two consecutive trials with the same filament, no further filaments were tested. Withdrawal responses were used to determine the absolute threshold, i.e. the 50% withdrawal threshold, by fitting to a Gaussian Integral Psychometric Function via a Maximum Likelihood method.

Thermal pain behavior

Thermal hyperalgesia was assessed using an Analgesiometer (Eddy's Hot Plate; Naugra Export, Ambala Cantt, Haryana, India) as described in our early studies (13, 16). Briefly, the temperature on the hot plate was set at 55±0.1 ℃, and the paw withdraw

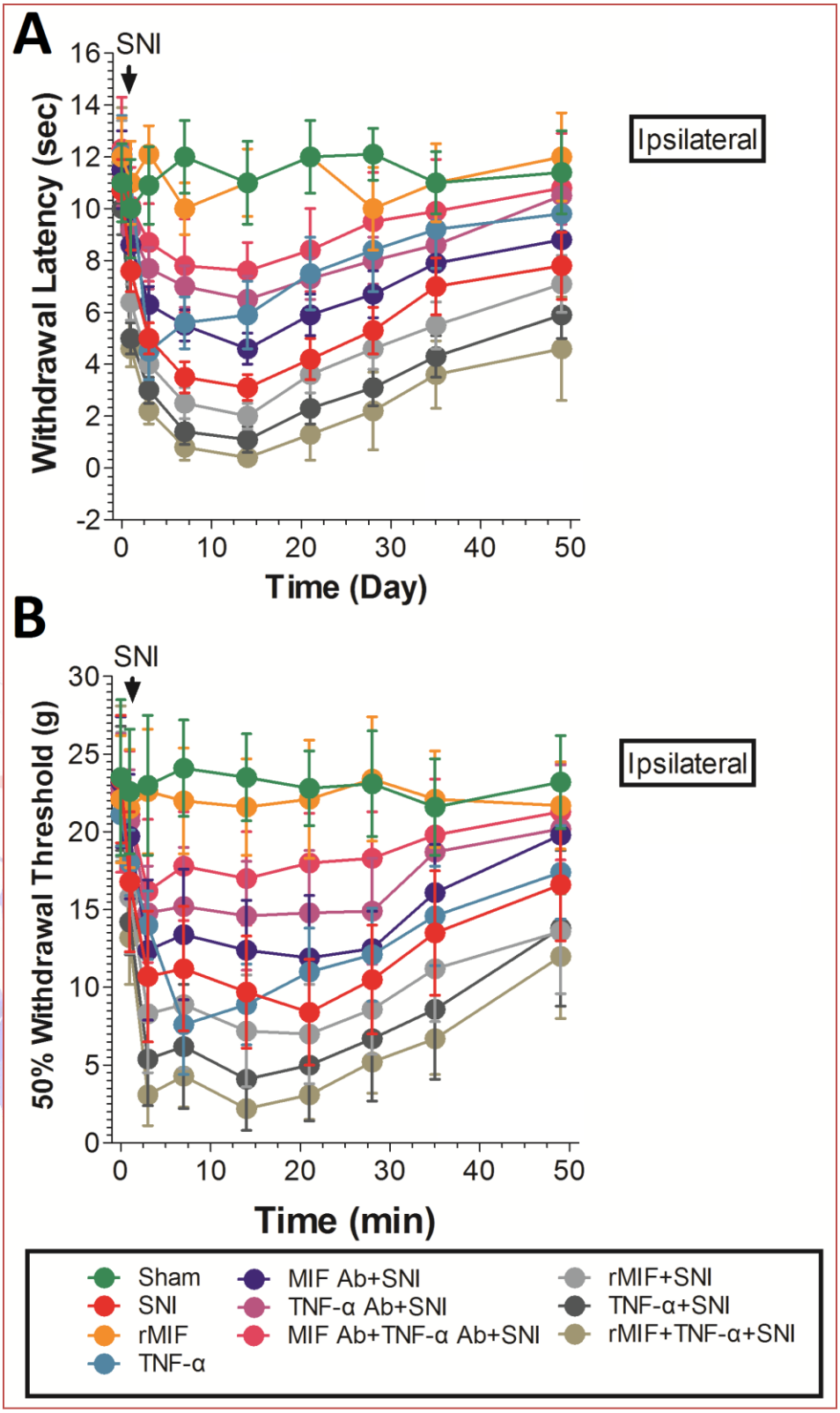

Figure 1. Nociceptive behavior responses after treatments. Experiments of thermal hyperalgesia (A) and tactile allodynia (B) were performed after SNI injury. MIF Ab plus TNF-α Ab produced the most significant alleviation of these two nociceptive behaviors than MIF Ab or TNF-α Ab alone ($P < 0.05$). The effect of TNF-α Ab on these behavior responses was more significant than MIF Ab ($P < 0.01$). Prophylactic rMIF or TNF-α amplified the pain behavior compared with sole SNI ($P < 0.05$), and an additive effect was produced after using rMIF and TNF-α together to the nerve injury ($P < 0.01$). Single use of rMIF without SNI did not produce any nociceptive effect, but TNF-α did.

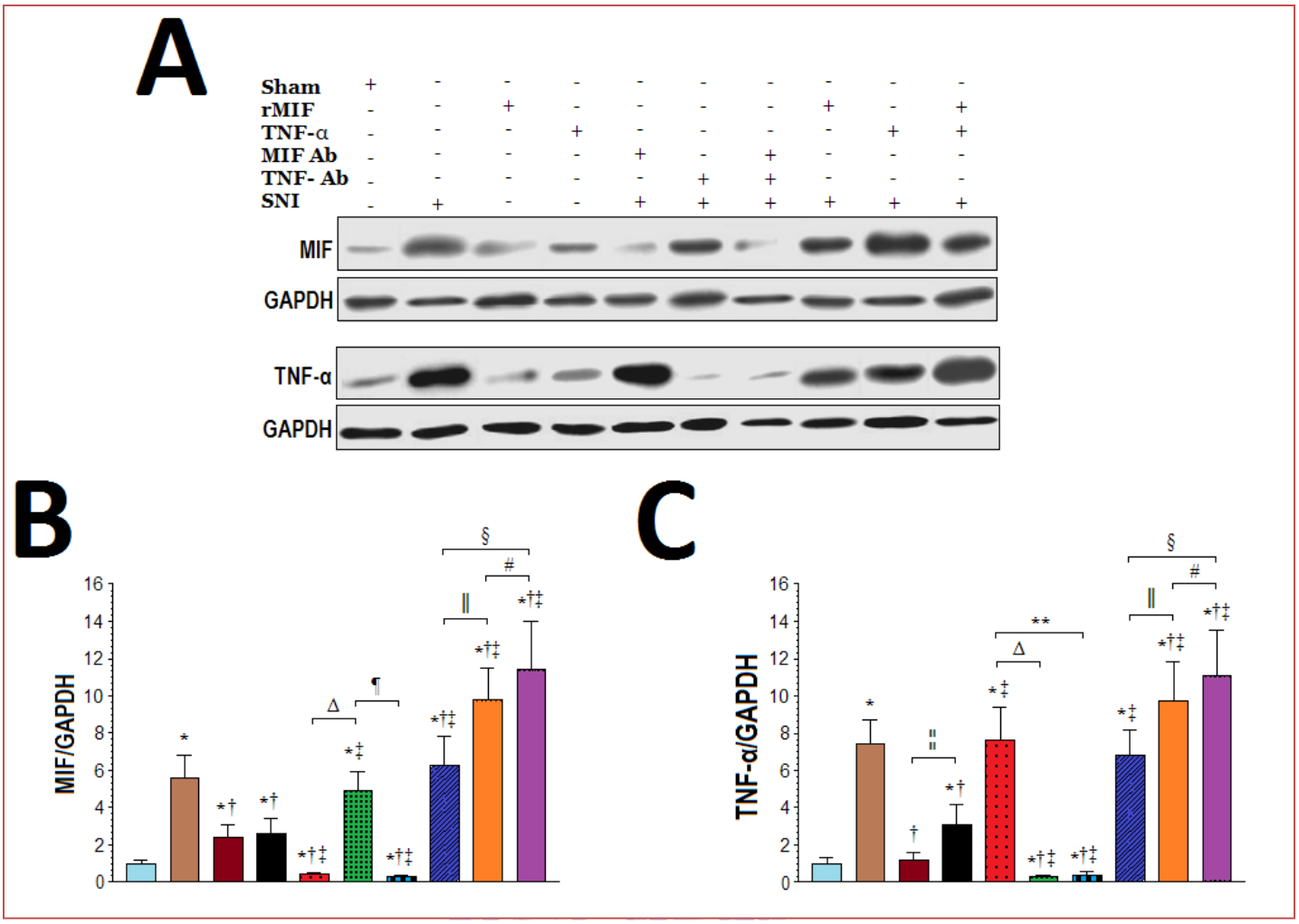

Figure 2. Immunobloting of MIF and TNF-α in the spinal cord. Spinal expression of MIF and TNF-α was detected by Western Blotting. MIF Ab could not inhibit TNF-α expression, and vise versa for TNF-α Ab on MIF expression after SNI. Nevertheless, rMIF or TNF-α combined to SNI produced more significant effect on the levels of MIF and TNF-α than SNI alone ($P < 0.001$), and this effect could be furthered by administering rMIF and TNF-α together ($P < 0.0001$). rMIF itself did not induce further expression of TNF-α, but TNF-α could increase spinal MIF. *, versus saline; †, versus SNI; ‡, versus single rMIF or TNF-α.

al latency of withdrawal were observed by recording the number of seconds required for reaction to the thermal stimuli. The maximal duration of stimulation was set at 22 s as a cut-off time to avoid tissue damage. Each animal was tested three times repeatedly at an interval of 15 min, and each time the test was carried out by a different investigator.

Immunoblotting for MIF and TNF-α expression

Lumbar spinal tissues were collected and the dorsal part was homogenized in buffer (10 mM Tris, 5 mM EDTA [ethylenediaminetetraacetic acid], 2% Triton X-100, 0.2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin) and mechanically disrupted. Samples were analyzed by SDS-PAGE by means of a transfer buffer (25 mM/l Tris, 192 mM/l glycine, and 20% methanol) in a wet-transfer apparatus. Blots were blocked with 5% non-fat dry milk in PBS with 0.1% Tweens-20 and then incubated with mouse anti-rat MIF or TNF-α Ab (all two were 1:1,000, Sigma-Aldrich, USA). After repeated washing, rabbit anti-mouse secondary antibody (1:4,000, horseradish peroxidase-conjugated) incubation was performed, developed with a chemiluminescence system, and followed with film exposure and relative intensity analysis with the Typhoon Imaging System (GE Healthcare, Piscataway, NJ, USA). The immuneblots were washed briefly and then incubated with a monoclonal mouse anti-rat GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (1:10,000, Abcam) for 40 min at room temperature followed by a horseradish peroxidase-conjugated rabbit anti-mouse antibody. GAPDH protein was then visualized and detected as the internal biomarker.

Immunohistochemistry

The L5 segment of the spinal cord was removed by laminectomy 20 min after the drug injection into the plantar surface of the hind paw and fixed with fresh 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at

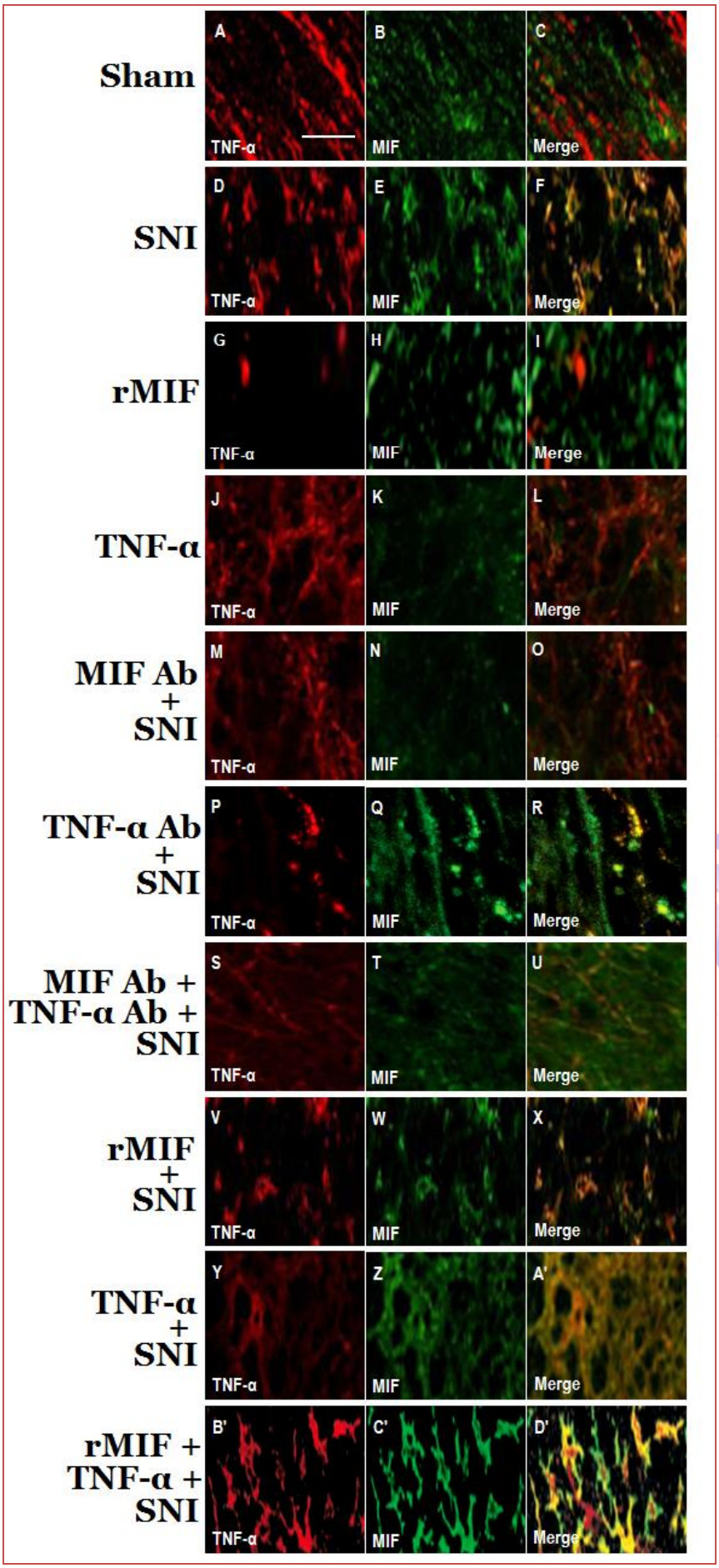

Figure 3. Immunolabeling of TNF-α and MIF expression in the ipsilateral spinal cord. In the Sham animals (A-C), MIF and TNF-α did not co-exist in the ipsilateral spinal dorsal horm. MIF was in glial cells, but TNF-α was in surrounding tissue of glial cells. In the SNI rats (D-F), TNF-α transferred into glial cells after peripheral nerve injury and co-located with MIF. In the rMIF injected animals (G-I), rMIF itself did not induce further expression of TNF-α in the dorsal horn. In the TNF-α-treated rats (J-L), TNF-α increased spinal level of MIF in part. In the MIF Ab + SNI animals (M-O), MIF Ab did not inhibit TNF-α expression in the ipsilateral spinal cord. In the TNF-α Ab + SNI animals (P-R), TNF-α Ab did not block MIF expression in the ipsilateral dorsal horn. In the MIF Ab + TNF-α Ab + SNI animals (S-U), MIF and TNF-α were blocked by both antibodies. In the rMIF + SNI (V-X), and the TNF-α + SNI (Y-A′) rats, prophylactic administration of rMIF or TNF-α to SNI produced additive effect on the spinal levels of TNF-α and MIF. In the rMIF + TNF-α + SNI animals (B′-D′), combined use of rMIF and TNF-α maximized the effect of SNI's role in the spinal levels of TNF-α and MIF.

4 ℃ overnight and then transferred to 30% sucrose in phosphate buffered saline for 48 h. Transverse sections (20-μm thick) of L5 spinal cord were prepared using a cryocut microtome (Leica CM3050S). The floating sections were incubated with 3% H_2O_2 for 30 min and blocked in solution containing 5% normal goat/horse serum, 5% fetal bovine serum, 2% bovine serum albumin, and 0.1% Triton X-100 for 2 h at room temperature. For double immunofluorescent staining, floating sections were incubated overnight at 4 ℃ with a mixture of mouse anti-rat TNF-α Ab (1:500, Santa Cruz Biotechnology, Santa Cruz, USA) and rabbit anti-rat MIF Ab (1:500, Sigma-Aldrich, USA), The sections were then incubated for 1 h at room temperature with a mixture of fluorescein isothiocyanate-conjugated anti-mouse IgG and cyanine 3 (Cy3)-conjugated anti-rabbit IgG antibodies (1:200, Jackson ImmunoResearch). The sections were mounted with Vectashield (Vector Laboratories). Fluorescent images

were viewed with an Olympus fluorescence microscope (Olympus America Inc., Center Valley, PA), and the images were shot with a digital camera.

Statistical analysis

Analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± standard error of the mean (SEM). Nociceptive data were analyzed with two-way ANOVA. The ANOVA tests were always followed by the Bonferroni *post hoc* tests for multiple comparisons. One-way ANOVA was used to analyze the intergroup difference in the tissue levels of MIF and TNF-α. All reported P values are two-sided and a P value of less than 0.05 was considered to be statistically significant.

RESULTS

Effect of prophylactic MIF and TNF-α blockades on the nociceptive behavior responses

The time course of the nociceptive responses to different treatments is presented in the Figure 1. Prophylactic administration of MIF Ab plus TNF-α Ab produced the most significant alleviation of tactile and thermal nociception than MIF Ab or TNF-α Ab alone ($P < 0.05$). The effect of TNF-α Ab on these behavior responses was more significant than MIF Ab ($P < 0.01$). If used rMIF or TNF-α simultaneously, the behavioral responses were amplified compared with those rats only with SNI injury ($P < 0.05$), and an additive effect was produced after using rMIF and TNF-α together ($P < 0.01$). Single use of rMIF without SNI did not produce any nociceptive effect. However, sole TNF-α injection evoked changes in rat behavior.

Spinal expression of MIF and TNF-α

Lumbar spinal cord was analyzed for protein expression of MIF and TNF-α depicted in the Figure 2. MIF Ab could not inhibit TNF-α expression in the experimental tissue, and TNF-α Ab also did not block MIF expression in the experimental spinal cord. Nevertheless, combined administration of rMIF or TNF-α produced additive effect on increasing tissue levels of MIF and TNF-α, and this effect could be furthered by adding rMIF and TNF-α together. Moreover, rMIF itself did not induce further expression of TNF-α, but TNF-α could increase spinal level of MIF.

Immunolabeling of TNF-α and MIF in the ipsilateral dorsal horn

Double staining of MIF and TNF-α in the ipsilateral dorsal horn demonstrated a strong fluorescence for the two proteins, but interestingly, MIF and TNF-α did not co-exist at the same site. MIF was in glial cells, but TNF-α was in surrounding tissue of glial cells. A more interesting thing was that TNF-α transferred into glial cells after SNI stimulation and co-located with MIF. MIF Ab could not inhibit TNF-α expression in ipsilateral spinal cord, and TNF-α Ab also did not block MIF expression in the ipsilateral dorsal horn. However, adding rMIF or TNF-α to SNI produced additive effect on increasing spinal levels of TNF-α and MIF, and this effect could be furthered by giving rMIF and TNF-α together. Finally, rMIF itself did not induce further expression of TNF-α in dorsal horn, but TNF-α could increase spinal level of MIF in part. (Figure 3)

DISCUSSION

We herein reported a pathologic role for MIF in facilitating TNF-α in sensitizing SNI-induced hypersensitivity. Rat recombinant MIF potentiates SNI-induced nociceptive behavior that were not evoked by single use of rMIF without SNI, and this potential effect could be blocked by MIF antibody in part. After giving rMIF simultaneously with TNF-α to SNI animals, the perception of exogenous stimuli was maximized. We report that underlying these behavior changes is the elevation of the levels of MIF and TNF-α in corresponding spinal cord tissues.

SNI-associated hypersensitivity was assessed through thermal and allodynia pain behaviors. These two types of behaviors were enhanced by sole intrathecal use of rMIF or TNF-α, and an additive effect on these behavioral responses would be evoked by giving rMIF and TNF-α together. On the contrary, these pain behaviors could be alleviated by MIF Ab or TNF-α Ab alone, and the blockade effect would reach maximal extent when combining MIF and TNF-α antibodies. Given rMIF itself did not evoke any changes in the nociceptive responses without SNI, but TNF-α was. Therefore, we conclude that spinal MIF functions as a promoting molecule to TNF-α in SNI-induced neuropathic hypersensitivity. Meanwhile, the SNI-induced nociceptive state is the prerequisite of MIF as a contributing factor to the pronociceptive role of TNF-α.

Although the effect of other proinflammatory cytokines including interleukin-1β (IL-1β) and interleukin-6 (IL-6) on central sensitization and hyperalgesia was evaluated and found IL-6 regulates inhibitory neurotransmission, and IL-1β regulates both excitatory and inhibitory neurotransmission, these effects are separate with TNF-α signaling (17, 18). In our study, we found a dependency link between MIF and TNF-α in sensitizing central perception of noxious stimuli. In the spinal cord, TNF-α evokes MIF release and MIF in turn promotes TNF-α production, TNF-α Ab cannot block MIF and MIF Ab cannot inhibit TNF-α.

Cytokine mechanisms of central sensitization give additional explana-

tion for the longstanding uncertainty surrounding the mechanism of this widely used pain model. Accumulating evidence suggests that TNF-α signaling plays an important role in central sensitization development. Overexpression or exogenous administration of TNF-α promotes excitatory neurotransmission (18, 19), inducing acute peripheral mechanical sensitization by acting on TNF receptor 1 (TNFR1) in primary afferent neurons which results in p38-dependent modulation of tetrodotoxin-resistant sodium channels (20), and inducing the functional expression of cyclooxygenase-II (COX-2) in dorsal root ganglion cells (21). TNF-α also enhanced AMPA-induced current and NMDA current in dorsal horn neurons (18) that was in agreement with a previous report from hippocampal neurons (22). These studies investigated the role of TNF-α in pain and potential mechanisms. In our study, we provide evidence that MIF facilitates TNF-α signaling by a feed-back modulation in SNI-induced hyperalgesia. Whether MIF also exerts effects on neurotransmission or on ion channels in the primary afferent nerve fibers and spinal dorsal horn neurons remain to be determined.

MIF and TNF-α inhibitors are effective for the treatment of inflammatory pain conditions, including rheumatoid arthritis (RA) and inflammatory bowel diseases (23-27). In addition, TNF-α blocking therapy reduced systemic MIF levels in RA (28). Our findings together with previous reports showed amelioration of pain in a variety of models suggested that agents that specifically target on MIF or TNF-α signaling may be effective in specifically targeting on pain hypersensitivity.

Some limitations should be acknowledged before concluding our study. First, previous data demonstrated that MIF is a subsequent product of the peripheral immune cells and the endocrine cells of the anterior pituitary gland (29). In our study, we did not seek the original source of MIF after nerve injury. Second, a change of gradient of the dosage of MIF and TNF-α or their antibodies delivered separately or in combination may be useful to analyze their precise relationship. Third, we only tested ipsilateral changes in MIF and TNF-α levels in spinal dorsal horn, how about the contralateral changes is meaningful for future studies. Therefore, further studies are needed to clarify these points.

In sum, MIF blockade alleviates SNI-induced thermal hypersensitivity and allodynia in accompanying with decrease in spinal TNF-α level. Neutralization of TNF-α produces identical effect on nociception as MIF inhibition did. However, MIF alone plays no role in nociceptive responses in normal animals. These data suggest that proinflammatory cytokine MIF involves in peripheral nerve injury-induced hypersensitivity by potentiating TNF-α signaling.■

Conflict of Interests

None

Acknowledgements

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Author Contributions

Maria L. Bolick, Qiangsong Zhao, and Shiqin Xu (Study Design, Data Collection, Data Interpretation, Manuscript Preparation, Literature Search); Mary K. Pathak and Aili Sunny (Data Collection, Data Interpretation, Manuscript Preparation, Literature Search); Fuzhou Wang (Study Design, Data Collection, Data Interpretation, Manuscript Preparation, Literature Search, Funds Collection)

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