Microglial Inhibition Influences XCL1/XCR1 Expression and Causes Analgesic Effects in a Mouse Model of Diabetic Neuropathy

Magdalena Zychowska, M.D., Ewelina Rojewska, Ph.D., Anna Piotrowska, M.D., Grzegorz Kreiner, Ph.D., Joanna Mika, Ph.D.

ABSTRACT

Background: Recent studies indicated the involvement of some chemokines in the development of diabetic neuropathy; however, participation of the chemokine-C-motif ligand (XCL) subfamily remains unknown. The goal of this study was to examine how microglial inhibition by minocycline hydrochloride (MC) influences chemokine-C-motif ligand 1 (XCL1)–chemokine-C-motif receptor 1 (XCR1)/G protein–coupled receptor 5 expression and the development of allodynia/hyperalgesia in streptozotocin-induced diabetic neuropathy.

Methods: The studies were performed on streptozotocin (200 mg/kg, intraperitoneally)-induced mouse diabetic neuropathic pain model and primary glial cell cultures. The MC (30 mg/kg, intraperitoneally) was injected two times daily until day 21. XCL1 and its neutralizing antibody were injected intrathecally, and behavior was evaluated with von Frey and cold plate tests. Quantitative analysis of protein expression of glial markers, XCL1, and/or XCR1 was performed by Western blot and visualized by immunofluorescence.

Results: MC treatment diminished allodynia (0.9 ± 0.1 g; n = 7 *vs.* 3.8 ± 0.7 g; n = 7) and hyperalgesia (6.5 ± 0.6 s; n = 7 *vs.* 16.5 ± 1 s; n = 7) in the streptozotocin-induced diabetes. Repeated MC administration prevented microglial activation and inhibited the up-regulation of the XCL1/XCR1 levels. XCL1 administration (10 to 500 ng/5 μl; n = 9) in naive mice enhanced nociceptive transmission, and injections of neutralizing XCL1 (4 to 8 μg/5 μl; n = 10) antibody into the mice with diabetic neuropathic pain diminished allodynia/hyperalgesia. Microglia activation evoked in primary microglial cell cultures resulted in enhanced XCL1 release and XCR1 expression. Additionally, double immunofluorescence indicated the widespread coexpression of XCR1-expressing cells with spinal neurons.

Conclusions: In diabetic neuropathy, declining levels of XCL1 evoked by microglia inhibition result in the cause of analgesia. The putative mechanism corroborating this finding can be related to lower spinal expression of XCR1 together with the lack of stimulation of these XCR1 receptors, which are localized on neurons. **(Anesthesiology 2016; 125:573-89)**

DIABETES is responsible for 60% of all deaths and is one of the world's major causes of premature illness.[1] The ability of glial cells and neurons to release immunologic factors is well documented.[2–4] Additionally, these factors seem to be responsible for the development of the neuropathic pain[5,6] that also accompanies diabetes.[7,8] The roles of activated microglia in pain models, including diabetic neuropathy, are well established,[7,9–13] and the substance that is most commonly used as microglial inhibitor is minocycline hydrochloride (MC).[7,11,12,14] MC treatment have been demonstrated to not only diminish allodynia/hyperalgesia, but also to prevent the up-regulation of proinflammatory and increase the antiinflammatory factors during diabetic neuropathy.[7,15] Recent studies indicate that chemokines from the C-X-C-motif-chemokine (CXC) subfamily participate in the development of diabetic neuropathy[8]; however, the roles of chemokine-C-motif ligand (XCL) subfamily in this process remain unknown.

The chemokines of the XCL subfamily consist of only two highly related members: chemokine-C-motif ligand 1 (XCL1; single cysteine motif [SCM]-1α) and XCL2 (SCM-1β).[16] Both of these chemokines exert their biologic effects through the G protein–coupled receptor chemokine-C-motif receptor 1 (XCR1; called G protein–coupled receptor 5).[17] XCR1 has been detected in many cell types,[18] but there is no existing evidence to prove its expression on neurons,

What We Already Know about This Topic

- Cytokines produced in spinal cord tissue are felt to be possible contributors to neuropathic pain
- Glial cells are a likely source of pain-related cytokines

What This Article Tells Us That Is New

- Using the mice streptozotocin model of diabetic neuropathy, it was observed that chemokine-C-motif ligand 1 was up-regulated in microglial cells, while chemokine-C-motif receptor 1 was found to be expressed on spinal neurons
- The administration of the microglial inhibitor minocycline hydrochloride or anti–chemokine-C-motif ligand 1 antibodies reduced tactile allodynia and thermal hyperalgesia

Submitted for publication September 11, 2015. Accepted for publication June 6, 2016. From the Departments of Pain Pharmacology (M.Z., E.R., A.P., J.M.) and Brain Biochemistry (G.K.), Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland.

 Anesthesiology 2016; 125:573-89

<zdoi;10.1097/ALN.0000000000001219>

astroglia, or microglia. The gene for its ligand *Xcl1* has been found in a variety of animal genomes, including rodents; however, the *Xcl2* gene is present in a limited range of species, and there is no evidence of the presence of this gene in the mouse genomes.[19] XCL1 is expressed by various immune cells[18] and has also been detected in astrocytes and monocytes.[20,21] The interaction between XCL1 and XCR1 plays a crucial role in the classical immunology response; however, the participation of this interaction in the diabetic neuropathy and the influences of MC on the expressions XCL1–XCR1 have not yet been studied.

We hypothesized that XCL1 and its receptor localized on glial and/or neuronal cells might play an important role in diabetic neuropathy development, and their pharmacologic modulation may have beneficial effects. Therefore, we determined the influence of twice-daily MC treatment on allodynia/hyperalgesia and glial activation in diabetic neuropathy model. We examined changes in the protein levels of XCL1–XCR1 during diabetic neuropathy and the modulation of these changes by MC administration. Furthermore, using primary glial cultures, we studied if XCL1–XCR1 is of cellular origin and examined the influence of MC treatment on these proteins' expression. We also verified the influence of intrathecal XCL1 administration on nociceptive transmission in naive mice and the influence of a XCL1 neutralizing antibody in mice with induced diabetic neuropathy. Finally, we made attempt to visualize cellular localization of XCR1 by immunohistochemistry.

Materials and Methods

Animals

Albino Swiss male mice (20 to 22 g) were purchased from Charles River (Germany) and housed in cages lined with sawdust under a 12/12-h light/dark cycle. Food and water were available without limitation. All experiments were performed in accordance with the Institute`s Animal Research Bioethics Committee (Poland), the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the International Association for the Study of Pain rules.[22] Upon their arrival to the institute, the animals were placed in randomly chosen cages by animal house staff and randomly distributed among experimental groups directly before the experiment was started.

Induction of the Type 1 Diabetes Model

To create the mouse model of type 1 diabetes, single intraperitoneal administrations of streptozotocin (200 mg/kg; Sigma Aldrich, USA) dissolved in water for injection[7,23,24] were performed. The control nondiabetic mice were injected with water. The blood glucose concentration was measured from the tail vein with an Accu-Chek Active glucometer (Roche, Switzerland). The mice were considered diabetic if the serum glucose levels were greater than 300 mg/dl. To observe the changes in the development of diabetes, behavioral tests were performed on days 7, 14, and 21 after streptozotocin administration.

Pharmacologic Study

Intraperitoneal MC Administration. MC was purchased from Sigma Aldrich, dissolved in water for injection, and administered at dose of 30 mg/kg. MC was first preemptively intraperitoneally injected at 16 and 1 h before streptozotocin injection and then twice daily for 21 days. The control groups received vehicle (water for injection; Polfa, Poland) injections according to the same schedule. Mice with developed neuropathy, which received vehicle or MC, revealed similar cage behavior and weight (please see our results). No adverse side effects of MC treatment were observed during the time of experiments. The behavioral tests were conducted on days 7, 14, and 21 after streptozotocin injection 30 min after intraperitoneal MC administration. In case of these experiments, there was no possibility to perform blind study due to physical properties of MC (yellow powder, clearly visible after reconstitution). Furthermore, it was administered twice daily for 21 days by the same persons who were carrying out the behavioral analysis. To compare the effectiveness of single intraperitoneal and single intrathecal administrations, another group of animals received single MC (30 mg/kg) injections on day 7 after streptozotocin administration. The behavioral tests were performed 1 and 4 h after the MC injection.

Intrathecal Administration. The intrathecal administrations were performed according to the methods of Hylden and Wilcox.[25] The XCL1 chemokine, its neutralizing antibody, or MC were injected in a volume of 5 μl using a Hamilton syringe with a thin needle between the L5 and L6 vertebrae in the lumbar portion of the spinal cord. The tail reflex was used as an indication of the proper administration of the drug.

Single MC Administration. Single intrathecal MC (60 μg/5μl) injections were performed on day 7 after streptozotocin administration, and the behavioral tests were conducted after 1 and 4 h after the MC injections.

Single XCL1/Lymphotactin Administration. The XCL1 was obtained from R&D Systems (USA) and dissolved in water for injection. The reconstituted chemokine was intrathecally injected into naive mice at the following concentrations: 10, 100, and 500 ng/5 μl. The behavioral tests were performed at 1, 4, 24 and 96 h after XCL1 administration.

Single XCL1/Lymphotactin Neutralizing Antibody Administration. The antimouse XCL1 neutralizing antibody was purchased from Amsbio (UK), reconstituted in water for injections, and singly intrathecally injected at day 7 after streptozotocin administration at the following concentrations: 500 ng, 1 μg, 2 μg, 4 μg, and 8 μg/5 μl. The XCL1/lymphotactin neutralizing antibody was used throughout the study and is referred to in the text as "nAb." The behavioral tests were performed at 1, 4, 24, and 96 h after nAb administration. Additionally, to eliminate the influence of

the antibody injection on the nociceptive transmission, a control antibody (normal rabbit IgG; R&D Systems) was intrathecally applied at a dose of 8 μg/5 μl followed by behavioral tests carried out according to the same experimental schedule.

Single Administration of XCL1/Lymphotactin Neutralizing Antibody on XCL1/Lymphotactin Effects in Naive Mice. To evaluate if the single intrathecal administration of XCL1 neutralizing antibody can influence the XCL1 effects in naive mice in this experimental schedule, the animals were divided into five groups as summarized in table 1. The first group consisted of the naive animals (control group) without any treatment. The next two groups consisted of the mice singly injected with vehicle (water for injections) 15 min before single vehicle or XCL1 (500 ng/5 μl) administration. The last two groups received antimouse XCL1 neutralizing antibody (8 μg/5 μl) singly injected 15 min before single vehicle or XCL1 (500 ng/5 μl) administration. The behavioral tests were conducted after 4 h after the second round of administration.

Behavioral Tests

Tactile Allodynia (von Frey Test). The reactions to nonnoxious stimuli were evaluated with von Frey filaments, which are calibrated nylon monofilaments of increasing strengths (from 0.6 to 6 g; Stoelting, USA). The filaments were applied sequentially to the plantar surfaces of the hind paws until withdrawal responses were observed.[7,26]

Thermal Hyperalgesia (Cold Plate Test). The reactions to noxious stimuli were assessed with the cold plate test (Cold/Hot Plate Analgesia Meter, Columbus Instruments, USA). The mice were placed on the cold plate at a temperature of 2°C. The latency of hind paw elevation was recorded. The cutoff latency was 30 s.[7,27]

Nociceptive Threshold (Tail-flick Test). Using a tail-flick analgesic meter (Analgesia Meter; Ugo Basile, Italy), the pain threshold in response to a thermal stimulus was assessed by focusing a beam of light on the dorsal tail surface 1 cm from the tip of the tail. The baseline was determined as 4 to 4.4 s, and cutoff time was 9 s.[7]

Measurement of Blood Glucose and Body Weight. Using an Accu-Chek Active glucometer, the blood glucose concentrations were determined during the body weight measurements on days 7, 14, and 21 after streptozotocin administration.

Primary Microglial and Astroglial Cultures

Primary cultures of microglia and astroglia were used in our *in vitro* studies. Both types of cell culture were prepared from Wistar rat pups (1 day old) as previously described.[28] The cells were isolated from the cerebral cortex and plated at a density of 3×10^5 cells/cm^2 in a culture medium composed of Dulbecco's Modified Eagle's medium/Glutamax/high glucose (Gibco, USA) supplemented with heat-inactivated 10% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 U/ml penicillin (Gibco). The cultures were maintained in poly-L-lysine-coated 75-cm^2 culture flasks at 37°C and 5% CO_2. After 4 days, the culture medium was changed. The next step involved the recovery of the loosely adherent microglial cells by gentle shaking and centrifugation at 37°C for 24 h (200 rpm) on day 9 and after replacing the medium on day 12. The medium was removed, and the astrocytes were replated in culture dishes that were maintained for 3 days and then trypsinized (0.005% trypsin ethylenediaminetetraacetic acid solution, Sigma-Aldrich). The microglia and astroglia were resuspended in culture medium and then plated at final densities of 2×10^5 cells on 24-well plates for mRNA analysis and 1.2×10^6 cells on 6-well plates for protein analysis and incubated for 48 h. The primary microglia and astrocyte cultures were treated with MC (20 μM) 30 min before the administration of lipopolysaccharide (100 ng/ml; Sigma-Aldrich) and left for 24 h. To identify the microglia and astrocytes in the *in vitro* cell cultures, we stained ionized calcium-binding adaptor molecule 1 (IBA1) as a microglial marker (anti-IBA1, 1:500, Santa Cruz, USA) and glial fibrillary acidic protein (GFAP) as an astrocyte marker (anti-GFAP, 1:500, Santa Cruz). We obtained highly homogeneous microglial and astroglial populations that were more than 95% positive for IBA1 and GFAP, respectively. The homogeneities of our cultures were similar to those of Zawadzka and Kaminska.[28]

Biochemical Tests

Analysis of Gene Expression by qRT-PCR. The primary microglial cultures were treated with MC (20 μM) 30 min before the administration of lipopolysaccharide (100 ng/ml) and stimulated for 24 h for mRNA analysis. The total RNA was extracted with TRIzol reagent (Invitrogen, USA) as previously described.[29] The RNA concentrations were measured using a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies, USA). Reverse transcription was

Table 1. Schedule of the Intrathecal Administration of XCL1 nAb (8 μg/5 μl) on XCL1 (500 ng/5 μl) Effects in Naive Mice

Experimental Groups	First Intrathecal Administration	Second (after 15 min) Intrathecal Administration	
Naive	—	—	Behavioral tests (von Frey and cold plate) were conducted 4 h after last administration
Vehicle + Vehicle	Vehicle (water for injection)	Vehicle (water for injection)	
nAb XCL1 + Vehicle	nAb XCL1 (8 μg/5 μl)	Vehicle (water for injection)	
Vehicle + XCL1	Vehicle (water for injection)	XCL1 (500 ng/5 μl)	
nAb XCL1 + XCL1	nAb XCL1 (8 μg/5 μl)	XCL1 (500 ng/5 μl)	

nAb = neutralizing antibody; XCL1 = chemokine-C-motif ligand 1.

performed on 500 ng of total RNA from the cultured cells using Omniscript reverse transcriptase (Qiagen Inc., USA) at 37°C for 60 min. The real-time reactions were performed in the presence of an RNAse inhibitor (Promega, USA) and oligo (dT16) primers (Qiagen Inc.). The cDNA was diluted 1:10 with H_2O, and for each reaction, approximately 50 ng of cDNA synthesized from the total RNA template was obtained from each individual animal and used for the quantitative real-time polymerase chain reaction (qRT-PCR) reactions. qRT-PCR was performed using Assay-On-Demand TaqMan probes (Applied Biosystems, USA) and run on an iCycler device (Bio Rad, USA). The amplification efficiency for each assay was determined by running a standard dilution curve. The following TaqMan primers were used: Rn01527840_m1 (*Hprt*, rat hypoxanthine guanine phosphoribosyl transferase), Rn00592605_m1 (*Xcl1*), and Rn03037149_s1 (*Xcr1*). The expressions of Hprt were measured in the vehicle with MC-, lipopolysaccharide- and both lipopolysaccharide- and MC-treated cells and quantified relative to the control (vehicle) to account for variations in the amounts of cDNA. The *Hprt* levels did not significantly differ across all groups, and *Hprt* was, therefore, used as a housekeeping gene control (data not shown). The cycle threshold values were calculated automatically with the iCycler IQ 3.0 software using the default parameters. The RNA abundance was calculated as $2^{-(\text{threshold cycle})}$.

Western Blot Protein Analysis. From the naive, vehicle (water for injection)-treated, and MC-treated streptozotcin-induced diabetic neuropathic mice, the lumbar (L4 to L6) parts of the spinal cords were removed at days 7, 14, and 21 after streptozotocin injection. Furthermore, the lumbar spinal cord (L4 to L6) from naive, vehicle + vehicle–treated, nAb + vehicle–treated, vehicle + XCL1–treated, and nAb + XCL1–treated mice was dissected after 4 h after last administration. The lysates from the cell cultures and tissues were collected using radioimmunoprecipitation assay buffer with inhibitor cocktails (Sigma-Aldrich) and cleared by centrifugation (14,000*g* for 30 min, 4°C). The protein concentrations in the supernatants were determined with bicinchoninic acid assay Protein Kits (Sigma). Samples containing 25 μg protein were heated in loading buffer for 8 min at 99°C and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 4 to 15% polyacrylamide Criterion TGX gels (Bio Rad). After gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Bio Rad). Using 2.5% blocking buffer (2.5% nonfat dry milk dissolved in Tris-buffered saline [TBS] with Tween 20 [TBST]), the blots were blocked at room temperature for 30 min and then incubated for 24 h at 4°C with antimouse XCL1 (LSBio, USA; 1:500), antimouse XCR1 (Santa Cruz; 1:200), antimouse cluster of differentiation (CD) 4 (CD4; Proteintech, USA; 1:500), antimouse CD8 (Abcam, UK; 1:500), antimouse IBA1 (Proteintech; 1:250), antimouse GFAP (Novus Biologicals, Inc., USA; 1:10,000), antirat XCL1 (BioVision Inc., USA; 1:500), and antirat XCR1 (Santa Cruz; 1:200) primary antibodies diluted in a SignalBoost Immunoreaction Enhancer Kit (Calbiochem, USA). After TBST washes, the blots were incubated with secondary antibodies conjugated to horseradish peroxidase diluted at 1:5,000 in a SignalBoost Immunoreaction Enhancer Kit for 1 h at room temperature. After three washes in TBST, the immunocomplexes were detected using a Clarity Western ECL Substrate (Bio Rad) and visualized with a Fujifilm Luminescent Image Analyzer LAS 4000 System (Fujifilm, China). Next, the blots were washed in TBS, stripped in stripping buffer (Thermo Scientific, USA), washed again in TBS, blocked, and reprobed with a mouse antibody against glyceraldehyde 3-phosphate dehydrogenase (1:5,000, Millipore, USA) as a loading control. The relative levels of immunoreactivity were quantified using Fujifilm Image Gauge software (Fujifilm).

Immunofluorescence Staining

Immunohistochemistry assays were performed on lumbar (L4 to L6) spinal cords that were removed from diabetic mice on day 7 after streptozotocin administration. Sections preparation and immunofluorescence staining were performed as described in the study by Chmielarz *et al.*[30] Briefly, after deparaffinization followed by antigen retrieval procedure (microwave method with citrate buffer), sections from streptozotocin-treated mice were incubated for 30 min in 5% normal pig serum (Vector Labs, USA) in PBST buffer (0.2% Triton X-100 in phosphate-buffered saline). Sections were incubated overnight at 4°C with following primary antibodies: anti-XCR1 (1:50, Abcam, ab188896), anti-NeuN (neuronal nucleus, 1:500, Millipore, MAB377), anti-IBA1 (1:50, Abcam, ab139590), and anti-GFAP (1:500, Millipore, AB5541). Antigen-bound primary antibodies were visualized with anti-rabbit Alexa-594–, anti-mouse Alexa-488–, and anti-chicken Alexa-488–coupled secondary antibodies. Stained sections were examined and photographed under fluorescent microscope (Nikon Eclipse 50i, Netherlands). Dorsal part of lumbar spinal cord was visualized on representative images.

Statistical Analysis

The behavioral data (in vivo studies) are presented as the means ± SD. Sample size determination was estimated according to our previous experiments.[7,8] *The biochemical data (ex vivo studies)* are presented as fold changes relative to the controls (naive) ± SD. The results of the qRT-PCR analyses are presented as normalized averages derived from the threshold cycle in the quantitative polymerase chain reaction. The protein analyses were performed using the Western blot technique. *The primary glial cell cultures data (in vitro studies)* are presented as the means ± the SD and represent the normalized averages derived from the analyses of three experiments.

The results were evaluated using one-way ANOVA followed by Bonferroni test for comparison of selected biologically relevant groups as visualized in figure 1 (*in vivo studies*;

naive *vs*. any of the other group; vehicle–streptozotocin– *vs*. MC–streptozotocin–induced neuropathy 1 h and MC–streptozotocin 1 *vs*. 4 h), figure 2 (*in vivo studies*; vehicle–streptozotocin– *vs*. MC–streptozotocin at each of the investigated time points: day 7, 14, and 21), figure 3 (*ex vivo studies*; vehicle–streptozotocin– *vs*. MC–streptozotocin at each of the three time points: day 7, 14, 21), figure 4 (*ex vivo studies*; the immunohistochemical study was not statistically analyzed), figure 5 (*in vitro studies*; unstimulated cells *vs*. all other groups and vehicle- *vs*. MC-treated lipopolysaccharide stimulated), figure 6 (*in vivo studies*; vehicle–naive– *vs*. XCL1–naive at each of the investigated time points: 1, 4, 24, and 96 h), figure 7 (*in vivo studies*; vehicle–streptozotocin– *vs*. nAb XCL1–streptozotocin at each of the investigated time points: 1, 4, 24, and 96 h), and figure 8, A and B (*in vivo studies*); figure 8, C–F (*ex vivo studies*; naive *vs*. any of the other group, vehicle–naive *vs*. any of the other group and vehicle + XCL1 – naive *vs*. nAb + XCL1–treated naive). Additionally, the results were evaluated using two-way ANOVA to determine the time × drug interaction, if applicable (figs. 2, 3, 6, and 7).

All statistical analyses mentioned in Materials and Methods were performed with GraphPad Prism version 5.04 (GraphPad Software, Inc., USA) involving automatic adjustment of the sheer number of each group being the subject of analysis (detailed values given in figure legends).

Results

The Influence of MC Administration on Allodynia and Hyperalgesia in the Mouse Model of Streptozotocin-induced Diabetic Neuropathy

Effects of Single Intrathecal or Single Intraperitoneal Administrations. An increase in the plasma glucose concentration (518 ± 11.4 mg/dl) was observed on day 7 after the single streptozotocin (200 mg/kg, intraperitoneally) administration compared with the naive animals (188 ± 7.4 mg/dl). Simultaneously, we observed the development of allodynia and hyperalgesia (fig. 1, A and B). Then allodynia and hyperalgesia were measured 1 and 4 h after single intrathecal (60 μg/ 5μl) or intraperitoneal (30 mg/kg) MC administration (fig. 1, A and B). The antiallodynic effect of MC to the nonnoxious stimuli in the von Frey test was observed at 1 h after both the intrathecal and intraperitoneal administrations, and this effect disappeared after 4 h (fig. 1A). Similarly, the antihyperalgesic effects to the noxious stimuli in the cold plate test were observed 1 h after both the intrathecal and intraperitoneal administrations, and these effects disappeared after 4 h (fig. 1B).

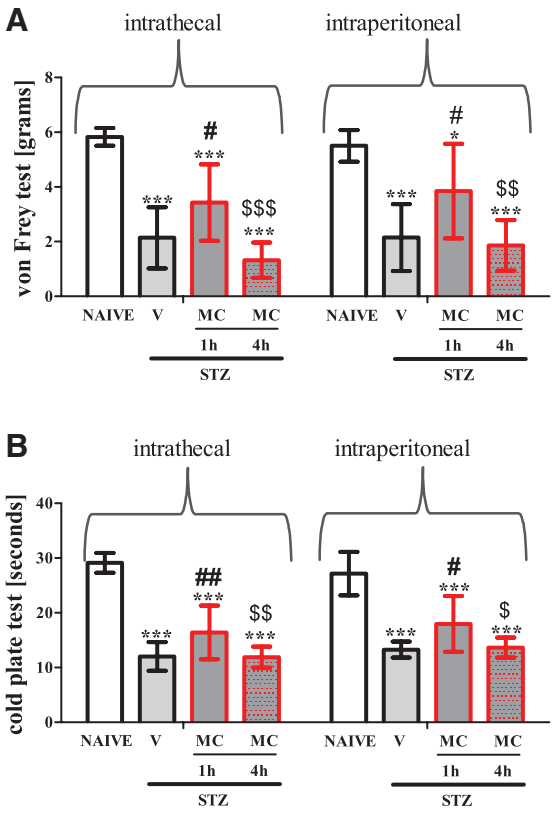

Fig. 1. The effects of single intrathecal and intraperitoneal minocycline hydrochloride (MC) administrations on allodynia and hyperalgesia in streptozotocin (STZ)-induced diabetic neuropathic pain in mice at day 7 after STZ administration. The effects of single intrathecal (60 μg/5 μl) or single intraperitoneal (30 mg/kg) MC administration on mechanical allodynia (von Frey test; *A*) and thermal hyperalgesia (cold plate test; *B*) were evaluated at 1 and 4 h after administration. Data are presented as the means ± SD (4 to 8 mice per group). The results were evaluated using one-way ANOVA followed by Bonferroni test for comparisons of selected pairs; **P* < 0.05 and ****P* < 0.001 compared naive mice *versus* any of the other group; #*P* < 0.05 and ##*P* < 0.01 compared the vehicle (V)- *versus* MC-treated streptozotocin-induced diabetic neuropathic pain in mice; $*P* < 0.05, $$*P* < 0.01, and $$$*P* < 0.001 comparison between 1 and 4 h in MC-treated STZ-induced diabetic neuropathic pain in mice.

Effect of Twice-daily Intraperitoneal Administration. The data for the naive groups at each time point were similar; therefore, for clarity of results presentation, we show them as average values. Single streptozotocin (200 mg/kg, intraperitoneally) administrations caused increases in the plasma glucose concentrations measured 7, 14, and 21 days after injection compared with the naive animals (fig. 2A). The blood glucose levels of the streptozotocin-injected mice with MC treatment (30 mg/kg, intraperitoneally; preemptively injected at 16 and 1 h before streptozotocin injection and twice daily for 21 days thereafter) were reduced at 14 and 21 days after streptozotocin administration compared with the streptozotocin-treated mice that received vehicle injections (fig. 2A). In this case, two-way ANOVA did not show time × drug interaction ($F_{2,71}$ = 0.1901; *P* = 0.8273); nevertheless, the glucose levels were significantly diminished after MC treatment to similar extent regardless of investigated time point ($F_{1,71}$ = 6.293; *P* = 0.0144).

Furthermore, streptozotocin administration resulted in decreases in body weight at each of the points of the experiment compared with the naive mice, whereas the MC treatment did not affect body weight (fig. 2B). Two-way ANOVA confirmed nonsignificant influence

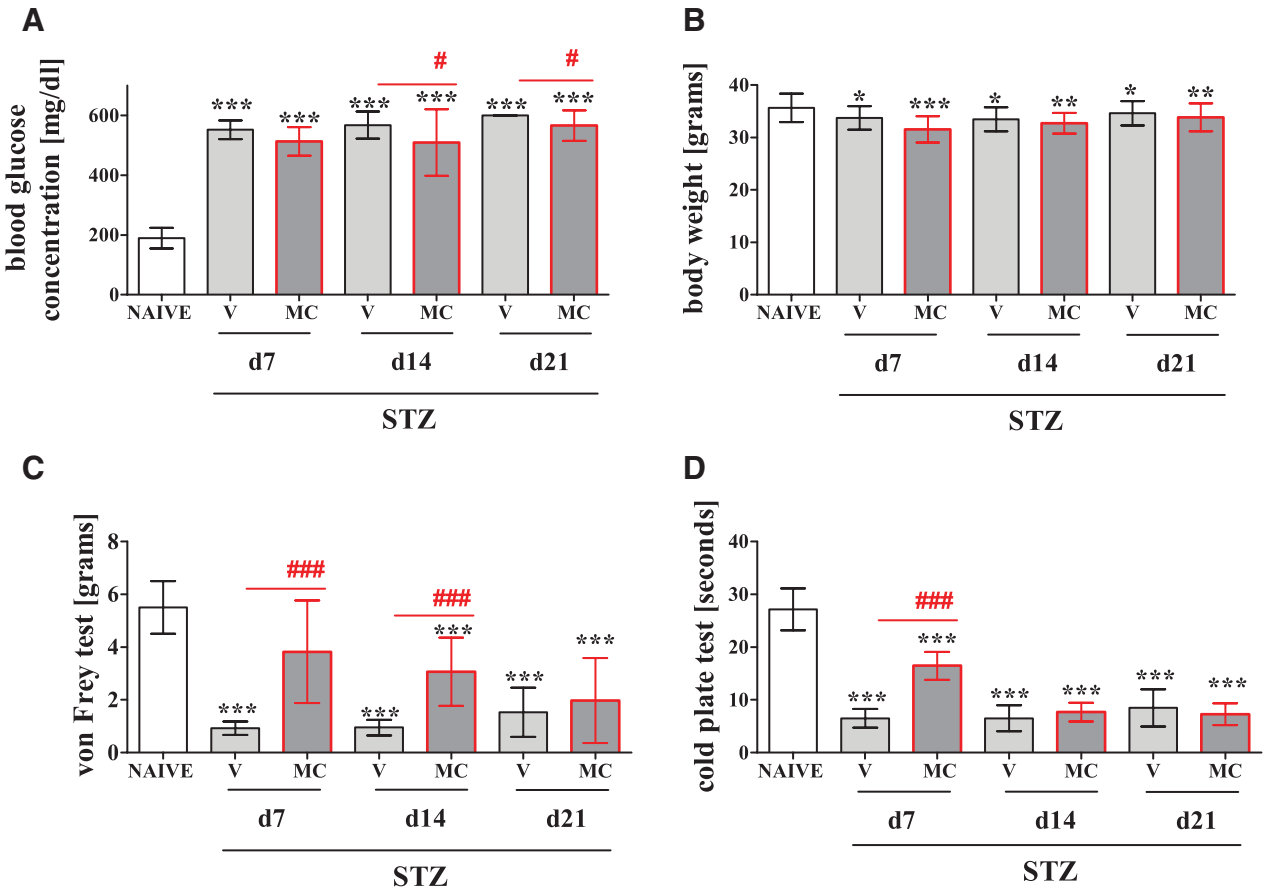

Fig. 2. The effect of twice-daily minocycline hydrochloride (MC; 30 mg/kg, intraperitoneally, for 21 days) treatment on streptozotocin (STZ; 200 mg/kg; intraperitoneally)–induced hyperglycemia, body weight changes, allodynia, and hyperalgesia measured at days 7, 14, and 21 after STZ administration. The effects of single STZ administrations and twice-daily vehicle (V) or MC treatment on plasma glucose (*A*), body weight (*B*), allodynia (von Frey test; *C*), and hyperalgesia (cold plate test; *D*) are shown. Data are presented as the means ± SD (7 to 30 mice per group). The results were evaluated using one-way ANOVA followed by Bonferroni test for comparisons of selected pairs. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with the naive animals; #*P* < 0.05 and ###*P* < 0.001 compared the V- *versus* MC-treated STZ-induced diabetic neuropathic pain in mice at respective time points.

($F_{2,115}$ = 1.009; *P* = 0.3679) of MC administration on body weight.

Allodynia and hyperalgesia were detected at 7, 14, and 21 days after streptozotocin administration. Mechanical allodynia was measured with von Frey filaments (fig. 2C). The mice with streptozotocin-induced diabetic neuropathy reacted to the nonnoxious stimuli delivered with lower strength nylon monofilaments compared with the control group (fig. 2C). MC administration delayed the development of mechanical allodynia until day 14 (*e.g.*, on day 7: 3.8 ± 0.7 g; n = 7 *vs.* 0.9 ± 0.1 g; n = 7) compared with the diabetic mice (fig. 2C). In this case, two-way ANOVA showed time × drug interaction ($F_{2,45}$ = 4.475; *P* = 0.0169), and the allodynia was significantly diminished after MC treatment ($F_{1,45}$ = 28.37; *P* < 0.0001).

Thermal hyperalgesia was assessed with the cold plate test (fig. 2D). The reactions to the noxious stimuli in the group of mice with streptozotocin-induced diabetic neuropathy were quicker than those of the naive animals at the subsequent time points (fig. 2D). MC administration delayed the development of thermal hyperalgesia only until day 7 (16.5 ± 1 s; n = 7) compared with the diabetic mice (6.5 ± 0.6 s; n = 7; fig. 2D). In this case, two-way ANOVA showed time × drug interaction ($F_{2,45}$ = 22.91; *P* < 0.0001), and the hyperalgesia was significantly diminished after MC treatment ($F_{1,45}$ = 21.75; *P* < 0.0001).

The Influence of Twice-daily MC Administration on IBA1, GFAP, CD4, CD8, XCL1, and XCR1 Protein Levels in the Lumbar Spinal Cord on Day 7, 14, and 21 in the Streptozotocin-induced Diabetic Neuropathic Pain in Mice

Changes in the protein levels in the lumbar spinal cord (L4 to L6) of the microglial (IBA1), astroglial (GFAP), and lymphoid cells ($CD4^+$ and $CD8^+$) activation markers during streptozotocin-induced diabetic neuropathy and the influence of MC treatment on these levels in the diabetic mice were evaluated using the Western blot technique (fig. 3, A–D). Single streptozotocin administration caused an increase in the IBA1 protein level that was detected after 7 (1.95-fold) and 14 days (1.90-fold)

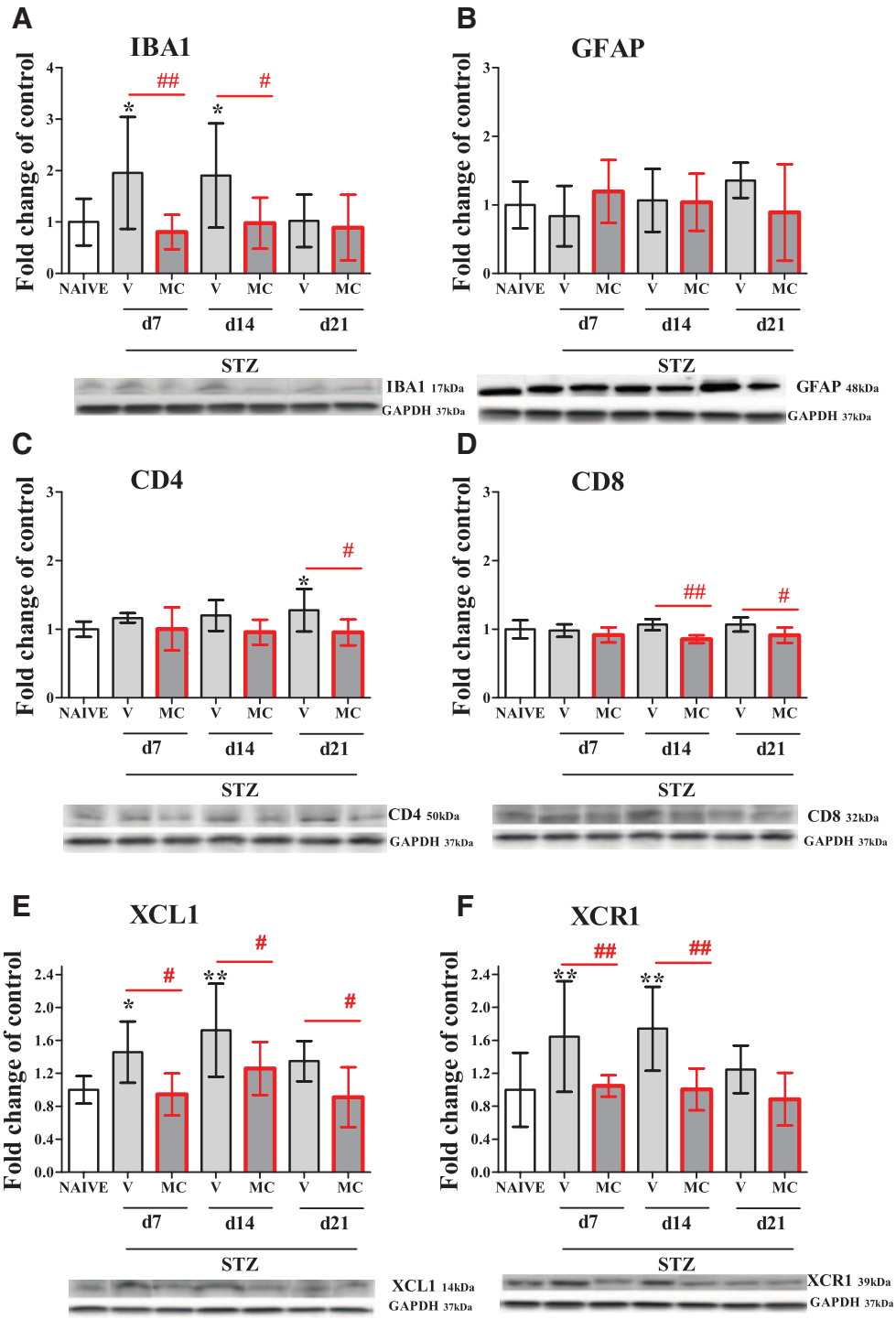

Fig. 3. The effect of twice-daily minocycline hydrochloride (MC; 30 mg/kg, intraperitoneally, for 21 days) treatment on glia and lymphoid cells activation parallel with the influence on the chemokine-C-motif ligand 1 (XCL1) and chemokine-C-motif receptor 1 (XCR1) protein levels in the streptozotocin (STZ; 200 mg/kg; intraperitoneally)-induced diabetic neuropathy model measured at days 7, 14, and 21 after STZ administration. Western blot analyses of ionized calcium-binding adaptor molecule 1 (IBA1; microglia marker, *A*), glial fibrillary acidic protein (GFAP; astroglia marker, *B*), cluster of differentiation (CD) 4 (CD4; T helper cell marker, *C*), CD8 (cytotoxic T cell marker, *D*), XCL1 (*E*), and XCR1 (*F*) in the lumbar spinal cords of naive, vehicle (V)- and MC-treated STZ-induced diabetic neuropathic pain in mice. Data are presented as fold changes relative to the control (naive) ± SD (4 to 8 samples per group). The results were evaluated using one-way ANOVA followed by Bonferroni test for comparisons of selected pairs; **P* < 0.05 and ***P* < 0.01 compared with the naive animals; #*P* < 0.05 and ##*P* < 0.01 compared the V-treated STZ-induced diabetic neuropathic pain in mice *versus* MC-treated STZ-induced diabetic neuropathic pain in mice at respective time points. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

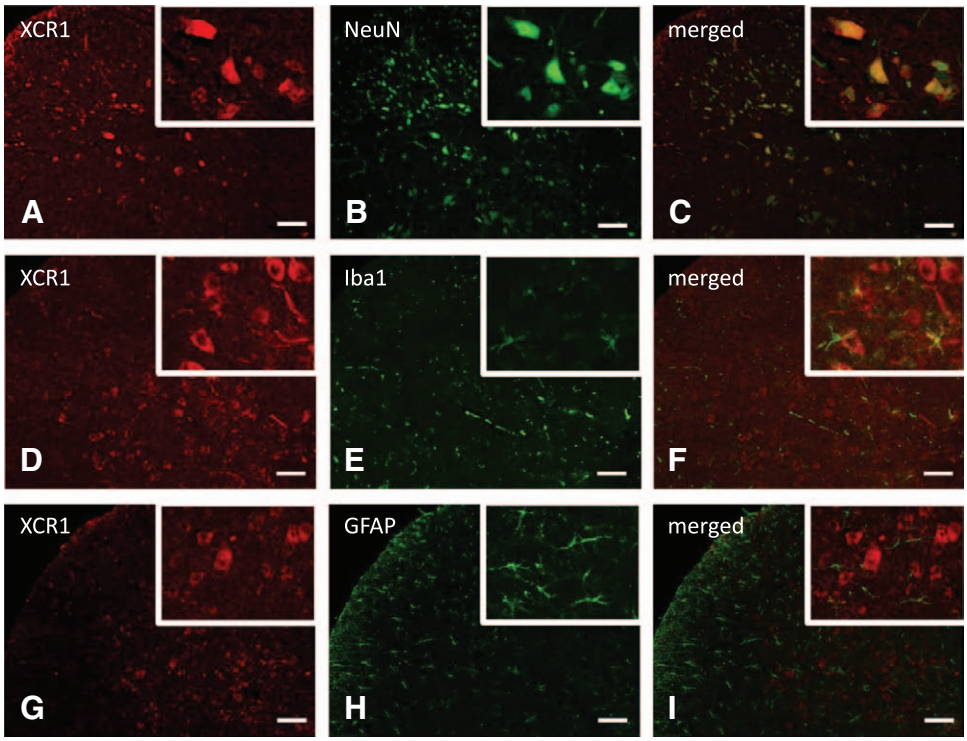

Fig. 4. Immunohistochemistry in the streptozotocin (STZ)-induced diabetic neuropathy in mice. Representative immunofluorescent images from colocalization analysis performed on spinal cord, paraffin-embedded 7-μM microtome slices from streptozotocin model: chemokine-C-motif receptor 1 (XCR1; *red*) with neuronal marker neuronal nucleus (NeuN; *green*; *A–C*), XCR1 (*red*) with microglia marker ionized calcium-binding adaptor molecule 1 (IBA1; *green*; *D–F*), and XCR1 (*red*) with astroglia marker glial fibrillary acidic protein (GFAP; *G–I*). Dorsal part of lumbar spinal cord (dissected at day 7 after STZ injection) was shown as a representative section on each of selected images. *Scale bars*: 25 μm.

but not 21 days after injection (fig. 3A). In the group that received MC, the IBA1 levels were similar to those of the control group (fig. 3A). In this case, two-way ANOVA did not show time × drug interaction ($F_{2,33}$ = 1.798; P = 0.1814); nevertheless, the IBA1 protein levels were significantly diminished after MC treatment to similar extent regardless of investigated time point ($F_{1,33}$ = 10.30; P = 0.0030).

Regarding the astroglial activation marker, neither single streptozotocin injection nor MC administration affected the GFAP protein levels (fig. 3B). Two-way ANOVA did not confirm any significant influence on GFAP protein level ($F_{2,32}$ = 2.562; P = 0.0929) of MC administration despite of investigated time points.

Single streptozotocin administration caused an increase in the CD4 protein level (1.27-fold) after 21 days after injection (fig. 3C), which was prevented by MC treatment. In this case, two-way ANOVA did not show time × drug interaction ($F_{2,28}$ = 0.3120; P = 0.7345); nevertheless, the CD4 levels were significantly diminished after MC treatment to similar extent regardless of investigated time point ($F_{1,28}$ = 8.345; P = 0.0074).

Single streptozotocin administration did not cause increase in the CD8 protein level (fig. 3D); however, the level of CD8 was considerably diminished in a group receiving MC administration on day 14. In this case, two-way ANOVA did not show time × drug interaction ($F_{2,30}$ = 1.902; P = 0.1669); nevertheless, the CD8 levels were significantly diminished after MC treatment ($F_{1,30}$ = 20.89; P < 0.0001) in all investigated time points.

The protein levels of XCL1 and XCR1 in the lumbar spinal cords (L4 to L6) of the streptozotocin-induced diabetic neuropathic pain mice with MC treatment were evaluated with the Western blot technique (fig. 3, E and F). Seven days after streptozotocin administration, the level of XCL1 was increased (1.45-fold), and this level further increased after 14 days (1.72-fold) compared with the control group (fig. 3E). MC injections prevented this up-regulation up to day 21 (fig. 3E). In this case, two-way ANOVA did not show time × drug interaction ($F_{2,30}$ = 0.03160; P = 0.9689), instead the XCL1 levels were significantly diminished after MC treatment ($F_{1,30}$ = 14.82; P = 0.0006) in all three investigated time points.

The XCR1 levels measured on days 7 and 14 were increased (1.65-fold), and MC treatment prevented this up-regulation (fig. 3F). No changes in the XCR1 protein level were detected on day 21 (fig. 3F). In this case, two-way ANOVA did not show time × drug interaction

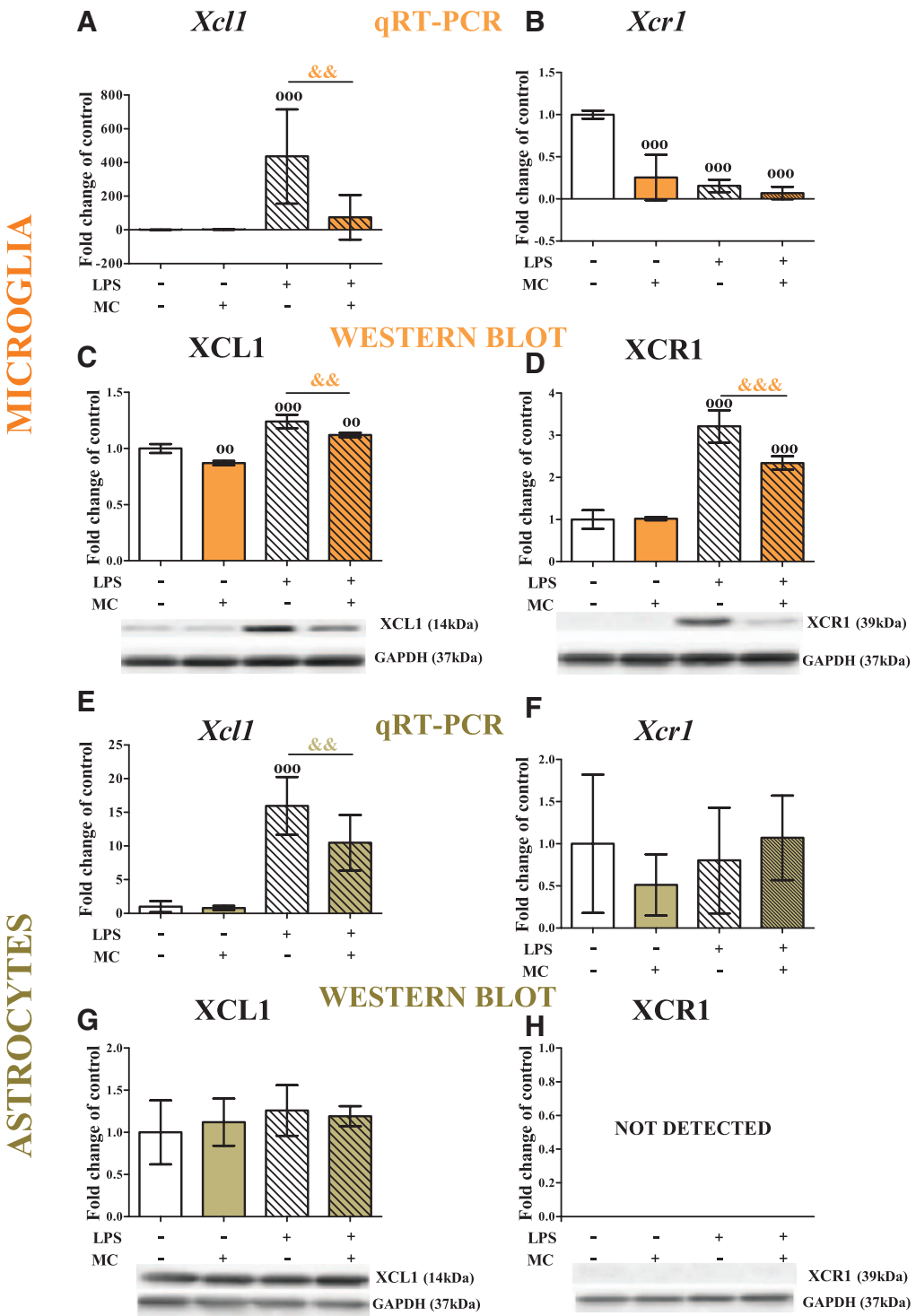

Fig. 5. The effects of minocycline hydrochloride (MC; 20 μM, 30 min before lipopolysaccharide [LPS]) treatment on the mRNA and protein levels of chemokine-C-motif ligand 1 (XCL1) and chemokine-C-motif receptor 1 (XCR1) in primary microglial and astroglia cultures after LPS (100 ng/ml) stimulation. The mRNA expressions of *Xcl1* (*A*, *E*) and *Xcr1* (*B*, *F*) were evaluated with the quantitative real-time polymerase chain reaction (qRT-PCR) technique, and the protein levels of XCL1 (*C*, *G*) and XCR1 (*D*, *H*) were determined by Western blot in the vehicle (–/–)-, vehicle with MC (–/+)–,LPS (+/–)-, and LPS with MC (+/+)–treated microglial (*A–D*) and astroglia (*E–H*) cells at 24 h after LPS stimulation. Data are presented as the means ± SD and represent the normalized averages derived from the analyses of three experiments (4 to 10 per group). The results were evaluated using one-way ANOVA followed by Bonferroni test for comparisons of selected pairs; $^{oo}P < 0.01$ and $^{ooo}P < 0.001$ compared the unstimulated cells *versus* any of the other group; &&$P < 0.01$ and &&&$P < 0.001$ compared the LPS-stimulated vehicle- *versus* MC-treated cells. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

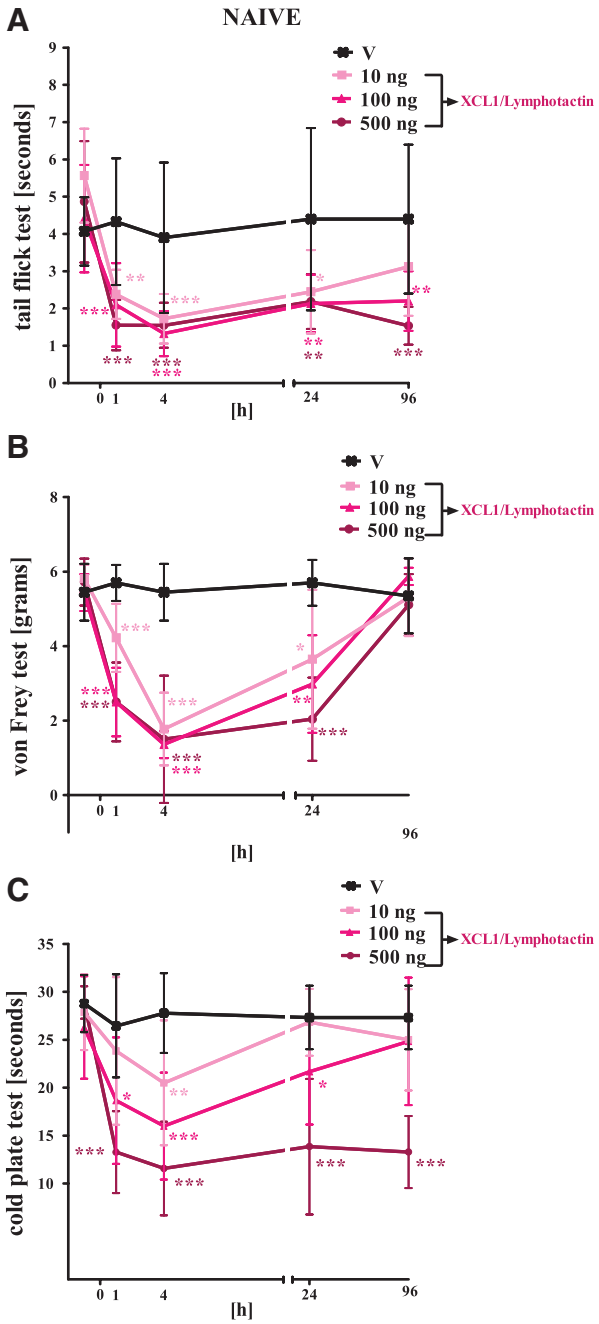

Fig. 6. The effects of single chemokine-C-motif ligand 1 (XCL1)/lymphotactin administration on nociceptive transmission in naive mice. The effects of single intrathecal XCL1 administrations (10 ng, 100 ng, or 500 ng/5 μl) on nociceptive threshold (tail-flick test; *A*), mechanical allodynia (von Frey test; *B*), and thermal hyperalgesia (cold plate test; *C*) were measured at 1, 4, 24, and 96 h after administration. Data are presented as the means ± SD (6 to 10 mice per group). The results were evaluated using one-way ANOVA followed by Bonferroni test for comparisons of selected pairs; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared the naive animals *versus* all groups at respective time points. V = vehicle.

($F_{2,35}$ = 0.7422; P = 0.4834); nevertheless, the XCR1 levels were again significantly diminished after MC treatment ($F_{1,35}$ = 19.96; P < 0.0001).

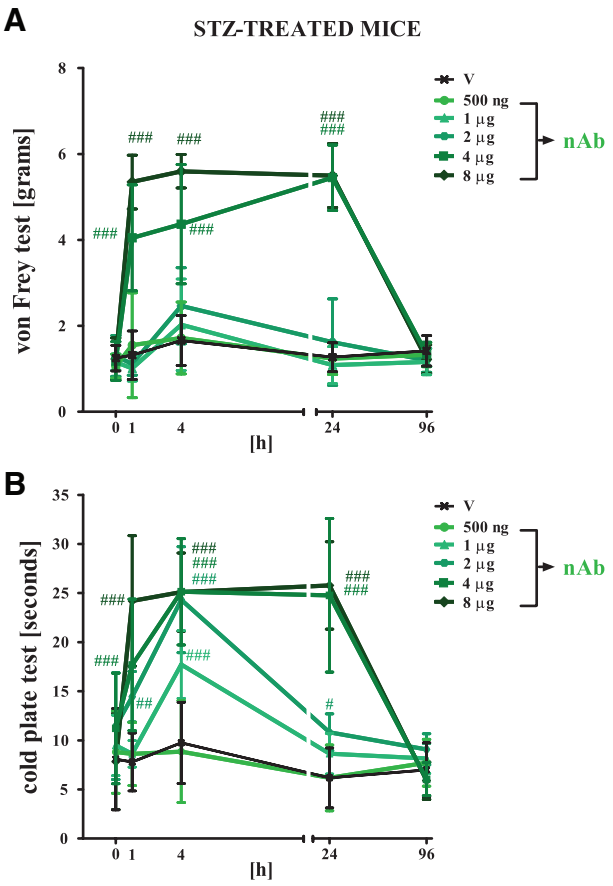

Fig. 7. The effects of single chemokine-C-motif ligand 1 (XCL1) neutralizing antibody (nAb) administrations on allodynia and hyperalgesia in streptozotocin (STZ; 200 mg/kg; intraperitoneal)-induced diabetic neuropathic pain in mice measured at day 7 after STZ injection. The effects of single intrathecal vehicle (V) or nAb XCL1 antibody administrations (500 ng, 1 μg, 2 μg, 4 μg, and 8 μg/5 μl) on developed mechanical allodynia (von Frey test; *A*) and thermal hyperalgesia (cold plate test; *B*) were measured at 1, 4, 24, and 96 h after nAb administration at day 7 after STZ injection. Data are presented as the means ± SD (6 to 13 mice per group). The results were evaluated using one-way ANOVA followed by Bonferroni test for comparisons of selected pairs; #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 compared the V- *versus* nAb-treated STZ-induced diabetic neuropathic pain in mice at respective time points.

Fluorescent Immunohistochemistry in the Streptozotocin-induced Diabetic Neuropathic Pain in Mice

Fluorescence immunohistochemical staining revealed clear neuronal but not astroglial localization of XCR1. Within investigated lumbar spinal cord sections of streptozotocin-treated mice, the NeuN (specific neuronal marker)-positive cells clearly colocalized with XCR1 (fig. 4, A–C), while no colocalization was observed with GFAP (specific astroglial marker; fig. 4, G–I). Determining of potential colocalization of XCR1 with IBA1 (microglia marker) was not conclusive. As shown on representative image, most of the XCR1 expressing cells most likely do not colocalize with IBA1 (fig. 4, D–F).

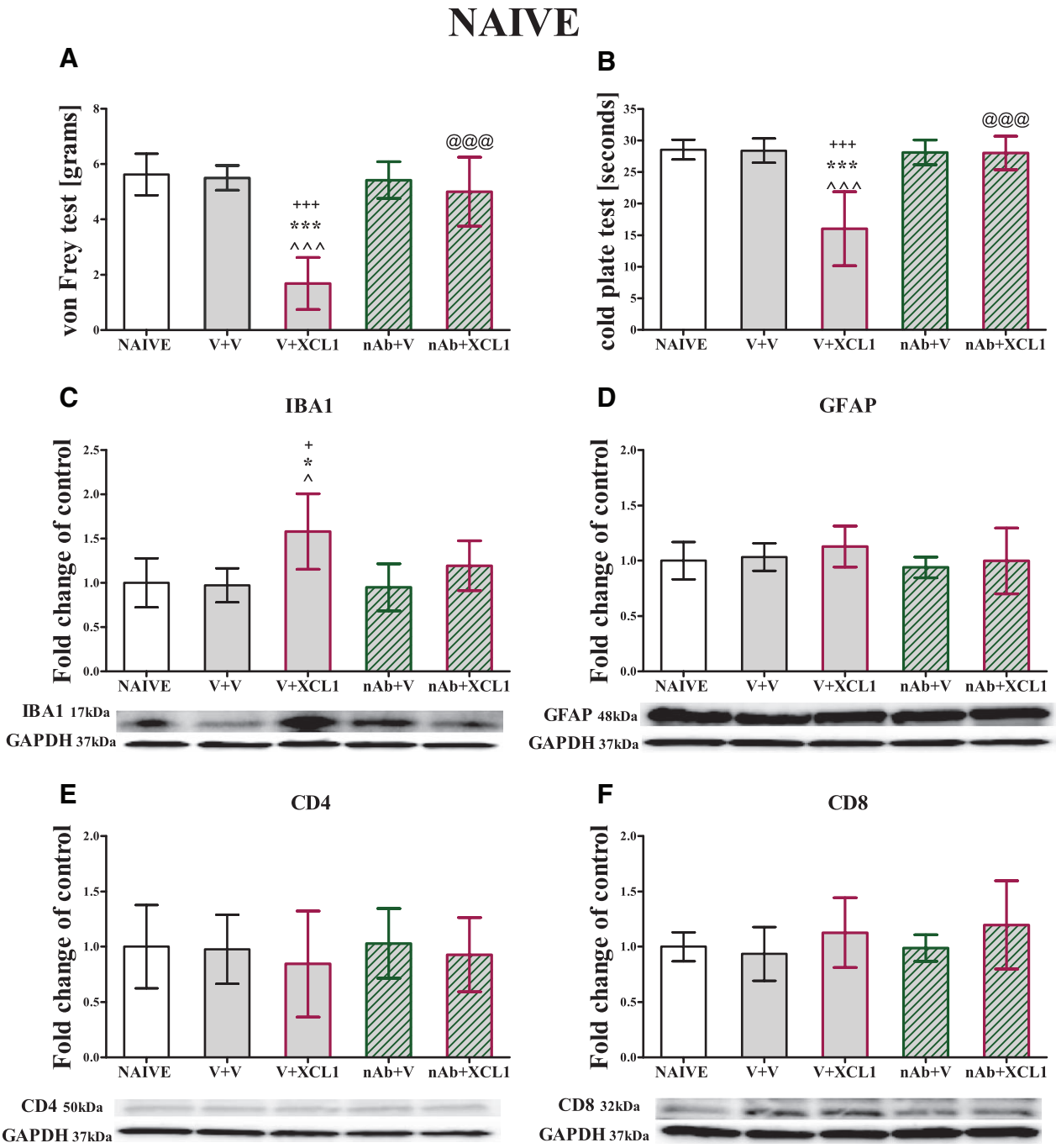

Fig. 8. The effects of single chemokine-C-motif ligand 1 (XCL1) neutralizing antibody administrations on a single XCL1/lymphotactin injection in naive mice (*A*, *B*) *on nociceptive transmission*. Single intrathecal administrations of vehicle (V) or neutralizing antibody (nAb) XCL1 (8 μg/5 μl) were performed 15 min before a single intrathecal administration of V or XCL1 (500 ng/5 μl). The effects of administration on mechanical allodynia (von Frey test; *A*) and thermal hyperalgesia (cold plate test; *B*) were measured 4 h after the last injections (*C–F*) *on spinal glial and lymphoid cells activation.* The lumbar spinal cord from naive, V + V–, nAb + V–, V + XCL1–, and nAb + XCL1–treated mice were dissected 4 h after the last administration. The protein analysis of ionized calcium-binding adaptor molecule 1 (IBA1; microglia marker, *C*), glial fibrillary acidic protein (GFAP; astroglia marker, *D*), cluster of differentiation (CD) 4 (CD4; T helper cell marker, *E*), and CD8 (cytotoxic T cell marker, *F*) was performed by Western blot technique. Data are presented as the means ± SD (3 to 12 mice per group). The results were evaluated with the use of one-way ANOVA followed by Bonferroni test for comparisons of selected pairs; $^{*}P < 0.05$ and $^{***}P < 0.001$ compared the naive animals *versus* V + XCL1–treated naive mice; $^{\wedge}P < 0.05$ and $^{\wedge\wedge\wedge}P < 0.001$ compared the V-treated naive mice *versus* V + XCL1–treated naive mice; $^{+}P < 0.05$ and $^{+++}P < 0.001$ compared the nAb-treated naive mice *versus* V + XCL1–treated naive mice; $^{@@@}P < 0.001$ indicate significant differences between the V + XCL1– and nAb + XCL1–treated naive mice. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

The Influences of Lipopolysaccharide Stimulation and MC Treatment on XCL1 and XCR1 mRNA and Protein Levels

Primary Microglial Cultures. The *Xcl1* and *Xcr1* mRNA levels in the vehicle-, vehicle with MC–, lipopolysaccharide-, and lipopolysaccharide with MC–treated microglial cells were evaluated with the qRT-PCR technique at 24 h after lipopolysaccharide (100 ng/ml) stimulation (fig. 5, A and B). The *Xcl1* mRNA level was increased after lipopolysaccharide stimulation, and the lipopolysaccharide with MC (20 μM) treatment prevents this up-regulation (fig. 5A). The expression of *Xcr1* mRNA was not changed after the lipopolysaccharide stimulation or the lipopolysaccharide with MC treatment (fig. 5B).

Using the Western blot technique, the XCL1 and XCR1 protein levels in the vehicle-, vehicle + MC–, lipopolysaccharide-, and lipopolysaccharide with MC–treated microglia were evaluated at 24 h after lipopolysaccharide (100 ng/ml) stimulation (fig. 5, C and D). The XCL1 and XCR1 protein levels in the microglia cultures were up-regulated after lipopolysaccharide stimulation, and lipopolysaccharide with MC (20 μM) treatment prevented these up-regulations (fig. 5, C and D).

Primary Astroglial Cultures. The *Xcl1* and *Xcr1* mRNA levels of the vehicle-, vehicle with MC–, lipopolysaccharide-, and lipopolysaccharide with MC–treated astroglia were evaluated with the qRT-PCR technique at 24 h after lipopolysaccharide (100 ng/ml) stimulation (fig. 5, E and F).The *Xcl1* mRNA level increased after lipopolysaccharide stimulation, and lipopolysaccharide with MC (20 μM) treatment prevented this up-regulation (fig. 5E). No changes in the *Xcr1* mRNA levels were observed (fig. 5F).

Using the Western blot technique, XCL1 and XCR1 protein levels in the vehicle-, vehicle + MC–, lipopolysaccharide-, and lipopolysaccharide with MC–treated astroglia were evaluated at 24 h after lipopolysaccharide (100 ng/ml) stimulation (fig. 5, G and H). The XCL1 level did not change after pharmacologic treatment and/or lipopolysaccharide stimulation (fig. 5G), and XCR1 protein was not detected (fig. 5H).

Effect of Single Intrathecal XCL1 Administrations on Nociceptive Transmission in Naive Mice

Single intrathecal administrations of XCL1 at each dose (10, 100, and 500 ng/5 μl) induced pronociceptive reactions as measured with the tail-flick test (fig. 6A) and caused the development of mechanical allodynia and thermal hyperalgesia as measured with the von Frey (fig. 6B) and cold plate (fig. 6C) tests, respectively.

Effect of XCL1 Administration on the Nociceptive Threshold. One hour after the injection of the highest dose (500 ng), the reactions to thermal stimuli peaked, and the two other doses produced similar but weaker responses. After 4 and 24 h, all of tested doses caused comparable reactions to heat stimuli. This effect was observed for up to 96 h in the cases of 100- and 500-ng doses but not in the case of 10-ng dose (fig. 6A). Two-way ANOVA confirmed significant interaction ($F_{12,144} = 2.277$; $P = 0.0112$) between investigated treatment and time points being subject of investigation. The XCL1 significantly enhanced nociceptive threshold ($F_{4,144} = 16.06$; $P < 0.0001$) showing pronociceptive dose-dependent effect of XCL1 in tail-flick test.

Effect of XCL1 Administration on Mechanical Allodynia. One hour after the injection of 10 ng, the reactions to nonnoxious stimuli were the weakest compared with the two other doses, which caused similar responses. The measurements that were performed after 4 h yielded the strongest responses and comparable results for all of the tested doses. These effects were dosage dependent, diminished after 24 h, and were completely reversed after 96 h (fig. 6B). Two-way ANOVA confirmed significant interaction ($F_{12,\ 142} = 8.776$; $P < 0.0001$) between investigated treatment and time points being subject of investigation. The XCL1 significantly enhanced allodynia ($F_{4,142} = 61.41$; $P < 0.0001$) showing pronociceptive dose-dependent effect of XCL1 in von Frey test.

Effect of XCL1 Administration on Thermal Hyperalgesia. The effect of a single 10-ng injection was observed after only 4 h, and the 100- and 500-ng doses elicited dose-dependent response to noxious stimuli for up to 24 h. Furthermore, the reaction after the highest dose (500 ng) was still observed 96 h after administration (fig. 6C). Two-way ANOVA confirmed significant interaction ($F_{12,119} = 3.613$; $P = 0.0001$) between investigated treatment and time points being subject of investigation. The XCL1 significantly enhanced hyperalgesia ($F_{4,119} = 15.51$; $P < 0.0001$) showing pronociceptive, dose-dependent effect of XCL1 in cold plate test.

Effect of Single Intrathecal XCL1 Neutralizing Antibody Administrations on Nociceptive Transmission in the Streptozotocin-induced Diabetic Neuropathic Pain in Mice

The XCL1 neutralizing antibody (500 ng, 1 μg, 2 μg, 4 μg, and 8 μg/ 5μl), the control antibody (normal rabbit IgG antibody; 8 μg/5 μl), and water for injections (vehicle) were administered once intrathecally on day 7 after the streptozotocin injection. Mechanical allodynia and thermal hyperalgesia were measured at 1, 4, 24, and 96 h after antibody injection using the von Frey (fig. 7A) and cold plate tests (fig. 7B), respectively. The control antibody administration did not influence the development of allodynia (*e.g.*, for 4 h: 1.5 ± 0.12 g *vs.* 1.6 ± 0.17 g) or hyperalgesia (*e.g.*, for 4 h: 10.6 ± 3.8 *vs.* 9.7 ± 1.2 s) at any time during the experiment (data not shown on the graph).

Effect of XCL1 Neutralizing Antibody Administration on Mechanical Allodynia Measured at Day 7 after Streptozotocin Injection. Single intrathecal injections of XCL1 neutralizing antibody at doses of 500 ng, 1 μg, and 2 μg did not influence the development of allodynia at any time during the experiment (fig. 7A). Effects were only observed after the administrations of the two highest doses (4 and 8 μg). These doses significantly prolonged the reactions to nonnoxious

stimuli; however, the reaction after the 4-μg dose was weaker than that after the 8 μg, which almost completely reversed the mechanical allodynia for up to 24 h (fig. 7A). Two-way ANOVA confirmed significant interaction ($F_{20,242}$ = 23.48; P < 0.0001) between investigated treatment and time points being subject of investigation in streptozotocin model. The XCL1 neutralizing antibody significantly diminished allodynia ($F_{4, 242}$ = 82.71; P < 0.0001) showing its antinociceptive dose-dependent effect in von Frey test.

Effect of XCL1 Neutralizing Antibody Administration on Thermal Hyperalgesia Measured at Day 7 after Streptozotocin Injection. Single intrathecal injections of the XCL1 neutralizing antibody at the dose of 500 ng did not influence the development of hyperalgesia at any time during experiment (fig. 7B). One hour after the administration of the 2-μg dose, the reaction to noxious stimuli was prolonged and at 4 h received similar value, which was obtained after highest dose injection. This effect was reversed by 24 h and returned to the control level after 96 h (fig. 7B). In the case of the 1-μg dose, the effect was only detected in the cold plate test at 4 h after administration (fig. 7B).The reactions obtained after the two highest doses (4 and 8 μg) were similar in that both doses significantly prolonged the reactions to noxious stimuli for up to 24 h (fig. 7B). Two-way ANOVA confirmed significant interaction ($F_{20,251}$ = 10.72; P < 0.0001) between investigated treatment and time points being subject of investigation in streptozotocin model. The XCL1 neutralizing antibody significantly diminished hyperalgesia ($F_{4, 251}$ = 52.19; P < 0.0001) showing its antinociceptive dose-dependent effect in cold plate test.

Effects of Single Intrathecal Administration of XCL1 Neutralizing Antibody on XCL1 Evoked Allodynia and Hyperalgesia and Glial and Lymphocyte Cells Activation in the Lumbar Spinal Cord in Naive Mice

The reactions to nonnoxious (fig. 8A) and noxious (fig. 8B) stimuli in naive, vehicle + vehicle–treated, and neutralizing antibody (nAb + vehicle)–injected mice were similar (fig. 8, A and B). The group vehicle + XCL1–treated developed allodynia and hyperalgesia, which was prevented by pretreatment with XCL1 neutralizing antibody (fig. 8, A and B).

The lumbar spinal cords from naive, vehicle + vehicle–, nAb + vehicle–, V + XCL1–, and nAb + XCL1–treated mice were dissected 4 h after the administration. The protein analysis of glial markers (IBA1 and GFAP) and lymphocyte ($CD4^+$ and $CD8^+$) was performed by Western blot technique (fig. 8, C–F). The increase of IBA1 protein level was only observed after XCL1 administration (fig. 8C), while the level of GFAP, CD4, and CD8 did not change in any experimental group (fig. 8, D–F).

Discussion

Our studies demonstrated that intraperitoneal/intrathecal MC treatments caused analgesic effects in a mouse model of diabetic neuropathy. MC administration prevented microglial activation and parallelly inhibited the up-regulation of XCL1 and XCR1. Primary glial culture findings provide evidence that activated microglial cells are responsible for XCL1 release and XCR1 expression, which is diminished by MC. Interestingly, immunofluorescent staining indicated that in the pathology of diabetic neuropathy, the XCR1 is expressed mainly on neurons. In behavioral studies, we demonstrated for the first time that intrathecal XCL1 administration in naive mice enhanced nociceptive transmission and that XCL1 neutralizing antibody diminished allodynia/hyperalgesia in streptozotocin-induced diabetic mice. Furthermore, administration of XCL1 neutralizing antibody prevented the development of allodynia/hyperalgesia and microglia activation induced by XCL1 injection in naive mice.

It has previously been shown that preventing microglial activation and the formation of proinflammatory interleukins with substances such as MC and pentoxifylline diminished neuropathy.[15,27,31–33] Mechanism of MC action has not been fully examined. Recent studies demonstrated that matrix metallopeptidase 9 and proinflammatory cytokines, including interleukin-6 and interleukin-18, are important in the development of neuropathic pain,[34,35] and Rojewska *et al.* demonstrated that MC treatment restores imbalances between pronociceptive and antinociceptive factors in a rat model of neuropathy.[12] It has been also reported that MC administration in diabetes models increases antinociceptive interleukins (interleukin-1α, -2, and -10)[7] and diminishes the up-regulation of pronociceptive factors tumor necrosis factor α, interleukin-1β, and inducible nitric oxide synthase.[36] Furthermore, MC attenuates pain by inhibiting spinal microglial activation,[36] which agrees with our results. The role of MC in the modulation of interleukin levels in diabetic neuropathy models has been established; however, its influence on XCL1–XCR1 expressions and the changes in their protein levels in diabetic neuropathy have not been verified.

The most commonly used substance to create type 1 diabetes models is streptozotocin, whose administration results in the development of neuropathy.[7,8,11,15] Current results correlate with our previous studies in which we demonstrated that single streptozotocin administrations result in the development of long-term diabetic neuropathy.[7] Here, we demonstrated that MC treatment prevents the development of allodynia for up to 14 days; however, the reductions in the hyperalgesic responses to noxious stimuli were only observed up to day 7. Those discrepancies may be correlated with the participation of different nerve fibers signal transduction and the unequal losses of these fibers after MC treatment during diabetes. The hyperglycemic states that occur in diabetes are responsible for disruptions of Schwann cell metabolic activity and myelin degeneration and the consequent development of neuropathic pain.[37] The reaction to noxious stimuli is transmitted by lightly myelinated A(δ) and unmyelinated C fiber, whereas the response to nonnoxious stimuli is

conducted by highly myelinated A(β) fiber.[38] In 2008, Keilhoff *et al.*[39] demonstrated that Schwann cells, which form myelin sheaths, are protected against oxygen glucose deprivation–induced cell death by MC treatment. This observation underlines our findings that MC treatment prolongs the development of allodynia but not hyperalgesia. Furthermore, MC acts as a cofactor to reduce nicotinamide adenine dinucleotide to inhibit poly(adenosine diphosphate-ribose) polymerase and prevent β cell necrosis,[40] which results in decreases in the blood glucose concentrations and indirectly delays neuropathy development.

It is known that glia play a crucial role in neuropathy development.[3,6,41] However, in the allodynia and hyperalgesia that occurs in type 1 diabetes models, microglia activation is observed,[7,42] and astrocytes are activated in type 2 diabetes models.[43] These findings correlate with our Western blot analysis, which indicated that the spinal microglia and not the astroglia were highly activated from the initial stage of diabetes until day 14. This proves that microglia are responsible for the initial neuropathy as has been previously demonstrated.[6,44] Interestingly, for up to 14 days after streptozotocin administration, the levels of XCL1–XCR1 are also increased, which highlight the important role of XCL1/XCR1 signaling in diabetic neuropathy. Moreover, MC injections not only prevented microglia activation but also prevented XCL1–XCR1 up-regulation. The role of MC in the prevention of microglia activation is established[7,11,12,14,36]; however, we first demonstrated the influence of MC on XCL1 and XCR1 expression in diabetes.

The XCL1–XCR1 interaction plays an essential role in the classical immunologic response.[18] For instance, the XCL1 has been identified to be released by natural killer, $CD8^+$ T, $CD4^+$ T helper 1 cells, astrocytes, and macrophages/microglia[20,21,45] and it seems that XCL1 participates in the T helper 1-type immune response.[45] According to our best knowledge, there is dearth of studies showing spinal infiltration by $CD4^+$ and $CD8^+$ T during diabetes. Our results suggest that the level of $CD4^+$ T and $CD8^+$ T is unchanged during first stage of diabetes, when we observed strong microglia activation. Therefore, we postulate that activated microglia are the main source of XCL1 during the development of diabetic neuropathy. The expression of XCL1 has been identified in patients in several inflammatory diseases, including Crohn disease[46] and rheumatoid arthritis,[47] however, not in diabetes. With the present diabetes model, we simultaneously demonstrated the up-regulation of spinal microglial activation and XCL1 up to day 14. Moreover, our primary cell culture studies provided the first evidence that mRNA and protein levels of XCL1 increase in the microglia after lipopolysaccharide stimulation; however, in the astroglia, the lipopolysaccharide stimulation had no influence on the protein level. Furthermore, MC treatment before lipopolysaccharide stimulation prevented the increases in mRNA expression in both glial cell cultures; however, the influence of MC on XCL1 protein levels was observed in microglia. These findings may suggest that microglia and not astroglia are primarily responsible for functional XCL1 release during inflammation.

Studies in naive animals have provided irrefutable evidence that XCL1 intrathecal administration can induce microglia activation and enhance nociceptive transmission. Interestingly, XCL1-induced pain behavior and microglia activation were abolished by pretreatment with XCL1 neutralizing antibody. Moreover, we have shown that the antibody neutralization of endogenous XCL1 results in reductions of allodynia and hyperalgesia in a model of diabetic neuropathy. In contrast, the single MC treatment diminished allodynia and hyperalgesia for only up to 1 h. It is documented that MC can reduce microglial activation by inhibiting mitogen-activated protein kinases in microglia.[48] It has been suggested that the analgesic effects of MC in neuropathy result from its ability to restore the equilibrium between the pronociceptive and antinociceptive factors,[12] thus implying a wide range of possible therapeutic effects of this drug. Contrary, the XCL1 antibody targets specific antigen, which seems to be very crucial for pain.

In 1995, Heiber *et al.*[49] identified the orphan receptor G protein–coupled receptor 5, which was subsequently demonstrated to be a high-affinity functional receptor for SCM-1 proteins,[17] and later officially designated as XCR1. XCR1 is the only receptor for XCL1 and XCL2; however, the genes for *Xcl1* and *Xcr1* but not X*cl2* have been found in mice.[19] Additionally, a viral macrophage inflammatory protein II[50] that acts as a XCR1 antagonist targets only the human and not the murine XCR1.[51] It has been suggested that XCR1 is expressed in murine $CD8^+$ dendritic cells,[52] T cells, B cells, and neutrophils[53] but not in macrophages.[54] Furthermore, the expression of XCR1 in infiltrating mononuclear cells has been detected in patients with rheumatoid arthritis[47,55] but was not studied in diabetes. The data obtained from our diabetic neuropathy model demonstrated the simultaneous activation of microglia and XCR1 up-regulation. Interestingly, our immunofluorescent staining distinctly revealed colocalization of XCR1 with NeuN. These results support hypothesis that XCL1 can directly influence neurons and not only microglial cells. Additionally, cell culture studies have demonstrated that glia express *Xcr1* mRNA and that pharmacologic treatment and/or lipopolysaccharide stimulation down-regulates this expression in microglia. Furthermore, we have demonstrated using anti-XCR1 antibody which is mapping the epitope within a cytoplasmic domain, that XCR1 is present in primary microglia and not in astroglia. The XCR1 level increases after lipopolysaccharide stimulation, and MC treatment prevents its up-regulation in microglial cultures. However, in MC-treated diabetic mice, the decreased level of XCR1 is correlated with lower level of XCL1 and microglia inhibition. This is phenomenon could be of great interest assuming that XCR1 is mainly expressed rather on neuronal cells; however, this issue needs further investigation.

Chemokines are pleiotropic factors that usually interact with several receptors. The chemokine (C-C motif)

ligand (CCL) 2 (CCL2) released from injured neurons activates chemokine (C-C motif) receptor 2-expressing microglia what leads to neuropathy development,[56] while anti-CCL2 antibody inhibits both microglia activation and behavioral changes.[57] CCL2 also activates chemokine (C-C motif) receptor 4 expressed in Th2 lymphocytes[58] and participates in macrophage recruitment.[59] Moreover, one receptor can be activated by several chemokines. It has been shown that CCL3, CCL4, and CCL5 participated in nociceptive transmission through chemokine (C-C motif) receptor 5.[60,61] However, there are known examples of chemokines interacting with one receptor only, CX3C-motif-chemokine-ligand-1 released from neuronal surface induces the activation of CX3-C-motif-chemokine-receptor-1–expressing microglia.[62] Another pair CXC-motif-chemokine-ligand-12/CXC-motif-chemokine-receptor-4 is responsible for hypersensitivity by neuronal sensitization, through CXC-motif-chemokine-ligand-12 released from astrocytes.[63,64] We have found out that XCR1 is expressed by both neurons and microglia; however, XCL1 is released by activated microglia. These findings indicate that the XCL1/XCR1 can participate in many aspects of neuroglial interaction, which seems to play a crucial role in the diabetic neuropathy.

Summarizing, neuropathy is serious and common complication of diabetes that is associated with increased risks of cardiovascular disease and mortality. In the light of our current results, we can confidently state that XCL1/XCR1 signaling plays an important role in diabetic neuropathy and that the prevention of microglial activation by MC treatment seems to strongly influence XCL1/XCR1 levels. Furthermore, the clinical studies have demonstrated that MC is safe and well tolerated[65] and that its administration improves diabetic neuropathy and visual acuity.[66] Taken together, our results provide novel insights into the participation of XCL1/XCR1 signaling in the diabetic neuropathy. Moreover, our data suggest that MC, by influencing far more immune factors than was previously thought, can have high analgesic potential for being an efficient pain relief agent in diabetic neuropathy.

Research Support

Supported by the National Science Centre grant 2012/05/N/NZ4/02416 (Krakow, malopolska, Poland); the statutory funds (Krakow, malopolska, Poland); and a scholarship from the KNOW sponsored by the Ministry of Science and Higher Education of the Republic of Poland (Warsaw, mazowieckie, Poland; to Drs. Zychowska and Piotrowska).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Mika: Department of Pain Pharmacology, Institute of Pharmacology, Polish Academy of Sciences, 12 Smetna Street, 31–343 Cracow, Poland. joamika@if-pan.krakow.pl.

References

1. World Health Organization: http://www.who.int/mediacentre/factsheets/fs312/en/. 2015. Accessed May 30, 2016
2. Boche D, Perry VH, Nicoll JA: Review: Activation patterns of microglia and their identification in the human brain. Neuropathol Appl Neurobiol 2013; 39:3–18
3. Ji RR, Xu ZZ, Gao YJ: Emerging targets in neuroinflammation-driven chronic pain. Nat Rev Drug Discov 2014; 13:533–48
4. Popiolek-Barczyk K, Kolosowska N, Piotrowska A, Makuch W, Rojewska E, Jurga AM, Pilat D, Mika J: Parthenolide relieves pain and promotes M2 microglia/macrophage polarization in rat model of neuropathy. Neural Plast 2015; 2015:676473
5. Liou JT, Lee CM, Day YJ: The immune aspect in neuropathic pain: Role of chemokines. Acta Anaesthesiol Taiwan 2013; 51:127–32
6. Mika J, Zychowska M, Popiolek-Barczyk K, Rojewska E, Przewlocka B: Importance of glial activation in neuropathic pain. Eur J Pharmacol 2013; 716:106–19
7. Zychowska M, Rojewska E, Kreiner G, Nalepa I, Przewlocka B, Mika J: Minocycline influences the anti-inflammatory interleukins and enhances the effectiveness of morphine under mice diabetic neuropathy. J Neuroimmunol 2013; 262:35–45
8. Zychowska M, Rojewska E, Pilat D, Mika J: The role of some chemokines from the CXC subfamily in a mouse model of diabetic neuropathy. J Diabetes Res 2015; 2015:750182
9. Jin D, Yang JP, Hu JH, Wang LN, Zuo JL: MCP-1 stimulates spinal microglia via PI3K/Akt pathway in bone cancer pain. Brain Res 2015; 1599:158–67
10. Mei XP, Zhou Y, Wang W, Tang J, Wang W, Zhang H, Xu LX, Li YQ: Ketamine depresses toll-like receptor 3 signaling in spinal microglia in a rat model of neuropathic pain. Neurosignals 2011; 19:44–53
11. Morgado C, Pereira-Terra P, Cruz CD, Tavares I: Minocycline completely reverses mechanical hyperalgesia in diabetic rats through microglia-induced changes in the expression of the potassium chloride co-transporter 2 (KCC2) at the spinal cord. Diabetes Obes Metab 2011; 13:150–9
12. Rojewska E, Popiolek-Barczyk K, Jurga AM, Makuch W, Przewlocka B, Mika J: Involvement of pro- and antinociceptive factors in minocycline analgesia in rat neuropathic pain model. J Neuroimmunol 2014; 277:57–66
13. Tsuda M, Inoue K: Neuron–microglia interaction by purinergic signaling in neuropathic pain following neurodegeneration. Neuropharmacology 2016;104:76–81
14. Mika J, Rojewska E, Makuch W, Przewlocka B: Minocycline reduces the injury-induced expression of prodynorphin and pronociceptin in the dorsal root ganglion in a rat model of neuropathic pain. Neuroscience 2010; 165:1420–8
15. Pabreja K, Dua K, Sharma S, Padi SS, Kulkarni SK: Minocycline attenuates the development of diabetic neuropathic pain: Possible anti-inflammatory and anti-oxidant mechanisms. Eur J Pharmacol 2011; 661:15–21
16. Kelner GS, Kennedy J, Bacon KB, Kleyensteuber S, Largaespada DA, Jenkins NA, Copeland NG, Bazan JF, Moore KW, Schall TJ: Lymphotactin: A cytokine that represents a new class of chemokine. Science 1994; 266:1395–9
17. Yoshida T, Imai T, Kakizaki M, Nishimura M, Takagi S, Yoshie O: Identification of single C motif-1/lymphotactin receptor XCR1. J Biol Chem 1998; 273:16551–4

18. Lei Y, Takahama Y: XCL1 and XCR1 in the immune system. Microbes Infect 2012; 14:262–7
19. Fox JC, Nakayama T, Tyler RC, Sander TL, Yoshie O, Volkman BF: Structural and agonist properties of XCL2, the other member of the C-chemokine subfamily. Cytokine 2015; 71:302–11
20. Kim BO, Liu Y, Zhou BY, He JJ: Induction of C chemokine XCL1 (lymphotactin/single C motif-1 alpha/activation-induced, T cell-derived and chemokine-related cytokine) expression by HIV-1 Tat protein. J Immunol 2004; 172:1888–95
21. Lisak RP, Nedelkoska L, Studzinski D, Bealmear B, Xu W, Benjamins JA: Cytokines regulate neuronal gene expression: Differential effects of Th1, Th2 and monocyte/macrophage cytokines. J Neuroimmunol 2011; 238:19–33
22. Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16:109–10
23. Lenzen S: The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia 2008; 51:216–26
24. Murakami T, Iwanaga T, Ogawa Y, Fujita Y, Sato E, Yoshitomi H, Sunada Y, Nakamura A: Development of sensory neuropathy in streptozotocin-induced diabetic mice. Brain Behav 2013; 3:35–41
25. Hylden JL, Wilcox GL: Intrathecal morphine in mice: A new technique. Eur J Pharmacol 1980; 67:313–6
26. Osikowicz M, Skup M, Mika J, Makuch W, Czarkowska-Bauch J, Przewlocka B: Glial inhibitors influence the mRNA and protein levels of mGlu2/3, 5 and 7 receptors and potentiate the analgesic effects of their ligands in a mouse model of neuropathic pain. Pain 2009; 147:175–86
27. Mika J, Osikowicz M, Makuch W, Przewlocka B: Minocycline and pentoxifylline attenuate allodynia and hyperalgesia and potentiate the effects of morphine in rat and mouse models of neuropathic pain. Eur J Pharmacol 2007; 560:142–9
28. Zawadzka M, Kaminska B: A novel mechanism of FK506-mediated neuroprotection: Downregulation of cytokine expression in glial cells. Glia 2005; 49:36–51
29. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–9
30. Chmielarz P, Kuśmierczyk J, Parlato R, Schütz G, Nalepa I, Kreiner G: Inactivation of glucocorticoid receptor in noradrenergic system influences anxiety- and depressive-like behavior in mice. PLoS One 2013; 8:e72632
31. Mika J, Osikowicz M, Rojewska E, Korostynski M, Wawrzczak-Bargiela A, Przewlocki R, Przewlocka B: Differential activation of spinal microglial and astroglial cells in a mouse model of peripheral neuropathic pain. Eur J Pharmacol 2009; 623:65–72
32. Liu J, Feng X, Yu M, Xie W, Zhao X, Li W, Guan R, Xu J: Pentoxifylline attenuates the development of hyperalgesia in a rat model of neuropathic pain. Neurosci Lett 2007; 412:268–72
33. Sweitzer SM, Schubert P, DeLeo JA: Propentofylline, a glial modulating agent, exhibits antiallodynic properties in a rat model of neuropathic pain. J Pharmacol Exp Ther 2001; 297:1210–7
34. Kawasaki Y, Xu ZZ, Wang X, Park JY, Zhuang ZY, Tan PH, Gao YJ, Roy K, Corfas G, Lo EH, Ji RR: Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. Nat Med 2008; 14:331–6
35. Miyoshi K, Obata K, Kondo T, Okamura H, Noguchi K: Interleukin-18-mediated microglia/astrocyte interaction in the spinal cord enhances neuropathic pain processing after nerve injury. J Neurosci 2008; 28:12775–87
36. Sun JS, Yang YJ, Zhang YZ, Huang W, Li ZS, Zhang Y: Minocycline attenuates pain by inhibiting spinal microglia activation in diabetic rats. Mol Med Rep 2015; 12:2677–82
37. Mizisin AP: Mechanisms of diabetic neuropathy: Schwann cells. Handb Clin Neurol 2014; 126:401–28
38. Neumann S, Doubell TP, Leslie T, Woolf CJ: Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 1996; 384:360–4
39. Keilhoff G, Schild L, Fansa H: Minocycline protects Schwann cells from ischemia-like injury and promotes axonal outgrowth in bioartificial nerve grafts lacking Wallerian degeneration. Exp Neurol 2008; 212:189–200
40. Alano CC, Kauppinen TM, Valls AV, Swanson RA: Minocycline inhibits poly(ADP-ribose) polymerase-1 at nanomolar concentrations. Proc Natl Acad Sci USA 2006; 103:9685–90
41. Old EA, Clark AK, Malcangio M: The role of glia in the spinal cord in neuropathic and inflammatory pain. Handb Exp Pharmacol 2015; 227:145–70
42. Wodarski R, Clark AK, Grist J, Marchand F, Malcangio M: Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats. Eur J Pain 2009; 13:807–11
43. Liao YH, Zhang GH, Jia D, Wang P, Qian NS, He F, Zeng XT, He Y, Yang YL, Cao DY, Zhang Y, Wang DS, Tao KS, Gao CJ, Dou KF: Spinal astrocytic activation contributes to mechanical allodynia in a mouse model of type 2 diabetes. Brain Res 2011; 1368:324–35
44. Tanga FY, Raghavendra V, DeLeo JA: Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem Int 2004; 45:397–407
45. Dorner BG, Scheffold A, Rolph MS, Huser MB, Kaufmann SH, Radbruch A, Flesch IE, Kroczek RA: MIP-1alpha, MIP-1beta, RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. Proc Natl Acad Sci USA 2002; 99:6181–6
46. Middel P, Thelen P, Blaschke S, Polzien F, Reich K, Blaschke V, Wrede A, Hummel KM, Gunawan B, Radzun HJ: Expression of the T-cell chemoattractant chemokine lymphotactin in Crohn's disease. Am J Pathol 2001; 159:1751–61
47. Blaschke S, Middel P, Dorner BG, Blaschke V, Hummel KM, Kroczek RA, Reich K, Benoehr P, Koziolek M, Müller GA: Expression of activation-induced, T cell-derived, and chemokine-related cytokine/lymphotactin and its functional role in rheumatoid arthritis. Arthritis Rheum 2003; 48:1858–72
48. Nikodemova M, Duncan ID, Watters JJ: Minocycline exerts inhibitory effects on multiple mitogen-activated protein kinases and IkappaBalpha degradation in a stimulus-specific manner in microglia. J Neurochem 2006; 96:314–23
49. Heiber M, Docherty JM, Shah G, Nguyen T, Cheng R, Heng HH, Marchese A, Tsui LC, Shi X, George SR: Isolation of three novel human genes encoding G protein-coupled receptors. DNA Cell Biol 1995; 14:25–35
50. Shan L, Qiao X, Oldham E, Catron D, Kaminski H, Lundell D, Zlotnik A, Gustafson E, Hedrick JA: Identification of viral macrophage inflammatory protein (vMIP)-II as a ligand for GPR5/XCR1. Biochem Biophys Res Commun 2000; 268:938–41
51. Lüttichau HR: The herpesvirus 8 encoded chemokines vCCL2 (vMIP-II) and vCCL3 (vMIP-III) target the human but not the murine lymphotactin receptor. Virol J 2008; 5:50
52. Dorner BG, Dorner MB, Zhou X, Opitz C, Mora A, Güttler S, Hutloff A, Mages HW, Ranke K, Schaefer M, Jack RS, Henn V, Kroczek RA: Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8+ T cells. Immunity 2009; 31:823–33
53. Huang H, Li F, Cairns CM, Gordon JR, Xiang J: Neutrophils and B cells express XCR1 receptor and chemotactically respond to lymphotactin. Biochem Biophys Res Commun 2001; 281:378–82
54. Yoshida T, Izawa D, Nakayama T, Nakahara K, Kakizaki M, Imai T, Suzuki R, Miyasaka M, Yoshie O: Molecular cloning of mXCR1, the murine SCM-1/lymphotactin receptor. FEBS Lett 1999; 458:37–40

55. Wang CR, Liu MF, Huang YH, Chen HC: Up-regulation of XCR1 expression in rheumatoid joints. Rheumatology (Oxford) 2004; 43:569–73
56. Zhang J, Shi XQ, Echeverry S, Mogil JS, De Koninck Y, Rivest S: Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. J Neurosci 2007; 27:12396–406
57. Peters CM, Eisenach JC: Contribution of the chemokine (C-C motif) ligand 2 (CCL2) to mechanical hypersensitivity after surgical incision in rats. Anesthesiology 2010; 112:1250–8
58. Frossard JL, Lenglet S, Montecucco F, Steffens S, Galan K, Pelli G, Spahr L, Mach F, Hadengue A: Role of CCL-2, CCR-2 and CCR-4 in cerulein-induced acute pancreatitis and pancreatitis-associated lung injury. J Clin Pathol 2011; 64:387–93
59. Liu Y, Chen K, Wang C, Gong W, Yoshimura T, Liu M, Wang JM: Cell surface receptor FPR2 promotes antitumor host defense by limiting M2 polarization of macrophages. Cancer Res 2013; 73:550–60
60. Kwiatkowski K, Piotrowska A, Rojewska E, Makuch W, Jurga A, Slusarczyk J, Trojan E, Basta-Kaim A, Mika J: Beneficial properties of maraviroc on neuropathic pain development and opioid effectiveness in rats. Prog Neuropsychopharmacol Biol Psychiatry 2016; 64:68–78
61. Matsushita K, Tozaki-Saitoh H, Kojima C, Masuda T, Tsuda M, Inoue K, Hoka S: Chemokine (C-C motif) receptor 5 is an important pathological regulator in the development and maintenance of neuropathic pain. Anesthesiology 2014; 120:1491–503
62. Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB, Feng L: Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. Proc Natl Acad Sci USA 1998; 95:10896–901
63. Hu XM, Liu YN, Zhang HL, Cao SB, Zhang T, Chen LP, Shen W: CXCL12/CXCR4 chemokine signaling in spinal glia induces pain hypersensitivity through MAPKs-mediated neuroinflammation in bone cancer rats. J Neurochem 2015; 132:452–63
64. Shen W, Hu XM, Liu YN, Han Y, Chen LP, Wang CC, Song C: CXCL12 in astrocytes contributes to bone cancer pain through CXCR4-mediated neuronal sensitization and glial activation in rat spinal cord. J Neuroinflammation 2014; 11:75
65. Yellowlees Douglas J, Bhatwadekar AD, Li Calzi S, Shaw LC, Carnegie D, Caballero S, Li Q, Stitt AW, Raizada MK, Grant MB: Bone marrow-CNS connections: implications in the pathogenesis of diabetic retinopathy. Prog Retin Eye Res 2012; 31:481–94
66. Syngle A, Verma I, Krishan P, Garg N, Syngle V: Minocycline improves peripheral and autonomic neuropathy in type 2 diabetes: MIND study. Neurol Sci 2014; 35:1067–73