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 naïve 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)
Zychowska

levels were greater than 300 mg/dl. to observe the changes in

land). The mice were considered diabetic if the serum glucose

cells18 and has also been detected in astrocytes and mono-

cytes.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 neu-

ropathy model. We examined changes in the protein levels of

XCL1–XCR1 during diabetic neuropathy and the modula-
tion 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 transmis-

sion 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 per-

formed 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 intra-

peritoneal administrations of streptozotocin (200 mg/kg; Sigma

Aldrich, USA) dissolved in water for injection7,23,24 were per-

formed. 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, switzer-

land). 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 1h before streptozoto-

cin 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 devel-

oped 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 vis-

ible after reconstitution). Furthermore, it was administered

twice daily for 21 days by the same persons who were carry-

ing out the behavioral analysis. To compare the effectiveness

of single intraperitoneal and single intrathecal administra-

tions, another group of animals received single MC (30 mg/

kg) injections on day 7 after streptozotocin administration.

The behavioral tests were performed 1 and 4h after the MC

injection.

Intrathecal Administration. The intrathecal administrations

were performed according to the methods of Hylden and

Wilcox.23 The XCL1chemokine, 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 strepto-

zotocin administration, and the behavioral tests were con-

ducted after 1 and 4h after the MC injections.

Single XCL1/Lymphotactin Neutralizing Antibody

Administration. The antimouse XCL1 neutralizing antibody

was purchased from Amsbio (UK), reconstituted in water for

injection, and singly intrathecally injected at day 7 after

streptozotocin administration at the following concentra-

tions: 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 behav-

ioral tests were performed at 1, 4, 24, and 96h after XCL1

administration.
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 antianimous 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

**Noxious 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-cm² culture flasks at 37°C and 5% CO₂. 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 MCC (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 Kamińska.28

**Biochemical Tests**

**Analysis of Gene Expression by qRT-PCR.** The primary microglial cultures were treated with MCC (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.28 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**

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

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 H2O, 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 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−(threshold cycle).

**Western Blot Protein Analysis.** From the naive, vehicle (water for injection)-treated, and MC-treated streptozotocin-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 (14,000g for 30 min, 4°C). The protein concentrations in the supernatants were determined with bicinchoninic acid assay Protein Kits (Sigma). Samples containing 25 μg of 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 antirat XCL1 (LSBio, USA; 1:500), antirat 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. 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. 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; in vitro studies).
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 4h after single intrathecal (60 μg/kg) 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 1h after both the intrathecal and intraperitoneal administrations, and this effect disappeared after 4h (fig. 1A). Similarly, the antihyperalgesic effects to the noxious stimuli in the cold plate test were observed 1h after both the intrathecal and intraperitoneal administrations, and these effects disappeared after 4h (fig. 1B).

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 1h before streptozotocin injection and twice daily for 21 days thereafter) were reduced at 14 and 21 days after streptozotocin administration compared with the streptozotocin-injected mice that received vehicle injections (fig. 2A). In this case, two-way ANOVA did not show time x drug interaction (F<sub>27,1</sub> = 0.1901; P = 0.8273); nevertheless, the glucose levels were significantly diminished after MC treatment to similar extent regardless of investigated time point (F<sub>1,24</sub> = 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. 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 4h 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) vs. MC-treated streptozotocin-induced diabetic neuropathic pain in mice; $P < 0.05, $$P < 0.01, and $$$P < 0.001 comparison between 1 and 4h in MC-treated STZ-induced diabetic neuropathic pain in mice.
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 x drug interaction (F2,45 = 4.475; P = 0.0169), and the allodynia was significantly diminished after MC treatment (F1,45 = 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 x drug interaction (F2,45 = 22.91; P < 0.0001), and the hyperalgesia was significantly diminished after MC treatment (F1,45 = 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 (1.9-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.
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. 3E 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; *<P < 0.01 and **<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.
Anesthesiology 2016; 125:573-89

Zychowska et al.

XCL1/XCR1 Expression in Diabetic Neuropathy

(F2,35 = 0.7422; P = 0.4834); nevertheless, the XCR1 levels were again significantly diminished after MC treatment (F1,35 = 19.96; P < 0.0001).

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 (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.

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.

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.
Fig. 8. The effects of single chemokine-C-motif ligand 1 (XCL1) neutralizing antibody administrations on a single XCL1/lympho-
tactin 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; ^P < 0.05 and ^^^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 < 0.01 indicate significant differences between the V + XCL1– and nAb + XCL1–treated naive mice. GAPDH = glycer-
aldehyde 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 + MC-, lipopolysaccharide-, and lipopolysaccharide with MC–treated microglia 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 + 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 (F12.144 = 2.277; P = 0.0112) between investigated treatment and time points being subject of investigation. The XCL1 significantly enhanced nociceptive threshold (F4.144 = 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 noxious 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 (F12.142 = 8.776; P < 0.0001) between investigated treatment and time points being subject of investigation. The XCL1 significantly enhanced allodynia (F4.142 = 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 (F12.119 = 3.6133; P = 0.0001) between investigated treatment and time points being subject of investigation. The XCL1 significantly enhanced hyperalgesia (F4.119 = 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/5 μl), 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 g) 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\text{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\text{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\text{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\text{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−, 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 parallely 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 metalloproteinase 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. In 2008, Keilhoff et al. 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, 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. However, in the allodynia and hyperalgesia that occurs in type 1 diabetes models, microglia activation is observed, and astrocytes are activated in type 2 diabetes models. 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. 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; 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. For instance, the XCL1 has been identified to be released by natural killer, CD8+ T, CD4+ T helper 1 cells, astrocytes, and macrophages/microglia and it seems that XCL1 participates in the T helper 1-type immune response. 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 and rheumatoid arthritis, 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 naïve 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. 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, 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. identified the orphan receptor G protein–coupled receptor 5, which was subsequently demonstrated to be a high-affinity functional receptor for SCM-1 proteins, and later officially designated as XCR1. XCR1 is the only receptor for XCL1 and XCL2; however, the genes for Xcl1 and Xcr1 but not Xcl2 have been found in mice. Additionally, a viral macrophage inflammatory protein II that acts as a XCR1 antagonist targets only the human and not the murine XCR1. It has been suggested that XCR1 is expressed in murine CD8+ dendritic cells, T cells, B cells, and neutrophils but not in macrophages. Furthermore, the expression of XCR1 in infiltrating mononuclear cells has been detected in patients with rheumatoid arthritis 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,\textsuperscript{56} while anti-CCL2 antibody inhibits both microglia activation and behavioral changes.\textsuperscript{57} CCL2 also activates chemokine (C-C motif) receptor 4 expressed in Th2 lymphocytes\textsuperscript{58} and participates in macrophage recruitment.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{65} and that its administration improves diabetic neuropathy and visual acuity.\textsuperscript{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. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

31. Mika J, Osikowicz M, Rojewska E, Korostynski M, Wawrzczak-Wójcik M, Prochazkova-Baucz J, Przewlocka B: Glial inhibitors influence the mRNA and protein levels of mGlul/2, 3 and 7 receptors and potentiate the analgesic effects of their ligands in a mouse model of neuropathic pain. Pain 2009; 147:175–86.


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
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