Neurokinin-1 Receptor Antagonists Inhibit the Recruitment of Opioid-containing Leukocytes and Impair Peripheral Antinociception

Heike L. Rittner, M.D., D.E.S.A.,* Christian Lux,† Dominika Labuz, Ph.D.,‡ Shaaban A. Mousa, Ph.D.,* Michael Schäfer, M.D.,§ Christoph Stein, M.D.,∥ Alexander Brack, M.D., D.E.S.A.*

Background: Neurokinins (e.g., substance P) contribute to pain transmission in the central nervous system, peripheral neurogenic inflammation, and leukocyte recruitment in inflammation. Leukocyte recruitment involves (1) up-regulation of adhesion molecule expression through neurokinin-1 (NK1) receptors on endothelial cells, (2) chemokine production, and (3) chemotaxis through NK1 receptors on leukocytes. In inflammation, leukocytes can trigger endogenous antinociception through release of opioid peptides and activation of opioid receptors on peripheral sensory neurons. The authors hypothesized that NK1 receptor antagonists impair recruitment of opioid-containing leukocytes and stress-induced antinociception.

Methods: Rats were treated intraperitoneally and intrathecally with peripherally restricted (SR140333) or blood-brain barrier–penetrating (L-733,060) NK1 receptor antagonists and were evaluated for paw pressure thresholds, numbers of infiltrating opioid-containing leukocytes and leucocyte subpopulations, expression of adhesion molecules, NK1 receptors, and chemokines 24–48 h after complete Freund adjuvant–induced hind paw inflammation.

Results: Systemic and peripherally selective, but not intrathecal, NK1 receptor blockade reduced stress-induced antinociception (control: 177 ± 9 g; L-733,060; 117 ± 8 g; and control: 166 ± 30 g; SR140333: 89 ± 3 g; both P < 0.05, test) without affecting baseline hyperalgesia. In parallel, local recruitment of opioid-containing leukocytes was decreased (L-733,060 and SR140333: 56.0 ± 4.3 and 59.1 ± 7.9% of control; both P < 0.05, test). NK1 receptors were expressed on peripheral neurons, infiltrating leukocytes and endothelial cells. Peripheral NK1 receptor blockade did not alter endothelial expression of intercellular adhesion molecule-1 or local chemokine and cytokine production, but decreased polymorphonuclear cell and macrophage recruitment.

Conclusions: Endogenous inhibition of inflammatory pain is dependent on NK1 receptor–mediated recruitment of opioid-containing leukocytes.

MANY cellular inflammatory mediators contribute to the generation of pain.1,2 However, leukocyte recruitment also contributes to peripheral antinociception.3 In complete Freund adjuvant (CFA)–induced inflammation, leukocytes containing opioid peptides (e.g., β-endorphin and metenkephalin) migrate to inflamed tissue. After exposure to a cold water swim stress (CWS) or local injection of various releasing agents, opioid peptides can be liberated from leukocytes and bind to nearby opioid receptors on peripheral sensory neurons resulting in unilateral antinociception.3-5 Both polymorphonuclear leukocytes (PMNs) and monocytes/macrophages cause opioid-mediated antinociception in different stages of inflammation.6-8 Blockade of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) or selectins inhibits the recruitment of opioid-containing leukocytes into the paw and CWS-induced antinociception.9-11 In early stages (i.e., 0–12 h after CFA injection), chemokines (i.e., CXCR2 ligands) are produced at the site of inflammation, mediate the recruitment of opioid-containing PMNs, directly trigger the release of opioid peptides from PMNs, and induce peripheral opioid-mediated antinociception.5,6 In contrast, at later stages (i.e., 24–96 h after CFA), molecular mechanisms for the recruitment of opioid-containing leukocytes have not yet been identified. PMNs are predominantly recruited in early inflammation (0–12 h after CFA), whereas macrophages are the principal leucocyte subpopulation at later stages (beyond 24 h after CFA).8

Leukocyte recruitment is classically mediated by chemokines, but other mediators, such as complement or neuropeptides, can also act as chemoattractants. Substance P is one of the neuropeptides and was originally described as a mediator in pain transmission in the central nervous system and in neurogenic inflammation in peripheral tissue.12,13 Three neurokinin (NK1,2) receptors have been identified. Substance P preferentially binds to NK1 receptors.14,15 NK1 receptors are expressed on neurons in the peripheral and central nervous system as well as on leucocytes, endothelial cells, and keratinocytes.16-18 Neurokinin receptor agonists enhance leukocyte migration by three distinct mechanisms: (1) direct chemokinetic effects on monocytes and PMNs,19-22 (2) binding to NK receptors on endothelial cells and increasing the expression of ICAM-1 and several selectins,17,19,24 and (3) augmentation of local chemokine production (e.g., CC chemokine ligand 2 [CCL2]; synonym: monocyte chemoattractant protein 1).25

In this study, we examined whether the blockade of NK1 receptors (1) impairs opioid-mediated peripheral antinociception; (2) reduces recruitment of opioid-containing leukocytes; and (3) influences endothelial adhe-
sion molecule expression, local chemokine production, and/or migration of PMNs or monocytes/macrophages at 24 or 48 h of CFA inflammation. To further characterize the site of action of NK<sub>1</sub> receptor agonists, we used systemic administration of blood-brain barrier–penetrating (L-733,060) and nonpenetrating NK<sub>1</sub> receptor antagonists (SR140333) as well as intrathecal injection.26–28

Materials and Methods

Animals and Model of Inflammation

Animal protocols were approved by local authorities and are in accordance with the guidelines of the International Association for the Study of Pain.29 Male Wistar rats weighing 180–240 g were injected with 150 μl CFA (Calbiochem, La Jolla, CA) into the right hind paw (intraplantar) during brief isoflurane anesthesia and developed an inflammation confined to the inoculated paw. Experiments were conducted at 0–48 h after inoculation of CFA. All further injections were also performed during brief isoflurane anesthesia.

Measurement of Paw Pressure Threshold

Mechanical nociceptive thresholds were assessed using the paw pressure algesiometer (modified Randall–Selitto test; Ugo Basile, Comerio, Italy).30 Rats were handled for 4 days before testing. On the day of testing, rats were held under paper wacling, and incremental pressure was applied via a wedge-shaped, blunt piston onto the dorsal surface of the hind paw by an automated gauge. The pressure required to elicit paw withdrawal, the paw pressure threshold, was recorded (cutoff at 250 g). The average of three trials, separated by 10-s intervals, was calculated. The same measurement was performed on the contralateral paw in alternated sequence to preclude order effects. To examine endogenous mechanisms of antinociception, paw pressure thresholds were determined at baseline conditions and 1 min after swimming in 2°–4°C cold water for 1 min.8 An increase in paw pressure threshold was interpreted as mechanical antinociception. All behavioral experiments were performed by an examiner blinded to the treatment protocol.

Implantation of Spinal Catheters

Implantation was performed under continuous isoflurane anesthesia as described.31 In brief, a 150-mm polyethylene tube (PE 10; Portex, Hythe, United Kingdom) was inserted intrathecally for 25 mm in cervical direction through an incision at the L3–L4 level. During the recovery period of 2 days, animals showing neurologic damage (e.g., paralysis of the hind limbs) were excluded from the study. To ensure intrathecal localization of the catheter, 10 μl lidocaine, 2% (Braun, Melsungen, Germany), followed by a 10-μl solvent flush was applied, and the animals were monitored for development and reversal of paralysis of both hind limbs.

Fluorescence-activated Cell Sorting

Antibodies. All hematopoietic cells were stained by mouse anti-rat CD45 Cy5 phycoerythrin monoclonal antibody (clone OX-1, identifies the leukocyte common antigen, 4 μg/ml; BD Biosciences, Heidelberg, Germany).32 PMNs were identified by mouse anti-rat RP-1 phycoerythrin monoclonal antibody (12 μg/ml). Macrophages were stained by mouse anti-rat CD68 fluorescein isothiocyanate monoclonal antibody (formerly called ED1; 2 μg/ml; Serotec, Oxford, United Kingdom). Opioid-containing cells were labeled by mouse 3E7 monoclonal antibody (recognizing the pan opioid sequence, 20 μg/ml; Gramsch Laboratories, Schwabhausen, Germany) followed by rabbit anti-mouse immunoglobulin (Ig)G<sub>2a</sub+b phycoerythrin antibody (15 μg/ml; BD Biosciences). NK<sub>1</sub> receptor was stained by rabbit anti-rat NK<sub>1</sub> receptor serum (1:100; Chemicon, Hampshire, United Kingdom) at 4°C for 30 min and subsequently by goat anti-rabbit IgG–fluorescein isothiocyanate antibody (15 μg/ml; Vector Laboratories, Burlingame, CA). Specificity of staining was controlled by isotype-matched control antibodies (mouse IgG<sub>2a</sub>, 20 μg/ml, BD Biosciences; and rabbit IgG 4 μg/ml, Santa Cruz, Santa Cruz, CA).

Leukocyte Staining. Cell suspensions from paw tissue were prepared and stained as described.5,7 Briefly, subcutaneous paw tissue was enzymatically digested and was pressed through a 70-μm nylon filter (BD Biosciences). Staining with anti-rat CD45 Cy5 phycoerythrin was done without permeabilization. For intracellular stains using 3E7, anti-rat–CD68 fluorescein isothiocyanate, and anti-rat RP-1 phycoerythrin, cells were fixed with 1% paraformaldehyde and permeabilized with saponin buffer. Permeabilized cells were incubated with the aforementioned primary and secondary antibodies. Replacement of the primary antibodies with isotype-matched irrelevant antibodies was used for negative controls.31

To calculate absolute numbers of cells per paw, fluorescence-activated cell sorting events from fluorescent TruCOUNT beads and CD45<sup>+</sup> stained cells were collected simultaneously, and the number of CD45<sup>+</sup> cells per tube was calculated accordingly. For quantification, 70,000 fluorescence-activated cell sorting events were acquired. Data were analyzed using CellQuest software (all BD Biosciences).

Enzyme-linked Immunosorbent Assay

All experiments were performed 0–48 h after intraplantar injection of CFA. Paw tissue was retrieved, the skin was removed, and subcutaneous tissue was cut into small pieces and processed as described.6 Substance P, CXC chemokine ligand 1 (CXCL1; synonym: keratinocyte-derived chemokine), CCL2, and interleukin-1β (IL-1β) concentrations were measured by commercially available sub-
stance P, mouse CXCL1, rat CCL2, and rat IL-1β enzyme-linked immunosorbent assay kits according to manufacturers’ instructions (Bachem/Peninsula, London, United Kingdom; Biosource International, Nivelles, Belgium; and R&D, Minneapolis, MN, respectively). To measure CXCL1, a rat CXCL1 peptide standard was used as described.\(^6\) Optical density was measured by Spectra Max (Molecular Devices, Ismaning, Germany). Data were analyzed by the Softmax program (Molecular Devices).

**Immunofluorescence**

The expression of ICAM-1 and NK\(_1\) receptor in inflamed paw tissue was analyzed in rats (\(n = 5/\text{group}\)) treated with NK\(_1\) receptor antagonists immediately before induction of inflammation and in control animals. Twenty-four hours later, rats were deeply anesthetized with halothane and perfused transcardially (0.1 m phosphate-buffered saline [PBS], followed by fixative solution: PBS containing 4% paraformaldehyde, pH 7.4). The skin with adjacent subcutaneous tissue was removed from both hind paws, postfixed for 30 min at 4°C in the fixative solution, and cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissue was embedded in Tissue Tek compound (OCT; Miles, Elkhart, IN), frozen, cut into 7-μm sections, mounted onto gelatin-coated slides, and processed for immunofluorescence.\(^3\) The sections were incubated with mouse anti-ICAM-1 (1-200) alone or in combination with rabbit polyclonal anti-NK\(_1\) receptor antibody (1-500) and then with secondary antibodies. After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with the appropriate secondary antibodies: a Texas red conjugated goat anti-rabbit antibody in combination with fluorescein isothiocyanate conjugated donkey anti-mouse. The sections were then washed with PBS, mounted in Vectashield or Vectashield mounting media containing 1.6 diamidino-2-phenylindole (DAPI) (both Vector Laboratories, Burlingame, CA), and viewed under Zeiss LSM510META confocal laser scanning system (Carl Zeiss Imaging, Thornwood, NY). To demonstrate specificity of staining, the following controls were included as mentioned in details elsewhere\(^10\) by omission of either the primary antiserum/antibodies or the secondary antibodies. Cell types were identified based on morphologic criteria. The number of ICAM and NK\(_1\) receptor-positive vessels per section was calculated from counting 3 sections per animal and 5 squares (384 mm\(^2\)).

**Experimental Protocols**

**Systemic Application of NK\(_1\) Receptor Antagonists.** Rats were injected intraperitoneally every 12 h with 20 mg/kg L-733,060 (dissolved in sterile water and further diluted in up to 500 μl normal saline; Tocris, Bristol, United Kingdom) or 10 mg/kg SR140333 (dissolved in dimethyl sulfoxide and further diluted in up to 500 μl normal saline; Sanofi, Paris, France). Doses were chosen based on previous publications\(^34\) and our pilot data. Control animals received solvent only (sterile water or dimethyl sulfoxide in normal saline, respectively). CFA was injected intraplantarly after the first injection, and experiments were performed 24–48 h later.

**Intrathecal Application of NK\(_1\) Receptor Antagonists.** Separate groups of rats were injected intrathecally with 0.5 mg/kg L-733,060 (20 μl every 12 h) followed by 10 μl NaCl (0.9%) flushes.\(^36\) Doses were chosen in pilot experiments and did not produce any toxic effects. Control animals received solvent only. Immediately after the first injection, paw inflammation was induced by CFA, and tissue was harvested 24 h later.

**Statistical Analysis**

Data are presented as raw values or percentage control of baseline (mean ± SEM). Missing values are due to injection-related hematoma formation distorting fluorescence-activated cell sorting quantification of leukocyte subpopulations or due to accidental death by isoflurane overdose. Normally distributed data were analyzed by \(t\) test. Otherwise, the Mann–Whitney rank sum test was used. Multiple measurements were analyzed by one-way analysis of variance for normally distributed data and by two-way repeated-measures analysis of variance when dependent and independent factors were present. *Post hoc* comparisons were performed by the Student-Newman-Keuls and Holm-Sidak method, respectively. Differences were considered significant if \(P < 0.05\).

**Results**

**Stress-induced Antinociception Is Reduced by NK\(_1\) Receptor Antagonists**

Neurokinin-1 receptor blockade did not significantly change baseline paw pressure threshold in inflamed (fig. 1) or noninflamed paws (data not shown). At 24 h of inflammation, CWS-induced antinociception was significantly reduced by both intraperitoneal L-733,060 and intraperitoneal SR140333 treatments (figs. 1A and B), but not by intrathecal L-733,060 (fig. 1C). Furthermore, systemic treatment with L-733,060 did not alter baseline hyperalgesia at 48 h of inflammation, whereas it significantly reduced CWS-induced antinociception (table 1).

**Recruitment of Opioid-containing Leukocytes Is Reduced by Blockade of NK\(_1\) Receptors**

Cold water swim stress-induced antinociception is mediated by opioid-containing leukocytes at the site of inflammation.\(^8\)\(^30\) In the inflamed paw, 213 ± 11 × 10\(^3\) 3E7\(^+\)CD45\(^+\) opioid-containing leukocytes per paw were
Expression of NK₁ Receptors and Substance P at the Site of Inflammation

Levels of substance P were low in noninflamed paws (fig. 3A). At 24 and 48 h of inflammation, substance P content in the paw was significantly increased, with a 17.5-fold increase at 24 h. NK₁ receptor expression was observed on endothelial cells as shown by colocalization with ICAM-1, peripheral neurons, and on CD45⁺ leukocytes in the inflamed paws (figs. 3B-D).

NK₁ Receptor Blockade Reduces Migration of PMNs and Macrophages

At 24 h of CFA, similar numbers of monocytes/macrophages and PMNs per paw were detected by flow cytometry (245 ± 18 × 10³ CD68⁺ macrophages per paw and 219 ± 16 × 10³ RP1⁺ PMNs per paw; fig. 5). Treatment with intraperitoneal L-733,060 or intrathecal SR140333 significantly reduced infiltrating PMNs and macrophages (CFA 24 h: figs. 5A, B, D, and E; CFA 48 h: table 4). CD3⁺ lymphocytes did not change (data not shown). Intrathecal administration of L-733,060 did not influence overall leukocyte migration or recruitment of leukocyte subpopulation at 24 h after CFA (figs. 5C and F).

Endothelial Adhesion Molecule Expression and Local Chemokine/Cytokine Content after NK₁ Receptor Blockade

Intercellular adhesion molecule 1 and NK₁ receptor immunoreactivities on endothelial cells were quantified. Both were significantly up-regulated at 24 h of inflammation (fig. 4, representative examples; and table 2). Blockade of NK₁ receptors using intraperitoneal SR140333 treatment did not alter ICAM-1 or NK₁ receptor expression at 24 h (table 2). At 24 h inflammation, intraperitoneal SR140333 treatment did not alter the content of CXCL1 (PMN specific), CCL2 (monocyte specific), or IL-1β (table 3).

Table 1. Neurokinin-1 Receptor Blockade Reduces Stress-Induced Antinociception but Not Baseline Hyperalgesia at 48 h of Inflammation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Intraperitoneal L-733,060</th>
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<tbody>
<tr>
<td>Baseline paw pressure threshold, g</td>
<td>36.9 ± 1.2</td>
<td>36.4 ± 1.3</td>
</tr>
<tr>
<td>CVS paw pressure threshold, g</td>
<td>170.3 ± 15.7*</td>
<td>125.0 ± 9.1*†</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.

* P < 0.05, baseline–cold water swim stress (CWS) (F = 172.2), † P < 0.05, drug × baseline-CWS (F = 6.9), two-way repeated-measures analysis of variance (n = 6/group).

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Fig. 3. Expression of substance P and neurokinin-1 (NK1) receptors in the inflamed hind paw. (A) Substance P content was measured in subcutaneous paw tissue 0–48 h after complete Freund adjuvant injection (CFA) by enzyme-linked immunosorbent assay (*P < 0.05, F = 7.6, analysis of variance, Student-Newman-Keuls method, n = 6/group). Data are presented as mean ± SEM. (B) NK1 receptor expression (red) in the inflamed paw was demonstrated by fluorescent immunohistochemistry 24 h after CFA injection. Arrows point to NK1 receptor immunoreactive endothelial cells, and arrowhead points to neurons (magnification 20×). In the inset, NK1 receptor expression is shown on infiltrating cells (nuclei stained in blue, magnification 40×). Representative sections are shown. (C) NK1 receptor expression on CD45+ cells in the paw was measured by flow cytometry 24 h after CFA injection (gray histogram = unstained control; dotted line = isotype control antibody; thick black line = anti-NK1 receptor antibody). To exclude nonhematopoietic cells and cell debris, cells were pregated on CD45+ leukocytes (data not shown). A representative histogram is shown. (D) To verify expression of NK1 receptor on endothelial cells, inflamed paw tissue was analyzed by fluorescent immunohistochemistry for NK1 receptor (red) and intercellular adhesion molecule 1 (green) 24 h after CFA injection. Coexpression of both markers is shown in yellow (magnification 20×).

Discussion

In this study, we demonstrated that NK1 receptor antagonists (1) impaired stress-induced peripheral opioid-mediated antinociception but did not alter baseline inflammatory hyperalgesia and (2) reduced migration of opioid-containing leukocytes without changing expression of ICAM-1 on endothelial cells or local chemokine/cytokine production. Systemic administration of blood-brain barrier–penetrating (SR140333) and nonpenetrating (L-733,060) NK1 receptor antagonists was equally effective, whereas intrathecal injection (L-733,060) was ineffective. Taken together, NK1 receptor antagonists seem to act peripherally by directly inhibiting the recruitment of opioid-containing leukocytes to the site of inflammation.

Baseline inflammatory hyperalgesia was not reduced by administration of NK1 receptor antagonists (figs. 1A and B and table 1). These findings are in line with previous animal studies as well as studies in human pain patients. Intrathecal application of NK1 receptor antagonists was also ineffective in our study (fig. 1C), but it has been reported to reduce hyperalgesia in other models. In those models, antihyperalgesic effects were only achieved by intrathecal injection of saporin-conjugated substance P that destroys NK1 receptor–expressing spinal neurons. Inflammatory hyperalgesia is induced by pronociceptive mediators, including cytokines (e.g., IL-1β and chemokines (e.g., CXCL1 and CCL2). In our studies, NK1 receptor blockade did not change the local expression of representative hyperalgesic cytokines or chemokines (table 3), in line with the unaltered baseline nociceptive thresholds (fig. 1 and table 1).

In contrast to this lack of change in baseline hyperalgesia, stress (CWS)–induced antinociception was reduced by NK1 receptor antagonists (figs. 1A and B and table 1). Our previous studies demonstrated that this form of antinociception is mediated by opioid peptide release from leukocytes at the site of inflammation and is fully blocked by opioid receptor antagonists in late stages of inflammation. In the current study, we observed a comparable decrease in opioid-containing leukocytes in the inflamed paw using both blood-brain barrier–penetrating (L-733,060) and nonpenetrating (SR140333) NK1 receptor antagonists, whereas intrathecal injection (L-733,060) was ineffective. The intrathecally injected dose of L-733,060 was previously used in rats and was shown to effectively inhibit NR2B tyrosine phosphorylation in the spinal cord in the CFA model. In addition to intrathecal injection of L-733,060, we also examined intrathecal injection of SR140333 (up to 6
against IL-1 or by an IL-1 receptor antagonist, indicating antinociception cannot be blocked by an antibody hot plate). In particular, in our model CWS-induced ous inflammation.30,47 In another paradigm, swim stress dominantly by locally secreted opioid peptides in subcutaneous sources of substance P are the peripheral sensory neu- trons.16,17,20,52 While neurogenic inflammation contributes to leukocyte infiltration, it is unclear whether blockade of NK1 receptors on peripheral sen- sory neurons influences leukocyte recruitment.53–55 However, NK1 receptors have previously been shown to enhance leukocyte recruitment by increasing adhesion molecule expression on endothelial cells,17,23,24 by augmenting chemokine production25 or by directly induc- ing chemotaxis of leukocytes.19–22

To further elucidate the mechanisms of reduced migra- tion of opioid-containing leukocytes, we measured substance P content and NK1 receptor expression in the inflamed paw. Local substance P content increased during CFA-induced inflammation (fig. 3A). Potential sources of substance P are the peripheral sensory neu- rons and infiltrating leukocytes.13,50,51 In addition to increased substance P amounts, we detected NK1 receptor expression on all infiltrating leukocytes, on neurons, and on endothelial cells (figs. 3B–D), confirming previous studies.16,17,20,52 While neurogenic inflammation contributes to leukocyte infiltration, it is unclear whether blockade of NK1 receptors on peripheral sen- sory neurons influences leukocyte recruitment.53–55 However, NK1 receptors have previously been shown to enhance leukocyte recruitment by increasing adhesion molecule expression on endothelial cells,17,23,24 by augmenting chemokine production25 or by directly induc- ing chemotaxis of leukocytes.19–22

Adhesion molecules (i.e., ICAM-1, selectins, and integ- rins) have previously been shown to mediate the re- cruitment of opioid-containing leukocytes, and their blockade can impair peripheral opioid-mediated antino- ciception during CFA-induced inflammation.9,11 In the current study, expression of ICAM-1 on endothelial cells was increased at later stages of inflammation (24 h after CFA; table 2). Neither the CFA-induced up-regulation of

Table 2. Intercellular Adhesion Molecule-1 and Neurokinin-1 Receptor Expression Are Up-regulated at 24 h of Inflammation but Unaltered by Concomitant Neurokinin-1 Receptor Blockade

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<td>Noninflamed, vessels/section</td>
<td>1.90 ± 0.19</td>
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<td>5.54 ± 0.32*</td>
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P < 0.05, differences between chemokines/cytokine, P > 0.05 for drug and drug × chemokines/cytokine, two-way repeated-measures analysis of vari- ance (F = 36.7, n = 6).

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CCL2 = CC chemokine ligand 2; CFA = complete Freund adjuvant; CXCL1 = CXC chemokine ligand 1; IL-1β = interleukin-1β.
Fig. 5. Peripheral neurokinin-1 receptor blockade decreased the number of infiltrating leukocytes in the inflamed paw. Rats were treated (intraperitoneal L-733,060 [control n = 8, drug n = 7], A and D; intraperitoneal SR140333 [n = 8/group], B and E; and intrathecal L-733,060 [n = 7/group], C and F) as described in figure 1. CD68<sup>+</sup>CD45<sup>+</sup> macrophages (A–C) and RP1<sup>+</sup>CD45<sup>+</sup> polymorphonuclear cells (D–F) were quantified by flow cytometry 24 h after complete Freund adjuvant injection (P < 0.05 for drug, not shown in the figure [F = 8.2 for intraperitoneal L773,060 and F = 8.6 for SR140333], P > 0.05 for drug intrathecal L-733,060, P > 0.05 for drug × leukocyte subpopulation for all drugs, two-way analysis of variance). Data are presented as mean of percentage control ± SEM.

ICAM-1 nor that of NK<sub>1</sub> receptors on endothelial cells was altered by peripheral NK<sub>1</sub> receptor blockade (fig. 4 and table 2). Previous in vitro studies have demonstrated that substance P increased the expression of ICAM-1<sup>16,17</sup> as well as NK<sub>1</sub> receptors.<sup>56,57</sup> In in vivo models of inflammation, the effects of NK<sub>1</sub> receptor antagonists vary between experimental models: They decreased NK<sub>1</sub> receptor but not ICAM-1 expression in a mouse model of pancreatitis.<sup>58</sup> In contrast, they reduced ICAM-1 expression in experimental autoimmune encephalomyelitis.<sup>59</sup> In our model, NK<sub>1</sub> receptor antagonists reduced the recruitment of opioid-containing leukocytes, but they did not alter the expression of ICAM-1 or NK<sub>1</sub> receptors on the inflamed endothelium. We cannot fully exclude the possibility that NK1 receptor antagonists alter the expression of other adhesion molecules such as selectins or integrins and, thereby, impair peripheral antinociception.

As a second hypothesis, NK<sub>1</sub> receptor antagonists might decrease local chemokine production and thereby impair selective leukocyte recruitment. Substance P has been shown to up-regulate chemokine production in leukocytes and epithelial cells in vitro.<sup>60,61</sup> In experimental pancreatitis, local chemokine expression was reduced by an NK<sub>1</sub> receptor antagonist.<sup>62</sup> We examined a PMN- and a monocyte/macrophage-specific chemokine in our model (i.e., CXCL1<sup>63</sup> and CCL2,<sup>64</sup> respectively), and their expression was not altered by NK<sub>1</sub> receptor antagonists (table 3). Multiple chemokines are responsible for PMN and monocyte/macrophage recruitment<sup>65</sup> and alterations in other chemokines might occur after treatment with NK<sub>1</sub> receptor antagonists. Although this possibility cannot be excluded, our previous study demonstrated that PMN-specific chemokines (i.e., CXCR2 ligands) were regulated as a group and did not show relevant differences between individual chemokines.<sup>6</sup>

Taken together, in our model NK<sub>1</sub> receptor antagonists reduce the migration of opioid-containing leukocytes, but this effect does not seem to be mediated through alterations in adhesion molecule expression on endothelial cells or in local chemokine production.

Third, substance P binding to NK<sub>1</sub> receptors on leukocytes might directly induce migration of opioid-containing leukocytes. This migration might be reduced by NK<sub>1</sub> receptor blockade. Several studies demonstrated that NK<sub>1</sub> receptor agonists induce chemotaxis of PMNs and monocytes/macrophages in vitro.<sup>19,60,67</sup> Previous studies have shown that PMNs and monocyte/macrophage migration to the site of inflammation is impaired after treatment with NK<sub>1</sub> receptor antagonists or in NK<sub>1</sub> receptor-deficient mice.<sup>20,24,68,69</sup> In line with these studies, the number of infiltrating macrophages and PMNs as well as of opioid-containing leukocytes was reduced in our model (figs. 2 and 5 and table 4).

In conclusion, we have shown that NK<sub>1</sub> receptor antagonists reduce the recruitment of opioid-containing leukocytes into the inflamed paw and thereby impair opioid-mediated antinociception in CFA inflammation. NK<sub>1</sub> receptor agonists such as substance P seem to directly induce preferential recruitment of opioid-containing monocytes/macrophages while no indirect effects (i.e., alterations in adhesion molecule expression or chemokine production) are observed. Most likely, NK<sub>1</sub> receptor antagonists act directly on NK<sub>1</sub> receptor-expressing, opioid-containing leukocytes, but the effects might also be mediated indirectly through NK<sub>1</sub> receptors on peripheral neurons. Taken together, NK<sub>1</sub> receptor agonists are important in the recruitment of opioid-con-

### Table 4. Neurokinin-1 Receptor Blockade Reduces Migration of Infiltrating Leukocytes at 48 h of Inflammation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-733,060</th>
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</thead>
<tbody>
<tr>
<td>Macrophages, %</td>
<td>100 ± 7.1</td>
<td>42.7 ± 9.8*</td>
</tr>
<tr>
<td>PMNs, %</td>
<td>100 ± 13.7</td>
<td>67.3 ± 10.4*</td>
</tr>
</tbody>
</table>

Data are presented as percentage control of baseline ± SEM.

* P < 0.05 for drug (F = 8.2), P > 0.05 for leukocyte subpopulation and drug × leukocyte subpopulation, two-way repeated-measures analysis of variance (n = 8/group).

PMNs = polymorphonuclear cells.
taining leukocytes and the generation of peripheral opioid-mediated antinociception. The impairment of endo-
genous opioid-mediated peripheral analgesia might be an additional explanation for the lack of efficacy of NK1 receptor antagonist in human studies.12

The authors thank Susanne Kotr{é} and Katharina Hopp (both Technicians, Department of Anesthesiology, Charité, Berlin, Germany) for expert technical assistance.

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